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Short Communication

The reversed-phase high-performance liquid chromatography of arylamine metabolites using mobile phases containing nickel and other divalent metal ions

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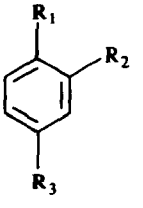
Introduction

Attempts to monitor the metabolism of potentially carcinogenic arylamines are complicated by the large number of metabolites and their closely similar chromatographic properties [1, 2]. Aniline (**1**), for example, is oxidized to phenylhydroxylamine (**2**), which in the presence of bisulphite, HSO_3^-, (present as a 'scavenging nucleophile' to simulate *in vivo* conditions), is converted to *o*- and *p*-aminophenol (**3** and **4** respectively), *o*- and *p*-aminobenzenesulphonate (**5**, **6**), and three oxidation products, nitrosobenzene (**7**), nitrobenzene (**8**) and azoxybenzene (**9**) [2, 3].



	R_1	R_2	R_3
1	NH_2	H	H
2	NHOH	H	H
3	NH_2	OH	H
4	NH_2	H	OH
5	NH_2	SO_3^-	H
6	NH_2	H	SO_3^-
7	N=O	H	H
8	NO_2	H	H
9	PhN=N↑O	H	H

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A brief earlier report [1] described the effects of adding nickel ions (Ni^{2+}) to the mobile phase in high-performance liquid chromatography (HPLC) to manipulate the retention of phenylhydroxylamine (**2**) and *o*-aminophenol (**3**), which were otherwise unseparated. Separation was achieved by virtue of the fact that **3** forms a chelate with Ni^{2+}, whereas **2** does not [1]. Since only certain functionalities with particular orientations bind to specific metal ions, such secondary equilibria can provide unique opportunities to achieve selective separations [4]. The addition of metal ions to the mobile phase has also been employed in the separation of olefins by argentation chromatography [5]; separation of amino acids and sulphonamides [6] using Zn^{2+}, Ni^{2+} or Cu^{2+} salts; and even in the resolution of enantiomeric mixtures of amino acids [7, 8] using an optically active metal chelate in the mobile phase.

This paper presents an investigation of the variables affecting the retention on octadecylsilane bonded silica of arylamine metabolites, including the use and limitations of Ni^{2+}, Hg^{2+} and Zn^{2+} ions in the mobile phase.

Experimental

Apparatus

Chromatography was performed using a Model 6000-A solvent delivery system, Model U-6K loop valve injector and Model 440 dual-channel absorbance detector operated at either 254 or 280 nm (all from Waters Associates, Milford, Mass., U.S.A.). The detector was linked to a Perkin–Elmer Sigma 15 chromatography data station (Palo Alto, CA, U.S.A.). The separation of aniline and related amines utilized a 250 × 4.6 mm i.d. LiChrosorb 10 μm RP18 column (E. Merck, Darmstadt, F.R.G.) operating at 2.0 ml/min with mobile phases of methanol–water or acetonitrile–water, containing 0.26 M ammonium acetate and Ni^{2+}, Hg^{2+} and Zn^{2+} as their diacetate salts. The ionic strength of the mobile phase was adjusted to 0.47 M with sodium perchlorate. After *ca* 30 injections, purging of the chromatographic system with 100% methanol removed condensation products which might otherwise have damaged the column. Glacial acetic acid (0.5 ml per 100 ml water) was added to the mobile phase for the studies on Hg^{2+}, to prevent hydrolysis of the diacetate salt.

