Journal of Chromatography, 552 (1991) 429–438
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2080

Possible role for stationary phase metal interactions in the chromatography of hydroxyamines on silica

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ABSTRACT

Problems associated with the non-elution or peak tailing of dihydroxyamines on silica with methanol–aqueous buffer mobile phases have been investigated. The problem is confined to molecules where the hydroxy groups are phenolic in nature and ortho to one another. Study of a controlled set of compounds shows that non-elution/elution with poor peak shape is a function of the distance between the dihydroxy and amine moieties. In terms of the stationary phase, the problem appears to be related to the metal content of the silica. Demineralisation of the silica by acid washing was ineffective, however, flushing the column with EDTA resulted in elution of all dihydroxyamines with improved peak shape.

INTRODUCTION

The chromatography of basic compounds on silica with buffered aqueous methanol eluents is usually associated with good efficiency and peak symmetry [1,2]. In a previous study [3] investigating the chromatographic characteristics of phenethylamine analogues, problems were encountered with certain compounds which gave assymmetric peaks (*e.g.*, adrenaline) or failed to elute (*e.g.*, noradrenaline). With a single exception these compounds possessed an *ortho* dihydroxy function in the aromatic ring. Such compounds are important biochemically, *e.g.*, catechol amines, and as drug metabolites.

The purpose of this present work was the elucidation of the mechanism of the secondary interaction responsible for the non-elution or poor symmetry. In so doing, it was hoped that the poor chromatographic characteristics of these compounds could be improved, thus further extending the applicability of this approach in drug analysis.

EXPERIMENTAL

Chromatography was carried out using a Water 6000A high-performance liquid chromatography (HPLC) pump which was used to deliver eluent at 1 ml/min to various columns (see below). Injections were made with a Rheodyne 7125 injection valve fitted with a 20-μl sample loop. Detection was carried out at 254 nm using an LDC III fixed-wavelength (254 nm) detector.

The eluent consisted of methanol–aqueous ammonium acetate buffer (9:1, v/v), pH 9.1, prepared as previously described [2]. This was also used with the addition of pentane-2,4-dione (Aldrich) (1% v/v) or ethylenediaminetetraacetic acid (EDTA, BDH) (2 to 10 m*M*).

Test compounds were obtained from a variety of commercial sources or from the ICI chemical collection. These were prepared in methanol at *ca.* 2 mg/ml, and 1–5 μl injected. The dead volume was determined with diphenylamine.

Columns were stainless-steel, 100 × 4.6 mm I.D., slurry packed in-house with different batches of Spherisorb S5W silica (5 μm). Similar columns, packed with Kromasil (100 A-SIL) 5-μm silica and YMC (A-001, 120A) 5-μm silica were obtained from Hichrom (Reading, U.K.). A glass-lined column (100 × 3 mm I.D.) with metal free frits was packed by Scientific Glass Engineering.

RESULTS AND DISCUSSION

The origin of secondary interactions

Initially, a wide range of non-phenethylamine compound types were examined. These compounds which possess significant structural dissimilarity to the dopamine type compounds studied previously [3], are shown in Fig. 1. As well as containing an amine function, the compounds contained a mixture of alcoholic, phenolic and alkoxy functions *ortho* to one another in a ring system.

Of this group, only one compound, 3,4-dihydroxyphenylpropylamine (A) was not eluted and a further two, apomorphine (C) and 3,4-dihydroxytamoxifen (D) were eluted with poor peak shape, asymmetry ($A_s > 8$). The remainder were eluted with high efficiency and good peak symmetry ($A_s < 1.2$). From these data, it would appear that elution problems only occur when the two oxygen atoms are phenolic in nature. The presence of *ortho*-dialcohols, *ortho*-dialkoxy or mixed *ortho*-phenolic alkoxy compounds, had no deliterious effect on chromatographic performance.

ortho-Diphenols are well known metal chelators [4], and these results suggest some form of secondary interaction with metals in the chromatographic system.

