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Effect of mobile phase acidic additives on enantioselectivity for phenylalanine analogs

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Abstract

The use of acidic mobile phase additives allows the chiral separation of underivatized phenylalanine analogs on a common amylosic column. In addition to decreasing retention and band-broadening arising from non-ideal interactions, acidic additives may also increase selectivity. This appears to be due to the minimization of non-selective binding in the recognition site. Effects of the additives are related to additive pK_a and size. Ethanesulfonic acid was typically the most effective additive with trifluoroacetic acid being one of the least effective.

Keywords: Enantiomer separation; Mobile phase composition; Phenylalanine analogues; Acidic additives

1. Introduction

Polysaccharide-based chiral stationary phases have proven to be highly versatile and rugged for a variety of chiral compounds [1–3]. Due to their practicality these columns are available in most laboratories. The chiral separation of underivatized amino acids has been achieved using less common chiral crown ether columns [4], antibiotic based columns [5,6] and by ligand exchange [7]. While viable means to separate underivatized amino acids are available, these analytes pose a difficult challenge to polysaccharide stationary phases. The highly polar acidic and basic portions of the amino acids lead to excessive retention on these phases. Mobile phase additives are often used to minimize peak distortion arising from unwanted interactions between polar solutes and the stationary phase. Perhaps additives could also be used to attenuate the binding of polar analytes to polysaccharide stationary phase, thus widening the application of these columns. Okamoto et al. [8] used acidic additives to elute and resolve carboxylic acids on such column, and also reported the separation of amino-protected amino acids with this approach. Techniques developed to use separate these analytes on polysaccharide stationary phases should be applicable to other charged analytes as well.

We report here a systematic study of acidic mobile phase additives resulting in the enantioseparation of a series of phenylalanine and tyrosine analogs on a ChiralPak AD column. The effect on retention, efficiency, selectivity and resolutions of different acidic additives were examined. The effect of acidic additive concentration is also examined.

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2. Experimental

2.1. Reagents

All reagents used in this study were reagent grade or better. Trifluoroacetic acid (TFA), difluoroacetic acid, dichloroacetic acid, chloroacetic acid, acetic acid, methanesulfonic acid (MSA) and ethanesulfonic acid (ESA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). *n*-Propanesulfonic acid, *n*-butanesulfonic acid were purchased from City Chemicals (West Haven, CT, USA), and used without further purification. The pK_a values of the acidic additives are listed in Table 1. HPLC-grade hexane was purchased from EM Sciences (Gibbstown, NJ, USA). Absolute ethanol was obtained from Aaper Alcohol and Chemical (Shelbyville, KY, USA).

The phenylalanine analogs used in this study are listed in Table 2. All were purchased from Sigma–Aldrich. Separate solutions of racemic mixtures and individual enantiomer of each phenylalanine analog were prepared according to the sample preparation procedures described below at a final concentration about 2 mg/ml. Underivatized amino acids do not dissolve in hexane, ethanol or their mixtures. It proved essential to incorporate acid into the sample diluent. About 20 mg of the phenylalanine analogs is weighed into a 10-ml volumetric flask. This was dissolved in 5 ml ethanol–TFA (9:1, v/v) with 5 min of sonication, and brought to volume with ethanol.

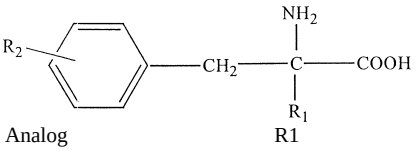
2.2. Chromatography

Chromatographic studies were performed on a HP 1100 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a vacuum degasser, a quaternary pump, an autosampler, a thermostated-column device and a variable-wavelength UV detector. Chromatographic data were acquired and processed with computer-based HP Chemstation software. A ChiralPak AD column (250×4.6 mm) was purchased from Chiral Technologies (Exton, PA, USA) and used as received. Chromatographic studies were performed at 40°C with a 1.0 ml/min flow-rate. The mobile phase consisted of hexane–ethanol (90:10, v/v) containing 0.2% (v/v) of acidic additive. After equilibration, 5-μl injections were made. Detection was achieved at 210 nm. Dead time was estimated from the first solvent disturbance peak.

