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COMPARISON OF THE ENANTIOSELECTIVITY OF β-CYCLODEXTRIN VS. HEPTAKIS-2,3-O-DIMETHYL-β-CYCLODEXTRIN LC STATIONARY PHASES

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ABSTRACT

The enantioselectivity of native β-cyclodextrin and its 2,3-methylated analogue were examined in both the "polar-organic" mode and the reversed phase mode. By comparing these two chiral selectors, the function of the secondary 2,3-hydroxyl groups at the mouth of the cyclodextrin can be examined (in regard to selectivity and retention). In the "polar-organic" mode, compounds that are easily resolved on the native β-cyclodextrin chiral stationary phase (CSP) cannot be resolved on the methylated CSP. These results, as well as the retention data, lend support to the previously proposed "noninclusion" mechanism. In the reversed phase mode, many more compounds could be resolved on the native β-cyclodextrin CSP than on the methylated analogue. However, a few compounds were resolved only on the 2,3-methylated cyclodextrin CSP. In these cases, steric interactions at the mouth of the cyclodextrin cavity may be more important to chiral recognition than hydrogen bonding.

INTRODUCTION

The β-cyclodextrin bonded stationary phase was the first broadly useful, reversed-phase chiral stationary phase (CSP).[1,2] When using aqueous or hydro-organic solvents the enantioselective retention mechanism was shown to involve inclusion complexation along with hydrogen bonding and/or steric interactions at the outer edge of the cyclodextrin cavity.[2-7] Conversely traditional normal phase enantioseparations with native β-cyclodextrin columns are quite rare.

Two very different approaches were utilized in order to extend the utility and selectivity of cyclodextrin-based CSPs. One approach was to use the "polar organic mode" in which the mobile phase consisted mainly of an aprotic polar organic solvent such as acetonitrile.[8-10] Smaller amounts of other nonaqueous additives (e.g., methanol, triethylamine, glacial acetic acid, etc.) were used to regulate retention and analyte charge. Inclusion complex formation was not thought to occur in this mode since the dominant acetonitrile component of the mobile phase occupied the cyclodextrin cavity.[8-10] Retention was thought to be due to hydrogen bonding and dipolar interactions between the analyte and the secondary hydroxyl groups at the mouth of the cyclodextrin cavity.[8-10] Steric interactions could also play a role in chiral recognition in this mode (see Figure 1). Since inclusion complexation does not occur, this mechanism is very different from that which predominates in the reversed-phase mode. This explains why:

1. The β-cyclodextrin enantioselectivity is different in the two modes,

2. non-hydrogen bonding solvents such as acetonitrile are necessary to accentuate retention and selectivity,

3. hydrogen bonding solvents such as methanol and water decrease retention and selectivity, and

4. analytes that are resolved in this mode usually have a minimum of two hydrogen bonding groups (one of them near to the stereogenic center) and an aromatic ring.[8-10]

A completely different way to alter the enantioselectivity of β-cyclodextrin involves derivatizing it with a variety of different groups.[11-14] Early on, both acetyl and hydroxypropyl derivatized cyclodextrin CSPs were shown to produce unique selectivities compared to native β-cyclodextrin.[11,12] These were followed by a variety of aromatic cyclodextrin derivatives and most recently with charged derivatives (e.g., sulfate, etc.).[13-15] The functionalized cyclodextrins greatly expanded the utility and expanded the enantioselectivity of this class of CSPs.

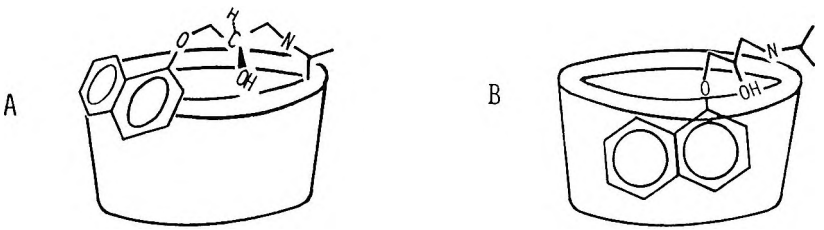


Figure 1. Simplified schematics illustrating two different enantioselective retention mechanisms for the native β-cyclodextrin/propanolol system. Case "A" is the polar-organic mode where acetonitrile occupies the hydrophobic cavity and the analyte is retained via a combination of hydrogen bonding and dipolar interactions at the mouth of the cyclodextrin. Steric interactions also can contribute to chiral recognition.[8,9] In case "B", (the reversed phase mode) retention is mainly due to hydrophobic inclusion complexation, while enantioselectivity also requires hydrogen bonding and steric interactions at the mouth of the cyclodextrin cavity.[1-4]

Subsequently, β-cyclodextrin and its derivatives were utilized as chiral run buffer additives in capillary electrophoresis.[16] One derivative that has been used in CE but not tested extensively in LC are simple methylated cyclodextrins. In this work we synthesized a heptakis-2,3-di-O-methyl-β-cyclodextrin bonded CSP and compared it to the native β-cyclodextrin CSP in both the reversed phase and "polar-organic" LC modes. This exercise was useful for at least three reasons: 1. It provided a stringent test for the proposed enantioselective retention mechanism in the "polar-organic mode", 2. it could help to elucidate the role of hydrogen bonding and steric interactions in the reversed phase mode, and 3. it indicated whether or not the selectivity of this particular derivatized cyclodextrin and its overall usefulness exceeds that of other cyclodextrins or cyclodextrin derivatives that are used as chiral selectors in LC.

EXPERIMENTAL

Materials

The analytes used in this study are given in the tables. Coumafuryl was obtained from Chem Service (West Chester, PA). Idazoxan and derivatives are drugs under investigation at Reckitt & Colman. They were supplied by N. A. Hyde (Danson Lane, Kingston-upon-Hull, UK). The crown ether analogues used were prepared for a previous study.[17] All other compounds were purchased from Sigma

Chemical Co. (St. Louis, MO) and Aldrich Chemical Co. (Milwaukee, WI). HPLC grade water acetonitrile, methanol, triethylamine, and glacial acetic acid were obtained from Fisher Scientific (St. Louis, MO).

The first step in the synthesis of heptakis-2,3-di-O-methyl-β-cyclodextrin was the protection of the primary hydroxy groups of β-CD with *t*-butyl dimethylsilylchloride in pyridine according to a procedure described by Fugedi.[18] The intermediate 6-O-*t*-butyldimethylsilyl β-cyclodextrin was made as described by Dietrich, et al.[19] The removal of the protecting group was achieved by refluxing 6-O-*t*-butyldimethylsilyl-2,3-di-O-methyl-β-cyclodextrin (25g) with ammonium fluoride (16 g) in methanol (250 mL) for 24 hours. The reaction was concentrated and ethyl acetate (150 mL) was added. The mixture was filtered through a pad of silica gel and the solvents were removed by distillation under vacuum. The resulting 2,3-di-O-methyl β-cyclodextrin was used without further purification. Binding of 2,3-di-O-methyled-β-cyclodextrin to epoxysilica was done as described previously.[1]

Methods

The Cyclobond I 2000 columns (25 cm x 4.6 mm i.d.) were obtained from Advanced Separation Technologies, Inc. (Whippany, NJ). All separations were done at room temperature using a Shimadzu model LC-6A solvent delivery module and SPD-6A UV detector.

Rheodyne's model 7125 sample injection valve (Cotati, CA) with a 20-μL loop was used. The flow rate was 1 mL/min. Supporting evidence for chiral separation was supplied by repeating the separation with detection accomplished at different UV wavelengths (e.g., 254 nm, 275 nm, etc.).

The mobile phases of reversed phase mode were mixtures of buffer and methanol or acetonitrile by volume ratios. The aqueous portion of the mobile phase (i.e., the buffer) was made by dissolving the desired amount of pure triethylamine in water and then adding glacial acetic acid to achieve the desired pH. The high water content of the mobile phase required the use of a silica pre-saturator column to saturate the mobile phase with silica in order to extend the life of the silica-based columns. The presaturated column consisted of a 10 cm x 0.46 stainless steel tube packed with 30-40 μm silica and was placed in-line before the injector.

The polar organic mode was also studied. The mobile phase conditions are given as the desired volume ratios acetonitrile/methanol/glacial acetic acid/triethylamine.

RESULTS AND DISCUSSION

Polar-Organic Mode

Native cyclodextrin bonded phase LC columns have been shown to resolve a large number of compounds in the "polar organic mode" even though inclusion complex formation is not believed to occur.[8-10] Table 1 lists typical compounds that have been resolved on β-cyclodextrin CSPs in the "polar organic mode." These include β-blockers, dansyl amino acids and a variety of other biologically active compounds that contain at least two hydrogen bonding groups and an aromatic moiety. However, none of these compounds could be resolved in the "polar organic mode" when using the 2,3-methylated-β-cyclodextrin CSP. They eluted as single peaks and their retention times were generally less than those for the equivalent separation done on native β-cyclodextrin CSPs. Thus far, no racemic compounds have been found that resolve on this methylated-β-cyclodextrin CSP in the "polar-organic-mode." This indicates that methylating the secondary hydroxyl groups at the mouth of the cyclodextrin cavity effectively eliminates chiral recognition and reduces retention for these compounds in the "polar organic mode." Further, this lends support to the proposed enantioselective retention mechanism in which chiral recognition and resolution is due to a combination of hydrogen bonding, dipolar and steric interactions at the mouth of the cyclodextrin cavity (see Figure 1A).

Reversed Phase Mode

Out of hundreds of compounds tested in the reversed phase mode, only a few were found that resolved exclusively on the 2,3-methylated-β-cyclodextrin CSP. These are listed in Table 2. About twice as many compounds could be resolved on both columns (Table 3). However, the greatest number and variety of compounds were found to resolve only on the native β-cyclodextrin CSP (Table 4). A number of interesting facts can be gleaned from this data. However, it would be useful to first briefly review and compare the factors that affect the strength of a cyclodextrin inclusion complex and chiral recognition.[27] (a) First and foremost (when in aqueous solution), organic solutes tend to form a hydrophobic association with the less polar interior of the cyclodextrin cavity (i.e., van der Waals-London dispersion forces). This can occur with both the native and 2,3-methylated β-cyclodextrin. However, the methyl groups at the mouth of the derivatized cyclodextrin would tend to enlarge the cavity somewhat and make the entire cyclodextrin more hydrophobic.[28,29] (b) Hydrogen binding between the guest molecule and the cyclodextrin (particularly the secondary hydroxyls at the mouth of the cavity) tends to enhance the strength of an inclusion complex. Clearly, this important interaction

Table 1

List of Racemic Compounds That Can Easily Be Resolved in the "Polar Organic" Mode on a Native β-Cyclodextrin CSP But Cannot Be Resolved on Its 2,3-O-Methylated Analogue

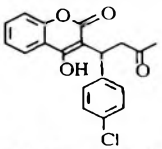
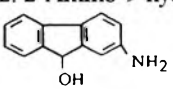
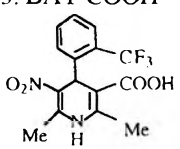
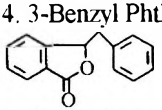
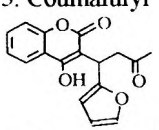
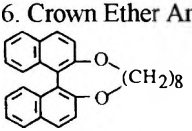
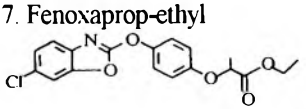
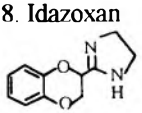
Compound	Reference
1. Alprenolol	8,9
2. Bendroflumethiazide	9
3. Homatropine	9
4. Metoprolol	8,9
5. Nadolol	8,9
6. Pindolol	8,9
7. Proglumide	9
8. Propranolol	8,9
9. Reuelene	9
10. Timolol	8,9
11. Trihexylphenidyl	9
12. Dansyl-D,L-phenylalanine	This work[a], 20
13. Dansyl-D,L-tryptophan	This work[a], 20
14. Dansyl-D,L-norleucine	20
15. Dansyl-D,L-norvaline	20

[a] The mobile phase condition for β-cyclodextrin was acetonitrile:methanol: acetic acid:triethylamine = 98:2:0.8:0.2 (v/v/v/v).

has been eliminated (in the case of the secondary hydroxyl groups) for the 2,3-methylated cyclodextrins. However, there is some possibility of hydrogen bonding with the remaining, less accessible primary hydroxyl groups at the bottom of the cyclodextrin cavity. (c) The release of "high energy water molecules" from the cyclodextrin cavity during complex formation also is beneficial.[27] This can occur for both the native and methylated cyclodextrins, however the number and energy of the water molecules may be different in the two cases. (d) Decreasing the strain energy of the cyclodextrin macrocyclic ring system upon complex formation can be a factor. However, it has been noted that these effects seem to be more important for the smaller α-cyclodextrin. (e) Steric effects (repulsion) tends to decrease the strength of an inclusion complex. These effects can be quite varied. One is an extreme case where the guest molecule is to large to form an inclusion complex. A more common scenario involves inclusion of part of a guest while other parts project out of the cyclodextrin cavity.

Table 2

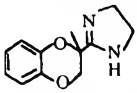
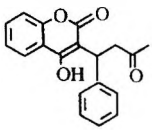
Compounds Resolved Only by 2,3-O-Methylated-β-Cyclodextrin CSP in the Reversed Phase Mode

Compound & Structure	k′[a]	α[b]	Rs[c]	Mobile Phase
1. Coumachlor 	3.06	1.37	3.38	A
2. 2-Amino-9-hydroxyfluorene 	2.40	1.29	2.36	B
3. BAY COOH 	1.06	1.18	1.0	C
4. 3-Benzyl Phthalide 	6.48	1.09	0.9	D
5. Coumafuryl 	3.08	1.20	1.25	E
6. Crown Ether Analogue #19 	1.25	1.30	2.06	F
7. Fenoxaprop-ethyl 	13.1	1.13	1.00	G
8. Idazoxan 	1.11	1.42	1.93	H

(continued)

Table 2 (continued)

Compounds Resolved Only by 2,3-O-Methylated-β-Cyclodextrin CSP in the Reversed Phase Mode

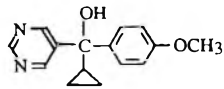
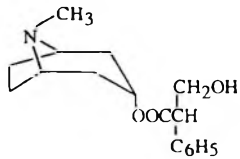
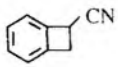
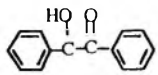
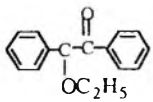
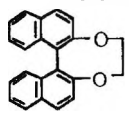
Compound & Structure	k'[a]	α[b]	Rs[c]	Mobile Phase
9. 1-Indanol 	1.34	1.18	1.25	I
10. Methylidazoxan 	1.31	1.26	1.03	D
11. 1,2,3,4-Tetrahydro-1-naphthol 	1.59	1.13	1.00	J
12. Warfarin 	2.72	1.58	2.11	A

[a] k' = capacity factor of the first eluted enantiomer. [b] The selectivity factor, α, = k_1'/k_2'. [c] The resolution = $2(tr_2 - tr_1)/(w_1 + w_2)$. [d] Mobile phase compositions:
A=MeOH:1% triethyammonium acetate in water, pH 4.1=30:70 (v/v).
B=MeOH: 1% triethyammonium acetate in water, pH 7.1=15:85 (v/v).
C=acetonitrile: 1% triethyammonium acetate in water, pH 4.1=15:85 (v/v).
D=MeOH: 1% triethyammonium acetate in water, pH 7.1=10:90 (v/v).
E=MeOH: 1% triethyammonium acetate in water, pH 4.1=20:80 (v/v).
F=acetonitrile: 1% triethyammonium acetate in water, pH 4.1=40:60 (v/v).
G=MeOH: 1% triethyammonium acetate in water, pH 7.1=20:80 (v/v).
H=1% triethyammonium acetate in water, pH 4.1=100%.
I=MeOH: 1% triethyammonium acetate in water, pH 7.1=3:97 (v/v).
J=MeOH: 1% triethyammonium acetate in water, pH 4.1=10:90 (v/v).

Depending on the size, nature, and location of the projecting group, it can associate with the mouth of the cyclodextrin (hydrogen bonding for example) be repelled from the cyclodextrin (steric interaction) or have no effect. The large number and greater size of the methyl groups on the derivatized cyclodextrin would

Table 3

Compounds Resolved on Both the Native and the 2,3-O-Methylated β-Cyclodextrin Columns in the Reversed Phase Mode

Compound & Structure	Column	k′[a]	α[b]	Rs[c]	Mobile Phase
1. Ancymidol	Methylated β-CD	7.92	1.08	0.90	A
	β-CD	3.21	1.09	0.80	B
					
2. Atropine	Methylated β-CD	1.16	1 12	0.40	A
	β-CD	6.83	1 04	0.6	C
					
3. 1-Benzocyclobutene Carbonitrile	Methylated β-CD	2.03	1.25	1.67	D
	β-CD	1.71	1.01	0.55	D
					
4. Benzoin	Methylated β-CD	1.71	1.08	0.9	B
	β-CD	3.17	1.08	1.0	E
					
5. Benzoin Ethyl Ether	Methylated β-CD	3.65	1.04	0.6	D
	β-CD	6.63	1.30	2.60	B
					
6. 2,2′-Binaphthyldiyl-8-crown-2	Methylated β-CD	5.16	1.16	1.60	F
	β-CD	4.18	1.24	1.60	F
					

(continued)

Table 3 (continued)

Compounds Resolved on Both the Native and the 2,3-O-Methylated β-Cyclodextrin Columns in the Reversed Phase Mode

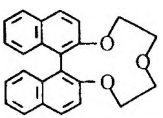
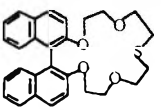
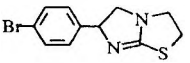
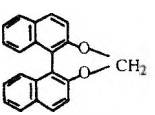
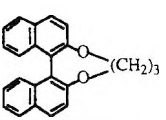
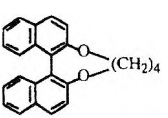
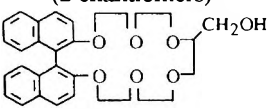
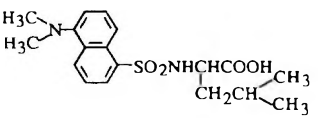
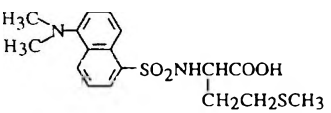
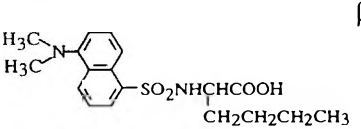
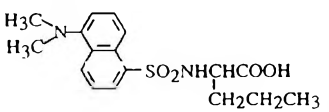
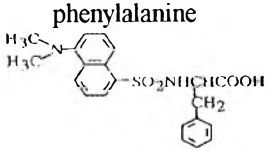
Compound & Structure	Column	k′[a]	α[b]	Rs[c]	Mobile Phase
7. 2,2′-Binaphthyldiyl-11-crown-3	Methylated β-CD	3.64	1.14	1.39	F
	Methylated β-CD	3.05	1.07	1.25	G
	β-CD	3.34-1.29	1.90		F
					
8. 2,2′-Binaphthyldiyl-17-thiacrown-5	Methylated β-CD	3.99	1.14	1.30	F
	β-CD	3.00	1.09	0.80	F
					
