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Detection of mercaptopyridines and mercaptopyrimidines in planar chromatography with iodine–azide reaction as a detection system

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Abstract

Reaction between iodine and azide ion induced by mercaptopyridines and mercaptopyrimidines was utilized as a detection system in TLC and HPTLC. The developed plates were sprayed with a freshly prepared mixtures of sodium azide and starch solution adjusted to pH 5.5, and exposed to iodine vapour. The spots became visible as white spots on violet-grey background. The iodine–azide detection system has been proved to be the most favourable and enabled to detect quantities per spot in the range of 1–20 pmol (HPTLC) and 1–60 pmol (TLC). The iodine–azide tests were compared with other visualizing techniques commonly used in planar chromatography (iodine vapour and UV_{254}). The developed method was applied to detection of thiopental in biological samples.

Keywords: Iodine–azide reaction; Mercaptopyridines; Mercaptopyrimidines; TLC; HPTLC

1. Introduction

The widespread use of thiols as drugs [1], cosmetics [2], corrosion inhibitors [3], agents in photographic [4] and vulcanization [5] processes and chemical analysis of varied metal ions [6] as well as different bio-oriented compounds [7,8], has created a demand for the selective detection of these compounds at trace levels in natural samples.

Planar chromatography is one of the oldest methods [9] but still one of the most widely applied for screening of many organic compounds using different detection methods [10]. Many applications for thiols detection exploited include varied spray reagent containing for example mercury(II) [11] and (I) [12], platinum(II) [11], iron(III) [13] permanganate [14] ions as well as sulphuric and phosphomolybdic acids [15]. To improve detection the prechromatographic derivatization reactions are sometime suggested [16]. The most common methods utilized for detection in TLC are visualization under 254 or 336 nm [17] and with a TLC/HPTLC scanner [18].

In this paper, we have focused on employing the iodine–azide reaction as the proposed method of detection of mercaptopyridines and mercaptopirymidines in order to improve their detection sensitivity at a pmol per spot level. In practice, azide reacts with iodine only in the presence of sulphur(II) compounds. It offers a mean of selective and sensitive detection of thiols as inductors of the iodine–azide reaction. This method bases on visual observation of the plates after spraying with freshly prepared solution of sodium azide and starch and exposure to iodine vapour [19]. The spots become visible as white spots on a violet-grey background. The proposed procedure can be applied to screening test of thiols in natural samples.

2. Experimental

2.1. Materials and apparatus

All thiols compounds were obtained from Aldrich, Fluka and Lancaster. Thiopental was purchased from Biochemie GmgH (Kundl, Austria).

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The plates were developed in a horizontal DS-chamber (Chromades, Poland) and sprayed with a TLC-sprayer (Merck).

2.2. Solutions and reagents

Thiols solutions were obtained by dissolving 100 μmol of particular reagent in 10 ml 0.1 M solution of sodium hydroxide and diluted with methanol to 100 ml. 10 ml 10% (v/v) aqueous sodium azide solution and 1 ml 0.025 M potassium iodide were added to 12.5 ml of 1% aqueous starch solution, adjusted to pH 5.5 with 0.1 M hydrochloric acid solution and diluted to 25 ml. When the dependence of detection limits of thiols was investigated, the amount of potassium iodide (0, 1, 10, 100, and 500 mM) was added into spray solution. All solutions were prepared fresh daily.

2.3. Planar chromatography

TLC silica gel 60 F_{254} aluminium sheets (Merc, Darmstadt, Germany; 10 cm × 5 cm, 0.2 mm thick layer), HPTLC silica gel 60 F_{254} aluminium sheets (Merck, Darmstadt, Germany; 5 cm × 5 cm, 0.2 mm thick layer) were used for the determination of detection limits of all thiols. The plates were spotted 1 cm (in TLC) or 0.5 cm (in HPTLC) from the edge of the plate with 1 μl of appropriate thiol solution (deposition area ≈0.2 cm^2) using 0.1–1 μl pipette (Brand). Distance between spots was 1 cm. The plates were developed using a horizontal DS-Chamber, which was ready for use 30 min after the solvent was poured into it. The developed distances were: 8 cm (for TLC) and 4 cm (for HPTLC). Methanol was used as a mobile phase. Then plates were air dried with a hair dryer. Spots were located by visualization with improved iodine–azide, iodine, UV_{254} procedures. For some thiols, non-improved iodine–azide technique was applied. The particular thiol concentration spotted decreases in following experiments like a calibration function. The detection limits were obtained in six experiments and was considered when the spot corresponding to the smallest amount of thiol appeared six times.