Table 1
pK_a values for acidic additives

Name	Abbreviation	pK_a value [10]
Methanesulfonic acid	MSA	−1.89
Ethanesulfonic acid	ESA	−1.61
n-Propanesulfonic acid	PSA	−1.62
n-Butanesulfonic acid	BSA	−1.41
Trifluoroacetic acid	TFA	0.67
Difluoroacetic acid	DFA	1.34
Dichloroacetic acid	DCA	1.37
Chloroacetic acid	CA	2.65
Acetic acid	AA	4.79

Table 2
Structures of phenylalanine analogs used in this study



Analog	R1	R2
1	Me	H
2	H	*p*-OCH_3
3	Me	*m*-OH
4	Me	*p*-OH
5	H	H
6	H	*p*-OH
7	H	*o*-OH
8	H	*m*-OH
9	H	*m*-F
10	H	*o*-F
11	H	*p*-F
12	H	*p*-CL
13	H	*p*-Br
14	H	*p*-I
15	H	*p*-NO_2

3. Results and discussion

None of the phenylalanine analogs eluted without acidic additive in the 10% ethanol mobile phase. Elution could be achieved with a 100% ethanol mobile phase, but solute peaks were broad and no enantiomer separation was observed. Fig. 1 shows chromatograms obtained with acetic, trifluoroacetic and ethanesulfonic acids. In addition to being necessary for elution of the phenylalanine analogs, the

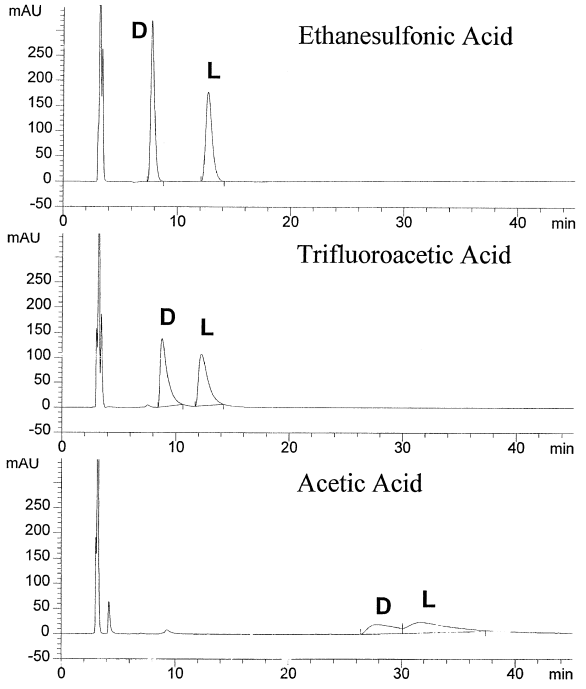


Fig. 1. Chromatograms obtained with different mobile phase composition. Mobile phase was hexane–ethanol (90:10, v/v) containing 0.2% (v/v) of the acidic additives. Flow-rate is 1 ml/min at 40°C with UV detection at 210 nm. The column was a ChiralPak AD.

additives had an unexpected impact on enantioselectivity. Results are summarized in Table 3.

3.1. Effect on retention

All of the analogs eluted within 60 min for the tested acidic additives. The retention factors of phenylalanine analog 15 are plotted as a function of the pK_a of the additive in Fig. 2. This plot is representative of similar plots for all analogs. Retention decreases sharply between acetic acid and dichloroacetic acid. The pK_a values of the analogs used in this study are around 2.2, between acetic acid (pK_a=4.79) and dichloroacetic acid (pK_a=1.37). Further increase of the additive's acidity has a relatively small effect on retention. These data strongly suggest that suppression of the ionization of phenylalanine analogs' carboxylic acid group is the most important mechanism for the retention characteristics of the acidic solutes.