9. p-Bromotetramisole Oxalate	Methylated β-CD	5.44	1.07	0.75	H
	β-CD	5.22	1.17	1.25	B
					
10. Crown Ether Analogue #15	Methylated β-CD	7.01	1.14	1.27	F
	Methylated β-CD	5.24	1.12	1.22	G
	β-CD	4.56	1.26	1.61	F
					
11. Crown Ether Analogue #17	Methylated β-CD	5.17	1.22	2.47	F
	Methylated β-CD	3.96	1.18	2.24	G
	β-CD	4.42	1.21	1.41	F
					
12. Crown Ether Analogue #18	Methylated β-CD	5.32	1.22	1.86	F
	Methylated β-CD	5.03	1.20	1.73	G
	β-CD	5.85	1.10	0.85	F
					

Table 3 (continued)

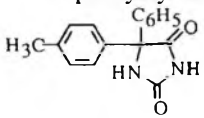
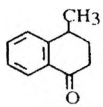
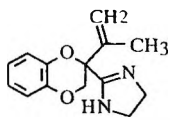
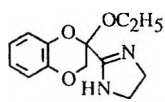
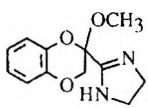
Compounds Resolved on Both the Native and the 2,3-O-Methylated β-Cyclodextrin Columns in the Reversed Phase Mode

Compound & Structure	Column	k'[a]	α[b]	Rs[c]	Mobile Phase
13. Crown Ether	Methylated β-CD	0.87	1.26	1.22	F
Analogue #24		1.93	1.22	1.36	
(2 enantiomers)	β-CD	1.81	1.10	0.80	F
					
14. Dansyl-D,L-Leucine	Methylated β-CD	2.52[D]	1.19	1.44	B
	β-CD	3.0[L]	1.4	2.4	I
					
15. Dansyl-D,L-Methionine	Methylated β-CD	2.29	1.06	0.6	B
	β-CD	3.18[L]	1.15	0.7	I
					
16. Dansyl-D,L-norleucine	Methylated β-CD	2.58	1.19	1.66	B
	β-CD	1.90[L]	1.26	2.30	I
					
17. Dansyl-D,L-norvaline	Methylated β-CD	2.08	1.06	0.6	B
	β-CD	2.80	1.13	0.83	I
					
18. Dansyl-D,L-	Methylated β-CD	1.61[D]	1.69	4.08	J
phenylalanine	β-CD	3.10[L]	1.23	1.10	K
					

(continued)

Table 3 (continued)

Compounds Resolved on Both the Native and the 2,3-O-Methylated β-Cyclodextrin Columns in the Reversed Phase Mode

Compound & Structure	Column	k′[a]	α[b]	Rs[c]	Mobile Phase
19. 5-(4-Methylphenyl)-5-phenylhydantoin 	Methylated β-CD	5.22	1.09	1.25	M
	β-CD	10.17	1.12	2.0	E
20. 1-Methyl-4-tetralone 	Methylated β-CD	4.61	1.04	0.70	D
	β-CD	10.34	1.02	0.60	D
21. RX 811005A 	Methylated β-CD	2.89	1.31	1.75	D
	β-CD	3.20	1.30	2.30	N
22. RX 811059A 	Methylated β-CD	1.49	1.54	2.83	D
	β-CD	1.62	1.40	2.50	O
23. RX 821002A 	Methylated β-CD	1.50	1.45	2.33	D
	β-CD	1.21	1.40	2.5	N

[a] k′ = capacity factor of the firsat eluted enantiomer; configuration indicated as a superscript when known. [b] The selectivity factor, α, is equal to k_1'/k_2'. [c] The resolution is equal to $2(tr_2-tr_1)/(w_1+w_2)$. [d] Mobile phase compositions:

A=1% triethylammonium acetate in water, pH 4.1 = 100% (v/v).
B=MeOH: 1% triethylammonium acetate in water, pH 7.1=20:80 (v/v).
C=acetonitrile: 1% triethylammonium acetate in water, pH 4.1=2:98 (v/v).
D=MeOH: 1% triethylammonium acetate in water, pH 7.1=10:90 (v/v).

Table 3 (continued)

Compounds Resolved on Both the Native and the 2,3-O-Methylated β-Cyclodextrin Columns in the Reversed Phase Mode

E=MeOH: 1% triethylammonium acetate in water, pH 4.1=30:70 (v/v).
F=MeOH: 1% triethylammonium acetate in water, pH 7.1=40:60 (v/v).
G=acetonitrile: 1% triethylammonium acetate in water, pH 4.1=25:75 (v/v).
H=MeOH: 1% triethylammonium acetate in water, pH 7.1=15:85 (v/v).
I=MeOH:water =50:50 (v/v).
J=MeOH: 1% triethylammonium acetate in water, pH 7.1=30:70 (v/v).
K=MeOH:water=55:45 (v/v).
L=MeOH: 1% triethylammonium acetate in water, pH 4.1=20:80 (v/v).
M=MeOH: 1% triethylammonium acetate in water, pH 4.1=15:85 (v/v).
N=MeOH: 1% triethylammonium acetate in water, pH 4.2=10:90 (v/v).
O=MeOH: 1% triethylammonium acetate in water, pH 4.1=10:90 (v/v).

tend to increase the steric repulsion at the mouth cavity for most included guest molecules. Steric repulsion can contribute to chiral recognition and enantioseparation, even though they may decrease the overall strength of the inclusion complex.

A minimum of three simultaneous interactions must occur for enantioselective recognition between the cyclodextrin host and at least one of the two enantiomeric guest molecules. The most likely interactions or combination of interactions are believed to be: hydrophobic inclusion, hydrogen bonding, and steric types. When comparing the association of a chiral compound with native β-cyclodextrin versus the 2,3-methylated cyclodextrin, the hydrophobic inclusion interaction may be somewhat analogous for the two. However, the hydrogen bonding and steric interactions at the mouth of the cavity must be very different. To a first approximation, we assume that many of the differences in enantioselectivity (in the reversed phase mode) indicated in Tables 2, 3, and 4 are the result of the nonequivalent hydrogen bonding and steric interaction between the native and derivatized cyclodextrin. Clearly, native β-cyclodextrin resolves a much greater number and variety of compounds (Table 4 and Table 1) than does its 2,3-methylated analogue (Table 2). Blocking the hydrogen bonding groups (via methylation) at the mouth of the cyclodextrin cavity interfers with chiral recognition for a large number of compounds (Table 4). However, there are a small number of compounds (Table 2) in which methylation enhances chiral recognition. Those particular compounds appear to be either neutral, nonionizible molecules or larger, bulky compounds, often containing three rings. In these cases, hydrogen bonding interactions are not as important as a tight fit in the cyclodextrin cavity and steric

Table 4

Racemic Compounds That Are Easily Resolved on the Native β-Cyclodextrin Column in the Reversed Phase Mode, But Which Could Not Be Resolved on the 2,3-O-Methylated β-Cyclodextrin CSP

Compound	Reference
1. Aminoglutethimide	4
2. 2,2′-Binaphthyldiyl-17crown-5	17,21
3. 2,2′-Binaphthyldiyl-20-crown-6	17,21
4. 2,2′-Binaphthyldiyl-23-crown-7	17,21
5. 2,2′-Binaphthyldiyl-17-thiacrown-5-sulphoxide	17,21
6. Crown Ether Analogue #9	17,21
7. Crown Ether Analogue #10	17,21
8. Crown Ether Analogue #11	17,21
9. Crown Ether Analogue #13	17,21
10. Crown Ether Analogue #14	17,21
11. Crown Ether Analogue #22	17,21
12. 1-[5-Chloro-2-(methylamino)-phenyl-1,2,3,4-tetrahydroisiquinoline	6
13. Chlorpheniramine	4
14. Chlorthalidone	4
15. Dansyl-D,L-α-amino-n-butyric Acid	22
16. Dansyl-D,L-serine	22
17. Dansyl-D,L-Threonine	22
18. Dansyl-D,L-valine	22
19. N-(3,5-Dinitrobenzoyl)-D,L-leucine	23
20. Ethylidazoxan	24
21. 5-Ethyl-5-(p-tolyl)-2-thiobarbituric Acid	6
22. Homatropine	25
23. 5-(4-Hydroxyphenyl)-5-phenylhydantoin	6
24. Ibuprofen	24
25. Ketoprofen	4
26. Methadone	4
27. 5-Methyl-phenylhydantoin	6
28. Metoprolol	4
29. Nicotine	26
30. Nisolidipene	4
31. Propranolol	4
32. Scopolamine	25
33. Tyrosine Methyl Ester	23
34. Verapramil	4

interactions at the mouth. It also is important to note that methylation of the 2- and 3-hydroxyl groups on β-cyclodextrin may not totally negate hydrogen bonding effects. It is still possible for an included compound to hydrogen bond with the primary hydroxyl groups at the narrow end of the cyclodextrin torus. This may be possible for smaller compounds such as 1-indanol (Table 2).

In some cases, chiral recognition was observed on both columns (Table 3). Compounds that contained hydrogen bonding or ionizible functional groups were usually retained longer on the native β-cyclodextrin CSP than on the 2,3-methylated analogue (when comparable mobile phases were used). However, neutral compounds containing no ionizible groups were usually retained longer on the 2,3-methylated-β-cyclodextrin column (see compounds 3, 6-8, and 10-11 in Table 3). Another interesting feature of the data in Table 3 is that the enantioselectivity of several compounds for which standards are available is reversed for the two columns. For example, the D-enantiomer of dansyl amino acids elute first on the methylated cyclodextrin column, but elute second on the native β-cyclodextrin column.

CONCLUSIONS

The 2 and 3-hydroxyl groups of β-cyclodextrin are essential for chiral recognition in the "polar-organic" LC mode. They are also very important for the enantioresolution of most compounds in the reversed phase mode. However, there are a few cases in the reversed phase mode where these hydroxyl groups are not essential. In fact, blocking them with methyl groups enhances the enantioseparation in these cases. The most likely reasons for the enhanced chiral recognition with 2,3-methylated-β-cyclodextrin are the increased steric interactions at the mouth of the cyclodextrin cavity and/or accentuated interaction with the primary 6-hydroxyl groups at the bottom of the torus (for those molecules with requisite geometry to reach them). Those compounds that can be resolved on both columns frequently show the opposite enantiomeric elution order. This indicates that there is a difference in some of the basic interactions that give rise to enantioselective retention.

REFERENCES

[1] Current Address: Advanced Separation Technologies, Inc., Whippany, NJ.

1. D. W. Armstrong, W. DeMond, J. Chromatogr. Sci., **22**, 411 (1984).

2. D. W. Armstrong, J. Liq. Chromatogr., 7, 353 (1984).

3. D. W. Armstrong, W. DeMond, B. P. Czech, Anal.Chem., **57**, 481 (1985).

4. D. W. Armstrong, T. J. Ward, R. D. Armstrong, T. E. Beesley, Science, **232**, 1132 (1986).

5. D. W. Armstrong, W. Li, Chromatography, **2**, 43 (1987).

6. S. M. Han, Y. I. Han, D. W. Armstrong, J. Chromatogr., **441**, 376 (1988).

7. J. A. Hamilton, L. Chen, J. Am. Chem. Soc., **110**, 5833 (1988).

8. D. W. Armstrong, S. Chen, C. Chang, S. Chang, J. Liq. Chromatogr., **15**, 545 (1992).

9. S. C. Chang, G. R. Reid, III, S. Chen, C. D. Chang, D. W. Armstrong, Trends Anal. Chem., **12**, 144 (1993).

10. M. Pawlowska, S. Chen, D. W. Armstrong, J. Chromatogr., **641**, 257 (1993).

11. D. W. Armstrong, Anal. Chem., **59**, 84A (1987).

12. A. M. Stalcup, S. Chang, D. W. Armstrong, J. Pitha, J. Chromatogr., **513**, 181 (1990).

13. D. W. Armstrong, A. M. Stalcup, M. L. Hilton, J. D. Duncan, J. H. Faulkner, S. C. Chang, Anal. Chem., **62**, 1610 (1990).

14. A. M. Stalcup, S. C. Chang, D. W. Armstrong, J. Chromatogr., **540**, 113 (1991).

15. A. M. Stalcup, K. H. Gahm, Anal. Chem., **68**, 1369 (1996).

16. T. J. Ward, Anal. Chem., **66**, 633A, (1994).

17. D. W. Armstrong, T. J. Ward, A. Czech, B. P. Czech, R. A. Bartsch, J. Organic Chem., **50**, 5556 (1985).

18. P. Fugedi, Carbohydr. Res., **192**, 366 (1989).

19. A. Dietrich, B. Maas, W. Messer, G. Bruche, V. Karl, A. Kaunzinger, A. Mosandl, J. High Res. Chrom., **15**, 590 (1992).

20. M. L. Hilton, S. C. Chang, M. P. Gasper, M. Pawlowska, D. W. Armstrong, A. M. Stalcup, J. Liq. Chromatogr., **16(1)**, 127 (1993).

21. D. W. Armstrong, H. Jin, Chinese J. Chromatogr., **8(1)**, 8 (1990).

22. D. W. Armstrong, W. DeMond, A. Alak, T. Ward, Anal. Chem., **57(1)**, 237 (1985).

23. D. W. Armstrong, Y. I. Han, S. M. Han, Anal. Chim. Acta, **208**, 275 (1988).

24. A. Berthod, H. L. Jin, T. E. Beelsey, J. D. Duncan, D. W. Armstrong, J. Pharm. & Biomed. Anal., **8(2)**, 123 (1990).

25. D. W. Armstrong, S. M. Han, Y. I. Han, Anal. Biochem., **167**, 261 (1987).

26. J. I. Seeman, H. V. Secor, D. W. Armstrong, K. D. Ward, T. J. Ward, J. Chromatogr., **483**, 169 (1989).

27. W. L. Hinze, Sep. Purif. Methods, **10**, 159 (1981).

28. E. Smolkova'-Keulemansova', H. Kralova', S. Krysl, L. Feltl, J. Chromatogr., **241**, 3 (1982).

29. J. Mraz, L. Feltl, E. Smolkova'-Keulemansova', J. Chromatogr., **286**, 17 (1984).

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COMPARISON OF THE SELECTIVITY AND RETENTION OF β-CYCLODEXTRIN VS. HEPTAKIS-2,3-O-DIMETHYL-β-CYCLO-DEXTRIN LC STATIONARY PHASES FOR STRUCTURAL AND GEOMETRIC ISOMERS

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ABSTRACT

Both β-cyclodextrin and 2,3-methylated-β-cyclodextrin bonded stationary phases effectively separate a variety of structural and geometrical isomeric compounds in the reversed phase mode. The retention of neutral structural isomeric compounds, as well as substituted phenols and anilines, was usually longer on the methylated cyclodextrin stationary phase. Conversely, the retention of all substituted carboxylic acids was greater on the native β-cyclodextrin stationary phase. The selectivity differences between the native β-cyclodextrin and its 2,3-methylated analogue were not as great as expected for the structural isomers. In general, the structural isomer that was retained longest on the native β-cyclodextrin stationary phase also was retained longest on the 2,3-methylated cyclodextrin stationary phase.

The *para* substituted isomer was often the longest retained, except for certain neutral compounds containing nitro substituents. In these cases, the *ortho* isomer was retained to the greatest extent. The greatest selectivity differences (i.e., retention reversals) were for geometrical isomers and the less retained structural isomers.

INTRODUCTION

β-Cyclodextrin bonded stationary phases are well known as one of the earlier and more successful chiral stationary phases for the resolution of enantiomers.[1-4] However, they also have been utilized as effective stationary phases for routine reversed phase separations.[5-10] The selectivity often is different from C_{18} and C_8 reversed phase columns. Also, it has been shown that β-cyclodextrin columns are usually more effective for the reversed phase separation of structural and geometrical isomers.[11-16] The formation of a host-guest inclusion complex is known to be the most important retention factor when using water-based mobile phase systems. However, the role (if any) of hydrogen bonding and/or steric interactions at the mouth of the cyclodextrin cavity is not well understood for achiral analytes and has only been considered in a few cases.[11,13]

In this work, a series of structural isomeric compounds and geometrical isomers are separated on both the native β-cyclodextrin bonded stationary phase and a 2,3-methylated-β-cyclodextrin bonded stationary phase. Differences in the reversed-phase selectivity and retention are examined and discussed.

EXPERIMENTAL

Methods

All separations were done at room temperature (21°C) either on a Shimadzu LC-6A or a Waters 590 liquid chromatograph. The compounds were detected with a Waters R401 Differential refractometer or, more frequently, with a variable-wavelength detector at 254 nm or 195 nm. All samples were dissolved in methanol prior to manual injection. The native β-cyclodextrin column (*i.e.*, Cyclobond I 2000) was obtained from Advanced Separation Technologies, Inc. (Whippany, NJ). The void volumes of the columns were determined by injecting neat methanol. The mobile phase consisted of mixtures (by volume) of methanol with water or 0.1 % buffer of pH 6.5 or 4.

The buffer solutions were made by dissolving pure triethylamine in water to form a 0.1% (by volume) solution. Then, glacial acetic acid was added drop-wise to achieve the desired pH. A silica-gel precolumn was used before the injector to saturate the mobile phase. The flow rate was 1 mL/min. for all separations.

Chemicals

HPLC-grade methanol, triethylamine, glacial acetic acid were obtained from Fischer Scientific (St. Louis, MO). All the compounds tested were purchased from Aldrich Chemical Co. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO) or Fluka Chemical Co. (Ronkonkoma, NY). The heptakis, 2,3-di-O-methyl-β-cyclodextrin was synthesized as follows. First, the primary hydroxy groups of β-CD were protected with *t*-butyldimethylsilychloride (in pyridine solution), and the methylated analogue was made as indicated in the previous paper of this series.[18] The removal of the protecting group was achieved by refluxing 6-O-*t*-butyldimethylsilyl-2,3-di-O-methyl-β-cyclodextrin (25g) with ammonium fluoride (16g) in methanol (250 mL) for 25 hours. The reaction was concentrated and ethyl acetate (150 mL) was added. The mixture was filtered through a pad of silica gel and the solvent was removed by distillation under vacuum. The resulting 2,3-di-O-methyl-β-cyclodextrin was used without further purification. Binding of 2,3-di-O-methylated-β-cyclodextrin to epoxy silica was done by the same method reported previously.[1]

RESULTS AND DISCUSSION

The strength of a cyclodextrin inclusion complex is determined by several factors including: the guest molecules's hydrophobic association with the less polar interior of the cyclodextrin cavity, hydrogen bonding between a guest and the cyclodextrin hydroxyl groups, the release of "high energy water molecules" from the cyclodextrin cavity during complex formation, and conformation changes in the cyclodextrin ring system upon complex formation.[17] Steric repulsion between the guest and the cyclodextrin can effect the strength of an inclusion complex as well (in a negative fashion). Even though steric repulsion can decrease the strength of an inclusion complex, it frequently increases the selectivity of a separation.[18]

When comparing the inclusion complex formed between a compound and native β-cyclodextrin or the 2,3-methylated cyclodextrin, major differences in hydrogen bonding effects at the mouth of the cyclodextrin cavity would be expected. Although inclusion complexation occurs with both β-cyclodextrin and its methylated analogue,[18] the 2,3-methylated cyclodextrin would be more hydrophobic.

