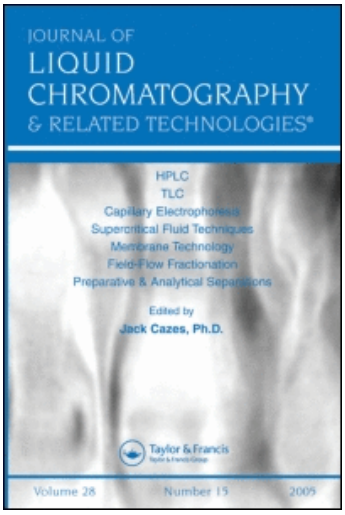

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POTASSIUM TETRAKIS-(1*H*-PYRAZOLYL)-BORATE: A MOBILE PHASE ADDITIVE FOR IMPROVED CHROMATOGRAPHY OF METAL CHELATING ANALYTES

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ABSTRACT

The use of potassium tetrakis(1*H*-pyrazolyl)borate $KB(Pz)_4$ as a mobile phase additive for reverse phase High Performance Liquid Chromatography of metal chelating compounds was investigated. The reason for its superiority over previously employed eluent modifiers is described.

INTRODUCTION

Rationalization of the puzzling retention behaviour of metal chelating substances is a particular challenge to the separation scientist. The development of High Performance Liquid Chromatographic (HPLC) analytical methods for these compounds has often met with difficulty due to band broadening, excessive tailing, and in the worst case, "chair-shaped" peaks.[1]

A number of strategies have been attempted in order to improve the chromatographic peak shape. Several research groups have added EDTA to the mobile phase to prevent complexation of the analytes with metal ions in the chromatographic system,[2-3] but an unfortunate consequence of its use is that EDTA "modifies" the packing material[4] because it is adsorbed by the stationary phase[5] which remains altered in its performance. Indeed, in order to restore the capacity ratio of different analytes, the column must be run for at least 3 h with strongly acidic eluents. The pH compatibility of silica makes this procedure inadvisable for routine analyses. Moreover, the EDTA, according to its ionisation pattern[6-7] and the well-known trends of the conditional formation constants of its metal complexes, is easily predicted to be nearly useless to this end at low pH values, as already reported.[1]

Some other investigators have recommended preequilibration of the column with another member of the analyte group which is able to presaturate the binding between analyte and active sites,[8-9] but the procedures are laborious and very time-consuming; moreover, it is not clear for how long, after preequilibration, active sites remain masked and how the original stationary phase can be restored.

Another approach to solving the problem of tailing is to add an unrelated compound, but, nevertheless, a complexing one, such as picolinic acid,[1] to the mobile phase in order to saturate the specific interaction with the chromatographic system. Such a modified eluent leads to an increase of the baseline noise and, consequently, a decrease of the signal to noise ratio; hence, higher detection limits are expected.

Since Trofimenko introduced the pyrazolylborato ligands in 1966, they are now among the most popular ligands in coordination chemistry.[10-13] Their outstanding complexing properties towards metal ions,[14-16] their much lower basicity in comparison with EDTA,[17] coupled with a wide range of transparency in the absorption spectra, make them highly eligible as mobile phase additives for improved chromatography of analytes which are prone to form metal complexes.

Pyridinedicarboxylic (PDAs) and pyridinecarboxylic (PMAs) acids can be considered model compounds in the chromatographic study of such kind of analytes because the performance of chelating isomers (see Fig. 1 for general structure) is much poorer than that of isomers whose complexing properties are low or non-existent.[18-19] Moreover, the findings reported here can be very useful for further implementing the already studied[20-23] chromatographic separation of PDAs and PMAs themselves.

Since good chromatographic performance is a prerequisite for high sensitivity, experiments designed to improve the peak shape of model metal chelating compounds via inclusion of $KB(Pz)_4$ in the mobile phase are here described.

MATERIALS AND METHODS

A Varian high pressure liquid chromatograph model 5000 equipped with a Rheodyne sample valve injector with 50 mL loop (Model 7125) was used. A Hewlett Packard 8452A diode array spectrophotometer equipped with a 30 μL flow cell (10 mm optical path) and with external computer control (HP 89531A MS-DOS - UV/VIS operating software) was used as detector. The analyses were run at room temperature under isocratic elution conditions. The eluent flow-rate was 0.9 mL/min. The detector was operated at 254 nm.

All experiments were carried out with a commercial stainless steel column (25 cm x 4.6 mm I.D.), packed with 5 mm Res Elut 5 C_{18}, for reverse phase chromatography, purchased from Varian.

All the isomers of pyridinedicarboxylic acid (3,4-, 2,5-, 2,6-, 3,5-, 2,3- and 2,4-PDAs), and pyridinecarboxylic acids (2-, 3-, 4-PMAs), EDTA disodium salt and $NaBH(Pz)_3$ were purchased from Aldrich. Potassium dihydrogen phosphate and disodium monohydrogen phosphate were purchased from Merck.

All chemicals were of the best available quality and used without further purification. Water was produced by a Milli Q 185 system (Millipore). $KB(Pz)_4$ was prepared according to Trofimenko.[24]

The best chromatographic performance was obtained with an aqueous mobile phase containing 0.2 mM $KB(Pz)_4$; the pH was maintained at 7.3 by 153.2 mM phosphate buffer.

