

# Separation of Inosine, Hypoxanthine, and Guanosine by High-Performance Liquid Chromatography on Silica

H. Kažoka

Latvian Institute of Organic Synthesis, 21 Aizkrakles str., LV-1006 Riga, Latvia

## Abstract

The separation of inosine (Ino), hypoxanthine (Hyp), and guanosine (Guo) on silica has been studied. In adsorption normal-phase systems the peak shapes are unsatisfactory; a low selectivity has been observed for the Ino–Hyp and Guo–Hyp pairs. Chromatograms can be significantly improved if systems with a mixed partition–adsorption retention mechanism are applied.

## Introduction

High-performance liquid chromatography (HPLC) has become a widely used chromatographic tool for the separation of complex organic mixtures. A majority of chromatographers practice separations using reversed-phase chromatography (RPC), and this mode has become a popular method for the separation of purine derivatives. However, some samples cannot be easily separated by RPC and must be handled in a different manner. When the separation of inosine (Ino) and guanosine (Guo) by RPC was investigated, a low selectivity (separation factor,  $\alpha$ , < 1.2) for the Guo–Ino pair was observed (1–8).

Normal-phase chromatography (NPC) is a powerful complement to RPC, and it is still of considerable importance (9). Polar organic substances require eluents of high polarity in order to be eluted from unmodified silica. Under these conditions, adsorption is usually the dominating retention mechanism; therefore, peak shapes of polar solutes are usually unsatisfactory. Peak asymmetry ( $A_s$ ) causes a decrease in column efficiency as well as a decrease in resolution and detection limits. In order to improve the peak shape in NPC, researchers rarely use mobile phases containing different additives (i.e., water). An efficient separation method of select purines on unmodified silica with aqueous dichloromethane–methanol (MeOH) mixtures as the mobile phase is known (10–12). The retention process cannot be considered as simple adsorption but rather a very complex mixed process of adsorption and liquid–liquid partitioning into a more polar, water-rich liquid stationary phase, which is developed from the eluent in situ in the column (10). These findings have demonstrated that some nonaqueous mixed partition–adsorption (MPA)

systems are applicable in the analytical practice of Ino (13–14). A mixture of two or three solvents with a limited mutual solubility was used as the mobile phase in MPA systems. Because the investigated homogeneous solutions contain poorly soluble polar components, such as ethylene glycol (EG) and formamide (FA), the dynamic generation of a stationary liquid phase on silica can be expected, even in the mobile phases not saturated with the polar component. If we assume that under conditions of typical adsorption NPC (A-NPC) having a strong enough eluent, such as ethyl acetate (EA), benzene is not adsorbed and its retention volume corresponds to the mobile phase volume ( $V_{m(a)}$ ) or total volume ( $V_{t(a)}$ ) within the column (apart from the silica), then it can be assumed that under A-NPC conditions  $V_{t(a)}$  equals  $V_{m(a)}$ . The formation of the dynamically generated stationary liquid phase in MPA–NPC conditions leads to a decrease in the mobile phase volume ( $V_{m(m)}$ ) and the total volume ( $V_{t(m)}$ ). It can be assumed that under MPA–NPC conditions:

$$V_{t(m)} = V_{m(m)} + V_s \quad \text{Eq. 1}$$

where  $V_s$  is the volume of the stationary phase. Thus accordingly:

$$V_s = V_{r(a)} - V_{r(m)} \quad \text{Eq. 2}$$

where  $V_{r(a)}$  is the retention volume of benzene under A-NPC conditions and  $V_{r(m)}$  is the retention volume of benzene under MPA–NPC conditions. The amount of a stationary liquid phase depends on the saturation of the mobile phase with polar components. If in an MPA mode the phase ratio of the column ( $V_s/V_{m(m)}$ ) reaches 0.1, then the partition retention mechanism dominates over the adsorption retention mechanism and it leads to the improvement of the peak shape of polar solutes (13–16).

The goal of this work was to study the separation of Ino and its typical impurities hypoxanthine (Hyp) and Guo on silica under A-NPC and MPA–NPC conditions.

## Experimental

The chromatographic measurements were performed on a Gilson Model 302 HPLC system equipped with a spectropho-

tometer at  $\lambda = 254$  nm for all of the chromatographic systems under study, except for the mobile phase with FA (which was  $\lambda = 265$  nm). The 150-  $\times$  4.6-mm column was packed with unmodified silica Zorbax Sil (5–6  $\mu$ m) (Du Pont). Mixtures of isopropanol (IPA), MeOH and/or dimethyl sulfoxide (DMSO), EG, FA with hexane (HEX), chloroform (CHCL), EA, or acetonitrile (ACN) were studied as the mobile phases. All of the solvents were purchased from commercial sources (such as Acros Organics and Merck) and were of analytical grade. They were used without any pretreatment. The flow rate was 1.5 mL/min. The samples (25- $\mu$ L injection volume, 0.1-mg/mL sample concentration in the mobile phase) were injected via a Rheodyne 7125 sampling valve.