Table 1

Comparison of Chromatographic Retention Data (k′s) for a Series of Structural Isomers on a Native β-Cyclodextrin Bonded Stationary Phase and a 2,3-Methylated-β-Cyclodextrin Bonded Stationary Phase[a]

No.	Compound	Mobile Phase Composition[b]					
		Methanol/H_2O		Methanol/pH6.5 Buffer		Methanol/pH4.0 Buffer	
		β-CD[c]	DM-β-CD[d]	β-CD[c]	DM-β-CD[d]	β-CD[c]	DM-β-CD[d]
1	Anthracene	1.59[e]	2.39[e]	1.47[f]	3.36[f]	0.90[f]	2.23[f]
	Phenanthrene	0.85[e]	1.62[e]	0.69[f]	1.94[f]	0.48[f]	1.50[f]
2	Benz[a]anthracene	2.88[g]	9.53[g]	2.88[g]	9.72[G]	3.41[G]	5.57[g]
	Benz[b]anthracene	2.15[g]	4.4[g]	1.91[g]	3.26[g]	1.14[g]	3.08[g]
	Chrysene	2.80[g]	9.53[g]	2.68[g]	8.57[g]	3.22[g]	4.81[g]
3	Benzo[a]pyrene	2.57[g]	14.58[g]	3.46[g]	13.28[g]	3.13[g]	13.46[g]
	Benzo[e]pyrene	2.36[g]	14.08[g]	3.01[g]	12.95[g]	2.65[g]	12.71[g]
4	Dibenz[a,c]-anthracene	1.62[f]	6.79[f]	4.14[g]	2.32[g]	4.40[g]	2.55[g]
	Dibenz[a,h]-anthracene	0.32[f]	1.08[f]	0.73[g]	0.79[g]	0.71[g]	0.68[g]
5	o-Nitrotoluene	1.27[h]	1.94[h]	0.88[h]	1.42[h]	0.79[h]	1.18[h]
	m-Nitrotoluene	0.99[h]	2.08[h]	0.66[h]	1.42[h]	0.59[h]	1.26[h]
	p-Nitrotoluene	2.10[h]	2.67[h]	1.43[h]	1.88[h]	1.22[h]	1.61[h]
6	o-Iodotoluene	0.36[e]	0.94[e]	1.19[g]	2.00[g]	4.70[h]	6.17[h]
	m-Iodotoluene	0.36[e]	1.09[e]	1.26[g]	2.00[g]	5.27[h]	7.34[h]
	p-Iototoluene	0.58[e]	1.50[e]	1.72[g]	2.26[g]	8.67[h]	9.63[h]
7	o-Xylene	1.97[h]	2.47[h]	1.23[h]	1.57[h]	1.16[h]	1.39[h]
	m-Xylene	1.97[h]	2.75[h]	0.96[h]	1.78[h]	1.10[h]	1.50[h]
	p-Xylene	3.09[h]	3.34[h]	2.01[h]	2.16[h]	1.81[h]	1.84[h]
8	o-Fluoronitrobenzene	1.10[h]	1.39[h]	0.77[h]	0.99[h]	0.69[h]	0.89[h]
	m-Fluoronitrobenzene	0.68[h]	1.21[h]	0.48[h]	0.85[h]	0.49[h]	0.78[h]
	p-Fluoronitrobenzene	0.54[h]	1.04[h]	0.43[h]	0.72[h]	0.40[h]	0.66[h]
9	o-Chloronitrobenzene	3.94[I]	4.34[I]	1.63[h]	1.93[h]	1.79[h]	1.91[h]
	m-Chloronitrobenzene	2.52[I]	3.08[I]	0.76[h]	1.22[h]	0.91[h]	1.28[h]
	p-Chloronitrobenzene	2.52[I]	2.83[I]	0.76[h]	1.22[h]	0.91[h]	1.28[h]
10	o-Bromonitrobenzene	2.70[h]	3.11[h]	1.67[h]	2.17[h]	1.60[h]	1.99[h]
	m-Bromonitrobenzene	1.30[h]	2.02[h]	0.81[h]	1.41[h]	0.81[h]	1.33[h]
	p-Bromonitrobenzene	1.30[h]	2.30[h]	0.81[h]	1.60[h]	0.87[h]	1.49[h]
11	o-Iodonitrobenzene	2.43[h]	3.24[h]	2.57[h]	3.17[h]	2.72[h]	3.12[h]
	m-Iodonitrobenzene	1.32[h]	2.31[h]	1.39[h]	2.23[h]	1.51[h]	2.27[h]
	p-Iodonitrobenzene	1.53[h]	2.91[h]	1.60[h]	2.23[h]	1.73[h]	2.87[h]
12	o-Dinitrobenzene	2.33[I]	2.67[I]	1.05[h]	1.24[h]	2.35[I]	2.67[I]
	m-Dinitrobenzene	0.79[I]	1.12[I]	0.27[h]	0.46[h]	0.79[I]	1.03[I]
	p-Dinitrobenzene	0.68[I]	1.01[I]	0.27[h]	0.46[h]	0.70[I]	0.94[I]
13	o-Nitroanisole	0.53[h]	0.89[h]	0.47[h]	0.83[h]	0.59[h]	0.88[h]
	m-Nitroanisole	1.29[h]	1.74[h]	1.08[h]	1.56[h]	1.35[h]	1.72[h]
	p-Nitroanisole	1.23[h]	1.88[h]	1.08[h]	1.69[h]	1.27[h]	1.86[h]
14	α-Naphthol	1.61[g]	1.64[g]	0.45[f]	1.01[f]	1.23[g]	2.19[g]
	β-Naphthol	2.38[g]	2.46[g]	0.32[f]	0.92[f]	0.93[g]	1.91[g]

Table 1 (continued)

Comparison of Chromatographic Retention Data (k′s) for a Series of Structural Isomers on a Native β-Cyclodextrin Bonded Stationary Phase and a 2,3-Methylated-β-Cyclodextrin Bonded Stationary Phase[a]

No.	Compound	Mobile Phase Composition[b]					
		Methanol/H_2O		Methanol/pH6.5 Buffer		Methanol/pH4.0 Buffer	
		β-CD[c]	DM-β-CD[d]	β-CD[c]	DM-β-CD[d]	β-CD[c]	DM-β-CD[d]
15	o,o'-Biphenol	0.51[e]	0.69[e]	0.23[f]	0.49[f]	0.40[g]	1.25[g]
	p,p'-Biphenol	0.78[e]	3.73[e]	0.85[f]	5.11[f]	2.47[g]	10.90[G]
16	o-Cresol	0.53[h]	1.16[h]	0.43[h]	1.00[h]	0.39[h]	0.86[h]
	m-Cresol	0.63[h]	1.25[h]	0.54[h]	1.10[h]	0.47[h]	0.95[h]
	p-Cresol	1.01[h]	1.55[h]	0.83[h]	1.36[h]	0.72[h]	1.16[h]
17	o-Nitrophenol	1.01[g]	0.86[g]	0.74[h]	0.50[h]	0.66[h]	0.88[h]
	m-Nitrophenol	0.61[g]	1.49[g]	0.74[g]	1.03[g]	0.59[h]	1.56[h]
	p-Nitrophenol	2.35[g]	1.82[g]	1,84[h]	1.03[h]	0.88[h]	1.95[h]
18	o-Bromophenol	0.72[h]	1.74[h]	1.00[h]	2.35[h]	1.20[h]	1.36[h]
	m-Bromophenol	1.32[h]	3.15[h]	1.62[h]	4.02[h]	2.06[h]	2.34[h]
	p-Bromophenol	1.74[h]	3.73[h]	1.99[h]	4.41[h]	2.52[h]	2.44[h]
19	o-Ethylphenol	0.69[h]	1.35[h]	0.72[h]	1.45[h]	0.82[h]	1.70[h]
	m-Ethylphenol	1.10[h]	1.99[h]	1.14[h]	2.12[h]	1.29[h]	2.42[h]
	p-Ethylphenol	1.80[h]	2.55[h]	1.93[h]	2.92[h]	2.21[h]	3.15[h]
20	o-Chlorophenol	0.60[h]	1.14[h]	0.89[h]	1.84[h]	1.08[h]	2.29[h]
	m-Chlorophenol	1.03[h]	2.32[h]	1.20[h]	2.89[h]	1.53[h]	3.51[h]
	p-Chlorophenol	1.22[h]	2.56[h]	1.37[h]	2.89[h]	1.77[h]	3.69[h]
21	o-Nitroaniline	0.85[h]	1.10[h]	0.59[h]	1.27[h]	0.55[h]	1.14[h]
	m-Nitroaniline	0.54[h]	1.59[h]	0.47[h]	1.67[h]	0.43[h]	1.49[h]
	p-Nitroaniline	1.17[h]	2.19[h]	1.48[h]	2.53[h]	1.34[h]	2.35[h]
22	o-Chloroaniline	0.74[h]	0.99[h]	0.67[h]	1.10[h]	1.23[j]	1.84[j]
	m-Chloroaniline	0.83[h]	1.48[h]	0.77[h]	1.68[h]	1.31[j]	2.62[j]
	p-Chloroaniline	1.22[h]	1.48[h]	1.12[h]	1.61[h]	1.70[j]	2.37[j]
23	o-Bromoaniline	1.06[h]	0.96[h]	0.79[h]	0.91[h]	1.00[h]	1.59[h]
	m-Bromoaniline	1.41[h]	1.46[h]	1.00[h]	1.51[h]	1.15[h]	2.92[h]
	p-Bromoaniline	2.41[h]	1.46[h]	2.41[h]	1.51[h]	1.71[h]	2.53[h]
24	o-Iodoaniline	0.46[h]	0.97[h]	0.15[f]	0.60[f]	0.36[f]	0.78[f]
	m-Iodoaniline	0.81[h]	2.99[h]	0.27[f]	1.37[f]	0.54[f]	2.09[f]
	p-Iodoaniline	1.65[h]	3.16[h]	0.58[f]	1.24[f]	1.04[f]	1.81[f]
25	o-Anisidine	1.05[g]	2.57[g]	0.94[h]	0.94[h]	0.24[j]	0.68[j]
	m-Anisidine	1.22[g]	1.51[g]	1.21[h]	1.11[h]	0.52[j]	0.85[j]
	p-Anisidine	1.56[g]	2.84[g]	1.48[h]	1.21[h]	0.00[j]	0.50[j]
26	o-Toluidine	0.51[h]	1.26[h]	0.87[i]	0.94[i]	0.49[i]	0.80[i]
	m-Toluidine	0.61[h]	1.97[h]	1.04[i]	1.14[i]	0.27[i]	0.80[i]
	p-Toluidine	1.02[h]	2.18[h]	1.92[i]	1.43[i]	0.27[i]	0.80[i]
27	o-Anisic Acid	0.67[i]	0.00[i]	0.29[h]	0.00[h]	3.97[h]	0.13[h]
	m-Anisic Acid	1.26[i]	0.10[i]	0.51[h]	0.04[h]	6.10[h]	0.79[h]
	p-Anisic Acid	1.85[i]	0.10[i]	0.68[h]	0.07[h]	5.29[h]	1.46[h]

(continued)

Table 1 (continued)

Comparison of Chromatographic Retention Data (k′s) for a Series of Structural Isomers on a Native β-Cyclodextrin Bonded Stationary Phase and a 2,3-Methylated-β-Cyclodextrin Bonded Stationary Phase[a]

No.	Compound	Mobile Phase Composition[b]					
		Methanol/H_2O		Methanol/pH6.5 Buffer		Methanol/pH4.0 Buffer	
		β-CD[c]	DM-β-CD[d]	β-CD[c]	DM-β-CD[d]	β-CD[c]	DM-β-CD[d]
28	o-Toluic Acid	0.48[h]	0.00[h]	0.88[i]	0.00[i]	2.25[f]	0.38[f]
	m-Toluic Acid	0.67[h]	0.07[h]	0.44[i]	0.14[i]	2.09[f]	0.62[f]
	p-Toluic Acid	0.81[h]	0.18[h]	2.57[i]	0.23[i]	2.39[f]	0.96[f]
29	o-Bromobenzoic Acid	0.84[h]	0.00[h]	1.42[i]	0.00[i]	6.84[f]	0.15[f]
	m-Bromobenzoic Acid	1.15[h]	0.11[h]	4.50[i]	0.30[i]	5.36[f]	0.73[f]
	p-Bromobenzoic Acid	1.15[h]	0.11[h]	6.06[i]	0.55[i]	5.63[f]	1.34[f]
30	o-Chlorobenzioc Acid	0.60[h]	0.00[h]	1.95[i]	0.00[i]	6.49[f]	0.13[f]
	m-Chlorobenzoic Acid	0.95[h]	0.00[h]	4.25[i]	0.24[i]	4.91[f]	0.63[f]
	p-Chlorobenzoic Acid	0.95[h]	0.13[h]	4.75[i]	0.35[i]	4.59[f]	0.91[f]
31	o-Nitrobenzoic Acid	1.23[h]	0.00[h]	2.13[i]	0.00[i]	7.61[f]	0.05[f]
	m-Nitrobenzoic Acid	1.37[h]	0.12[h]	3.08[i]	0.13[i]	5.84[f]	0.22[f]
	p-Nitrobenzoi c Acid	1.84[h]	0.15[h]	3.88[i]	0.21[i]	6.74[f]	0.40[f]

[a] Both columns were25x0.44 [i.d.] cm. The silica gel support consisted of identical 5μ spherical particles. The linkage chain and bonding chemistry were identical (see Experimental section).

[b] Three compositions of mobile phase were used: methanol with water; methanol with 0.1% triethylammonium acetate buffer, pH 6.5 and methanol with 0.1% triethylammonium acetate buffer, pH4.0.

[c] Native β-Cyclodextrin Bonded Stationary Phase.

[d] 2, 3-methylated-β-Cyclodextrin Bonded Stationary Phase.

[e-j] Mobile phase ratios (methanol to water or 0.1% triethylammonium acetate buffer, pH6.5 and 4.0, v/v): [e]55/45; [f]50/50; [g]40/60; [h]30/70; [i]10/90; [j]20/80.

The methyl groups at the mouth of the cyclodextrin cavity can be thought of as extending the size of the cavity and/or providing sites for steric repulsion at the mouth of the cyclodextrin cavity. These differences have been shown to significantly alter the enantioselectivity of β-cyclodextrin.[18] However, the effect on the retention and selectivity of other achiral isomeric compounds has not been considered.

Table 1 gives the separation data for a series 31 different structural isomeric compounds separated on both the native β-cyclodextrin bonded stationary phase and its 2,3-methylated analogue. The compounds are listed in groups according to their class (*i.e.*, from top to bottom: polycyclic aromatic hydrocarbons, neutral disubstituted benzenoid compounds, substituted phenolic compounds, substituted

Table 2

Comparison of Chromatographic Retention Data (k's) for a Series of Geometric Isomers on a Native β-Cyclodextrin Bonded Stationary Phase and a 2,3-Dimethylated β-Cyclodextrin Bonded Stationary Phase[a]

No.	Compound	Mobile Phase Composition[b] Methanol/H_2O		Methanol/pH6.5 Buffer		Methanol/pH4.0 Buffer	
		β-CD[c]	DM-β-CD[d]	β-CD[c]	DM-β-CD[d]	β-CD[c]	DM-β-CD[d]
1	Cis-stilbene	1.63[e]	1.63[e]	0.90[e]	1.07[e]	0.26[f]	0.44[f]
	Trans-stilbene	0.84[e]	3.93[e]	0.58[e]	2.59[e]	0.15[f]	1.02[f]
2	Cis-3-hexene-1-ol	0.95[g]	0.65[g]	0.85[h]	0.69[h]	1.03[h]	0.72[h]
	Trans-3-hexene-1-ol	0.65[g]	0.57[g]	0.56[h]	0.46[h]	0.65[h]	0.63[h]
3	Cis-1,2-bis(phenyl sulfonyl) ethylene	2.47[i]	1.62[i]	2.15[i]	1.57[i]	1.39[i]	1.18[i]
4	trans-cis-decahydro-naphthalene	1.82[f]	1.16[f]				
5	trans-cis-1,4-dimethyl-cyclohexane	0.80[f]	0.52[f]				
	trans-	0.51[f]	0.36[f]				

[a] Both columns were25x0.44 [i.d.] cm. The silica gel support consisted of identical 5μ spherical particles. The linkage chain and bonding chemistry were identical (see Experimental section).

[b] Three compositions of mobile phase were used: methanol with water; methanol with 0.1% triethylammonium acetate buffer, pH 6.5 and methanol with 0.1% triethylammonium acetate buffer, pH4.0.

[c] Native β-Cyclodextrin Bonded Stationary Phase.

[d] 2, 3-methylated-β-Cyclodextrin Bonded Stationary Phase.

[e-i] Mobile phase ratios (methanol to water or 0.1% triethylammonium acetate buffer, pH6.5 and 4.0, v/v): [e]55/45; [f]70/30; [g]15/85; [h]10/90; [i]30/70.

anilines and substituted benzoic acids). Table 2 gives comparable separation data for several geometric isomers. Since all separations were done in the reversed phase mode (using hydro-organic solvents) it is assumed that inclusion complexation takes place.[1-4,18]

Retention

A number of interesting trends are evident from the data in Table 1. First, the retention of all neutral, nonionizable compounds (No.'s 1-13 in Table 1) is greater on the 2,3-methylated cyclodextrin stationary phase than on the native β-cyclodextrin stationary phase (when using comparable mobile phases and experimental conditions). Clearly, the methylated cyclodextrin stationary phase is more hydrophobic and nonpolar. Conversely, the retention of all carboxylic acid compounds (No.'s 27-31, Table 1) was greater on the native β-cyclodextrin

stationary phase than on its 2,3-methylated analogue. It is apparent that the interactions between the carboxylic acid moiety of the guest and the 2- or 3-hydroxyl groups at the mouth of the cyclodextrin cavity contributes significantly to the stability of these inclusion complexs.

The retention behavior of the aromatic amines (No.'s 21-26 Table 1) and the phenolic compounds, (No.'s 14-20, Table 1) is not as consistent as that of the aforementioned neutral compounds or the substituted carboxylic acids. Since the substituted phenols and anilines have ionizable functional groups that are able to act as hydrogen bond acceptors or donors, one might assume that their retention behavior would more closely resemble that of the substituted benzoic acids. However, this does not seem to be the case. Most of the time, the relative retentions of the substituted phenols and anilines resemble the neutral analytes in that they are retained to a greater extent on the 2,3-methylated-β-cyclodextrin stationary phase than on the native β-cyclodextrin stationary phase (Table 1). The main exceptions to this are a few *para* substituted compounds at specific pHs (see compounds 18, 25, and 26). It is not surprising that pH can have an effect, since it controls whether the compound is ionized or neutral. The pH effects on binding constants of substituted phenols and anilines has been studied previously.[19]

Adding buffer to the mobile phase can produce at least three different effects. First, it controls the ionization of analytes that have weak acidic or basic functional groups. Both the retention and selectivity of a compound depend on whether it is neutral or in an ionized state. For example, the retention of the substituted benzoic acids increases at pH 4.0 (when using comparable mobile phases). The opposite trend or variable retentions are observed for the substituted anilines (Table 1). If the ionization of a solute is not a factor, added buffer still sometimes reduces retention, in effect acting like additional organic modifier (see compounds 5-13, Table 1, where identical mobile phases are used). Finally, the buffer can sometimes enhance efficiency by interacting with and masking strong adsorption sites on the stationary phase. This trend was particularly evident for the separation of enantiomers[4,18] but did not seem to be as significant in this work for structural and geometrical isomers.

