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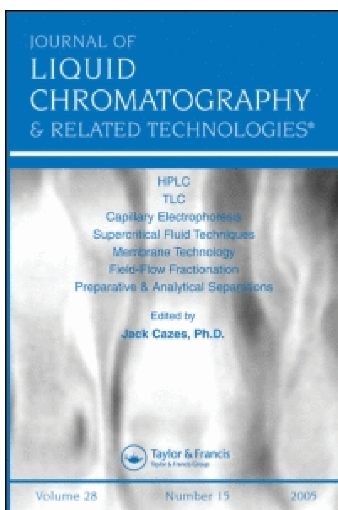

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SEPARATION OF ALL ISOMERS OF PYRIDINEDICARBOXYLIC ACIDS BY ION-PAIRING CHROMATOGRAPHY

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ABSTRACT

The separation and quantitation of the six isomers of pyridinedicarboxylic acids (PDAs) can be achieved rapidly utilizing High Pressure Liquid Chromatography (HPLC). Optimal separation is accomplished using reverse phase chromatography with an aqueous mobile phase mantained at a pH of 7.3 by 153.2 mM phosphate buffer, containing 15 mM tetrabutylammonium phosphate as ion-pairing agent and 2 mM EDTA as mobile phase additive.

The influence of the eluent parameters on retention of PDAs has been investigated in order to elucidate the separation mechanisms involved in the ion pair chromatography of these ionizable substances.

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INTRODUCTION

The pyridine derivatives, whose separation is described in this paper, are very important in biochemistry. The six isomers (2,3-; 2,4-; 2,5-; 2,6-; 3,4-; 3,5-PDAs) have been studied as a class of compounds in order to investigate their role in the neurotransmission process as triggers of burst firing,[1] and in altered physical state of erythrocyte membrane proteins responsible for hippocampal neurotoxicity;[2] 2,5- and 2,3-PDA's are reported to have occurred as a result of the putrefaction of human viscera.[3]

Moreover, 2,3-; 2,5-; 2,6-; 3,5-PDA's are important compounds obtained by oxidative degradation of humic acids derived from coal.[4]

Earlier analytical work mostly regarded quinolinic acid (2,3-PDA), whose presence in biological samples was determined in order to monitor altered aromatic acids metabolism. The determination of quinolinic acid has been carried out by gas chromatography,[5,6] gas chromatography-mass spectrometry,[7,8] ion-exchange liquid chromatography,[9] liquid chromatography with fluorometric detection,[10] thin layer chromatography,[11] and radioenzymatic assay.[12] 2,6-PDA has been characterized as a fungal metabolite by HPLC.[13]

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Previous attempts to separate some of the isomers relied on the use of ion-exchange chromatography on anionic resins[4] and on the use of HPLC with an amino stationary phase,[14] but no attempts to separate all the isomers have ever been made.

Here, a simple and rapid separation procedure, based on ion-pairing in reverse phase HPLC with a buffered isocratic elution is reported.

The chromatographic behaviour of PDA isomers was studied in order to elucidate their separation mechanism.

MATERIALS AND METHOD

A 1090 series II Hewlett Packard high pressure liquid chromatograph equipped with a Rheodyne sample valve injector with 25 mL loop (Model 7125) was used.

The analyses were run at room temperature under isocratic elution condition. 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 μm Res Elut 5 C_{18}, for reverse phase chromatography, purchased from Varian.

A Wescan conductivity detector (Model 213-505) monitored the eluent conductivity.

All the isomers of pyridinedicarboxylic acid, uracil, EDTA disodium salt, tetraethyl ammonium bromide, and tetrabutylammonium phosphate 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).

The best chromatographic performance was obtained with an aqueous mobile phase containing 15 mM tetrabutylammonium phosphate and 2 mM EDTA; the pH was manteined at 7.3 by 153.2 mM phosphate buffer. Eluent parameters were varied and the k' values of PDA's in each system determined (uracil retention time was used as column hold-up time).

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All isomers were dissolved in mobile phase at a final concentration of 0.20 μg/μl each for 3,4-; 2,5-; 2,6-; 3,5-PDA's, 0.25 μg/μl for 2,3-PDA and 0.30 μg/μl for 2,4-PDA.

All solutions were filtered through a 0.2 μm 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.

RESULTS AND DISCUSSION

Preliminary attempts to separate PDA isomers by reverse phase ion pair chromatography, without EDTA as mobile phase additive, met with difficulty due to the poor peak shape of some isomers. The chromatographic peak of 2,4-PDA was the most tailed and asymmetric ($AF_{10} = 10$). A severe retention time increase upon dilution was observed for this isomer, so that simultaneous quantitation of all the PDAs was impossible. The tailing bettered with increasing medium basicity but was still very unsatisfactory at pH 7.3. The pH of the buffered mobile phase was chosen to be 7.3 as a compromise between the necessary predominance of the dianionic protolytic