2.3.1. Detection with the improved iodine–azide procedure (improved I_2–N_3^-)

After drying, the developed plates were sprayed with a freshly prepared mixture of sodium azide, potassium iodide and starch solution adjusted to pH 5.5 and were exposed to iodine vapour for 5 s. Due to catalytic effect of the C–S or C=S bounds, the spots became visible as white spots on a violet-grey background and they were stable for several minutes. The spots were read after 2 min since they were taken out from iodine chamber. When concentration of potassium iodide into a spray solution was 0.5 M, the spots were white on a brown background. They were stable for some hours.

2.3.2. Detection with the non-improved iodine–azide procedure (non-improved I_2–N_3^-)

After drying, the developed plates were sprayed with a freshly prepared 1:1 (v/v) mixture of 1 M sodium azide solution and 1 M iodine solution in 1 M potassium iodide solution adjusted to pH 6.0. Due to catalytic effect of the C–S or C=S bound, the spots which were read after 2 min, became visible as white spots on a yellow background and they were stable for several minutes.

2.3.3. Detection with the iodine procedure (I_2)

After drying, the developed plates were exposed to iodine vapour for 10 min. The spots became visible as brown spots on a yellow background.

2.3.4. Detection with the UV_{254} procedure (UV_{254})

After drying, substances were visualized under a UV lamp (254 nm) using TLC or HPTLC plates with a fluorescent indicator.

2.4. Analytical application of developed procedure

Developed procedure was applied to detect thiopental in blood serum and urine samples.

2.4.1. Detection of thiopental in a spiked blood serum sample

A 300 μl of serum with appropriate amount of thiopental (at the level of μM) was placed in the conical flask and diluted to 1 ml with methanol. If necessary, the precipitation was filtered. The 1 μl of the filtrate, standard and blank solutions were spotted on the HPTLC plates. Then, the plate was developed with dichloromethane as a mobile phase and the spots were visualized with iodine–azide reaction as a detection system.

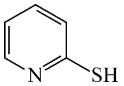
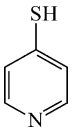
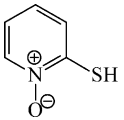
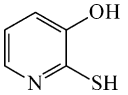
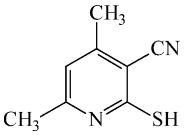
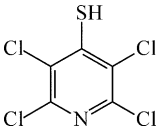
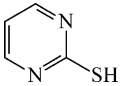
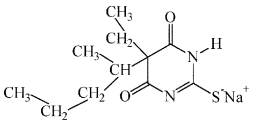
2.4.2. Detection of thiopental in a spiked urine sample

A 4 ml of urine with appropriate amount of thiopental (at the level of μM) was placed in the conical flask with 750 μl 0.1 M EDTA and fix up to 10 ml with methanol. If necessary the precipitation was filtered. The 1 μl of the filtrate, standard and blank solutions were spotted on the HPTLC plate. Then, the plate was developed with methanol as a mobile phase and the spots were visualized with iodine–azide reaction as a detection system.

2.4.3. Detection of thiopental in a real world urine sample

4 ml of a urine sample of a 54-year patient (dosage: 140–160 mg thiopental/h) was collected. Thiopental was detected in the same way as with the spiked urine sample.

Table 1
Detection limits of mercaptopirydines and mercaptopyrimidines in TLC and HPTLC using methanol as a mobile phase

Number	Compound	Formula	Method	Detection limit (pmol/spot)								R_F
				Improved I_2–N_3^-					Non-improved I_2–N_3^-	I_2	UV	
				c(KI) = 0 mM	c(KI) = 1 mM	c(KI) = 10 mM	c(KI) = 100 mM	c(KI) = 500 mM				
1	2-Mercaptopyridine		TLC	20	20	20	20	80	2000	300	400	0.86
			HPTLC	*7*	*7*	*7*	*7*	*80*		*200*	*200*	*0.82*
2	4-Mercaptopyridine		TLC	20	20	20	20	200	6000	500	400	0.83
			HPTLC	*20*	*20*	*20*	*20*	*200*		*200*	*100*	*0.81*
3	*N*-Oxo-2-mercaptopyridine		TLC	1	1	1	1	4		500	100	0.85[a]
			HPTLC	*1*	*1*	*1*	*1*	*2*		*200*	*50*	*0.85*[a]
4	3-Hydroxy-2-mercaptopyridine		TLC	60	60	80	80	400		500	500	0.88
			HPTLC	*20*	*20*	*70*	*70*	*300*		*300*	*400*	*0.91*
5	4,6-Dimethyl-2-mercaptonicotine nitrile		TLC	60	60	60	60	90		600	500	0.88
			HPTLC	*20*	*20*	*20*	*20*	*20*		*300*	*300*	*0.86*
6	4-Mercapto-2,3,5,6-tetrachloropyridine		TLC	20	20	20	20	20		30	700	0.99
			HPTLC	*20*	*20*	*20*	*20*	*20*		*20*	*600*	*0.99*
7	2-Mercaptopyrimidine		TLC	7	7	20	20	20	2000	70	500	0.83
			HPTLC	*2*	*2*	*7*	*7*	*7*		*30*	*200*	*0.79*
8	Thiopental		TLC	4	4	4	4	7	2000	300	300	0.96
			HPTLC	*2*	*2*	*2*	*2*	*5*		*150*	*200*	*0.96*