Selectivity

The *para*-isomer was generally the most retained isomer on both columns for the substituted phenols, anilines and carboxylic acids (Table 1). The one exception to this was for a few of the substituted anilines mainly at pH 4.0. Presumably, the shorter relative retention of the *para*-substituted anilines at this pH was because they are protonated, cationic species which do not form as strong inclusion complexes.

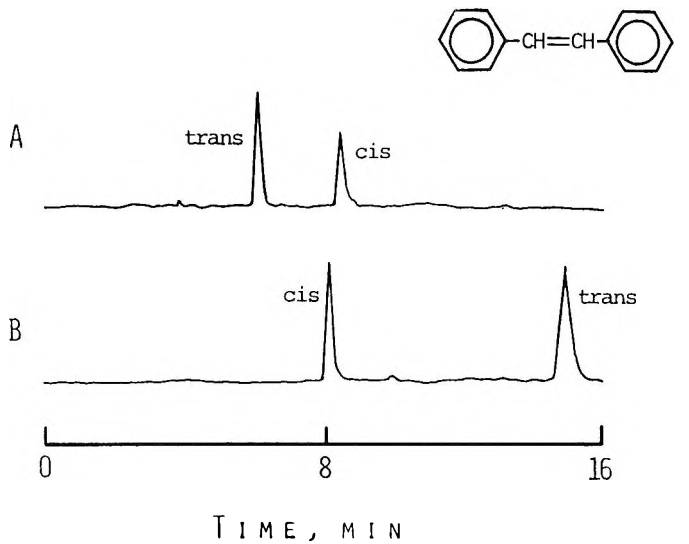

Figure 1. Chromatograms showing the difference in reversed phase selectivity for *cis* and *trans* stilbene of the A) native β-cyclodextrin bonded stationary phase, and the (B) 2,3-methylated-β-cyclodextrin bonded stationary phase. The mobile phase consisted of 45:55 (v:v) methanol:water in both cases. The flow rate was 1.0 mL/min. and UV detection (254 nm) was used.

Halogenated nitrobenzene and the dinitrobenzene (compounds 8-12, Table 1) were unique in that the *ortho* isomer was always the most strongly retained on both columns. In fact, the differences between the 2,3-methylated β-cyclodextrin and the native β-cyclodextrin column (in retention and selectivity) were not substantial for this particular group of neutral molecules. Apparently, shape discrimination during the hydrophopic inclusion complex process is the dominant factor determining selectivities for these compounds. For most of the other structural isomers, the ortho isomer was the least retained.

The *para*-isomers of most ionizable compounds (No.'s 15-31, Table 1) were retained to a greater extent than the ortho and meta isomers. The main exceptions to this were for some of the substituted anilines which were protonated at pH 4.0 (as discussed previously). It is interesting that the most retained structural isomer on the native β-cyclodextrin column also was most retained on the 2,3-methylated-β-cyclodextrin stationary phase. Selectivity differences between the two columns were more frequently observed for the less retained structural isomers (Table 1).

Geometrical isomers (Table 2) often gave the opposite retention order when separated on the native β-cyclodextrin column versus it 2,3-methylated analogue (see Figure 1). The retention trends for geometrical isomeric compounds were somewhat similar to that seen for the structural isomers. Neutral, hydrophobic compounds (stilbene, Table 2) tended to be retained more on the 2,3-methylated β-cyclodextrin stationary phase, whereas more polar or ionizable compounds were retained relatively longer on the native β-cyclodextrin stationary phase.

CONCLUSIONS

Methylation of the 2- and 3- hydroxyl groups on β-cyclodextrin produces a more nonpolar, hydrophobic stationary phase. Most of the isomeric compounds in this study were retained to a greater extent (in the reversed phase mode) on the methylated cyclodextrin stationary phase. The main exception to this was the substituted benzoic acids which were more strongly retained on the native β-cyclodextrin columns regardless of mobile phase conditions. The selectivity differences between the methylated and native β-cyclodextrin were not as significant for structural isomers as they were for the previously observed optical isomers (enantiomers).[18]

In general, the isomer that was retained longest on the native β-cyclodextrin stationary phase was also retained longest on its 2,3-methylated analogue. Retention reversals were most frequently seen for the less retained isomers. Retention reversals were also observed for pairs of geometrical isomers.

It appears that hydrogen bonding at the mouth of the cyclodextrin cavity is not as crucial to the selectivity of many structural isomers as it is for enantiomers. However, in some cases (*i.e.*, compounds with carboxy-functional groups) this hydrogen bonding, rim interaction remains very important.

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REFERENCES

† Current address: Advanced Separation Technologies, Inc., Whippany, NJ.

1. D. W. Armstrong, W. DeMond, J. Chromatogr. Sci., **22**, 411 (1984).

2. D. W. Armstrong, J. Liq. Chromatogr., 7, 353 (1984).

3. D. W. Armstrong, W. DeMond, B. P. Czech, Anal. Chem., **57**, 481 (1985).

4. D. W. Armstrong, T. J. Ward, R. D. Armstrong, T. E. Beesley, Science, **232**, 1132 (1986).

5. D. W. Armstrong, A. Alak, W. DeMond, W. L. Hinze, T. E. Riehl, J. Liq. Chromatogr., **8**, 261 (1985).

6. C. A. Chang, Q. Wu, D. W. Armstrong, J. Chromatogr., **354**, 454 (1986).

7. H. J. Issaq, J. Liq. Chromatogr., **9**, 229 (1986).

8. H. J. Issaq, J. H. McConnel, D. E. Weiss, D. G. Williams, and J. E. Saavedra, J. Liq. Chromatogr., 9, 1791 (1986).

9. M. Paleologou, S. Li, W. C. Purdy, Can. J. Chem., **68**, 1208, (1990).

10. A. J. Packham, P. R. Fielden, J. Chromatogr., **552**, 575 (1991).

11. D. W. Armstrong, W. DeMond, A. Alak, W. L. Hinze, T. E. Riehl, K. H. Bui, Anal. Chem., **57**, 234 (1985).

12. B. G. Snider, J. Chromatogr., **351**, 548 (1986).

13. R. D. Armstrong, T. J. Ward, N. Pattabiraman, C. Benz, D. W. Armstrong, J. Chromatogr., **414**, 192 (1987).

14. G. W. Tindall, J. Liq. Chromatogr., **10**, 1077 (1987).

15. C. A. Chang, Q. Wu, J. Liq. Chromatogr., **10**, 1359 (1987).

16. C. A. Chang, Q. Wu, Anal. Chim. Acta, **189**, 293 (1986).

17. W. L. Hinze, Sep. Purif. Methods, **10**, 159 (1981).

18. D. W. Armstrong, L. W. Chang, S. C. Chang, X. Wang, H. Ibrahim, G. R. Reid, III, T. E. Beesley, J. Liq. Chromatogr., **20(20)**, 3279-3295 (1996).

19. G. L. Bertrand, J. R. Faulkner, Jr., S. M. Han, D. W. Armstrong, J. Phys. Chem., **93**, 6863 (1989).

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A DIRECT INJECTION ASSAY OF ANGIOTENSIN CONVERTING ENZYME IN TISSUE EXTRACTS

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ABSTRACT

A rapid, sensitive and selective method for the determination of angiotensin converting enzyme (ACE) activity in tissue extract and serum is described. The procedure is based on the high performance liquid chromatographic separation of the synthetic substrate furylacryloylphenylalanyl-glycyl-glycine (FAPGG) from the hydrolysis product furylacryloylphenylalanine (FAP). Baseline separation is accomplished in ten minutes by direct injection of biological assay mixtures onto a 15 cm shielded hydrophobic phase column with isocratic elution using 180 mM ammonium acetate/ 15% acetonitrile as the mobile phase. Both substrate and product are detected by absorbance at 305 nm.

INTRODUCTION

Angiotensin-converting enzyme (ACE) is a key component of the renin-angiotensin system, an important regulator of blood pressure and fluid balance in mammals.[1] Physiologically, ACE is a dipeptidyl carboxypeptidase (EC 3.4.15.11) which cleaves the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II, an octapeptide. ACE is known to participate in the degradation of bradykinin and other neuropeptides. [2] Specific inhibitors of ACE have been used for the control of hypertension in man.[3,4]

ACE is a zinc metallopeptidase which occurs in both soluble and membrane-bound forms. The enzyme has been detected in a wide variety of tissues and organisms.[5-9] The determination of ACE activity is clinically significant in human serum, as well as physiologically important in many other tissues.[10] Currently, assays of ACE are carried out using synthetic substrates, most often hippuryl-histidyl-leucine (HHL).[11,12] Here, we describe an HPLC based assay using an alternative substrate FAPGG which has several advantages over HHL.[13] Chromatography is performed on a shielded hydrophobic phase (SHP) column which allows for direct injection of assay mixtures without prior protein precipitation or product extraction. The procedure is simple, rapid, and more sensitive than the spectrokinetic assay using this substrate.[14]

EXPERIMENTAL

Materials

FAP, FAPGG, captopril, and purified rabbit lung ACE were obtained from Sigma. All solvents were HPLC grade and other chemicals were of reagent grade. Human serum was obtained fresh in-house. The marine worm, *Nereis diversicolor* was obtained from Marine Biological Laboratory, Woods Hole and crude extracts were prepared by homogenization of tissue in 20 volumes of ice-cold 50 mM phosphate buffer, pH 8.3.

Methods

Chromatographic analysis

Shielded hydrophobic phase high performance liquid chromatography (SHP-HPLC) was carried out using a Varian Model 2510 liquid chromatograph

equipped with a 250 mm x 4.6 mm I.D. HISEP SHP column with a 20 mm x 4.6 mm I.D. HISEP guard column (Supelco) and a Rheodyne Model 7125 injection valve with a 20 μL injection loop. The mobile phase used for isocratic elution at a flow rate of 1.5 mL/min was 180 mM ammonium acetate-acetonitrile (85:15, V/V). Absorbance at 305 nm was measured using a Varian Model 2550 spectrophotometer with an 8 μL flow cell and peak areas were determined using a Spectra-Physics Model 4270 integrator.

Measurement of ACE activity

Angiotensin-converting enzyme (ACE) activity was determined using a modification of the spectrophotometric method of Baudin and Giboudeau[13] in which the hydrolysis of the substrate FAPGG to FAP is measured. Each assay had a total volume of 650 μL containing FAPGG (1 mM), phosphate buffer (100 mM, pH 8.3), NaCl (276 mM), and either human serum, crude *Nereis* extract, or purified rabbit lung ACE solution, and was incubated at 37°C in a 1.5 mL polyethylene centrifuge tube in a shaker bath. After a suitable incubation period (15-60 min), the incubation mixture for crude extacts was centrifuged for 2.0 min at 735 g prior to removing a 20 μL aliquot for HPLC analysis. For serum assays, a 20 μL aliquot was removed from the incubate and injected directly onto the chromatographic column.

RESULTS

A series of chromatograms of standard assay mixtures containing increasing amounts of human serum are shown in Fig. 1. Baseline separation of the substrate FAPGG and the product FAP of the ACE catalyzed reaction is accomplished in 10 minutes. ACE activity is detectable in as little as 1.0 μL of human serum if an incubation time of 60 min is utilized. Of course, much shorter incubation times may be used if larger volumes (up to 100 μL) are available. A typical assay of ACE activity in 100 μL of human serum is accomplished in 15 min.

Note that although the peak at 10.40 min is not obvious in Frame A of Fig. 1 at 1.0 aufs, the signal was well above background for the electronic integrator.

It can be seen in Fig. 2 that the production of FAP was linear with respect to time for at least 60 min over a ten-fold range of enzyme activity units and that 0.5 mU ACE was reliably detected.

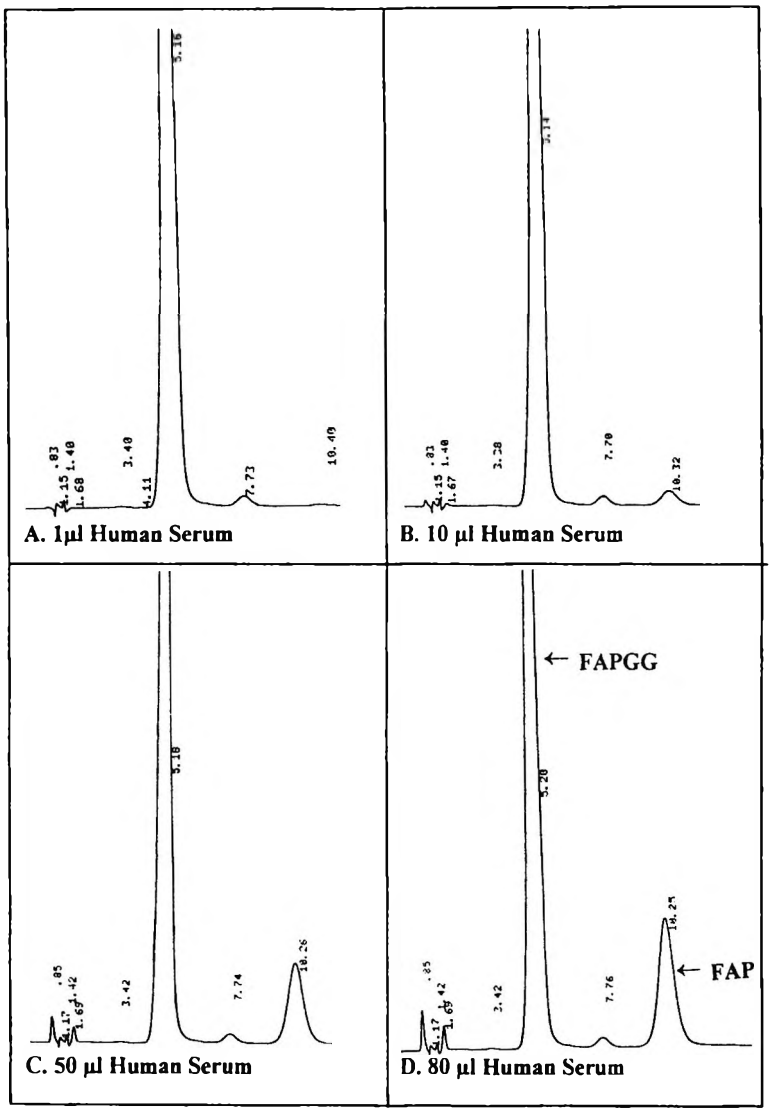

Figure 1. Chromatograms of ACE assay mixtures containing 1, 10, 50, and 80 µL human serum (panels A-D, respectively) showing development of the FAP peak with a retention time of approximately 10 minutes. Assays mixtures were incubated for 60 min at 37°C prior to chromatography. Chromatographic conditions: column, HISEP (4.6 x 20 mm); mobile phase, 180 mM ammonium acetate:acetonitrile (85:15); flow rate , 1.5 mL/min; temperature, ambient; detection, UV at 305 nm; injection volume, 20 µL; aufs = 1.0.

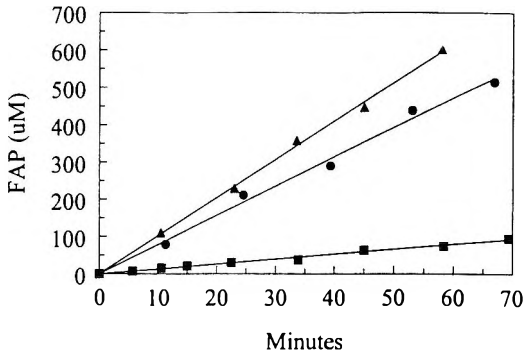

Figure 2. Assay linearity over time with three different concentrations of purified rabbit lung ACE . Each value is the mean of replicate assays. In no instance is the variance greater than 10%. ■ = 5 x 10^{-4} U ACE, ● = 1 x 10^{-3} U ACE, ▲ = 5 x 10^{-3} U ACE.

Production of FAP was also linear with respect to the amount of enzyme (Fig. 3) for all three sources of enzyme. Captopril (data not shown) at a concentration of 4 x 10^{-4} M completely inhibited the production of FAP by each of the ACEs.

DISCUSSION

The present study was prompted by a need for a sensitive assay for ACE that could be applied to crude extracts of biological tissues from diverse sources. These extracts are complex mixtures that often contain high concentrations of light-absorbing species and enzymes, other than ACE, which have proteolytic activity. The combination of the substrate FAPGG with SHP-HPLC analysis obviates many of the problems associated with the determination of ACE activity in such extracts.

A major advantage of the substrate FAPGG over the most frequently used synthetic substrate HHL is that both FAPGG and the hydolysis product FAP absorb maximally above 300 nm. Therefore, the assay using FAPGG and monitoring absorbance at 305 nm is less subject to interferences encountered with HHL and the hydrolysis product hippurate which have absorbance maxima at 238 nm and are usually monitored at 254 nm.

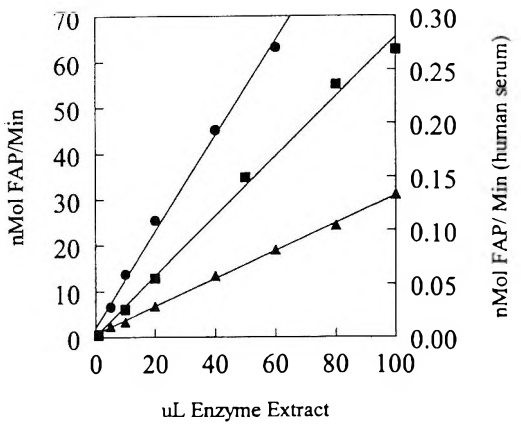

Figure 3. FAP production as a function of enzyme concentration from three different tissue sources. Each value is the mean of replicate assays. In no instance was the variance greater than 10%. ● = rabbit lung ACE, ▲ = *Nereis diversicolor* crude extract, ■ = human serum. The left Y axis is scaled for FAP production by rabbit lung and *Nereis diversicolor* ACE and the right Y axis is scaled for FAP production by human serum ACE

Additional advantages of FAPGG are that stock solutions of the substrate are stable at 4°C for more than a year, the rate of hydrolysis is considerably faster, and, most importantly, FAPGG is a more specific substrate for ACE in that it is not cleaved by carboxypeptidase A as is HHL[13]. This is particularly important in studies involving tissues from the digestive tract.

Although FAPGG is the preferred substrate for ACE activity measurement because of its specificity and lack of background interference problems, the poor sensitivity of the spectrophotometric assay has limited its use. This assay is based on a blue shift in the absorbance spectrum upon substrate hydrolysis,[14] but the difference absorption spectrum is relatively small at its maximum at 328 nm. In the modified procedure reported here, separation of the substrate and product by HPLC allow for a 23-fold increase in sensitivity by utilizing the absorbance maximum at 305 nm and 60 minute incubation times. An additional advantage of the HPLC-based assay is that both the disappearance of substrate and the appearance of product are simultaneously monitored. Moreover, the use of SHP columns allows for direct injection of assay mixtures without need for prior protein precipitation or product extraction.[15] Thus, the assay is simple, rapid, sensitive, and applicable to a wide range of tissues without interference.