Reagents

Reagent grade aniline and nitrobenzene (J. T. Baker, Phillipsburg, NJ, U.S.A.) were purified by vacuum distillation. Practical grade *p*-aminophenol was purified by three successive recrystallizations from ethanol–water. Practical grade *o*-aminophenol (Eastman Kodak, Rochester, NY, U.S.A.) was purified by sublimation. Phenylhydroxylamine was synthesized by reduction of nitrobenzene with zinc and ammonium chloride [9]. Sulphanilic acid (Matheson, Coleman & Bell, East Rutherford, NJ, U.S.A.) and *o*-aminobenzenesulphonic acid (Eastman Organic Chemicals, Rochester, NY, U.S.A.) were recrystallized from water. Reagent grade *o*-methoxyaniline, *o*-phenetidine and *o*-hydroxybenzylamine were used as received from Aldrich Chemical Company (Milwaukee WI, U.S.A.). Deuterium oxide (isotopic purity 99.8%) was obtained from Stohler Isotope Chemicals (Waltham, MA, U.S.A.). Analytical reagent grade nickel acetate (E. Merck, Rahway, NJ, U.S.A.), zinc acetate (Fisher Scientific Co.) and mercuric acetate (Matheson, Coleman & Bell) were used as received. HPLC methanol and acetonitrile were obtained from the Fisher Scientific Co. Sodium perchlorate A.R. grade was obtained from the Aldrich Chemical Co.

Procedures

Retention time measurements were made using 10^{-4} M solutions of the individual analytes freshly prepared in deoxygenated HPLC grade methanol. Injections of 5–10 μl were made for each component. The elution time (t_0) of deuterium oxide was used as a measure of hold-up time for the calculation of the capacity factor, k'. The dual channel absorbance detector was operated at 280 nm for mobile phases containing Hg^{2+} and at 254 nm for mobile phases containing Ni^{2+} and Zn^{2+}. Stock solutions of **2**, **3** and **4** were freshly prepared in deoxygenated methanol and stored in an ice bath.

Results and Discussion

The chromatographic behaviour of the potential products of aerobic oxidation of carcinogenic arylhydroxylamines was evaluated on a reversed-phase column with respect to the concentration of transition metal ions in the mobile phase, mobile phase pH, solvent composition and column temperature.

Mobile phases with added nickel ion

The addition of up to 0.07 M nickel ion to the mobile phase produced quite different effects on retention depending on the analyte, the volume fraction of the organic

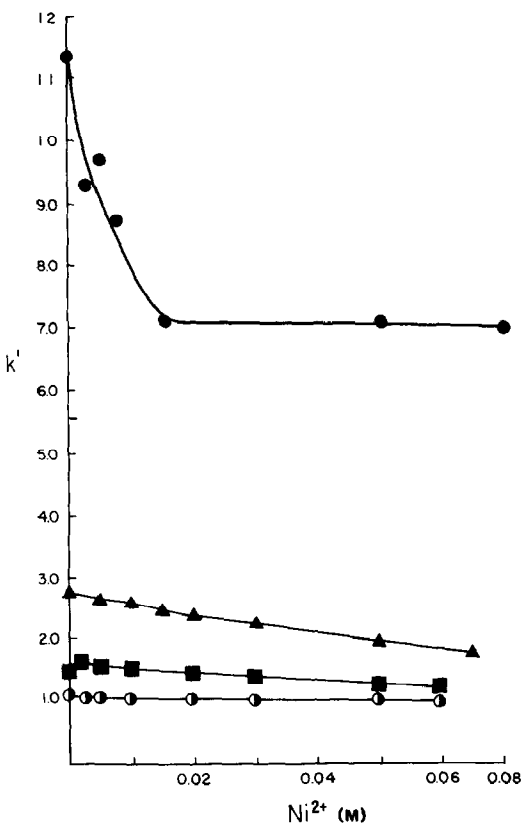
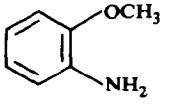


Figure 1
Retention (as capacity factor, k') of *o*-aminophenol on a reversed-phase column, with aqueous eluents containing 0% (●), 15% (▲), 23% (■) and 30% (◑) methanol, determined as a function of nickel acetate concentration in the eluent.