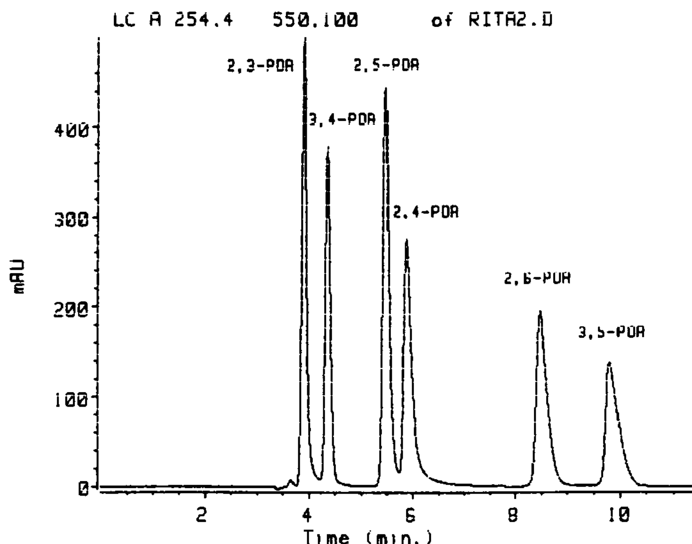

Figure 1. Separation of all PDAs isomers. Condictions: column, 5 µm Res Elut 5 C_{18} (25 cm x 4.6 mm I.D.); mobile phase: 153.2 mM phosphate buffer, pH 7.3 , 15 mM tetrabutylammonium phosphate and 2 mM EDTA.

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species[15-16] and the pH compatibility of silica. A pH higher than 7;3 is not advisable for routine analysis because quaternary ammonium salts in an alkaline medium tend to be harmful for silica of the bonded stationary phase base.

Figure 1 illustrates the excellent separation of the isomers achieveable in the presence of tetrabutylammonium phosphate 15mM and EDTA 2mM: all of the isomers were chromatographed in a matter of minutes. Since some of the PDA's isomers readly form complexes with metal impurities in the chromatographic system, EDTA was included in the mobile phase in order to minimize tailing[17-19] and retention time increase upon dilution. Its addition to the mobile phase improves the partition isotherms parameters and this results in a dramatic improvement of the chromatographic performance.

Mobile phase buffer and tetrabutylammonium concentration were important parameters for optimizing the chromatographic performance; the effect of their variation on capacity factors evidenced the complex nature of ion-pair equilibria.

Table 1

Effect of Ion-Pair Reagent Concentration on the Capacity Ratio of PDAs

	Tetrabutylammonium Concentration		
	15mM	**10mM**	**5mM**
k' 2,3-PDA	0.202	0.168	0.123
k' 3,4-PDA	0.346	0.292	0.227
k' 2,5-PDA	0.687	0.586	0.476
k' 2,4-PDA	0.814	0.679	0.562
k' 2,6-PDA	1.616	1.459	1.226
k' 3,5-PDA	2.029	1.810	1.48

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Table 1 details the dependence of k' at a fixed EDTA (2mM) and buffer concentration (152.2 mM) upon tetrabutylammonium concentration. The regular increase of the capacity factors with tetrabutylammonium concentration points to the formation of ion pairs between analyte dianions and the lipophilic reagent. Since the dependence of k' upon its concentration is not altered by removing EDTA from the mobile phase, it follows that the ion pairing between PDA isomeric ions and tetrabutylammonium is operating with or without EDTA in the mobile phase at a pH of 7.3.

The best selectivity was achieved with the addition of 15 mM tetrabutylammonium phosphate to the mobile phase (Figure 1). Higher concentration of the lipophilic reagent did not appreciably improve chromatographic retention, hence 15mM represents a kind of saturation concentration. The advantage of working at this saturation level is that we have very high capacity factors, which are not very sensitive to small errors in tetrabutylammonium concentration.

Tetrabutyl ammonium was selected as ion pair agent because its high hydrofobicity served to increase retention. When tetrabutyl ammonium was replaced by tetraethyl ammonium, only three peaks could be resolved, thereby indicating that some isomers co-eluted as a result of lower retention due to the lesser lipophilicity of such an ion pairing agent.

Table 2 shows that k' values for PDAs, at fixed tetrabutylammonium (15mM) and EDTA (2mM) concentrations, decrease regularly with increasing buffer concentration. This can be explained by assuming that a stationary phase is being increasingly blocked by ion-pairs from

Table 2

Effect of Phosphate Buffer Concentration on the Capacity Ratio of PDAs

	Phosphate Concentration		
	76.1 mM	152.2 mM	228.3 mM
k' 2,3-PDA	0.323	0.202	0.158
k' 3,4-PDA	0.483	0.346	0.295
k' 2,5-PDA	0.973	0.687	0.614
k' 2,4-PDA	1.018	0.814	0.719
k' 2,6-PDA	2.070	1.616	1.403
k' 3,5-PDA	2,417	2.029	1.855

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tetrabutylammonium and buffer ions. The experimental results can be explained by the physical-chemical retention model proposed by Bidlingmeyer,[20-21] according to which the lipophilic reagent dynamically adsorbs at the surface of stationary phase, forming an electrical double layer. The retention of the sample results from both electrical and Van der Waals forces.

High phosphate concentration limits the formation of ion-pairs between analyte and tetrabutylammonium because buffer ions are involved in a competing equilibrium with the analyte ones for adsorbed lipophilic ions, and this causes a retention time decrease as is seen from Table 2. Adsorption of the lipophilic reagent at the surface of stationary phase is consistent with the long time the eluent conductivity took to decrease when the column was washed with Milli Q water after use: where tetrabutylammonium phosphate was not present in the same mobile phase, the washing time was considerably shorter.

The final parameter investigated was the limit of detectability via absorption of UV light. By calibrating the integrator with known concentrations of the various compounds and obtaining peak height for 2,4-PDA and peak area for all the other isomers it was possible to estimate the ultimate limits of detection. For 2,4-PDA the level of detection was 8.0 ng; for 3,5-PDA this value was 2.5 ng, while for 2,3-; 3,4-; 2,5-; 2,6-PDA it was 1.5 ng.

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