The column was conditioned before each series of retention measurements. It included flushing with 50 mL of a 50% IPA solution in HEX followed by the mobile phase under study. Usually, 50 mL of the latter eluent was sufficient to obtain constant retention values in A-NPC and MPA-NPC conditions except when the mobile phase contained FA (in this case 150 mL was necessary). The eluents for MPA systems were prepared by slowly

adding the polar component (DMSO, EG, or FA) to the vigorously mixed binary solvent alcohol-HEX or -CHCL and EA in order to ensure a complete homogenization.

The capacity factors of the solutes under study ( $k'$ ), the  $\alpha$  values, and the theoretical plate number ( $N$ ) were calculated according to the usual expressions (17). The system's mobile phase volume ( $V_{m(a)}$  and  $V_{m(m)}$ ) was regarded as equal to the retention volume of benzene. The peak width used for the column efficiency calculation was measured at half height. The peak  $A_s$  was calculated by the determination of the  $A/B$  ratio at 10% peak height (18).

## Results and Discussion

A-NPC systems with silica and binary mobile phases (both solvents completely miscible in all proportions, shown in Table I) were investigated for the separation of Ino, Hyp, and Guo (Table II).

The lower aliphatic alcohols (IPA and MeOH) are the most frequently used as the polar component of the mobile phase for the elution of polar analytes from unmodified silica (9). Tables I and II demonstrate that all systems with alcohols (mobile phases for

N	Mobile phase (v/v)	Ino		Theoretical plate
		$k'$	$\alpha$	
1	IPA-HEX (50:50)	6.0	5.0	200
2	IPA-CHCL (40:60)	2.2	3.0	250
3	IPA-EA (25:75)	2.0	3.0	250
4	IPA-ACN (20:80)	1.6	3.0	250
5	MeOH-CHCL (16:84)	4.0	2.5	300
6	MeOH-EA (16:84)	1.6	3.0	250
7	MeOH-ACN (6:94)	1.7	3.0	250
8	DMSO-CHCL (16:84)	2.5	2.5	300
9	DMSO-EA (10:90)	1.5	3.0	250
10	DMSO-ACN (5:95)	1.6	3.0	250
11	EG-ACN (10:90)	2.0	2.0	500
12	FA-ACN (10:90)	2.0	2.0	600

Mobile phase* for N	$\alpha$		
	Guo-Ino	Ino-Hyp	Guo-Hyp
1	1.1	1.6	1.8
2	1.5	0.7	1.1
3	1.8	0.9	1.2
4	1.5	0.7	1.0
5	1.6	1.1	1.8
6	1.7	1.0	1.7
7	1.8	0.9	1.7
8	2.3	1.5	3.4
9	7.0	1.0	7.0
10	9.4	1.0	9.4
11	1.8	0.7	1.1
12	1.8	0.9	1.6

\* A-NPC systems listed in Table I.

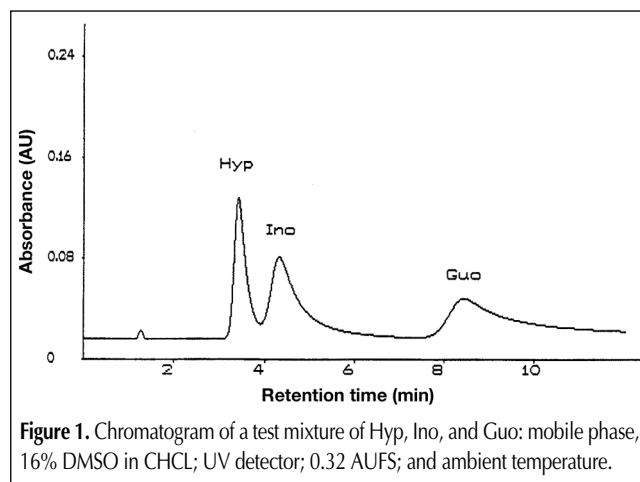


Figure 1. Chromatogram of a test mixture of Hyp, Ino, and Guo: mobile phase, 16% DMSO in CHCL; UV detector; 0.32 AUFS; and ambient temperature.

N	Mobile phase (v/v)	Ino		Theoretical plate
		$k'$	$\alpha$	
1	DMSO-IPA-HEX (13:19:68)	4.8	1.2	4000
2	EG-IPA-HEX (8.5:30:61.5)	6.2	1.2	4000
3	EG-IPA-CHCL (13:6:81)	4.1	1.2	4000
4	EG-MeOH-CHCL (10:2:88)	6.3	1.2	4000
5	EG-IPA-EA (13.5:6:80.5)	7.5	1.1	5500
6	EG-MeOH-EA (12:4:84)	8.0	1.1	5500
7	FA-IPA-HEX (7:40:53)	13	1.2	4000
8	FA-IPA-CHCL (15:20:65)	6.4	1.3	4000
9	FA-MeOH-CHCL (10:10:80)	7.4	1.3	4000
10	FA-IPA-EA (16:3:81)	10	1.1	5500
11	FA-MeOH-EA (15:1.5:83.5)	7.5	1.1	5500

N 1–7) were applicable for the analysis of the solutes under study. At the same time, an unsatisfactory peak shape (As factor > 2.5) and column plate number (< 300) were observed for Ino. As previously shown, the main disadvantage of RPC is a low selectivity for the Guo–Ino pair. Under A–NPC conditions poor separation for the Guo–Ino pair was observed only in a chromatographic system having a mobile phase of IPA–HEX (mobile phase for N 1). However, the main problem for A–NPC systems was a low selectivity ( $\alpha < 1.2$ ) for the Ino–Hyp (mobile phases for N 3 and 5–7) and Guo–Hyp pair (mobile phases for N 2–4).