REFERENCES

1. M. J. Peach, Physiol. Rev., **57**, 313-370 (1977).

2. P. Corvol, T. A. Williams, F. Soubrier, "Peptidyl Dipeptidase A: Angiotensin I-Converting Enzyme," in **Methods in Enzymology, Vol. 248**, A. J. Barrett, ed., Academic Press, San Diego, 1995, pp. 283-305.

3. C. M. Ferrario, J. Cardiovasc. Pharmacol., **15**, S1-S5 (1990).

4. H. Gavras, Circulation, **81**, 381-388 (1990).

5. D. W. Lipke, K. R. Olson, Physiol. Zool., **61**, 420-428 (1988).

6. M. E. Ibarra-Rubio, J. C. Peña, J. Pedraza-Chaverri, Comp. Biochem. Physiol., **92B**, 399-403 (1989).

7. J. W. Smiley, M. T. Doig, Comp. Biochem. Physiol, **108A**, 491-496 (1994).

8. B. Bénéteau-Burnat, A. Tahraoui, F. Barbut, J. Giboudeau, B. Baudin, Comp. Biochem. Physiol., **109B**, 623-635 (1994).

9. M. E. van Sande, S. L. Scharpe, H. M. Neels, K. O. Van Camp, Clin. Chim. Acta, **147**, 255-260 (1985).

10. P. R. Studdy, R. Bird, Ann. Clin. Biochem., **26**, 13-18 (1989).

11. D. W. Cushman, H. S. Cheung, Bichem Phramacol., **20**, 1637-1647 (1971).

12. M. T. Doig, J. W. Smiley, J. Chromatogr., **613**, 145-149 (1993).

13. B. Baudin, J. Giboudeau, Enzyme Protein, **48**, 81-89 (1994-95).

14. B. Holmquist, P. Bünning, J. F. Riordan, Anal. Biochem., **95**, 540-548 (1979).

15. D. J. Gisch, B. T. Hunter, B. Feibush, J. Chromatogr., **433**, 264-268 (1988).

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LIQUID CHROMATOGRAPHIC ANALYSIS OF ALL-RAC-α-TOCOPHERYL ACETATE, TOCOPHEROLS, AND RETINYL PALMITATE IN SRM 1846

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ABSTRACT

A liquid chromatographic method is described for the analysis of all-rac-α-tocopheryl acetate, tocopherols, and retinyl palmitate in the infant formula standard reference material 1846. The vitamins are extracted in isopropanol and hexane/ethyl acetate without saponification and quantitated by normal phase chromatography with fluorescence detection. Retinyl palmitate, all-rac-α- tocopheryl acetate, and naturally occurring tocopherols are quantitated isocratically with a mobile phase of 0.5% isopropanol in hexane. The results were within the certified ranges for all-rac-α-tocopheryl acetate and retinyl palmitate. Recoveries averaged 97.5% for retinyl palmitate and 101% for all-rac-α- tocopheryl acetate (n = 20).

The method provides a rapid, specific, and easily controlled assay approach to the analysis of vitamin A and vitamin E in fortified infant formula. Additionally, the method eliminates the use of chlorinated solvents.

INTRODUCTION

Since the passage of the Infant Formula Act in 1980 by Congress, much interest has been generated in the area of nutrient analysis of formulas and other infant foods. Many methods have been developed for fat soluble vitamins. Most methods for analysis of vitamins A and E use saponification in the extraction procedure.[1-6] In fact, some type of saponification technique was used by each of the laboratories involved in the certification of the National Institute of Standards and Testing (NIST) milk based infant formula Standard Reference Material (SRM 1846). The current AOAC Official Methods for vitamins A and E in milk based infant formula (methods 50.1.02, 50.1.03, and 50.1.04) utilize saponification followed by hexane/ethyl ether or hexane/methylene chloride extraction.[7] The AOAC methods, however, do not adequately describe precautionary steps for saponification. Failure to blanket the sample with nitrogen prior to saponification, or use of a condenser on top of the saponification tube, can lead to vitamin loss. Furthermore, a saponification technique usually requires parititioning of the analyte into an organic solvent which may result in emulsions that are difficult to break and, thus, increasing the possibility of low analyte recoveries. The organic solvent, methylene chloride, used in the AOAC methods 50.1.03 and 50.1.04[7] can have a negative environmental impact.

In addition, saponification converts the all-rac-α-tocopherol acetate to all-rac-α-tocopherol which cannot be resolved from naturally occurring RRR-I-tocopherol. On a biological activity basis, all-rac-α-tocopherol has only 74% of the activity of RRR-α-tocopherol.[7] Therefore, coelution of the synthetic all-rac-isomer, together with the naturally occurring RRR-isomer, decreases the overall accuracy of the assay when vitamin E activity is expressed in α-tocopherol equivalents (α-TE where 1 α-TE = 1 mg RRR-α-tocopherol).[9]

Other methods have used direct solvent extraction with various solvent mixtures and LC with UV or fluorescence detection.[10-14] All-rac-α-tocopheryl acetate fluoresces weakly compared to free tocopherols. In the past, this property was a hindrance to quantitation of the ester by fluorescence. Fortunately, present day fluorescence detectors are sufficiently sensitive to allow for routine detection of α-tocopheryl acetate.[14,15]

An alternative technique eliminating the saponification step was developed by Landen[11] for the analysis of vitamins A and E in infant formula. Samples were dehydrated with magnesium sulfate and extracted with isopropanol and methylene chloride. The fat was removed via gel permeation chromatography followed by quantitation on reverse phase LC with UV detection. This method was used extensively in this laboratory; however, due to environmental concerns with methylene chloride usage, the technique has all but been abandoned.

The objective of this study was to develop a simplified, specific assay procedure for vitamin A and vitamin E in fortified infant formulas that does not require saponification. The newly developed NIST Infant Formula SRM (SRM 1846) was used as the test matrix for this study. An extraction scheme derived from the procedure of Landen[11] was developed, however, it eliminates the use of methylene chloride, a gel permeation chromatographic clean up, reverse phase chromatography, and UV detection. The extraction procedure in this study, uses sample dehydration with magnesium sulfate and extraction with isopropanol and hexane/ethyl acetate (85:15 v/v). After evaporation and filtration, the sample extract is injected directly into a normal phase LC system using more specific and sensitive fluorescence detection.

MATERIALS

Reagents

Hexane: LC grade (Baxter Healthcare Corp., Muskegon, MI, USA); Isopropanol: LC grade (EM Science, Gibbstown, NJ, USA); Ethyl Acetate: LC grade (Baxter Healthcare Corp.); Magnesium Sulfate: Anhydrous (Fisher Chemical, Fairlawn, NJ, USA); Mobile Phase: Isopropanol @ 0.5% in hexane. Vitamin A Standard: approximately, 50 mg of retinyl palmitate (Fluka Bio Chemika, Switzerland) was accurately weighed and dissolved in 50.0 mL of hexane. The exact concentration was determined by the $E^{1\%}$ value of 975. Appropriate dilutions were made with the mobile phase to give five working standard concentrations ranging from 0.5 to 6.0 μg/mL. Vitamin E Standard: approximately 200 mg of all-rac-α-tocopheryl acetate (Fluka Bio Chemika) was accurately weighed and dissolved in 50.0 mL of hexane. Gamma and δ-tocopherol were obtained from the University of Georgia, Athens, GA, USA. The tocopherols were purified by preparatory LC. RRR-α-tocopherol was obtained from Fluka. The concentration of each tocopherol was calculated from their respective $E^{1\%}$ values of 42, 75.8, 91.4, and 87.3 for all-rac-α-

tocopheryl acetate, RRR-α, γ and δ-tocopherol, respectively. The appropriate dilutions were made with the mobile phase to give five working standards, each containing the four combined tocopherols, with the following range of concentrations: RRR-α-tocopherol (0.8 to 10 μg/mL), γ-tocopherol (2.6 to 33 μg/mL), δ-tocopherol (1.3 to 13 μg/mL) and all-rac-α-tocopheryl acetate (8.0 to 106 μg/mL). Butylated hydroxytoluene (BHT): approximately 9 mg of BHT (Sigma, St. Louis, MO, USA) was dissolved in 25.0 mL of hexane to give a concentration of 360 μg/mL.

Apparatus

Analytical LC: retinyl palmitate, all-rac-α-tocopheryl acetate, and the tocopherols were quantitated separately. One 50 μL injection was made for vitamin E and a separate 50 μL injection was made for retinyl palmitate on a Lichrosorb Si 60 (5μm, 4.6mm x 25 cm) column (E. Merck, Darmstadt, Germany). The LC consisted of a LDC Analytical Constametric 3200 pump (Thermo Separation Products, Riviera Beach, FL, USA), Waters 715 auto injector (Waters Inc., Milford, MA, USA), a Hewlett Packard (Avondale, PA, USA) 1046A fluorescence detector (Ex=285 nm, Em=310 nm for all-rac-α-tocopheryl acetate and the tocopherols, and an Ex=325 nm, Em=470 nm for retinyl palmitate), and a Hewlett Packard Model 3396 integrator. Flow rates were 1.0 mL/min and 0.5 mL/min, respectively, for the analysis of vitamin E and retinyl palmitate. Polytron^{R} Homogenizer: Kinematica PT10-35 (Brinkman Instruments, Westbury, NY, USA). Rotary Evaporator: Buchi Rotavapor EL 130 (Brinkman Instruments). Turboevaporator: TurboVap II (Zymark, Hopkinton, MA, USA).

METHODS

The standard reference material for milk based infant formula, (SRM 1846) was obtained from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA). The contents of each packet (approximately 28 g) were reconstituted with approximately 145 g of hot water (70-80°C) and thoroughly mixed.

Approximately 6.5 g of the reconstituted infant formula was accurately weighed into a 100 mL wide mouth graduated cylinder. Isopropanol was heated to near boiling before adding 15 mL to the sample and mixing thoroughly with a spatula.

A 7.5 g portion of magnesium sulfate was added, followed by 30 mL of the extraction solution (hexane/ethyl acetate, 85:15, v\v) and 1.0 mL of the BHT solution, with mixing by spatula after each addition.

The mixture from above was homogenized with a Polytron Homogenizer for 1 min at medium speed prior to filtering the mixture through a coarse porosity fritted glass filter into a 125 mL Philips beaker using a vacuum bell jar filtration apparatus. The vacuum was then released, the magnesium sulfate cake was broken up with the aid of a spatula and washed twice with 15 mL of the extracting solution.

The extraction process was repeated by transferring the magnesium sulfate cake back to the original graduated cylinder and adding 20 mL of the extracting solution plus 5 mL of isopropanol. The procedure was then repeated for the once extracted mixture beginning with "homogenized with a Polytron Homogenizer..."

The combined filtrate was transferred to a 500 mL round bottom flask containing 1 g of magnesium sulfate and evaporated to dryness using the rotary evaporator. The residue was immediately dissolved in 10 mL of hexane and filtered through a 0.45 μm Nylon 66 filter (Alltech Associates, Deerfield, IL, USA) into a 125 mL Philips beaker using a bell jar filtration apparatus. The flask was washed with three 7 mL portions of hexane. The hexane wash was filtered into the flask containing the dissolved extract. The combined filtrate was evaporated to a volume of less than 5 mL using a turboevaporator at 45°C with a nitrogen pressure of 10 psi. The evaporated filtrate was diluted to 10 mL with the mobile phase and injected.

RESULTS AND DISCUSSION

Figure 1a illustrates the LC chromatogram of all-rac-α-tocopheryl acetate and the natural vitamin E homologs in a sample extract of SRM 1846. The all-rac-α-tocopheryl acetate is resolved from the γ and δ-tocopherol. Since the SRM 1846 does not contain oils that contain appreciable amounts of naturally occuring levels of RRR-α-tocopherol, it was not necessary to be able to quantitate both the RRR-α-tocopherol and all-rac-α-tocopheryl acetate. However, the all-rac-α-tocopheryl acetate is also easily separated from the RRR-α-tocopherol as illustrated in the standard chromatogram of Figure 1b, thereby making this method capable of routine quantitation of all vitamin E homologs.

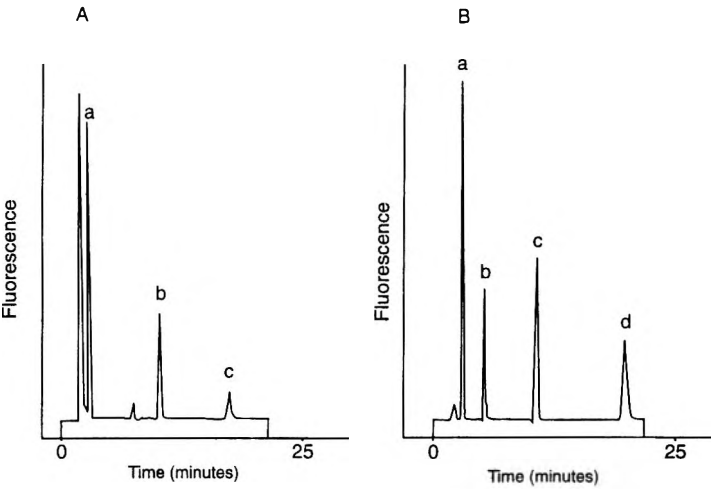

Figure 1. LC chromatogram of all-rac-α-tocopheryl acetate using fluorescence detection (Ex 285 nm, Em 310 nm), flow rate of 1.0 mL/min, injection volume of 50 µL, and mobile phase of 0.5% isopropanol in hexane. 1A is the extract of an infant formula where (a) is all-rac-α-tocopheryl acetate, (b) is γ tocopherol and (c) is delta tocopherol. 1B is the vitamin E standard where (a) is all-rac-α-tocopheryl acetate (42.4µg/mL), (b) is RRR-α-tocopherol (4.02 µg/mL), (c) is γ-tocopherol (13.3 µg/mL) and (d) is δ-tocopherol (6.54 µg/mL).

Fluorescence response for all-rac-α-tocopherol acetate, RRR-α-tocopherol, γ-tocopherol and δ-tocopherol were linear (r=0.999) for the 8.0-106 µg/mL, 0.8-10 µg/mL, 2.6-33 µg/mL, and 1.3-13 µg/mL ranges, respectively. The limit of detection was calculated to be 3.14 µg/mL for all-rac-alpha-tocopheryl acetate. The calculation was based on the equation, $y-a=3(s_{y/x})$, where a is the y-intercept in the linear expression $y=bx+a$ and $s_{y/x}$ is equal to, $\Sigma\{(y_i-y_i)^2/n-2\}^{1/2}$, where y_i is the actual peak response and y_i is the peak response as calculated from the linear expression of $y=bx+a$.[16] Once y is determined, x, which is then the estimated value of the limit of detection, can be calculated.

Figure 2a and 2b illustrate the LC chromatogram for retinyl palmitate in the sample and standard, respectively. The flow rate is reduced for the analysis of the vitamin A ester resulting in a run time of 8 min. The retinyl palmitate differs in its fortified concentration as compared to the tocopherols, thus, the gain was increased to aid in the detection of vitamin A. Two injections from

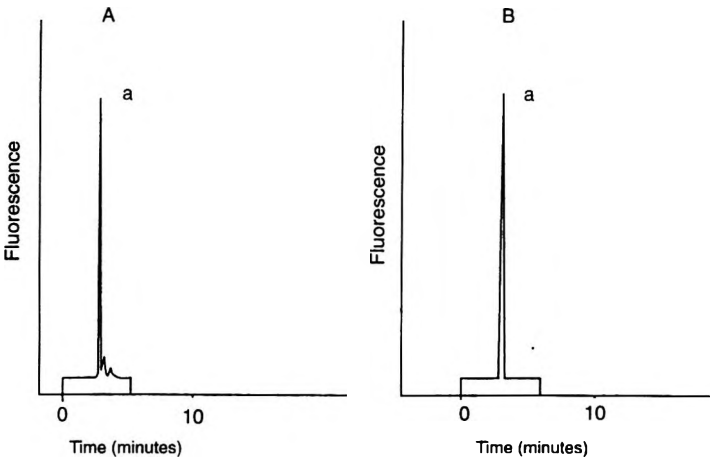

Figure 2. LC chromatogram of retinyl palmitate using fluorescence detection (Ex 325 nm, Em 470 nm), flow rate of 0.5 mL/min, injection volume of 50 μL, and a mobile phase of 0.5% isopropanol in hexane. 2A is the extract of an infant formula where (a) is the retinyl palmitate. 2B is the retinyl palmitate (a) standard (1.25 μg/mL).

the same extract were used to simplify the overall method. For example, one injection was for the vitamin E determination and, a second injection was for the vitamin A determination. The vitamins of interest can easily be quantitated from a single injection by using a programmable pump in conjunction with the programmable fluorescence detector. The retinyl palmitate fluorescence response was linear ($r=0.999$) from 8.0 to 106 μg/mL. The limit of detection, calculated in a manner as previously described for all-rac-α-tocopheryl acetate, was 0.187 μg/mL.

Ten replicates of SRM 1846 were assayed for retinyl palmitate, all-rac-α-tocopheryl acetate and natural tocopherol content. Since vitamins A and E are expressed as the alcohol form in the SRM 1846 certificate, ester forms of the vitamins were converted to the alcohol forms. Table 1 illustrates the NIST ranges for all rac-α, γ and δ-tocopherol and vitamin A, as well as the results obtained in this study. The all-rac-α-tocopherol and retinol values are certified values; whereas, the γ and δ-tocopherol values are non-certified. The all-rac-αl-tocopherol and retinol data obtained utilizing our method approximate those of the SRM certified values. The γ-tocopherol level meets the non-certified specifications, but the δ-tocopherol level exceeds the non-certified specification

Table 1

Assay Values of SRM 1846

	NIST Value (mg/kg)	Found (mg/kg) (n = 10)
α tocopherol*	246 - 296	281 ± 6.15 (cv = 2.19)
γ tocopherol	70.5 - 76.5	76.2 ± 4.62 (cv = 6.06)
δ tocopherol	17.53 - 18.45	19.1 ± 0.81 (cv = 4.25)
retinol*	5.16 - 6.52	5.37 ± 0.26 (cv = 4.79)

* certified values.

slightly. NIST used saponification techniques to quantitate the γ and δ-tocopherols; whereas, this study does not. The higher δ-tocopherol level found with the present method could indicate higher tocopherol stability and/or better extraction efficiency compared to the saponification method.

Initial attempts using hexane as the extraction solvent resulted in low recoveries for retinyl palmitate. Substitution of 100% hexane with hexane:ethyl acetate (85:15 v/v); to increase the polarity of the solvent; resulted in increased recoveries for all-rac-α-tocopheryl acetate and retinyl palmitate. Studies by Ueda and Igarashi[17] indicated the usefulness of ethyl acetate for vitamin E extractions. Using the hexane:ethyl acetate mixture, recoveries were run in five replicates at four different levels. The mean certified value plus approximately 50, 100, 150, and 300% of the certified value was studied. The levels of 50, 100, 150, and 300 % were obtained by using the process of standard additions to SRM 1846. For example, since α-tocopherol is certified at a mean level of 271 mg/kg, 50% of that value would be the standard addition level of 135.5 mg/kg, thus giving a total of 406.5 mg/kg.