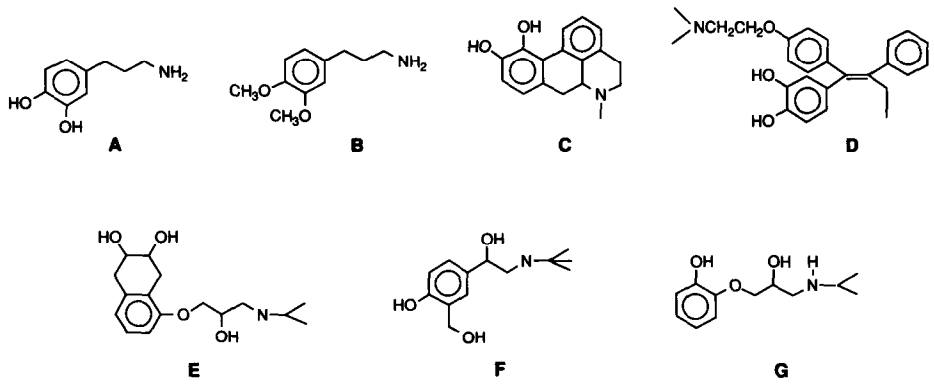


Fig. 1. Structures of various solutes studied, A = 3,4-dihydroxyphenylpropylamine, B = 3,4-dimethoxyphenylpropylamine, C = apomorphine, D = 3,4-dihydroxytamoxifen, E = nadalol, F = salbutamol and G = ICI 46040.

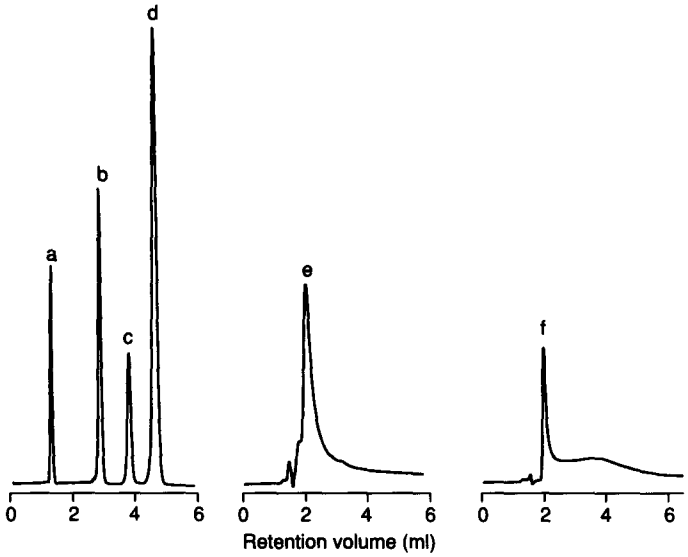


Fig. 2. Chromatograms showing simple alkylamines. a = Diphenylamine, b = benzylamine, c = phenethylamine, d = phenylpropylamine, e = ICI 83378 and f = 6-hydroxydopamine.

However, they do not explain the reason why some compounds elute easily, albeit with poor peak shape (*e.g.*, apomorphine), but other compounds (*e.g.*, 3,4-dihydroxyphenylpropylamine) do not elute at all.

To address this problem, further analysis was carried out using a more controlled set of test compounds, where the length of the amine side chain and the number and position of the ring hydroxyls was systematically varied. The structure and retention characteristics of these compounds are shown in Table I and some typical chromatograms for simple solutes and ring hydroxylated amines which exhibit poor peak shape are shown in Fig. 2.

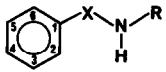
As expected, the compounds with the *ortho* ring hydroxyls failed to elute, or eluted with poor symmetry. The compound with the tailing peak also showed variable retention, k' increasing with decreasing mass injected and also a non-linear peak height response with mass injected. These phenomena, which were also observed with the compounds mentioned earlier, are common where more than one retention mechanism operates.

Analysis of this data indicated that the compounds which failed to elute completely (**2**, **5**, **6**, **9** and **12**) could be distinguished through having a small chain (three atoms or less) between the phenyl ring and the amino function. The only 3,4-dihydroxy compound which did elute, although with poor peak shape, was compound **14** which had a longer side chain with four atoms between the ring and the amine function. It is unlikely that the N-isopropyl or the side chain hydroxyl functions in this compound played any role, as both these groups were present in compound **9**, and the N-isopropyl group was also present in compound **6**, both of which did not elute. It would appear therefore that it is the close proximity of the amine and dihydroxy function which is responsible for the non-elution of compounds **2**, **5**, **6**, **9** and **12**, as well those such as adrenaline in the previous study [3].