It is known that DMSO (19,20), FA (21), and EG (22) are capable of moderating a silica surface and increasing the selectivity for various functional classes. DMSO is completely miscible with all of the less polar components of the mobile phase under study except HEX. The substitution of alcohols by DMSO in A–NPC conditions (mobile phases N 8–10; Table I) resulted in a strong retention of Guo ( $\alpha > 2.3$  for the Guo–Ino and Guo–Hyp pair; Table II). However, only the chromatographic system N 8 (DMSO–CHCL) had a reasonable separation for all of the solutes under study. It is known (24) that for good quantitative analysis an As factor of less than 1.5 is preferred. Unfortunately, an unsatisfactory peak shape was observed for Ino and especially for Guo (Figure 1). EG and FA were either immiscible or limitedly miscible with all of the less

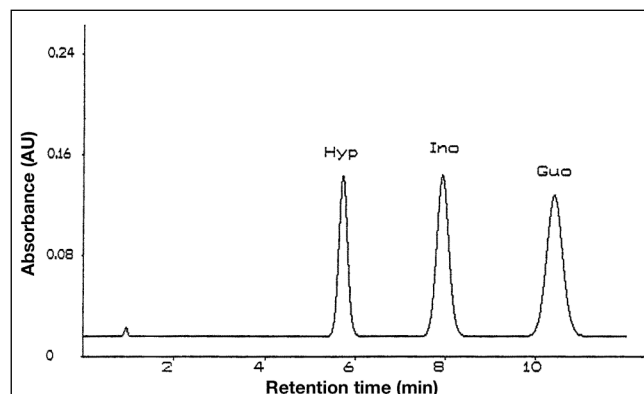
polar components of the mobile phase under study except ACN. The chromatographic systems N 11–12 in Table I showed a relatively high measurement for Ino for the column efficiency under A–NPC conditions (N > 500). However, system N 11 (EG–ACN) unfortunately had a poor selectivity for the Guo–Hyp pair ( $\alpha$  value only 1.1), and the chromatographic system N 12 (FA–ACN) had a poor selectivity for the Ino–Hyp pair ( $\alpha$  value only 0.9).

MPA–NPC systems with silica and ternary mobile phases (shown in Table III) were also investigated for the separation of the solutes under study (Table IV). A ternary mobile phase consists of an alcohol (completely miscible with all solvents in the mobile phase) and two solvents with limited mutual solubility. For the creation of MPA–NPC conditions (13–16) with a high column efficiency and good peak shape, the composition of the mobile phase was chosen to reach approximately 70% saturation with the polar component (DMSO, EG, or FA). It can be seen (Table III) that all of the MPA systems under study revealed high column efficiency (N > 4000) and good peak symmetry for Ino (As factor < 1.3). Data in Table IV showed that for the Guo–Ino pair the  $\alpha$  value is less than 1.4, but also among eleven MPA systems only two systems (N1 and 7) possessed an  $\alpha$  value of less than 1.2. The separation of the Ino–Hyp pair and the Guo–Hyp pair was good ( $\alpha > 1.3$ ) in all of the MPA systems. According to Figures 1 and 2 the separation was much better under MPA–NPC (As factor < 1.1) than A–NPC conditions (As factor > 2.5).

**Table IV. Separation Factor for the Solute Studied Under MPA–NPC Conditions**

Mobile phase* for N	$\alpha$		
	Guo–Ino	Ino–Hyp	Guo–Hyp
1	1.1	1.4	1.4
2	1.2	1.6	1.8
3	1.3	1.4	1.9
4	1.3	1.3	1.7
5	1.4	1.5	2.0
6	1.4	1.5	2.2
7	1.0	2.3	2.3
8	1.4	2.5	3.3
9	1.4	2.2	3.1
10	1.4	2.4	3.4
11	1.3	2.2	2.9

\* MPA–NPC systems listed in Table III.



**Figure 2.** Chromatogram of a test mixture of Hyp, Ino, and Guo: mobile phase, 13.5% EG and 6% IPA in EA; UV detector; 0.32 AUFS; ambient temperature.

## Conclusion

In A–NPC systems the peak shapes of the solutes under study were unsatisfactory. A low selectivity was observed for the Ino–Hyp and Guo–Hyp pairs. The substitution of the adsorption retention mechanism (A–NPC conditions) for the MPA retention mechanism (MPA–NPC conditions) resulted in a specific selectivity and good peak symmetry.

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