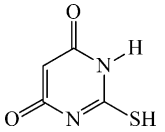
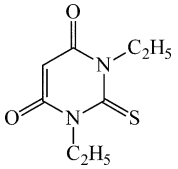
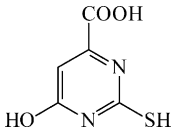
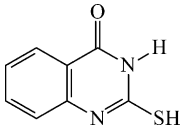
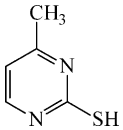
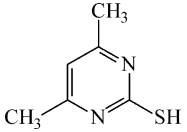
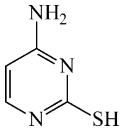
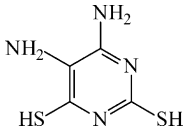
9	2-Thiobarbituric acid		TLC	4	4	4	4	7	80	100	0.93
			HPTLC	*2*	*2*	*2*	*2*	*2*	*30*	*60*	*0.92*
10	1,3-Diethyl-2-thiobarbituric acid		TLC	25	15	15	250	3000	80	500	0.98
			HPTLC	*10*	*10*	*10*	*150*	*700*	*50*	*150*	*0.97*
11	2-Thioorotic acid		TLC	10	6	4	4	8	150	250	0.95
			HPTLC	*8*	*2*	*2*	*2*	*2*	*80*	*100*	*0.90*
12	2-Mercapto-4(3H)-quinazolinone		TLC	1	1	2	2	8	150	400	0.87
			HPTLC	*1*	*1*	*1*	2	2	*80*	*100*	*0.87*
13	2-Mercapto-4-methyl-pyrimidine		TLC	3	3	2	2	20	400	500	0.80
			HPTLC	*2*	*2*	*2*	*2*	*7*	*30*	*200*	*0.79*
14	4,6-Dimethyl-2-mercaptopyrimidine		TLC	3	2	2	2	30	200	500	0.80
			HPTLC	2	2	2	2	*8*	*30*	*100*	*0.76*
15	4-Amino-2-mercaptopyrimidine		TLC	10	10	10	80	300	100	200	0.80
			HPTLC	*10*	*10*	*10*	*60*	*200*	*30*	*70*	*0.80*
16	4,5-Diamino-2,6-dimercaptopyrimidine		TLC	10	10	20	20	100	70	400	0.80
			HPTLC	*8*	*10*	*20*	*20*	*70*	*30*	*150*	*0.82*

Table 1 (*Continued*)

Number	Compound	Formula	Method	Detection limit (pmol/spot)							R_F
				Improved I_2–N_3^-				Non-improved I_2–N_3^-	I_2	UV	
				c(KI) = 0 mM	c(KI) = 1 mM	c(KI) = 10 mM	c(KI) = 100 mM	c(KI) = 500 mM			
17	2-Mercapto-3,4,5,6-tetrahydropyrimidine		TLC	30	10	7	20	90	300	3500	0.81
			HPTLC	*10*	*3*	*3*	*7*	*30*	*200*	*700*	*0.81*
18	2,4-Dimercaptopyrimidine		TLC	15	3	3	15	30	250	500	0.80
			HPTLC	*15*	*3*	*3*	*7*	*3*	*80*	*250*	*0.80*
19	6-Aza-2-thiotymine		TLC	2	2	2	2	10	300	300	0.94
			HPTLC	*1*	*1*	*1*	*1*	*3*	*80*	*200*	*0.94*

[a] Using methanol–dichloromethan mixture as a mobile phase (1:3).

3. Results and discussion

There are parameters common to TLC with iodine–azide detection system which control the optimum performance of thiols detection. Some of them have impact only on the separation process; others influence only iodine–azide detection procedure. However, a large number of parameters usually affect them both. We checked the influence of iodine, pH of spray solution, concentration of sodium azide and induction time on the detection limits of thiols listed in Table 1. The different plates were tested.