All analytes were dissolved in mobile phase to give a final concentration of 0.28 mg /mL. All solutions were filtered through a 0.2 mm pore size cellulose nitrate filter (Whatman).

Prior to use, the reverse phase column was equilibrated with the solvent system to be used in the separation for 30 min. Equilibration was established by obtaining similar results in duplicate runs at a 15 min interval.

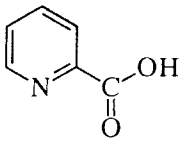

Figure 1. Part structure of heterocyclic acid series of metal chelating analytes.

RESULTS AND DISCUSSION

The observed tailing and asymmetry were specific for analytes featuring carboxylic acids functional group adjacent to heterocyclic, pyridine-type nitrogens, as shown in Figure 1. It was then hypothesised that the complex forming properties were responsible for the poor peak shape which has been reported[1] to be common to a wider range of compounds including tetracyclines: their complexation with metal impurities in the chromatographic system has been claimed[2,3,25] to worsen their chromatographic performance. A slow, reversible chemical reaction between chromatographic migrants has been reported to result in kinetic tailing, even in presence of linear partition isotherms.[26]

An example of the worst peak shape of a typical heterocyclic acid compound from these series, obtained with an otherwise unmodified mobile phase, is shown in Figure 2. The chromatographic peak was very tailed and asymmetric and a severe retention time increase upon dilution was observed.

In order to rule out the influence of residual silanols remaining on the surface of the silica based packing material, up to 8 mM of a good Brönsted base was added to the mobile phase and the pH readjusted to the original value with potassium dihydrogen phosphate 0.5 M. Since no improvement in the peak shape was observed upon addition of triethylamine or diethylamine, it seemed clear that the tailing could not be related to simple analyte-silanols interactions.

Conversely, the asymmetry factor could be progressively reduced in presence of ever larger amounts of etylendiamine, whose metal chelating activity differentiates it from the other two organic bases used. The chance to use it as a mobile phase additive for improving the chromatographic behaviour of metal chelating analytes is limited by the low efficiency of such a bidentate ligand in competing with the analytes for metal ions.

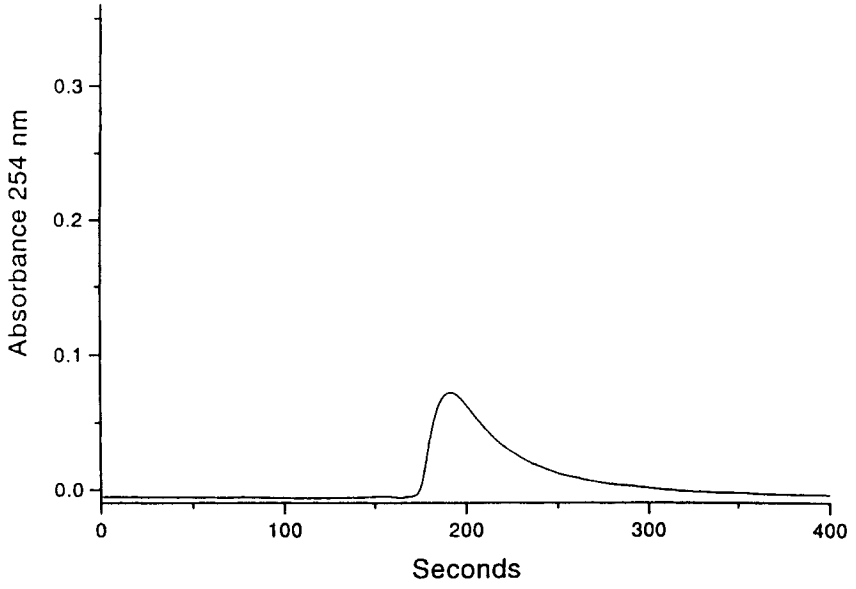

Figure 2. Typical chromatogram obtained for a member of the heterocyclic acid series. Conditions: column, 5 mm Res Elut 5 C_{18} (25 cm x 4.6 mm I.D.); mobile phase: 153.2 mM phosphate buffer, pH 7.3; flow rate 0.9 mL/min at ambient temperature.

The tailing could be reduced by increasing the mobile phase pH, but an alkaline medium is not advisable for routine analysis because it tends to be harmful for silica of the bonded stationary phase base. The observed improvement of peak shape can be explained by the fact that the increased hydroxide ion concentration can promote the formation of hydroxocomplexes and reduce the effective concentration of metal ions in the eluent. This results in a decrease of the negative influence exerted by metal ions on the peak shape of metal complexing analytes.