Since infant formulas are fortified with all-rac-α-tocopheryl acetate and retinyl palmitate, the ester forms were used for the recovery studies. Alpha, γ and δ-tocopherols vary in concentration according to the type of oil used in the formulation. Table 2 illustrates the recoveries obtained in this study. The recoveries were fairly constant for the 50-150% levels for both the retinyl palmitate and all-rac-α-tocopheryl acetate, however, at 300% the recoveries decreased to 91.8 and 94.2%, respectively. The 300% level for retinyl palmitate corresponds to almost twice the legal limit allowed (750 IU/100 kcal).

Table 2

Recovery Results of all Rac-α-Tocopheryl Acetate and Retinyl Palmitate

Level (%)*	% Recovered Vitamin A	% Recovered Vitamin E
50 (n = 5)	98.6 ± 5.7 (cv = 5.8)	103 ± 4.3 (cv = 4.2)
100 (n = 5)	100 ± 4.0 (cv = 4.0)	103 ± 3.5 (cv = 3.4)
150 (n = 5)	99.4 ± 4.6 (cv = 4.6)	103 ± 1.1 (cv = 1.1)
300 (n = 5)	91.8 ± 3.3 (cv = 3.6)	94.2 + 2.6 (cv = 2.7)

* Corresponds to percentage of mean certified value added to the sample.

Recovery studies utilizing the method of standard additions for a matrix already containing a known certified amount of analyte, cannot show how the analytes would behave at levels less than the certified value. Since a true sample blank does not exist for infant formula, recoveries in this study are only for values exceeding the certified value. Work is currently being conducted in this laboratory to develop a "zero control" infant formula or a true matrix blank. A "zero control" reference material would enable one to determine the method's performance as the analyte of interest is reduced in quantity to near zero.

Peak purity was established using a peak ratioing technique described by Haroon et al.[18] The emission wavelength was kept constant for the analytes while the fluorescence was measured at three different excitation wavelengths. The fluorescence emission of retinyl palmitate at 470 nm was determined at excitation wavelengths of 315, 325, and 335 nm. Ratios were calculated for 315/325 and 335/325. For all-rac-α-tocopheryl acetate, the emission wavelength was held at 310 nm and the excitation was at 275 and 295 nm. Ratios were calculated for 275/285 and 295/285. These ratios were compared for the standard and sample as illustrated in Table 3. Good agreement was obtained for the ratios of the standard and sample for both retinyl palmitate and all-rac-α-tocopheryl acetate, indicating the purity of the peaks.

This method presents an alternative technique to saponification for analysis of infant formulas and provides data in agreement with SRM certified values. Elimination of saponification permits quantitation of the added ester forms and natural vitamin E homologs. Fat removal from the extract is not necessary, since normal phase chromatography on silica can accomodate up to 2 mg of fat per injection.[10] Analysis of a milk based formula with the

Table 3

Peak Purity Evaluation[a]

Nutrient	Excitation Wavelength	Peak Response Ratios Standard	Sample
retinyl palmitate	315/325	1.15	1.13
	335/325	0.86	0.82
all-rac-α-tocopherol acetate	275/285	0.66	0.69
	295/285	0.52	0.47

[a] Emission wavelengths were constant for vitamins A (470 nm) and E 3310 nm) (n = 2).

procedure will load approximately 1.6 mg of fat per 50 μL injection on to the column. Stability of the analytes is increased when working with the ester forms of vitamins A and E. A true quantitation of vitamin E content can be established to include natural tocopherols and added all-rac-α-tocopheryl acetate. The use of chlorinated solvents is eliminated. The method is simple and rapid as an experienced analyst can assay up to 10 samples per day.

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REFERENCES

1. H. E. Indyk, Analyst, **113**, 1217-1221 (1988).

2. S. Tuan, T. F. Lee, C. C. Chou, Q. K. Wei, J. Micronut. Anal., **6**, 35-45 (1989).

3. J. T. Tanner, S. A. Barnett, M. K. Mountford, J. AOAC Int., **76**, 399-413 (1993).

4. E. J. deVries, Ch. C. J. Odding, U. Manz, E. Tagllaferri, Dmt. Dairy Fed, IDF Bulletin 285, 1993, pp. 53-64.

5. S. K. Jensen, J. Dairy Res., **61**, 233-240 (1994).

6. E. J. M. Konings, H. H. S. Roomans, P. R. Beljaars, J. AOAC Int., **79**, 902-906, (1996).

7. AOAC International, "Infant Formula and Medical Diets, Methods 50.1.02, 50.1.03, and 50.1.04," **AOAC Official Methods of Analysis**, 16 th ed., AOAC International, Gaithersburg, MD., 1995, pp. 50-1-50-4.

8. M. K. Horwitt, **Vitamin E Abstracts,** VERIS, LaGrange, IL, (1987).

9. **National Research Council, Recommended Dietary Allowances**, 10th ed., National Academy Press, Washington, D.C., 1989, p.100.

10. J. N. Thompson, G. Hatina, J. Liq. Chromatogr., **2**, 327-344 (1979).

11. W. O. Landen, J. AOAC Int., 65, 810-813 (1982).

12. D. C. Woollard, A. D. Blott, J. Micronut. Anal., **2**, 97-115 (1986).

13. A. D. Blott, D. C. Woollard, J. Micronut. Anal., **2**, 259-274, (1986).

14. M. K. Balz, E. Schulte, H. P. Thier, Fat Sci. Tech., **95**, 215-220 (1993).

15. D. C. Woollard, A. D. Blott, H. Indyk, J. Micronut. Anal., **3**, 1-14 (1987).

16. J. C. Miller, J. N. Miller, **Statistics for Analytical Chemistry**, Ellis Horwood Limited, West Sussex, England, 1989.

17. T. Ueda, O. Igarashi, J. Micronut. Anal., 7, 79-96 (1990).

18. Y. Haroon, D. S. Bacon, J. A. Sadowski, Clin. Chem., **32**, 1925-1929, (1986).

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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.

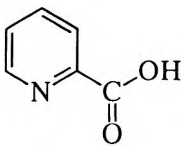

Figure 1. Part structure of heterocyclic acid series of metal chelating analytes.

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.

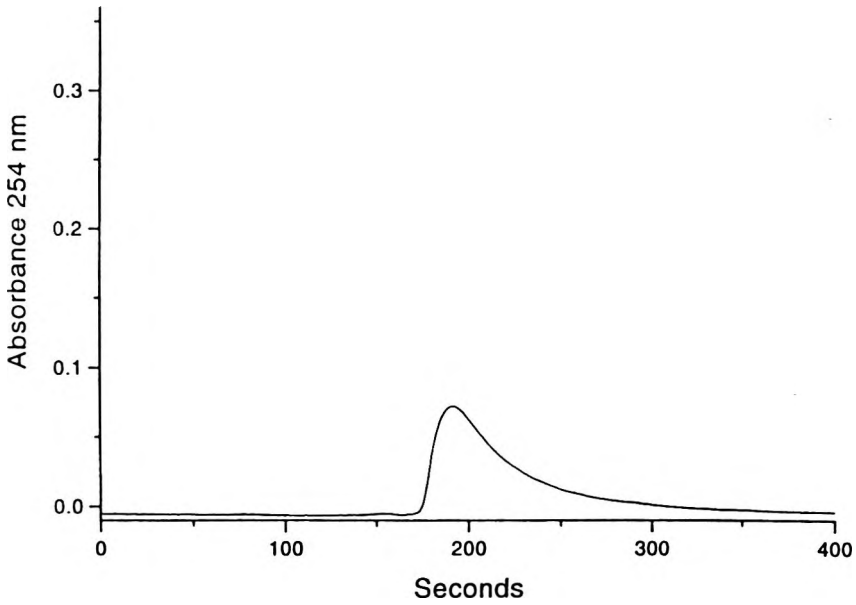

Figure 2. Typical chromatogram obtained for a member of the heterocyclic acid series. Conditions: column, 5 mm Res Elut 5 C_{18} (25 cm x 4.6 mm I.D.); mobile phase: 153.2 mM phosphate buffer, pH 7.3; flow rate 0.9 mL/min at ambient temperature.

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

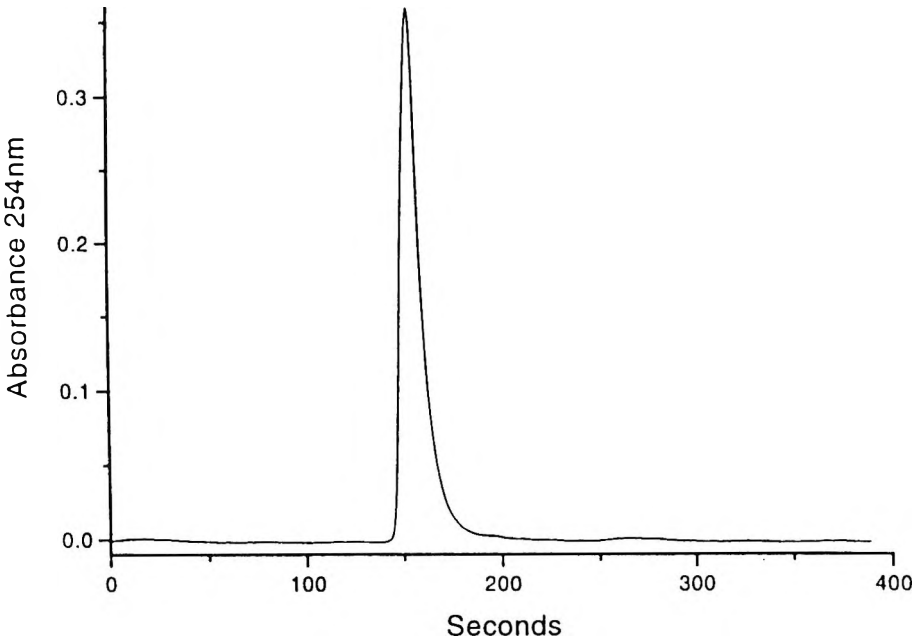

Figure 3. As Fig. 2 except addition of 0.2 mM $KB(Pz)_4$ to the mobile phase.

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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REFERENCES

1. D. W. Roberts, R. J. Ruane, I. D. Wilson, J. Chromatogr., **471**, 437-441 (1989).

2. G. D. Mack, R. B. Ashworth, J. Chromatogr. Sci., **16**, 93-101 (1978).

3. G. Chevalier, C.Bollet, P. Rohrbach, C. Risse, M. Claude, R. Rosset, J. Chromatogr., **124**, 343-349 (1976).

4. A. P. Leenheer, H. J. C. F. Nelis, J. Chromatogr., **140**, 293-299 (1977).

5. J. H. Knox, J. Jurand, J. Chromatogr., **186**, 763-782 (1979).

6. F. F. Carini, A. E. J. Martell, J. Amer. Chem. Soc., **74**, 5745-5748 (1952).

7. F. F. Carini, A. E. J.Martell, J. Amer. Chem. Soc., **75**, 4810-4813 (1953).

8. R.Bocker J. Chromatogr., **187**, 439-441 (1980).

9. J. P.Sharma, R. P. Bevill, J. Chromatogr., **166**, 213-220 (1978).

10. S. Trofimenko, Chem. Rev., **93**, 943-980 (1993).

11. S. Trofimenko, Progr. Inorg. Chem., **34**, 115-210 (1986).

12. K. Niedenzu, S. Trofimenko, Top. Curr. Chem., **131**, 1-37 (1986).

13. S. Trofimenko, Chem. Rev., 72, 497-509 (1972).

14. S. Trofimenko, J. Am. Chem. Soc., **89**, 3170-3177 (1967).

15. P. Cecchi, G. Gioia Lobbia, F. Marchetti, G. Valle, S. Calogero, Polyhedron, **13**, 2173-2178 (1994).

16. S. Calogero, G. Gioia Lobbia, P. Cecchi, G. Valle, J. Friedl, Polyhedron, **13**, 87-97 (1994).

17. Y. Sohrin, H. Kokusen, S. Kihara, M. Matsui, Y. Kushi, M. Shiro, J. Amer. Chem. Soc., **115**, 4128-4136 (1993).

18. L. G. Sillen, A. E. Martell, **Stability Constants**, Special Publication n° 17, London Chemical Society, 1964.

19. A. E. Martell, R. M. Smith, **Critical Stability Constants**, Vol. 1, Plenum Press.

20. F. Pucciarelli, P. Passamonti, T. Cecchi, J. Liq. Chrom., in press.

21. C. Davies, R. D. Hartley, G. J. Lawson, J. Chromatogr., **18**, 47-52 (1965).

22. J.Královsky, M. Kalhousová, K. Placek, Chem. Prum., **10**, 537-540 (1987).

23. J. Cazes, Am. Biotechnol. Lab., **6 (2A)**, 20 (1988).

24. S. Trofimenko, Inorg. Synth., **12**, 99-109 (1970).

25. H. J. E. M. Reeuwijk, U. R. Tjaden, J. Chromatogr., **353**, 339-350 (1986).

26. R. A. Keller, J. C. Giddings, J. Chromatogr., **3**, 205-220 (1986)

27. L. Thunus, Il Farmaco - Ed. Sc., **24**, 1082-1104 (1969).

28. L. Thunus, J. Pharm. Belg., **22**, 379-386 (1967).

29. L. Thunus, J. Pharm. Belg., **21**, 491-504 (1966).

30. C. Lopez, R. M. Claramunt, D. Sanz, C. Foces Foces, F. H. Cano, R. Faure, E. Cayon, J. Elguero, Inorg. Chim. Acta, **176**, 195-204 (1990).

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A RAPID MONITORING METHOD OF PARAQUAT AND DIQUAT IN SERUM AND URINE USING ION-PAIRING BARE-SILICA STATIONARY PHASE HPLC FOLLOWING A SINGLE ACIDIFICATION STEP OF SAMPLE PRETREATMENT

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ABSTRACT

Intoxication by paraquat, an agricultral chemical, is frequently reported in Asian and eastern European countries because of its availablity by general population. Because paraquat is not significantly biotransformed in man, it requires an intensive monitoring of paraquat level during both stages of rescue and recuperation. It is critical to design an effective technique to eliminate the paraquat from the patient at the earlier stage of the therapy, and a rapid method is required for monitoring the paraquat concentration. Diquat is a less toxic analog of paraquat, and is often mixed with paraquat for agricultural usage. Recently, a high rate of fatal cases were reported on the mixture of diquat and paraquat among all paraquat incidences. In this study, a rapid HPLC method has been developed for monitoring paraquat and diquat in serum and urine samples.

The total analysis is less than 6 minutes. It only requires a minimal sample pretreatment, including acidification and centrifugation of sample at 9500 x g for 1 minute. This method utilizes ion-pairing HPLC with bare silica (150 mm X 4.6 mm) as stationary phase. After acidification with 2M phosphoric acid, the supernatant of samples can be injected directly into the HPLC system. The mobile phase was 25/75 acetonitrile/water (V/V); containing 10 mM 1- Heptanosulphonic Sodium Salt, 4 mM potassium phosphate, 10 mM potassium chloride, pH 3.0. The analysis was performed at room temperature with a flow rate of 1.0 mL/min. The optimum UV wavelength for paraquat and diquat were 257 nm and 310 nm, respectively; the corresponding detection limits were measured at 63 ng/mL (1.25 ng on column) for diaquat and 125 ng/mL (2.5 ng on column) for paraquat, respectively. For simultaneous monitoring of both compounds, a common wavelength of 290 nm can be used; with an injection volume of 20 μL, paraquat could be detected at 500 ng/mL(10 ng on column) and 125 ng/mL (2.5 ng on column) for diquat in both urine and serum. A lower detection limit at 290 nm can be achieved by using a larger injection volume. The CV% of retention data are less than 1.2% for both compounds. Interference studies were also conducted for common drugs and metabolites. Additional tests were conducted on drugs which are strongly retained on cation exchange methods: chloroquine, strychnine and nicotine. All these three compounds did not interfere with the method.

INTRODUCTION

Agriculture is a major industry in Asian and eastern European countries. Intoxication by agricultural chemicals is frequently reported in these regions because of the easy accessibility by general population. Among different agricultural chemicals, paraquat intoxication is a major concern because of its toxicity and its frequent use for a suicidal agent.

Paraquat (Gramoxone W, Weedol, methyl viologen) is a bis-quaternary ammonium compound that has been widely used since 1962 as a domestic and commercial herbicide.[1] The dichloride salt is supplied as a 5% powder for domestic use or a 10% to 30% aqueous concentrate for agricultural purposes. The compound is known to be absorbed via dermal contact, inhalation and ingestion.

Diquat is a less toxic analogue of paraquat (an ortho-bipyridyl analogue), and it is usually supplied as the dibromide in a 2% concentrate for spraying. Combinations of paraquat and diquat are also avaliable for agricultural usage.

There were many death since the introduction of this compound.[1,2] From 1964 to 1974, over 200 fatal cases were reported from ingestion of paraquat in all countries of use.[1] In recent years, higher incidence frequencies were reported. For example, in Japan alone,[3] 355 fatal cases of paraquat (PQ) and diquat (DQ) were reported in 1994 (93 PQ, 1 DQ, 261 mixture); 361 cases in 1993 (88 PQ, 5 DQ, 268 mixture).

Because paraquat is not significantly biotransformed in man, it requires intensive monitoring during both stages of rescue and recuperation. It is reported that patients with plasma concentrations in excess of 0.25 μg/mL from 12 to 68 hours after ingestion, with associated renal failure, usually do not survive.[1] Another report indicated that patients whose plasma concentrations do not exceed 2.0, 6.0, 0.3, 0.16, and 0.1 μg/mL at 4, 6, 10, 16, and 24 hours, respectively, are likely to survive.[4] Therefore, it is critical to design an effective technique to eliminate the paraquat from patient at the earlier stage of the therapy, and a rapid method is useful for monitoring the paraquat concentration at 0.1 μg/mL or above.

Early colorimetric methods[5,6] were developed with a detection limit of 5 μg/mL. Later, several modifications were able to improve detection limit to 1 μg/mL, including isolation by cation-exchange chromatography,[7,8] protein precipitation,[9] ion-pairing extraction,[10] or direct addition of a reagent to urine.[11] A thin layer chromatography (TLC) method was developed for detection of residue remaining on vegetation,[12] which also required extensive sample preparation.

There were also several gas chromatography (GC) methods initially developed for environmental and safety concerns.[13,14] Later, a GC method for biological fluid had a detection limit of 0.5 μg/mL for paraquat and 1.0 μg/mL for diaquat.[14] However, the sample pretreatment method (1 hour) was complex and impractical. Another GC method claimed a sensitivity of 0.025 μg/mL with nitrogen-selective detector,[1] and again the sample preparation procedure was long (>1.5 hours) and a large volume of patient sample was required (3 mL).

A GC/MS method for forensic tissue samples was able to detect paraquat at 10 ng/mL when operating at selected ion monitoring (SIM) mode.[1] Either thermospray[16,17] or particle beam techniques[18] were used for the interface of

LC/MS for the detection of diquat and paraquat in soil and water, with the detection limits of 5 ng/mL to 20 ng/mL in SIM mode. Sample volumes were from 10 grams of soil to 4 liters of water.