In this part of our article, we would like to present the most relevant points of our study we managed to establish.

3.1. Separation

The R_F data (TLC and HPTLC) are summarized in Table 1. There was no need to find new chromatographic systems because several different ones had been proposed for standards [13–15].

It was necessary to establish good resolution of thiopental in biological samples since the iodine–azide detection system is sensitive towards other thiols component of the samples (e.g. cysteine, glutathione).

It appeared that methanol was sufficient enough as a mobile phase to separate thiopental from other sulphur(II) compounds (e.g. cysteine, glutation) in urine sample but it was necessary to find another solvent to separate thiopental in serum sample. Acetonitryl as a mobile phase was also useless since the spots with great tailing overlapped. The use of dichloromethane was found highly satisfactory for discrimination between all spots in serum sample and there was no interference. Cysteine, cystine and glutathione are natural compounds of blood and urine present in a relatively large scale in those biological samples. When methanol was used as a mobile phase the R_F value for all compounds mentioned above was nearly 0. However, there were still two dots in planar chromatograms (R_F = 0.78 and 0.71 in urine sample separated with methanol as a mobile phase and R_F = 0.2 and 0.03 for blood sample separated with dichloromethane as a mobile phase) that corresponded to compounds sensitive toward iodine–azide detection system. Since the R_F values were different from the case of thiopental (R_F = 0.96 and 0.27 for urine sample with methanol as a mobile phase and for blood serum sample with dichloromethane as a mobile phase, respectively) the presence of these compounds did not interfere with detection of thiopental in biological samples.

In order to show the usefulness of the improved iodine–azide detection system, three mixtures of pirymidines and pirydines were separated in HPTLC and detected with the proposed method. The obtained outcome of the resolution is depicted in Table 2.

3.2. Detection

The optimum conditions of the improved detection method using iodine–azide reaction for thiouracils were pre-

Table 2
HPTLC analysis of mixtures of thiols (developed distance 4 cm); solvent methanol:dichloromethane 1:3 (v/v)

Mixture of compounds	Spotted amount (pmol/spot)		R_F	
	I_2–N_3^-	UV_{254}	I_2–N_3^-	UV_{254}
2-Mercaptopyrimidyne	9	70	0.62	0.60
2-Mercapto-4-methylpyrimidyne	3	210	0.75	0.74
4,6-Dimethyl-2-mercaptopyrimidyne	10	100	0.80	0.81
4,5-Diamino-2,6-dimercaptopyrimidyne	10	200	0.25	0.26
N-Oxo-2-mercaptopyridine	7	50	0.95	0.95
4-Mercaptopyridine	50	100	0.66	0.67
2-Mercaptopyridine	25	200	0.86	0.87
4,6-Dimethyl-2-mercaptonicotine nitrile	60	600	0.75	0.76
2-Thiobarbituric acid	6	200	0.70	0.72
Thiopental	6	600	0.95	0.95
1,3-Diethyl-2-thiobarbituric acid	30	500	0.88	0.88
2-Thioorotic acid	6	300	0.08	0.07
2-Mercaptopyrimidyne	9	70	0.62	0.60

sented in a previous report [19]. In this report, we have expanded the application of the method to the group of mercaptopyridines and mercaptopyrimidines and the influence of iodide ions on detection limits was checked. All studied thiols listed in Table 1 were checked considering optimum conditions for the detection method of the described thiols and they remained the same as the previous ones. The 4% sodium azide solution, pH 5.5 was applied to detect all thiols listed in Table 1 using iodine–azide reaction as a detection system. As in the previous report [19], the plates were exposed to iodine vapour for 5 s. This is the time within which iodine is adsorbed on the TLC plate. The exposure time is so short due to adsorption of iodine on the plate and vanishing of white spots. The spots became visible as white dots on a violet-grey background within 2 min due to catalytic effect of the C−S and C=S bonds. The spots were stable within several minutes due to sublimation of iodine from the plate. Appearance of white spots could take some time, due to the induction time which depended on by induction properties of a particular thiol. It is the time within which the induction reaction has finished. This is less than 2 min in each case.

3.2.1. Comparison of the detection limits using different detection methods

The comparison of the detection limits of thiols achieved by using different detection systems (iodine–azide procedure, iodine vapour and UV) used routinely in laboratories is listed in Table 1. The presented outcome indicates that the iodine–azide method is the most favorable one. In general, the detection limits were established at pmol per spot for almost all of the analysed sulphur(II) compounds using iodine–azide detection system.