modifier and the pH of the mobile phase. The addition of nickel ion only led to measurable changes in retention with mobile phases containing ≤ 23% methanol (or ≤ 20% acetonitrile), the effect of the metal ion being more pronounced as the fraction of organic modifier decreased. Ionic strength was kept constant in all cases, in order to ensure that any effects observed were attributable to the cation and not to the general influence of salt on retention, as illustrated in Fig. 1. With no organic modifier in the mobile phase, addition of increasing amounts of Ni^{2+} resulted in progressively decreased retention. The slopes of plots of k' vs Ni^{2+} concentration decreased as organic modifier concentration in the mobile phase increased up to 30% MeOH (or 20% acetonitrile). The retention behaviour of *p*-aminophenol, phenylhydroxylamine, aniline and *o*- and *p*-aminobenzene-sulphonate was not affected by Ni^{2+}, even in a pure aqueous mobile phase. Thus, the effect of Ni^{2+} was attributed to chelation by *o*-aminophenol to form a charged five-membered ring complex [10]. Complexation constants for such chelates decrease rapidly as the organic composition of the solvents is increased [11]. This model would account for the loss in effect at higher concentrations of organic modifier. If it is assumed that the other compounds tested cannot form stable complexes with Ni^{2+}, their unaffected retention is explained. To examine further the property of Ni^{2+} complexation to modify retention, the chromatographic behaviour of a series of disubstituted benzenes was investigated. Introduction of a methylene group between the amine and phenyl ring yields *o*-hydroxybenzylamine (**10**), which could interact with Ni^{2+} to form a six-membered ring. The retention of this compound, however, was little affected by Ni^{2+}, even in totally aqueous mobile phases, although it was poorly retained by the column under all the mobile phase conditions studied. This suggests that a five-membered ring chelate (as formed with *o*-aminophenol) may be optimal for stability. In general, substitution at either the amine nitrogen or the phenolic hydroxyl of *o*-aminophenol sterically restricts chelation with Ni^{2+}, as evidenced by the minimal effect of Ni^{2+} on the retention of compounds **11**–**13**.



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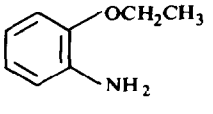

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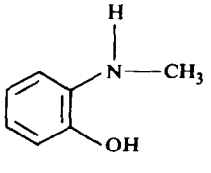

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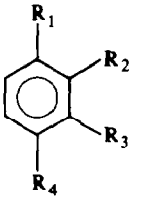
To define further the limitations of Ni^{2+} as a mobile phase modifier, various dihydroxybenzenes, halophenols, haloanilines and aminophenols (listed in Table 1) were examined chromatographically. Catechol, resorcinol and *o*- and *m*-halophenols exhibited no change or a slight increase (≤ 8%) in k' as the Ni^{2+} concentration was increased in a mobile phase of methanol–water (15:85 v/v).

Retention of *o*-haloanilines, however, decreased only slightly (≤ 4%) as Ni^{2+} was added to the mobile phase. The retention of *m*-haloanilines was unaffected by Ni^{2+}, as expected, since these compounds are structurally incapable of chelation with simple metal ions. The k' values for *o*-halophenols and *o*-haloanilines were lower than those for the corresponding meta isomers. The influence of Ni^{2+} on retention is thus most

Table 1
Capacity factors of disubstituted benzenes on LiChrosorb 10 μm RP-18 column using methanol–water (15:85 v/v) mobile phase in the presence (50 mM) and absence of nickel ion.



R_1	R_2	R_3	R_4	k', $[Ni^{2+}] = 0$	k', $[Ni^{2+}] = 50$ mM
HO	HO	H	H	3.6	3.8
HO	H	HO	H	2.6	2.7
HO	F	H	H	11.0	12.2
HO	H	F	H	17.0	17.0
HO	Cl	H	H	30	32
HO	H	Cl	H	52	55
HO	Br	H	H	45	47
HO	H	Br	H	75	81
H_2N	F	H	H	9.9	9.5
H_2N	H	F	H	9.9	9.9
H_2N	Cl	H	H	29.8	28.8
H_2N	H	Cl	H	31.6	31.7
H_2N	Br	H	H	42.0	41.2
H_2N	H	Br	H	45.0	44.8
H_2N	I	H	H	72.0	72.0
H_2N	H	I	H	82.0	82.0
H_2N	OH	H	H	2.7	2.0
H_2N	H	HO	H	1.4	1.4
H_2N	H	H	HO	0.42	0.43

noticeable for *o*-aminophenol, suggesting that, of the solutes examined, this compound forms the only effective complex with Ni^{2+}. These experiments suggest that the incorporation of Ni^{2+} into the mobile phase may be of limited utility in modifying retention behaviour.