There are two kinks in the ''titration'' curve of Fig. 2. Retention factors increased between dichloroacetic acid and difluoroacetic acid despite a small decrease in pK_a (1.37 to 1.34). Retention also increased between ethanesulfonic acid (pK_a=−1.61) and methanesulfonic acid (pK_a=−1.89). Retention of phenylalanine analogs is not exclusively governed

Table 3
Effects of acidic additives on retention and selectivity of phenylalanine analogs

Analog No.	TFA			DFA			MSA			ESA			CA			DCA			AA		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
1	1.19	1.19	1.00	1.42	1.42	1.00	1.22	1.31	1.07	0.91	0.95	1.04	1.43	1.43	1.00	1.33	1.33	1.00	4.25	4.25	1.00
2	1.90	3.05	1.61	2.21	3.89	1.76	1.94	3.65	1.88	1.41	2.94	2.09	2.22	4.07	1.84	2.13	3.88	1.82	8.11	9.39	1.16
3	1.50	2.19	1.46	1.56	2.41	1.54	1.48	1.90	1.29	1.04	1.65	1.59	1.50	2.37	1.57	1.49	2.35	1.58	4.45	4.85	1.09
4	1.95	1.95	1.00	2.23	2.23	1.00	1.82	1.97	1.08	1.37	1.37	1.00	2.15	2.15	1.00	2.14	2.14	1.00	7.74	7.74	1.00
5	1.45	1.78	1.23	1.81	2.31	1.28	1.50	2.01	1.34	1.06	1.54	1.44	1.75	2.31	1.32	1.69	2.23	1.32	6.35	6.67	1.05
6	2.63	3.85	1.46	2.94	4.77	1.63	2.20	3.67	1.67	1.53	3.10	2.03	2.80	4.77	1.70	2.78	4.70	1.69	6.76	9.42	1.39
7	1.66	1.85	1.12	1.95	2.24	1.15	1.52	1.84	1.21	1.06	1.35	1.28	1.86	2.19	1.17	1.85	2.17	1.17	10.6	10.6	1.00
8	2.37	2.37	1.00	2.71	2.71	1.00	2.05	2.31	1.12	1.58	1.71	1.09	2.62	2.62	1.00	2.58	2.58	1.00	6.80	6.80	1.00
9	1.44	1.65	1.15	1.90	2.27	1.19	1.46	1.83	1.26	1.02	1.36	1.34	1.90	2.29	1.20	1.71	2.10	1.23	4.89	5.11	1.04
10	1.47	1.82	1.24	2.05	2.60	1.27	1.66	2.21	1.33	1.19	1.70	1.42	2.15	2.72	1.27	1.89	2.48	1.32	5.18	5.60	1.08
11	1.45	1.89	1.30	1.94	2.66	1.37	1.46	2.16	1.48	1.01	1.67	1.65	2.06	2.78	1.35	1.72	2.45	1.42	5.20	5.81	1.12
12	1.50	2.01	1.34	2.03	2.90	1.43	1.51	2.35	1.56	1.06	1.87	1.76	2.24	3.12	1.39	1.82	2.72	1.50	5.58	6.34	1.13
13	1.61	2.18	1.36	2.19	3.18	1.45	1.61	2.58	1.60	1.15	2.07	1.81	2.46	3.47	1.41	1.95	2.98	1.53	6.40	7.26	1.14
14	1.76	2.43	1.38	2.41	3.57	1.48	1.76	2.88	1.63	1.28	2.37	1.86	2.75	3.94	1.43	2.15	3.35	1.56	7.43	8.42	1.13
15	3.21	4.27	1.33	4.29	6.14	1.43	2.98	4.69	1.58	2.11	3.71	1.76	4.88	6.73	1.38	3.86	5.73	1.48	16.53	18.30	1.11

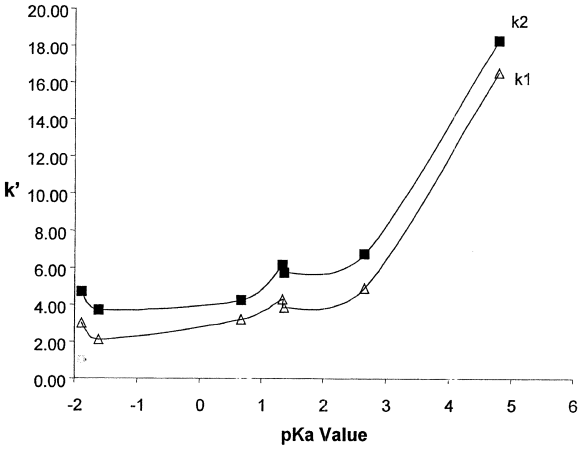


Fig. 2. The effect of additive pK_a on retention for analog 15.