Many HPLC methods were developed for paraquat formulation analysis,[19] urinary analysis (1 mL urine),[20] direct injection (3 μL to 5 μL) of spiked urine, spiked water, and gastric aspirate,[21] column switching (40 minutes run time),[22] ion pairing reversed phase HPLC,[23] and post column reaction.[24]

Different HPLC techniques were used in these studies.[19-24] For example, reversed phase stationary phase (Silica base ODS C_{18}) with ion-pairing reagent at low pH (pH 2-3) is the most commonly reported method.[20,23] Other types of stationary phases were also used, such as ion-exchange resin,[19,22] γ-aminopropyltriethoxysilane bonded alumina,[21] and bare silica.[24] However, these methods all required tedious off-line extractionprocedure for removing interference materials from matrix, except Pryde's study.[21] A column switching technique was reported[22] for on-line extraction procedure by using gel filtration.

Other techniques such as capillary electrophoresis (CE), radioimmunoassay (RIA), and fluoresence-polarization immunoassay (TDx) were also reported.[23,25,26] For example, a CE method was reported on using acetic acid-sodium acetate (pH 4.0) with 100 mM sodium chloride as buffer and electrokinetic injection;[23] an RIA method was able to detect paraquat;[25] a TDx was reported to have a detection limit of 5 ng/mL of paraquat in serum.[26]

In this study, we developed a rapid direct injection method to meet the needs of clinical treatment situation. Both serum and urine samples can be analyzed directly folowing a single step of acidification, and the total analysis time is less than 6 minutes with a detection limit of 63 ng/mL. The method only requires a single pump isocratic elution and a single analytical cartridge. The separation is based on the ion-pairing chromatography with bare silica.

MATERIALS AND METHODS

Materials

Standards of paraquat (1,1'-Dimethyl-4,4'-bipyridinium dichloride) and diquat dibromide (6,7-Didropyridol[1,2-a:2'1'-c]pyrazidinium dibromide) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Chemical

Service Inc. (West Chester, PA, USA), respectively. 1- Heptanesulphonic Sodium Salt, 1-hydrate (HPLC grade) was from Eastman Kodak Company (Rochester, NY, USA). Drugs used for interference studies were obtained from Sigma, Alltech (State College, PA, USA), or their respective manufacturers.

A total of 18 compounds were tested, including diazepam, amphetamine, imipramine, morphine, hydrocodone, benzoylecgonine, methamphetamine, methadone, codeine, trimethoprim, pseudoephedrine, nortriptyline, propoxyphene, EDDP (methadone metabolite), dextromethorphan, strycinine, chloroquine, and nicotine.

HPLC

An HPLC system was consisted of a 1350 HPLC pump, a UV monitor (UV-1816 UV/Vis Detector), and an ALR 486 computer. The BDS software (BarSpec, Israel) was used for data collection. An automated sample injector equipped with 20 μL sample loop (Model AS-100 HPLC Automatic Sampling System) was used. All above mentioned HPLC modules and software were avaliable from Bio-Rad Laboratories (Hercules, California 94547, USA).

Separation conditions

A single bare silica cartridge (150 mm X 4.6 mm I.D.) was used (Bio-Rad Laboratories, part # 1957145). The mobile phase was 25/75 acetonitrile/water (V/V; containing 10 mM 1- Heptanosulphonic Sodium Salt, 1-hydrate, 4 mM KH_2PO_4, 10 mM KCl, pH 3.0 adjusted by H_3PO_4). The mobile phase was filtered by 0.45 μm Nylon 66 filter membrane. The flow rate was set at 1.0 mL/min. The analysis was performed at room temperature.

The optimum UV wavelength for paraquat and diquat were 257 nm and 310 nm, respectively. For simulataneous monitoring, a common wavelength of 290 nm can be used for these two compounds. The samples was kept at 4°C in the sample tray.

Sample Preparation (Acidification)

Minimal sample preparation is required. 50 μL of phosphoric acid (2 M) was added to 1 mL sample to acidify the sample. Then it was centrifuged at 9500 x g for 1 minute at room temperarture. After the centrifugation, 20 μL of the supernatant was injected to the system directly.

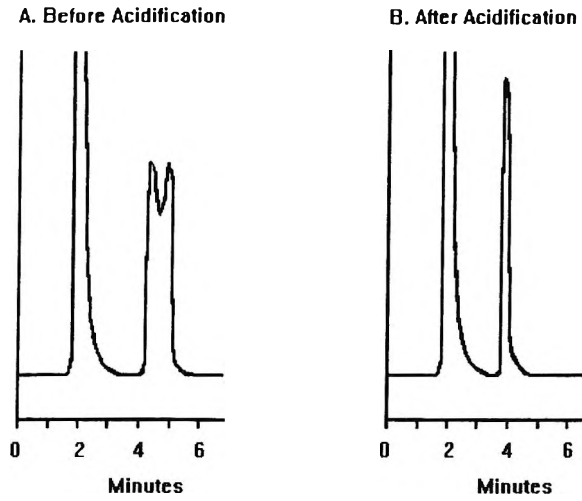

Figure 1. (A) A splitting peak of diquat was observed when urine sample was not acidified. (B) Splitting peak of diquat became a single peak after addition of 50 μL of 2 M phosphoric acid.

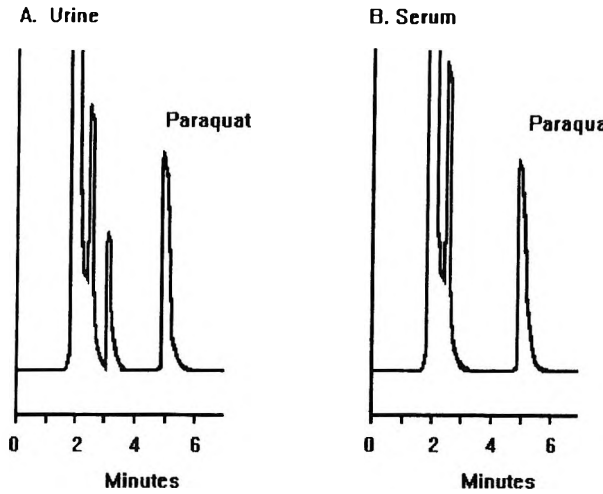

Figure 2. Chromatograms of paraquat in urine (A) and serum (B) with a detection at a UV wavelength of 257 nm.

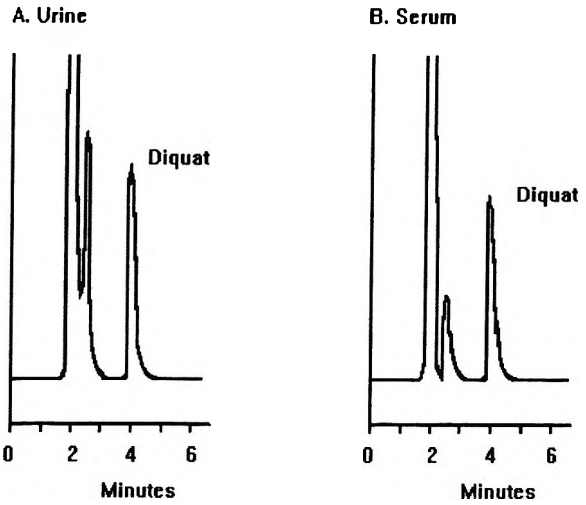

Figure 3. Chromatograms of diquat in urine (A) and serum (B) with a detection at a UV wavelength of 310 nm.

RESULTS AND DISCUSSION

Acidification of Sample

As shown in Figure 1-a, a splitting peak was observed when the spiked diquat in drug free urine was analyzed without any treatment. This might be due to the fact that there are components in the sample matrix which are adsorbed to the front end of the silica cartridge.[27] This problem was resolved by acidification of the sample matrix. As depicted in Figure 1-b, after an addition of 50 μL of 2 M phosphooric acid to 1 mL of sample, the split diquat peak became a single and sharp peak.

Analytical Separation

Figures 2-a and 2-b are chromatograms of paraquat standard at UV detection of 257 nm in urine and serum. Figures 3-a and 3-b are chromatograms of diquat at 310 nm in urine and serum.

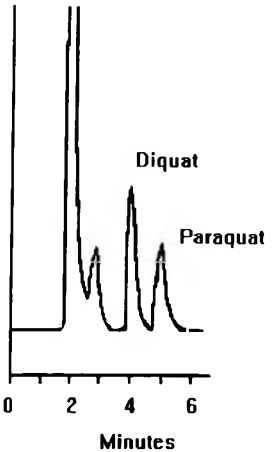

Figure 4. Chromatogram of mixture of diquat and paraquat at a UV wavelength of 290 nm in serum.

As shown in these four chromatograms, the endogenous components in both urine and serum matrices did not interfere with the detection of paraquat and diquat at either 257 nm or 310 nm. Figure 4 shows that a simultaneous monitoring can be conducted at a wavelength of 290 nm.

Linearity

A linearity study was conducted at the concentration range of 0.5 - 10 μg/mL (a total on-column amount of 10 - 200 ng). As shown in Table 1, a good linear coefficient is observed for paraquat and diquat in urine and serum samples. The peak height in serum was about 20% less than urine samples, and this was due to a slight broad peak for serum sample.

Reproducibility

As shown in Table 2, within-run study was performed by 10 consecutive runs at 5 μg/mL in both urine and serum. The CV% of retention data are less than 1.2% for both compounds. Paraquat showed a higher CV% of peak height in serum (8.18%).

Table 1

Linearity*

	Linearity	r=
Paraquat in Urine	Y = 7.6368X - 2.0199	0.99961
Paraquat in Serum	Y = 9.1716X + 0.4478	0.99971
Diquat in Urine	Y = 5.9876X - 0.9527	0.99989
Diquat in Serum	Y = 7.8980X - 0.5124	0.99999

*0.5 μg/mL to 10.0 μg/mL; = 5

Table 2

Within-Run Reproducibility*

Peak Height (Absorbance Unit)	**PQ in Urine**	**PQ in Serum**	**DQ in Urine**	**DQ in Serum**
Average	0.018	0.014	0.022	0.018
STDV	0.0003	0.0012	0.0003	0.0005
CV%	1.77	8.18	1.43	2.73
Retention Time (Minutes)				
Average	4.97	5.05	4.01	4.03
STDV	0.0350	0.0359	0.0467	0.0459
CV%	0.70	0171	1.16	1.14

* n = 10, 5μg/mL
Note: PQ: Paraquat, DQ: Diquat

Detection Limits

Clinical requirement

For paraquat, in most cases, blood concentrations greater than 2 μg/mL at 4 hours and 0.1 μg/mL at 24 hours are likely to be lethal, but individual

response is variable and some cases with much higher concentration have recovered. Therefore, the detection limit of paraquat must be at least 100 ng/mL or 2 ng on column.

Analytical detection

The detection limits were measured at 63 ng/mL (1.25 ng on column) and 125 ng/mL (2.50 ng/mL) for diaquat and paraquat, respectively. The detection wavelength for paraquat and diquat was 257 nm and 310 nm at 0.01 absorbance range, respectively. The detection limit was defined with a signal to noise ratio of 10. For a simultaneous monitoring of two compounds at 290 nm, paraquat could be detected at 10 ng (20 μL of 500 ng/mL) and 2.5 ng for diquat (20 μL of 125 ng/mL) in both urine and serum. A lower detection limit can be achieved by using a larger injection volume such as 50 μL.

Drug Interference Studies

The analytical cartridge of bare silica has been used for the separation of common drugs and metabolites with mobile phases at pH of 6.7[28] and 6.5[29] because of its weak cation exchange chromatographic behavior. In this study, with the adjustment of a low pH (3.0) and the addition of an ion pairing reagent to the mobile phase, all the common drugs and metabolites lost retention on the bare silica. They all eluted at the endogenous region (before 3 minutes) of sample matrices for this HPLC assay, and they did not cause any interference for the detection of paraquat and diquat. A total of 15 compounds were tested, including diazepam, amphetamine, imipramine, morphine, hydrocodone, benzoylecgonine, methamphetamine, methadone, codeine, trimethoprim, pseudoephedrine, nortriptyline, propoxyphene, EDDP (methadone metabolite), and dextromethorphan.

Additional tests were conducted on drugs which were strongly retained under the cation exchange chromatographic conditions;[28,29] chloroquine, strychnine, and nicotine. Again, all these three compounds eluted at the endogenous region (before 3 minutes) in this HPLC method, and they did not interfere with the detection of paraquat and diquat.

REFERENCES

1. G. H. Draffan, R. A. Clare, D. L. Davies, G. Hawksworth, S. Murray, D. S. Davis, J. Chromtogr., **139**, 311 (1977).

2. J. J. Haley, Clin. Toxicol., **14**, 1-46 (1979).

3. "Annual Case Reports of Drugs and Toxic Poisoning in Japan," Japan National Research Institue of Police Science, Tokyo, Japan, 1993 & 1994.

4. A. T. Proudfoot, M. S. Stewart, T. Levitt, B. Widdop, Lancet, **2**, 330-332 (1979).

5. K. Fletcher, **Forensic Sciences**, B. Ballantyne, ed., John Wright, Bristol, p.86, 1974.

6. S. L. Tompsett, Acta. Pharmacol. Toxicol., **28**, 346 (1970).

7. P. F. Lott, J. W. Lott, D. J. Doms, J. Chromatogr. Sci., **16**, 390-395 (1978).

8. T. L. Kuo, For. Sci. Int., 33, 177-185 (1987).

9. J. Knepil, Clin. Chim. Acta, 79, 387 (1977).

10. D. R. Jarvie, M. J. Stewart, Clin. Chim. Acta., **94**, 241-251 (1979).

11. B. Widdop, Brit. Med. J., **2**, 1135 (1976).

12. N. H. Choulis, J. Chromatogr., **168**, 562 (1979).

13. C. J. Soderquist, D. C. Crosby, Bull. Environ. Contam. Toxicol., **83**, 63 (1972).

14. S. Ukai, H. Hirose, S. Kawase, J. Hyg. Chem., **19**, 281 (1973).

15. Shozi Kawase, Susumu Kanno, Shigeo Ukai, J. Chromatogr., **283**, 231-240 (1984).

16. M. Yoshida, T. Watabiki, T. Tokiyasu, N. Ishida, J. Chromatogr., **628**, 235-239 (1993).

17. D. Baecelo, G. Durand, J. Chromatogr., **647**, 271-277 (1993).

18. I. Kambhampati, K. S. Roinsetad, T. G. Hartman, J. D. Rosen, E. K. Fukuda, R. L. Lippincott, R. T. Rosen, J. Chromatogr. A., **688**, 67 (1994).

19. Y. Kawano, J. Audino, M. Edlund, J. Chromatogr., **115**, 289-292 (1975).

20. R. Gill, S. C. Qua, A. C. Moffat, J. Chromatogr., **255**, 483-490 (1983).

21. A. Pryde, F. J. Darby, J. Chromatogr., **115**, 107-116 (1975).

22. I. Nakagiri, K. Suzuki, Y. Kuroda, N. Takatsu, A. Kohama, J. Chromatogr., **481**, 434-438 (1988).

23. M. C. Cameriro, L. Puignou, M. T. Galcern, J. Chromatogr., **669**, 217-224 (1994).

24. V. A. Simon, A. Taylor, J. Chromatogr., **479**, 153-158 (1989).

25. D. Fatori, W. M. Hunter, Clin. Chim. Acta., **100**, 81-90 (1980).

26. D. L. Colbert, R. E. Coxon, **34**, 1948-1949 (1988).

27. T. Fornstedt, J. Chromatogr., **612**, 137-144 (1933).

28. I. Jane, A. McKinnon, R. J. Flanagan, J. Chromatogr., **323**, 191 (1985).

29. S. R. Binder, M. Regalia, B. McEachern, M. Mazhar, J. Chromatogr., **473**, 325-341 (1989).

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IRON ASSAY AND SIZE EXCLUSION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF FERRITIN AND MAGNETOFERRITIN

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ABSTRACT

A size-exclusion HPLC method was developed for analysis of the ferritin and magnetoferritin samples. In the ferritin sample three peaks corresponding to aggregate and monomer were observed, which are in contrast to the magnetoferritin sample in which only two peaks corresponding to aggregate and monomer were observed. A sensitive spectrophotometric method was developed for assay of iron in the ferritin and magnetoferritin samples. The method involves solubilization of iron core by HCl or acetic acid, reduction of Fe^{3+} to Fe^{2+} with sodium dithionite or sodium sulfite, and reaction of Fe^{2+} with 2, 2' bipyridyl. Increasing concentration of acetic acid or sodium sulfite increased the iron content, suggesting that higher concentration of these reagents are needed to solubilize the iron core and to reduce Fe^{3+} to Fe^{2+}, respectively. An HPLC analysis of the protein after the experiment demonstrated that the proteins do not survive the analysis conditions.

INTRODUCTION

There are several proteins which contain iron or have strong affinity towards iron, e.g. the iron and oxygen transport proteins, Transferrin and Hemoglobin, respectively, and the iron and oxygen storage proteins, Ferritin and Myoglobin, respectively.[1] Ferritin, a water soluble protein, is synthesized in almost all tissues but is most abundant in liver, spleen, and bone marrow. Ferritin consists of a spherical polypeptide shell (apoferritin with molecular weights of 21 kDa and 19 kDa, respectively. The polypeptide shell surrounds a 450 kDa molecular weight), which has two different types of subunits, heavy (H) and light (L), crystalline hydrated iron oxide/phosphate core (6 nm in size), $(FeOOH)_8 .FeOPO_3H_2$, and can accommodate up to 4500 iron atoms.[2]

Methods for modification of the protein, Ferritin, such as changing the charge, introducing reactive and biologically active groups, radioiodination, and removal of iron from the protein pocket have been published.[3-9] Mann and coworkers[10] changed ferritin to magnetoferritin by replacing the iron core. In their experiments, they[10] removed the iron oxide/phosphate core from the ferritin sample by dialysis of the sample against thioglycolic acid, added Fe(II) slowly, followed by air oxidation to a mixture of Maghemite (γ-Fe_2O_3) and Magnetite (Fe_3O_4). Magnetoferritin has superparamagnetic character, high T_2 relaxivity, which is useful in MRI as a contrast agent.[11,12]

There are numerous reports on the characterization and analysis of ferritin. The structure of horse spleen apoferritin at 2.8 Å resolution and the amino acid sequence have been determined.[13,14] It is possible to isolate different iron containing fractions from a single ferritin preparation by density gradient centrifugation[15] and to determine the average molecular iron content by spectroscopic methods.[16] High Performance Liquid Chromatography (HPLC) was used[17] to analyze subunits in human liver and spleen ferritins and HPLC coupled with ICP-MS[18] and ICP-AES[19] were used to quantitate iron and protein. The average number of iron atoms were determined by an X-radiation absorption technique.[20] However, information on characterization and analysis of magnetoferritin is lacking.

Analysis of ferritin and magnetoferritin for their iron content and their purity is essential for their evaluation and characterization. Gravimetric, solvent extraction, emission spectrography, complexometric, and redox titrations, ICP analysis, and spectrophotometric methods can be used for iron assay of these proteins.[21] Among all of these methods, the spectrophotometric method is by far easiest and sensitive, accurate and precise. In the method, iron in the 2+ or 3+ oxidation state is complexed or chelated to form a highly

colored species which is measured using its visible absorbance spectrum. In the higher oxidation state iron may be complexed with excess sodium thiocyanate. There are two potential problems in using the method to analyze magnetoferritins: (1) The method requires working at low pH due to hydrolysis of Fe^{3+} above pH 2. Under these conditions the protein may decompose into small fragments, and (2) The iron in ferritin or magnetoferritin is a mixture of 2+ and 3+ oxidation states, consequently, one has to use an oxidizing agent such as hydrogen peroxide. An excess of the oxidizing agent may also oxidize the coordinating ligand, thiocyanate, and may give erroneous results.