The effect of Ni^{2+} on retention is also pH dependent. In the presence of Ni^{2+}, k' increases < 10% with pH, comparable with the response observed in the absence of Ni^{2+}. Similar observations were also made for *o*-anisidine, *o*-phenetidine, phenylhydroxylamine, aniline, and *o*- and *p*-aminobenzenesulphonate. However, *o*-aminophenol exhibits different behaviour (Fig. 2), in that retention increases up to pH 5.8 and then decreases. The increased retention would be expected to result from deprotonation of the anilinium ion to yield the less polar amine [12]. However, as this basic form increases in concentration, it can chelate with Ni^{2+} to form a charged hydrophilic species, which elutes more rapidly. Prior to deprotonation, the nitrogen electron pair is unavailable for interaction with Ni^{2+}. Thus, it is to be expected that maximum chelation should be observed when the amine is in the free base form.

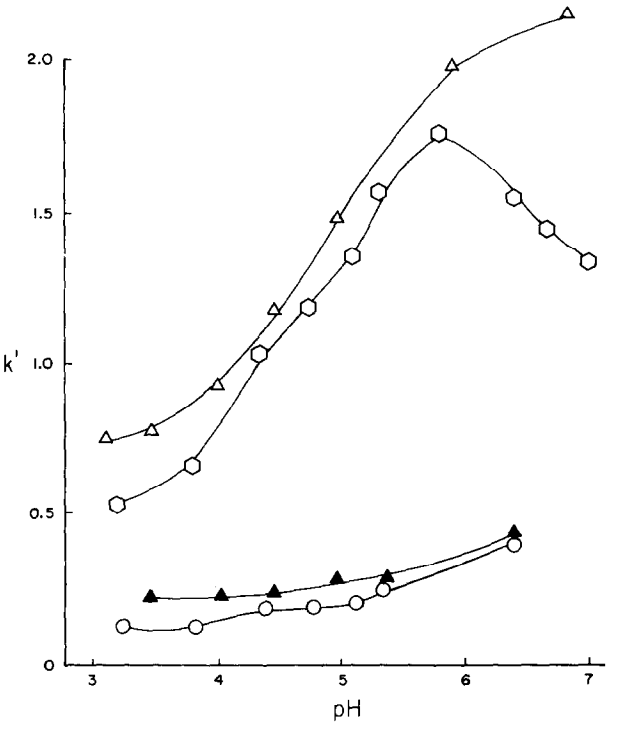


Figure 2
Retention (as capacity factor, k') of *o*- and *p*-aminophenol with methanol–water (15:85 v/v) as eluent in the presence (50 mM) and absence of nickel acetate, determined as a function of pH; the ionic strength remained constant at 0.47 M. *o*-Aminophenol: no Ni^{2+} △, with Ni^{2+} ⬡; *p*-aminophenol: no Ni^{2+} ▲, with Ni^{2+} ○.

Temperature effects

Over the range 20–60°C, no significant difference in the retention of *o*-anisidine, *o*-phenetidine, aniline, phenylhydroxylamine, *p*-aminophenol and *o*- and *p*-aminobenzenesulphonate was observed in the absence or presence of Ni^{2+} (0.02 M) with methanol–water (15:85 v/v) as the mobile phase. Van't Hoff plots of k' vs T^{-1} were linear for all solutes. The enthalpy of transfer ($\triangle H^0$), determined from the slopes of these plots, is shown in Fig. 3. As expected, *o*-aminophenol shows a significant difference in ln ($k'_{313°K}$) measured in the presence and absence of Ni^{2+}. Applying the enthalpy–entropy compensation technique to this data [13–15], all points follow a linear regression (Fig. 3). This suggests that a common retention mechanism could exist which permits specific changes in selectivity due to the presence of Ni^{2+}.