TABLE I

STRUCTURES AND RETENTION DATA FOR VARIOUS RING SUBSTITUTED PHENYLALKYLAMINES

Column: 100 × 4.6 mm I.D. Spherisorb S5W silica batch F5387; eluent: methanol–ammonium acetate buffer (9:1).



No.	Compound	k'[a]	3	4	5	6	*X*	*R*
1	Benzylamine	0.96	H	H	H	H	CH_2	H
2	3,4-Dihydroxybenzylamine	NE[b]	OH	OH	H	H	CH_2	H
3	Phenethylamine	1.61	H	H	H	H	$CH_2\ CH_2$	H
4	4-Hydroxyphenethylamine (tyramine)	1.74	H	OH	H	H	$CH_2\ CH_2$	H
5	3,4-Dihydroxyphenethylamine (dopamine)	NE	OH	OH	H	H	$CH_2\ CH_2$	H
6	3,4-Dihydroxynorephedrine	NE	OH	OH	H	H	$CH(OH)CH(CH_3)$	H
7	5-Hydroxydopamine	3.9[c]	OH	OH	OH	H	$CH_2\ CH_2$	H
8	6-Hydroxydopamine	1.4[c]	OH	OH	H	OH	$CH_2\ CH_2$	H
9	ICI 46399	NE	OH	OH	H	H	$CH(OH)\ CH_2$	$CH(CH_3)_2$
10	Phenylpropylamine	2.19	H	H	H	H	$CH_2\ CH_2\ CH_2$	H
11	3,4-Dimethoxyphenylpropylamine	3.52	OCH_3	OCH_3	H	H	$CH_2\ CH_2\ CH_2$	H
12	3,4-Dihydroxyphenylpropylamine	NE	OH	OH	H	H	$CH_2\ CH_2\ CH_2$	H
13	ICI 45849	0.91	H	H	H	H	$OCH_2CH(OH)CH_2$	$CH(CH_3)_2$
14	ICI 83378	0.42[d]	OH	OH	H	H	$OCH_2CH(OH)CH_2$	$CH(CH_3)_2$
15	ICI 101812	0.84	OH	H	OH	H	$OCH_2CH(OH)CH_2$	$CH(CH_3)_2$

[a] k' = Capacity factor.
[b] NE = not eluted.
[c] Approximate value due to poorly defined tailing peak.
[d] Poor peak shape.

It is possible to rationalise these date through postulation of a two-point interaction between the dihydroxyamine and the silica surface. Given that ion-exchange is the major mechanism of retention [5], then the ionised amine function will undergo a charge-charge interaction with the ionised silanol groups on the silica surface. The distance between the amine and the ring hydroxyls then controls the degree of the secondary interaction. When the side chain is short ($\leqslant 3$ atoms) it can be envisaged that the dihydroxy group is brought into close proximity to the silica surface leading to a relatively strong secondary interaction and hence infinite retention. When the side chain is long, however (>3 atoms), the ring hydroxyls are less likely to come into close contact with the surface. In these latter circumstances, the strength of the secondary interaction is reduced and manifested only in poor peak symmetry.

This discrimination between compounds which failed to elute and those which eluted asymmetrically would also explain the characteristics of apomorphine and 3,4-dihydroxytamoxifen discussed earlier, where the rigid structure results in a greater distance between the amine and dihydroxy group than that seen in the simpler compounds.

It is also interesting to note (Table I), the influence of the addition of a third ring hydroxyl. Thus, 5- and 6-hydroxydopamine (trihydroxyphenethylamines) both eluted but gave very distorted peaks, whereas dopamine (3,4-dihydroxyphenethylamine) failed to elute. It would appear, therefore, that the presence of an additional third phenolic hydroxyl can turn a non-eluting compound into one which elutes, albeit with poor peak shape. Since the phenolic groups are acidic, the addition of a third such group to the molecule will increase the overall negative charge of the molecule in the alkaline eluent (pH 9.1). This will therefore increase the electrostatic repulsion between the hydroxy amine and the ionised silanols resulting in shortened retention compared with the dihydroxy compound.