As the pH increases, a decrease of the capacity factor was observed and this can be accounted for by considering that the more free metal impurities are present, the more retention increases and vice versa.[25]

We tested EDTA as a mobile phase additive. The efficiency of 2 mM EDTA modified mobile phase compares to the 0.2 mM $KB(Pz)_4$ one only at high pH. Furthermore, it alters the stationary phase in such a way that the capacity factors of related and non related compounds can be restored only by

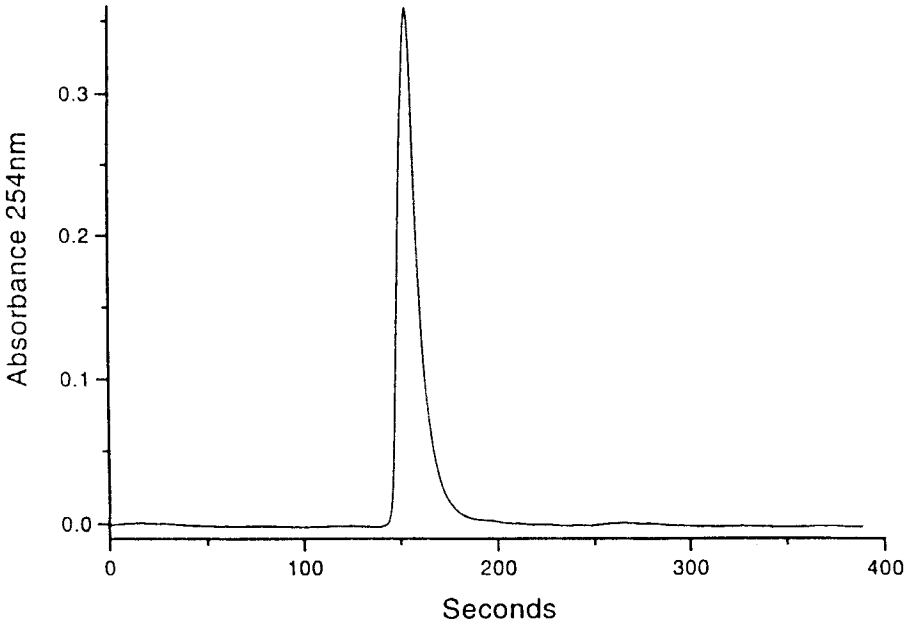

Figure 3. As Fig. 2 except addition of 0.2 mM $KB(Pz)_4$ to the mobile phase.

eluting the column with HCl (pH 2.4), for at least 3 h, thereby confirming that EDTA was adsorbed by the silica base of the packing material: this procedure is not advisable for routine analyses because silica based packing materials are unstable at pH < 2.5.

In order to saturate the binding between analytes and metal impurities we added $KB(Pz)_4$ 0.2 mM to the mobile phase. This resulted in a dramatic improvement of the peak shape (Fig. 3) without a corresponding increase of the baseline noise provided by the eluent, because the mobile phase modifier is almost transparent in the UV region (the cut-off wavelength of such a mobile phase being 212 nm). The retention time increase upon dilution could not be observed anymore thus enabling, together with more than satisfactory peak shape, trace analysis of the compound of interest at the ppm level.

It is very unlikely that the observed improvements in the chromatographic performance of isomeric metal chelating analytes are due to the action of $B(Pz)_4^-$ as an ion-pair reagent, as analytes are anions or dianions themselves at the mobile phase pH selected.[27-29] Instead, it is very probable that the

mechanism by which $KB(Pz)_4$ causes the observed improvement in peak shape is that it saturates dinamically generated active sites on the stationary phase, since its effectiveness progressively increased and reached the steady-state only after *ca* 80 column void volumes were eluted.

$KB(Pz)_4$ proved to be much more efficient than $NaBH(Pz)_3$ in reducing peak asymmetry. In order to obtain an improvement in peak shape similar to that one of 0.2 mM $KB(Pz)_4$, 7 mM $NaBH(Pz)_3$ had to be added to the same mobile phase (pH 7.3). Since $BH(Pz)_3^-$ is a much better complex forming agent than $B(Pz)_4^-$[17,30] and a major difference between the two modifiers is the higher lipophylicity of the latter, it follows that it subtracts metal impurities from analyte equilibria better than $BH(Pz)_3^-$ because the complexes it forms are better retained by the stationary phase.

Such hypothesis underscores the importance of having no complexation equilibria to compete with the chromatographic one.

In a mobile phase at a pH of 2.5 $KB(Pz)_4$ 1 mM was able to reduce the AF_{10} of the analytes by at least 60%, thereby indicating that even at the lowest pH that can be reached, taking into account the pH compatibility of silica, this mobile phase additive is able to prevent interaction between analytes and metal ions by complexing the latter.

The slight decrease in retention that was observed when $KB(Pz)_4$ was included in the mobile phase was also observed for EDTA (tested by the present Authors) and picolinic acid[1] modified mobile phases, and can be explained by taking into account ion-exclusion phenomena on the stationary phase modified by the presence of the negatively charged adsorbed additive. In order to improve analyte retention, $KB(Pz)_4$ was also successfully used in presence of an ion-pairing agent.

While EDTA could be displaced from the stationary phase only by eluting the column with a harmful very acidic mobile phase, the displacement of $KB(Pz)_4$ can be obtained by running the column with a mixture MeOH:H_2O (35:65 vol/vol) for half an hour at a flow rate of 0.9 mL/min: this procedure makes the use of $KB(Pz)_4$ safe for column life.

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