by the acidity of the additives. In addition to ion suppression of residual silanols and solutes' carboxylic acids the additive may also form an ion-pair with the primary amine group of the phenylalanine analogs. *n*-Propane- and *n*-butanesulfonic acids were tested as additives to examine the impact of ion-pair formation on retention behavior. The concentration of alkylsulfonic acid additives was held constant at 25 m*M*. In the series methyl–ethyl–propyl–butylsulfonic acid, the trend in pK_a values would predict an increase in retention. In Fig. 3 it is shown that

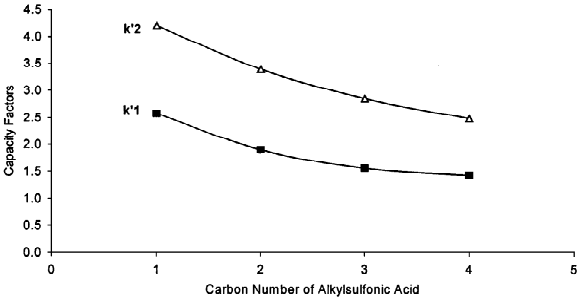


Fig. 3. The effect of chain length of different straight-chain alkylsulfonic acids on retention.

retention of phenylalanine analogs decreased with the increase of carbon number of alkylsulfonic acid. The polarity of the solute ion-pairs will decrease with increasing chain length, thereby decreasing retention.

3.2. Effect on efficiency and resolution

Additives are typically used to enhance column efficiency. An increase in theoretical plates due to the use of various additives as shown in Table 4 along with resulting resolution values. A 10-fold increase in theoretical plates for ethanesulfonic acid relative to acetic acid translated to increase in resolution from partial resolution to values as high as

Table 4
Number of theoretical plates[a] and resolution for phenylalanine analogs

Analog No.	MSA		ESA		TFA		DFA		DCA		CA		AA	
	N	R_s	N	R_s	N	R_s	N	R_s	N	R_s	N	R_s	N	R_s
1	2573	1.00	2683	0.35	1336	0.00	1160	0.00	1146	0.00	1217	0.00	288	0.00
2	2480	5.83	2522	6.24	1126	2.76	1308	3.62	1587	4.08	1659	4.50	217	0.56
3	2929	2.16	3293	3.72	1135	2.00	1672	2.76	2037	3.02	2382	3.38	229	0.38
4	1866	0.61	1421	0.00	820	0.00	842	0.00	980	0.00	1091	0.00	63	0.00
5	2993	2.52	3022	2.81	856	0.94	1051	1.33	1111	1.43	1432	1.71	193	0.25
6	1917	4.16	2004	5.52	466	1.52	694	2.34	1170	3.16	932	2.92	52	0.62
7	2596	1.49	2941	1.79	517	0.50	594	0.61	585	0.65	1059	0.88	121	0.00
8	2207	0.97	2057	0.62	464	0.00	579	0.00	583	0.00	719	0.00	148	0.00
9	2840	1.91	2984	2.12	615	0.58	749	0.83	859	0.97	1966	1.43	206	0.22
10	2913	2.61	2990	2.86	862	1.00	912	1.24	1175	1.50	2059	1.89	192	0.35
11	2854	3.40	2965	3.75	726	1.12	843	1.54	1260	1.83	1795	2.18	223	0.45
12	2909	3.89	2974	4.42	822	1.33	895	1.82	1337	2.27	1507	2.25	233	0.50
13	2883	4.16	2936	4.81	840	1.44	907	1.97	1408	2.55	1366	2.26	206	0.50
14	2877	4.54	2826	5.17	847	1.56	945	2.15	1511	2.89	1369	2.40	210	0.49
15	2640	4.59	2816	5.50	437	1.22	580	1.72	1184	2.64	1404	2.51	299	0.51

[a] N = Number of theoretical plates for the second eluting enantiomer.

6.2. Baseline separations (R_s>1.5) were obtained for 12 of the studied analogs using ethanesulfonic acid. ESA and MSA gave the highest efficiency for all probes but a relationship between efficiency and additive pK_a was not apparent.

3.3. Effect on selectivity

Additives are typically incorporated into chiral mobile phases to sharpen tailing peaks or aid in elution. Table 3 shows that the choice of acidic additives can also affect enantioselectivity. Selectivity for analog 2 ranged from 1.16 to 2.09 depending on additive. No relationship between additive pK_a or chain length and selectivity was apparent. Baseline separation for 12 of the phenylalanine analogs was obtained with ethanesulfonic acid additive (selectivity>1.25). TFA gave lower selectivity for all analogs relative to other acidic additives except for acetic acid.