In an attempt to rationalise the elution/non-elution characteristics of the compounds studied, the Functional Group Contribution (FGC) approach was applied. In a previous study [5], using a similar HPLC system, it was shown that retention increments for various substituents (N-alkylation, ring hydroxylation, etc.) on phenylalkylamines are additive. In the same study, the non-basic solute phenol was also shown to have minimal retention ($k' \approx 0.02$). In order to account for the non-elution of the short chain dihydroxyamines, it would be predicted, on the basis of the additivity rules, that catechol (1,2-dihydroxybenzene) despite its non-basisity would have significant retention in this cation-exchange system.

Catechol and other simple hydroxybenzenes were analysed and the results are shown in Table II. As expected, most of the compounds had relatively short retention eluting at or near the void volume. Catechol and pyrogallol, however, the two compounds having *ortho*-dihydroxy groups, showed somewhat greater retention and poor peak shape, with asymmetry values of around 7.

Although catechol has only slightly greater retention than phenol, the effect of the second hydroxyl group is very significant when considered in FGC terms. The FGC value (τ) for the second hydroxyl group is defined as:

$$\tau = \log\left(\frac{k'_s}{k'_p}\right)$$

TABLE II

RETENTION CHARACTERISTICS FOR HYDROXYBENZENE DERIVATIVES

Column: 100 × 4.6 mm I.D. Spherisorb S5W silica batch F5387; eluent: methanol–ammonium acetate buffer (9:1).

Compound	k'	Peak symmetry
Phenol	0.00	good
Catechol (1,2-dihydroxybenzene)	0.11[a]	poor
Resorcinol (1,3-dihydroxybenzene)	0.00	good
Pyrogallol (1,2,3-trihydroxybenzene)	0.75[a]	poor
Phlorglucinol (1,3,5-trihydroxybenzene)	0.00	good

[a] Approximate value.

where k'_s and k'_p refer to the capacity factors for the substituted and parent compounds, respectively, catechol and phenol in this instanse. As k' for phenol is zero, the τ value for a second *ortho*-hydroxyl is infinite. Thus, the FGC approach explains the observed effect in that addition of a second hydroxyl to a monohydroxy compound (*e.g.*, tyramine) would increase retention from k' 1.74 (Table I) to infinity as observed for dopamine.

The results for pyrogallol and phlorglucinol, the two trihydroxybenzenes, however, cannot be rationalised in the same manner.

Elimination of secondary interactions

Trace metal impurities in silica are believed to be the cause of problems in the chromatography of a number of substance such as hop acids [6], and they are apparently the cause of the problem in the case of the catcholamines studied here. Metals in silica can exert their influence either directly, or indirectly through their effects on silanols, which can be made highly acidic and hence very reactive [7]. In the case of the catecholamines a direct interaction is more likely suggesting that the offending metals are situated on the silica surface and hence easily removed.

Various treatment processes have been proposed for the removal of trace impurities, such as acid washing [8,9] or washing with specific metal complexing agents

TABLE III

METAL CONTENT OF SILICA BATCHES USED IN THIS STUDY

All samples Spherisorb S5W from Phase Separations. Results obtained following digestion in HF and analysis by inductively coupled plasma atomic emission spectroscopy.

Batch No.	Element concentration (ppm)							
	Fe	Al	Ca	Ti	Zr	Na	K	Mg
F5387	551	304	117	283	104	6008	<25	37
5068	175	241	57	88	25	5880	<25	30
Acid washed	65	176	23	44	11	318	<25	<10

[6,10]. Initially we investigated acid washing which has been previously shown to reduce the metal content of silica significantly. An acid-washed silica with relatively low metal content (Table III) was obtained from Phase Separations. This material was packed into a glass lined column complete with metal free frits. To further minimise possible interactions with metals other than those in the column, as observed by Trumbore *et al.* [11], PTFE tubing was used for the injection loop, column to valve and column to detector connections. Analysis using this "metal free" system failed to give any improvement in the chromatography of the dihydroxy compounds. These results confirm the view that the offending metals are present in the silica and not in the column tubing or frits as indicated previously [11], and that acid washing, at least in this instance, is ineffective.