Another advantage of iodine–azide reaction as a detection system in planar chromatography over other examined methods of detection was the quality of obtained chromatograms. Spots of all the thiols detected with the proposed method were compact with sharp edges against the violet-gray background of the plate and provided an accurate measurement of R_F value.

Applications of the iodine–azide procedure in detection of thiopental in biological samples are shown in Table 3. The detection limits of thiopental in spiked blood serum and urine samples are 30 and 0.5 nmol/ml sample, respectively. The detection limits in spiked samples corresponds to the detection limits for standards. To confirm the proposed procedure, thiopental was detected in a real world urine sample. The R_F value for thiopental, and other sulphur(II) compounds in the sample are in accord with the one for the spiked sample.

The optimum conditions of the improved method of detection using iodine–azide reaction were checked in comparison with non-improved detection method for some studied mercaptopyridines and mercaptopyrimidines. The method involving iodine–azide reaction was based on spraying the plates with a mixture of sodium azide and iodine solution (in potassium iodide solution) [20]. As a result, white spots on a yellow background appeared. However, it was observed that the excess of iodine made the white spots vanish and consequently, this was the reason of the greatly increased detection limits. The phenomenon does not generally occur when others visualization techniques of TLC plates are applied. The

Table 3
Detection limits of thiopental in biological samples; HPTLC (developed distance 4 cm) with improved iodine–azide detection system

Sample	Solvent	Detection limit (pmol/spot)	Detection limit (nmol/1 ml sample)	R_F
Blood serum	Dichloromethane	3	30	0.27
Urine	Methanol	2	0.5	0.96

excess of a visualization solution does not make detection higher. It is important and difficult to spray the plates with finely divided spray solution for optimum staining of the TLC plates. It is overcome by applying iodine vapour in the proposed procedure. Some examples are shown in Table 1. The detection limits were established at nmol per spot level for each compound.

3.2.2. Influence of iodide ions on detection limits obtained with iodine–azide procedure

The present potassium iodide solution in the spraying mixture also adversely affected the detection limits. Hence, the introduced improvements to the method made it more favorable. The hampering effect of the iodide ions in iodine–azide reaction induced by many thiols or organothiophosphorus compounds is intensed with increasing iodide ions concentration [21]. In the previous report, the effect of potassium iodide concentration on detection limits was not examined [19]. According to the Kurzawa's report [22], an increase of potassium iodide concentration makes an increase of iodine consumption into iodine–azide reaction induced with mercaptopyrimidines and an increase of induction time. We investigated the influence of iodide ions on the detection limits obtained with improved iodine–azide procedure (Table 1). We sprayed TLC plates with solution containing proper amounts of potassium iodide (1, 10, 100, 500 mM). When the influence of potassium iodide concentration into a spray solution on detection limits was investigated, the background changes from violet-grey into brown with an increase of potassium iodide concentration. The higher concentration of iodide ions into a spray solution, the more stable background is. In volumetric titration, the presence of iodide ions in a reagent solution slower down the course of iodine–azide reaction [22]. In planar chromatography, the appearance of spots takes more time in the case of iodide ions presence in spray solution compared with their absence in spray solution.

The presence of high iodide ions concentration (which correspond the iodide ions concentration in spray solution applied in non-improved iodine–azide procedure) makes detection limits higher in the cause of the most thiols (see Table 1). Sometimes the small amount of iodide ions in a spray solution in improved iodine–azide procedure improved detection limits (2,4-dimercaptopyrimidine, 2-thioorotic acid, 2-mercapto-3,4,5,6-tetrahydropyrimidine). We chose potassium iodide concentration equal to 1 mM as the optimal one for detection of mercaptopyridines and mercaptopyrimidines series in TLC and HPTLC methods. Because of the difficulty with spraying plates with finely divided spray solution, excess of iodine in sprayed plates and the presence of iodide ions in spray solution, the non-improved procedure cannot be proposed in detection of mercapropyridines and mercaptopyrimidines since relatively high detection limits obtained with the procedure.

4. Conclusion

The discussed results confirm the potential and the beneficial effects of iodine–azide reaction as a detection system in planar chromatography. The proposed detection system allows selective and the most sensitive detection for mercaptopyridines and mercaptopyrimidines at pmol per spot level. The other applied detection methods routinely used in TLC-iodine vapour and UV gave a positive but less sensitive test. Iodine–azide detection system for detection of thiopental in biological samples is simple, with inexpensive, readily available chemicals and with short analysis time. The non-improved methods has not been wildly applied since relatively high detection limits obtained with the procedure.

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