Other cations

Up to 0.47 M zinc ion added to the mobile phase did not significantly affect the retention of any of the solutes investigated, except perhaps *o*-aminophenol (Fig. 4). Mercuric ion also exerted a significant effect on the retention of *o*-aminophenol, k' decreasing from 1.25 in the absence of Hg^{2+} to 0.30 at 0.07 M Hg^{2+}. Moreover, in the presence of Hg^{2+}, the retention of *o*-anisidine, *o*-phenetidine, *o*-hydroxybenzylamine,

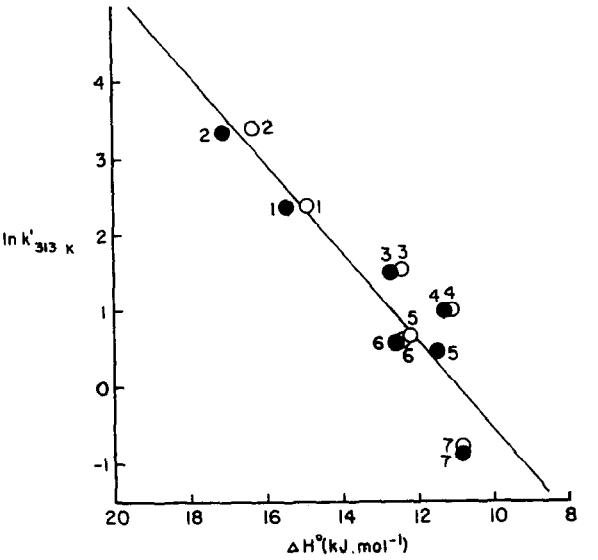


Figure 3
Chromatographic conditions: as described in text, with methanol–water (15:85 v/v) as eluent in the absence (○) and presence (●) of nickel acetate (15 mM).
Enthalpy–entropy compensation plot of ln ($k'_{313°K}$) vs △ H^0 (enthalpy of transfer). 1, *o*-anisidine; 2, *o*-phenetidine; 3, aniline; 4, phenylhydroxylamine; 5, *o*-aminophenol; 6, *o*-aminobenzenesulphonate; 7, *p*-aminophenol.

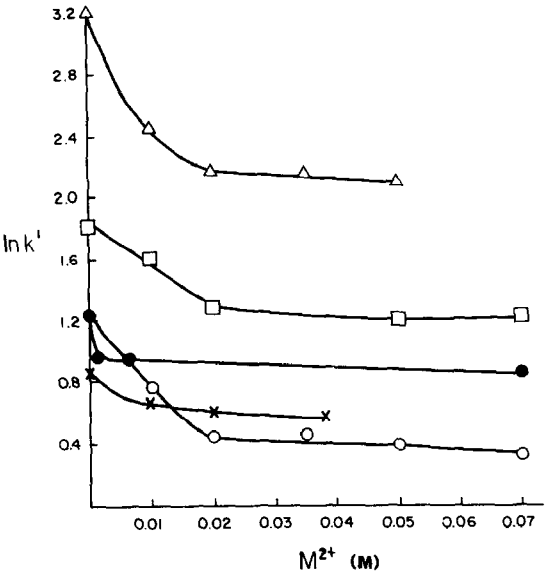


Figure 4
Retention (as ln k') on a reversed-phase column with methanol–water (15:85 v/v) as eluent in the presence of Zn^{2+} or Hg^{2+}. *o*-Aminophenol (Zn^{2+} ●; Hg^{2+} ○); aniline with Hg^{2+} (□); phenetidine with Hg^{2+} (△); *o*-hydroxybenzylamine with Hg^{2+} (×).

aniline, *p*-aminophenol and phenylhydroxylamine also decreased (Fig. 4). The degree to which Hg^{2+} facilitated elution varied, reflecting differences in the extent to which the solutes complex this ion. Mercuric ion appears to be less selective than either Ni^{2+} or Zn^{2+}, even complexing with analytes incapable of chelation (e.g. *p*-aminophenol and aniline). The decrease in retention with increasing concentration of mercuric acetate in dilute acetic acid is unlikely to be attributable to the consequent increase in pH of the mobile phase, since it was shown previously that pH increase led to increased retention.

These studies suggest that the use of Ni^{2+} in the mobile phase to modify retention is limited to unhindered *ortho*-substituted arylamines. It appears that the mercuric ion may offer more general applicability as a retention modifier in reversed-phase chromatography.

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