An interesting solution to the problem of metal interactions applied by Roberts *et al.* [12], involved inclusion of the analyte or an analyte analogue in the mobile phase to block the active metal sites. It was not possible to use this approach in the present study due to the unstable nature of catechol compounds which undergo rapid oxidation in the basic eluent (pH 9.1). An alternative approach involving EDTA, a well known metal-complexing agent which has also been used very effectively in the demineralisation of silica [10] was evaluated. This compound (in the free acid form) was introduced into the eluent up to a concentration of 10 m*M*, and 500 ml passed through a column packed with the acid-washed material. The column was tested before, and after washing with the EDTA. The post-wash test was carried out with EDTA in the eluent. The test compounds were those listed in Tables I and II. In this instance there was a dramatic effect, with EDTA washing resulting in an improvement

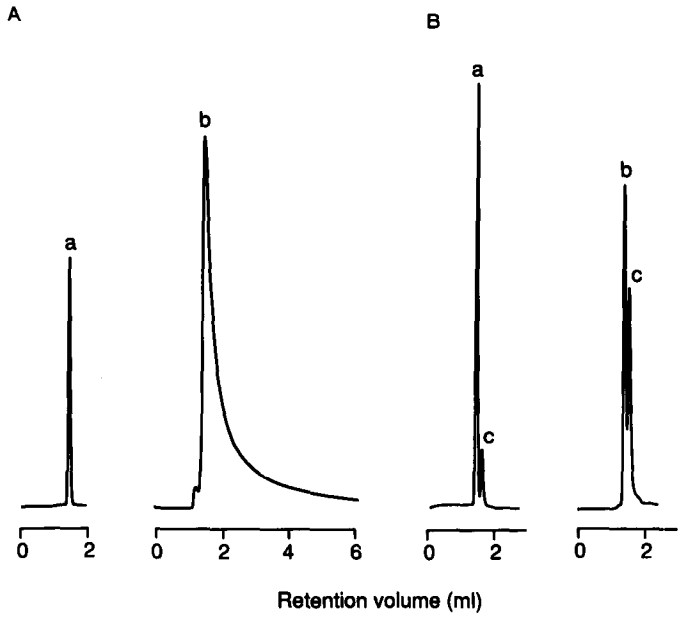


Fig. 3. Chromatograms showing a = phenol, and b = catechol chromatographed on silica (batch F5387) (A) before and (B) after washing with EDTA. Peak c is a system peak due to the inclusion of EDTA in the eluent.

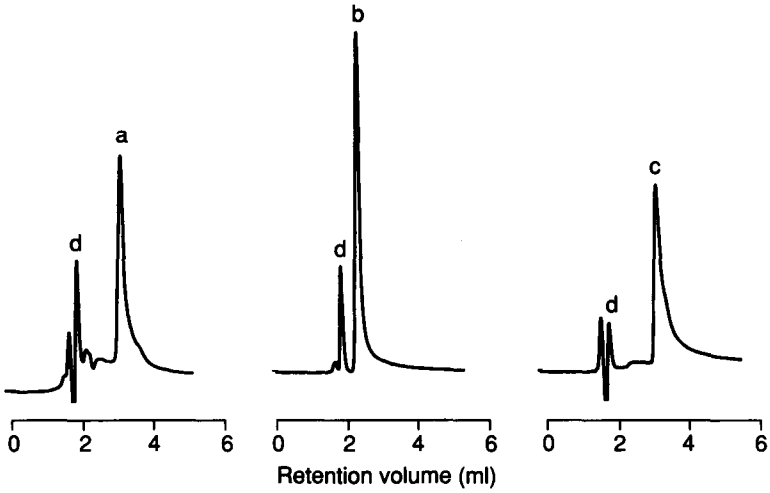


Fig. 4. Chromatograms showing a = ICI 83378, b = 6-hydroxydopamine and c = 3,4-dihydroxybenzylamine on silica (batch F5378) after washing with EDTA. Peak d is a system peak due to the presence of EDTA in the eluent.

in peak shape (Figs. 3 and 4) and elution of all the dihydroxy compounds but with poor symmetry. One side effect of chromatographing with EDTA in the eluent was the reduction in the retention of all the compounds eluted. This reduction increased with k' ranging from 5% for benzylamine (k' *ca.* 0.9) to 56% for 5-hydroxydopamine (k' *ca.* 3.9).