It has long been recognized that the binding of an analyte to a chiral stationary phase is a combination of chiral and non-selective interactions. Pirkle et al. [9] noted that non-specific retention would attenuate observed enantioselectivity. Schurig and Weber [11] observed that measured enantioselectivity in gas chromatography was confounded by non-specific retention. Measured enantioselectivity is thus an empirical value without chemical meaning. They prepared a blank column to approximate the non-specific retention contributions. Recently, Guiochon's laboratory [12] has distinguished non-specific from enantiospecific interactions. Observed selectivity is a function of true selectivity (k'_2/k'_1) offset by the strength of the non-selective interactions (k'_{ns}):

$$\alpha_{obs} = \frac{[k'_2 + k'_{ns}]}{[k'_1 + k'_{ns}]} \quad (1)$$

A plot of observed selectivity against observed retention ($k'_2 + k'_{ns}$) should yield a curve that approaches 1.0 as k'_{ns} increases. If k'_{ns} could be minimized α_{obs} would approach a maximum of true selectivity at k'_2. Acid additives decrease retention of the phenylalanine analogs and as retention decreases selectivity increases. If these additives act by minimizing non-specific retention a plot of observed selectivity against observed retention ($k'_2 + k'_{ns}$) should yield such a curve. The representative plot in Fig. 4 shows results consistent with this mode of action except for TFA.

3.4. Acidic additive concentration effect

If acidic additives increase selectivity by minimizing non-specific retention it is reasonable to expect that increasing the concentration of additive in the mobile phase should further increase selectivity. Analog 15 was chromatographed with increasing levels of ESA. It was found that retention decreased with increasing additive level between 0.025 and 0.05% but only slight decreases were observed above 0.05% ESA. Selectivity increased between 0.025 and 0.05% ESA but was fairly constant with higher levels of additive. Column efficiency increased up to 0.15% ESA.

The fact that acidic additives effects on efficiency and selectivity show different concentration responses suggests that additives exert their effects on different interactions. Typically additives are viewed as minimizing interactions outside of the chiral binding site such as between analytes and residual silanols of the stationary phase. These interactions are expected to contribute more to band broadening than to overall retention. Once the pK_a of the additive is below that of silanol there should be little

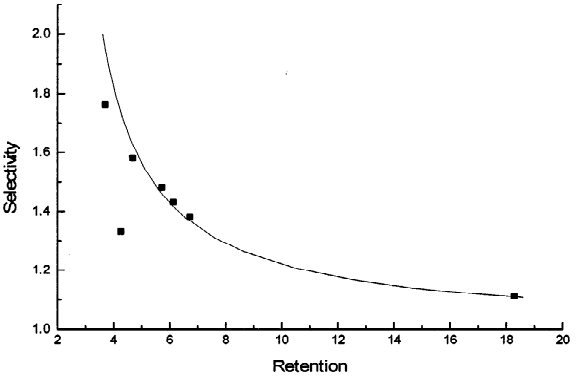


Fig. 4. Effect of retention for selectivity of analog 15. The line represents $\alpha_{obs} = [k'_2 + k'_{ns}]/[k'_1 + k'_{ns}]$ where $k'_2 = 3.6$; $k'_1 = 1.8$ and k'_{ns} varies between 0 and 15. Squares represent results obtained with different additives. The point off the line is for TFA.

relationship between efficiency and additive pK_a as observed.

The non-specific retention of Eq. (1) may also arise from within the chiral recognition site. Chiral recognition is typically represented as arising from three points of interaction. In many cases the interactions are suggested to be two hydrogen bonds plus an interaction between aromatic groups. The least retained enantiomer has two interactions in common with the more retained enantiomer, but experiences a much weaker third interaction. Since two of the interactions are common to both enantiomers, the third interaction is the chirally selective interaction. The common interactions will be the stronger interactions, often the hydrogen bonds. Agents that weaken the common, non-selective interactions relative to the third interaction will decrease overall retention and increase observed selectivity. Acidic additives can be envisioned as attenuating hydrogen bonds involved in chiral recognition of amino acids. The lack of additive concentration effect suggests saturation of this effect at very low levels.

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