Removal of EDTA from the eluent not only corrected the decrease in retention but the dihydroxy compounds were still eluted or gave the improved peak symmetry seen with EDTA in the eluent.

EDTA is a charged polar species and its observed effect may have been due to factors other than washing of metals from the stationary phase, *i.e.*, ion-pairing with the protonated amine or modification of the silica surface. A second less polar metal chelating agent, namely pentane-2,4-dione used at a concentration of 1% was therefore evaluated. However, because of its high UV absorbance, its effect could only be evaluated after washing of the column unlike EDTA which was actually included in the eluent during chromatography. On a mol-per-mass basis the dione seemed much less efficient in demineralisation of the silica. Typically, chromatographic performance was improved by passing approximately 6 mmol of EDTA or 60 mmol pentane-2,4-dione/1.5 g silica through the column. As expected, there appeared to be a relationship between the total metal content and the mass of chelating agent producing an effect. On the acid-washed silica (Table III), elution of the hydroxyamines such as 3,4-dihydroxybenzylamine was brought about with a total wash of only 1.1 mmol EDTA/1.5 g silica. The more heavily metal loaded silica, *i.e.*, batch F5387 required considerably more EDTA, typically 6 mmol EDTA/1.5 g silica.

It is unclear which of the metal impurities in the silica are responsible for the tailing or non-elution of these dihydroxyamines. Verzele *et al.* [8], determined 19 metals in silica in the ppm range, although it is unlikely that all of these are capable of forming organic complexes. Of those that do, Al and Fe figure highly as impurities in

many chromatographic silicas, and both are capable of forming complexes with EDTA, diketones and *ortho*-diphenols [4].

To further understand this problem, two silicas from different manufacturers were examined; these were Kromasil and YMC silica. The Kromasil silica is claimed to have typical levels of Al and Fe of 20 ppm. The data for YMC silica indicates Al levels to range from 16.8 to 5.4 ppm and Fe levels 18 to 0.5 ppm (data from five batches).

Both Kromasil and YMC silica showed similar selectivity and retention properties to the Spherisorb material, although both columns without any pretreatment eluted all the dihydroxyamines mentioned previously. The YMC column, however, with the lowest metal content, showed peak shape and symmetry comparable to the Spherisorb silica after EDTA washing.

Differentiating between the effects of Al and Fe is difficult since most silicas contain similar quantities (within a factor of 2) of these two elements. Furthermore, acid or EDTA washing appears effective at removing them to the same degree (Table III and refs. 9 and 10).

CONCLUSIONS

These data show that the non-elution or poor peak shape seen with aromatic dihydroxyamines on silica is due to an interaction of the diphenol and metal atoms in the silica matrix. The distance between the amine function and the diphenol appears to control the chromatographic characteristics. A short distance gives non-elution, whilst a large distance results in elution with poor peak shape.

In terms of the stationary phase, the problem appears to be related to the metal content of the silica. Acid washing of the silica although reducing the metal content, was ineffective in improving chromatographic performance. Washing the column with specific metal-chelating agents, *i.e.*, EDTA or pentane-2,4-dione, especially the former, was very effective.

It is unclear which of the metals are causing the secondary interaction, although Al and Fe with their well-known chelation properties, are the likely candidates. From the data generated here, it would appear that chromatographic silicas need to be very pure with much less than 20 ppm of the aforementioned elements in order to give good chromatography with the catechol amines.

ACKNOWLEDGEMENTS

We wish to thank Peter Myers of Phase Separations for the gift of the acid-washed silica and the elemental analysis, and Jane Durham for expert typing.

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