Iron in the lower oxidation state, Fe^{2+}, is usually determined by its reaction with 2,2' bipyridyl to form $Fe(bpy)_3^{2+}$ followed by it absorbance measurement at 522 nm. Drysdale and Munro[22] used this method to determine iron in ferritin samples. Acetic acid rather than H_2SO_4, $HClO_4$, or HNO_3 was used in the analysis to avoid acid-catalyzed cleavage of the protein as the recovery of the protein after analysis was desirable. No validation of the method or proof of recovery of the protein was given in the report.

The goals of the present work were: (a) to reexamine and develop analytical methods to determine iron content in ferritin and magnetoferritin samples, respectively, (2) to develop a size-exclusion chromatographic (HPLC) method to investigate aggregation of the protein, and (3) to investigate if the protein can cope with these analysis conditions and can be recovered after the iron assay for further use.

MATERIALS AND METHODS

Horse spleen ferritin, (lot # 33H 70302) was purchased from Sigma Chemicals (St. Louis, MO) with 12.0 and 108 mg/mL iron and protein concentrations, respectively. A sample of horse spleen ferritin (100 times diluted Sigma horse spleen ferritin which would correspond to 0.12 and 1.08 mg/mL iron and protein concentrations, respectively) was used in the present work. The magnetoferritin samples were received from Dr. S. Mann of the University of Bath (England). Sodium Chloride, Sodium Hydroxide, Sodium Dihydrogen Phosphate, Sodium Acetate, Acetic Acid, 1.0 N Hydrochloric Acid (all from Fisher), Sodium Azide, Sodium Dithionite, Sodium Sulfite (all from Aldrich), and HEPES, and 2, 2' Bipyridyl (all from Sigma) were used as is. The pH of the buffer was adjusted with Hydrochloric Acid and Sodium Hydroxide (both from Fisher) in the case of HEPES and phosphate buffers, respectively. Distilled deionized water was used for all solution preparations.

Solution pH values were measured with an Orion combination glass electrode and an Orion pH meter model 740. All spectrophotometric measurements were made with an HP 8452A diode-array spectrophotometer interfaced to an HP-310 data station. Solutions for iron content determination were heated in a Fisher heating bath.

The HPLC was a two-pump system (Rainin Instruments) with a Rheodyne injection valve containing a 50 μL loop. The HPLC system was interfaced with a Macintosh SE computer. An Applied Biosystem UV-Vis detector at 280 nm was used. Integration of peak areas was performed using Dynamax software. A silica based size-exclusion column, Bio Sil SEC-400 (300 x 7.8 mm, Bio-Rad), with a molecular weight range of 5,000 to 1,000,000, was used. A Bio Rad protein standard was used for size (molecular weight) vs. retention time calibration curve. The mobile phase condition used was: 0.15 M NaCl, 0.050 M Na_2HPO_4/ NaH_2PO_4 at pH 7.0 (containing 0.01% Sodium Azide). In some experiments HEPES buffer was used in the mobile phase. In all experiments the flow rate was 1.0 mL/min.

For iron assay, experiments were carried out using the literature method[22] (Method I) under variable conditions to validate the method (*vide infra*). The method requires the presence of 75 mM sodium sulfite, 6% acetic acid (1.04 M), and 0.05% 2,2' bipyridyl and heating at 100°C in a water bath for 1 h. The literature method was also modified to determine iron content in the protein samples (method II). In method II: 1.0 mL protein was mixed with 0.1 mL of 1.0 M HCl solution in a scintillation vial. The mixture was incubated at 80°C for 1 h and then cooled to room temperature. 0.5 mL buffered (0.5 M Sodium Acetate, pH 5.3) oxygen free $Na_2S_2O_4$ (10 mM) solution was added to the mixture followed by addition of 0.5 mL 2,2' bipyridyl (15 mM in 0.05 M HCl) solution. The mixture was heated again at 80°C for 1 h to ensure the complete formation of $Fe(bpy)_3^{2+}$. The solution was taken into a volumetric flask and the final volume was made up to 25.0 mL with water and the pH was adjusted to 4.0. The iron content was calculated from a knowledge of the absorbance at 522 nm and the molar extinction coefficient of $Fe(bpy)_3^{2+}$ ($\varepsilon = 8.65 \times 10^3\ M^{-1}\ cm^{-1}$).[23]

RESULTS AND DISCUSSION

Size-Exclusion Chromatography of Ferritin and Magnetoferritin

A Bio-Rad standard was initially injected to calibrate the peak positions as a function of size (molecular weight) of the macromolecules (Figure 1). The

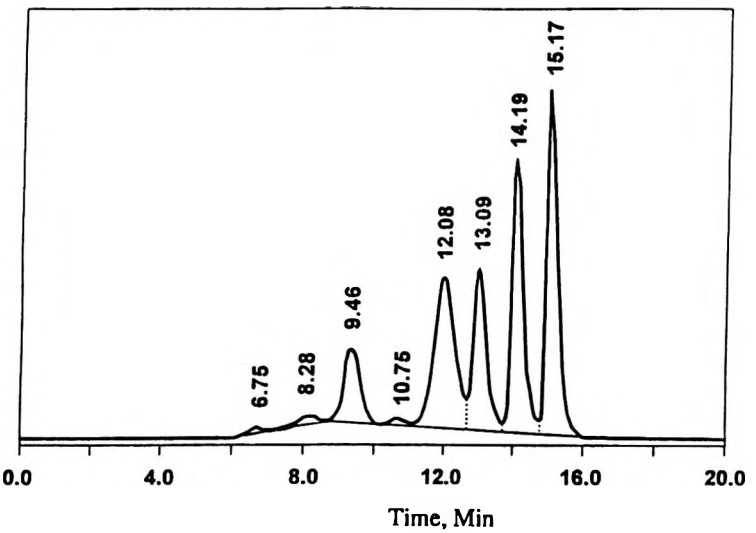

Figure 1. HPLC chromatogram of the Bio Rad standard. The HPLC conditions used are: Column: Bio-Sil SEC-400, Mobile Phase: 0.15 M NaCl, 0.050 M Na_2HPO_4/NaH_2PO_4 at pH 7.0 (containing 0.01% sodium azide), Detector: UV/Vis at 280 nm, and Flow Rate: 1.0 mL/min.

Bio Rad standard is a mixture of five proteins (Mwt in kDa and retention times in minutes are given in parenthesis) and these are: Thyroglobulin (670, 9.5), Immunoglobulin (158, 12.1), Ovalbumin (44, 13.1), Myoglobin (17, 14.2), and Vitamin B_{12} (1.35, 15.1). Smaller peaks at 6.7 and 8.25 minutes were also observed; they are due to aggregates of proteins. When a horse spleen ferritin sample was injected onto a size-exclusion column, three peaks were observed, a first peak at 8.7 min (~35%), a second peak at 9.7 min (~20%), and followed by a third peak at 11.2 min (~45%) (Figure 2). The size of ferritin or magnetoferritin is the same as the size of apoferritin. This is due to the fact that all the iron, $(FeOOH)_8.FeOPO_3H_2$ in the case of ferritin and a mixture of Fe_2O_3 and Fe_3O_4 in the case of magnetoferritin, is inside the pocket of the protein. This suggests that the peaks eluting at 8.8 and 9.8 minutes are probably due to the mixtures of aggregates which could not be separated by this column, and the last peak is a monomer of horse spleen ferritin. Similar chromatographic behavior was observed in the presence of HEPES buffer. Aggregation of ferritin is not reported in the past. The source of aggregation may be the sample. More work is in progress to resolve this. Experiments were carried out with the magnetoferritin samples also, and two peaks were observed (Figure 3). The first peak which appeared at 6.6 minutes corresponds to the aggregate and the monomer of the protein eluted at 11.2 minutes (450

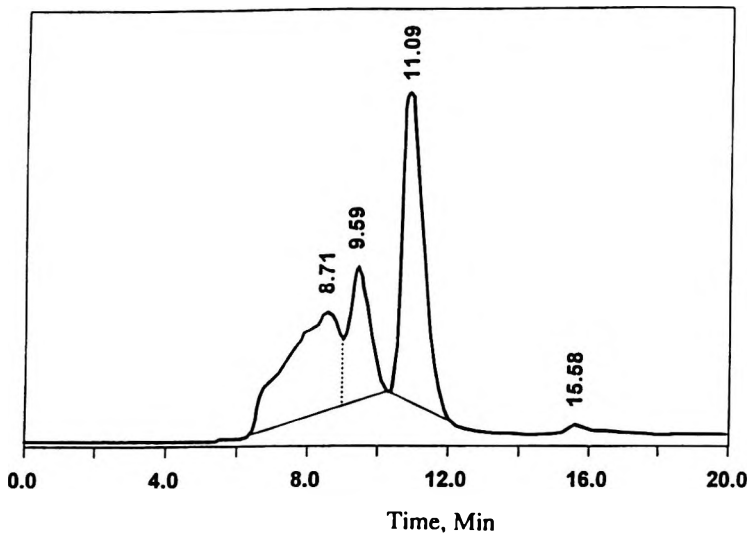

Figure 2. HPLC chromatogram of ferritin, the HPLC conditions are same as in Figure 1.

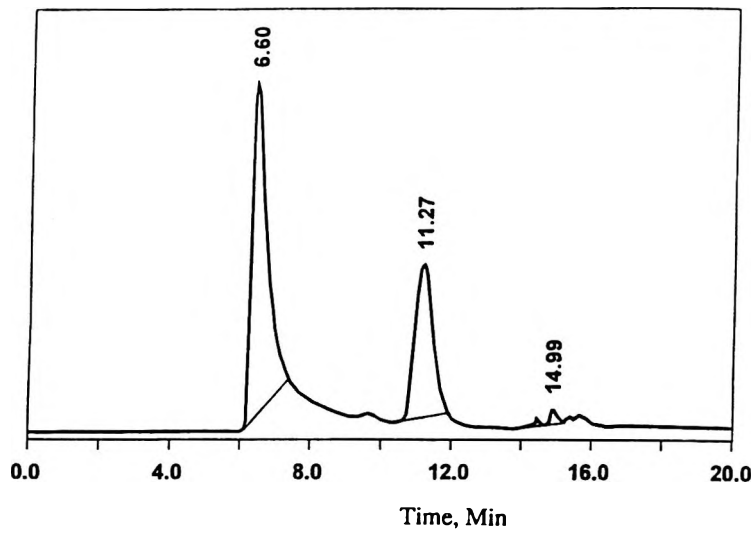

Figure 3. HPLC chromatogram of magnetoferritin, the HPLC conditions are are same as in figure 1.

kDa molecular weight). The ratio of the peak areas was 65:35. Some HPLC experiments with magnetoferritin were carried out at a lower concentration of salt or in the absence of sodium chloride in the mobile phase. The monomer peak was less retained by lowering the concentration of salt. In the absence of salt both peaks coeluted at 6.6 min. This observation is consistent with the previous results that many proteins can undergo hydrophobic interaction with the stationary phase at high ionic strength.[24,25]

Ultrasonication of magnetoferritin was attempted to disrupt the aggregation of the protein. The ratio of the aggregate and monomer remained the same, however, several minor peaks with retention times between 15 and 20 minutes were seen. This suggests that the ultrasonication breaks protein subunits into smaller molecules which have molecular weights <1 kDa as the retention time of Vitamin B_{12} (with molecular weight of 1.35 kDa) is 15.1 minutes.

Iron Assay

Initially a sample of ferritin and four samples of magnetoferritin with variable amounts of iron were analyzed by method I.[22] The pH of the mixture was measured as 3.6. The same samples were analyzed by method II and the values are compared in Table 1. From the table two observations can be made: (1) the iron content determined by method II is consistently higher than the values determined by method I and (2) the difference is lower in the case of ferritin (<5%) than magnetoferritin (11-30%).

The difference in the final pH (3.6 vs. 4.0) in the two methods was thought to be responsible for this discrepancy. The ligand protonation constant of 2,2' bipyridyl is reported as 4.47 at 25°C and $\mu = 0.1$ (KCl).[26] The stepwise stability constants (log K_1, log K_2, and log K_3) of its Fe^{2+} complexes are known to be 4.20, 3.70, and 9.55, respectively.[26] These constants were used to calculate the percentage of $Fe(bpy)_3^{2+}$ complex formed under different pH conditions.[27] Under the experimental conditions (for example: 0.019 mM Fe^{2+}, 0.30 mM bpy, pH 3.6) these calculations revealed 99.9% formation of $Fe(bpy)_3^{2+}$. In other cases the ratio of 2,2' bipyridyl to Fe^{2+} was much higher than this experiment and the percentage of $Fe(bpy)_3^{2+}$ formed should be 100%, suggesting that the pH difference can not explain the difference in iron values determined by the two methods. Moreover, additional experiments were carried out in which pH was raised from 3.6 to 4 and no change in the absorbance was observed.

Table 1

Analysis of Ferritin and Magnetoferritin Samples for the Iron Content by Method I[22] and Method II

Sample #	Method I, μg/mL	Method II, μg/mL
Ferritin	113.8 ± 0.9	119.4 ± 0.7
Magnetoferritin Sample #1	20.7 ± 1.0	26.9 ± 0.3
Magnetoferritin Sample # 2	20.9 ± 0.2	27.0 ± 0.6
Magnetoferritin Sample # 3	27.8 ± 1.0	30.8 ± 0.7
Magnetoferritin Sample # 4	25.6 ± 0.9	29.6 ± 0.9

The iron concentration by the two methods differed by only 4.6% in the case of ferritin, while the difference was as high as 30% in magnetoferritin samples (Table 1). This was thought to be due to the different iron cores in the two proteins, i.e. ferritin contains $(FeOOH)_8.FeOPO_3H_2$ whereas a mixture of Fe_2O_3 and Fe_3O_4 was introduced in the modified protein, magnetoferritin. It is possible that 6% acetic acid can not solubilize iron core in ferritin and, less so, in magnetoferritin. The iron content in ferritin and magnetoferritin samples were determined under variable acetic acid concentrations. The percentage of acetic acid was varied between 0.175 M and 2.58 M (1% to 15% volume by volume). The iron content was found to increase with the increased acetic acid concentration (Table 2, Figure 4). The increase in Fe content was 33.3% for ferritin samples and 59.3% in the magnetoferritin samples. This is probably due to the difference in the nature of iron cores in the two proteins. The iron content in the two proteins at higher acetic acid concentration agrees with the iron content determined by method II. For example, the values of iron content in the ferritin sample are: 119.4±0.7 μg/mL and 122.7±0.8 μg/mL by method II and by method I (with high concentration of acetic acid), respectively. Similarly, the iron content in the magnetoferritin sample are 30.8±0.7 μg/mL and 29.51±0.08 μg/mL by these two methods, respectively. In summary, the data given in Table 2, suggest that a higher concentration of acetic acid is required for solubilization of all iron in the protein cores rather than just 6%.

Experiments were also carried out to investigate the effect of reducing agent Na_2SO_3 on the iron concentration (Table 2, Figure 5) at 0.975 M acetic acid and 3.2 mM 2,2` bipyridyl concentration. The amount of iron increased gradually from 95.9±0.4 μg/mL to 113.8±0.9 μg/mL (15.7%) as the concentration of Na_2SO_3 increased from 10 mM to 75 mM. Under similar conditions, the iron concentration of the magnetoferritin sample increased from

Table 2

Iron Content Determination in Ferritin and Magnetoferritin Samples Under Different Experimental Conditions

S.N.	pH Range	$[CH_3CooH]$[a]	$[Na_2SO_3]_b$	[Bipyridyl][b]	Ferritin		Magnetoferritin	
					Absorbance	[Fe], μg/mL[c]	Absorbance	[Fe], μg/mL[c]
1	3.12-3.19	2.58	75.0	3.20	0.380 ± 0.003	122.7 ± 0.8	0.1828 ± 0.0005	29.51 ± 0.08
2	3.28-3.40	1.72	75.0	3.20	0.371 ± 0.006	119.6 ± 1.8	0.170 ± 0.002	27.43 ± 0.24
3	3.54-3.63	0.975	75.0	3.20	0.352 ± 0.003	113.8 ± 0.9	0.164 ± 0.003	26.5 ± 0.5
4	3.69-3.79	0.65	75.0	3.20	0.347 ± 0.009	112.0 ± 2.9	0.136 ± 0.003	22.01 ± 0.52
5	3.88-4.05	0.335	75.0	3.20	0.253 ± 0.015	81.8 ± 4.8	0.106 ± 0.005	17.17 ± 0.77
6	4.20-4.26	0.175	75.0	3.20	0.222 ± 0.008	71.8 ± 2.5	0.0744 ± 0.004	12.0 ± 0.6
7	3.54-3.63	0.975	75.0	3.20	0.352 ± 0.003	113.8 ± 0.9	0.164 ± 0.003	26.5 ± 0.5
8	3.35-3.50	0.975	50.0	3.20	0.343 ± 0.005	110.6 ± 1.5	0.167 ± 0.003	27.0 ± 0.5
9	3.21-3.30	0.975	25.0	3.20	0.323 ± 0.001	104.4 ± 0.3	0.167 ± 0.002	26.9 ± 0.3
10	2.74-2.85	0.975	10.0	3.20	0.297 ± 0.001	95.9 ± 0.4	0.150 ± 0.001	24.2 ± 0.2
11	3.50-3.60	0.975	75.0	12.8	0.358 ± 0.006	115.5 ± 1.9	0.163 ± 0.003	26.3 ± 0.4
12	3.38-3.53	0.975	75.0	6.20	0.352 ± 0.001	113.5 ± 0.4	0.161 ± 0.002	26.0 ± 0.4
13	3.40-3.50	0.975	75.0	1.60	0.347 ± 0.007	112.1 ± 2.2	0.162 ± 0.002	26.1 ± 0.4
14	3.42-3.53	0.975	75.0	0.80	0.344 ± 0.005	110.9 ± 1.5	0.157 ± 0.002	25.3 ± 0.3

[a] Concentration in M
[b] Concentration in mM
[c] Calculated iron content in the stock solution, N = 4

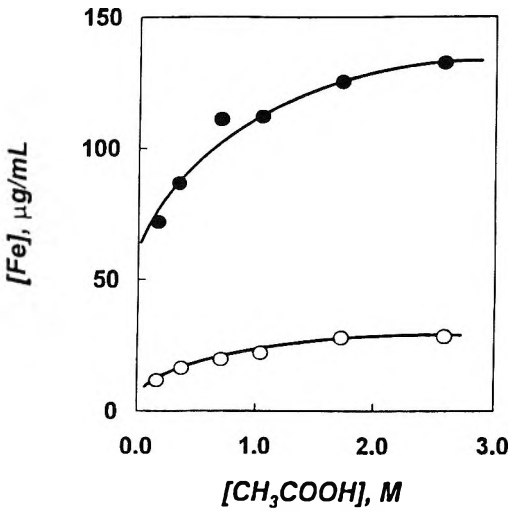

Figure 4. Plot of Fe content in the ferritin (●) and magnetoferritin (o) samples vs. [Acetic Acid].

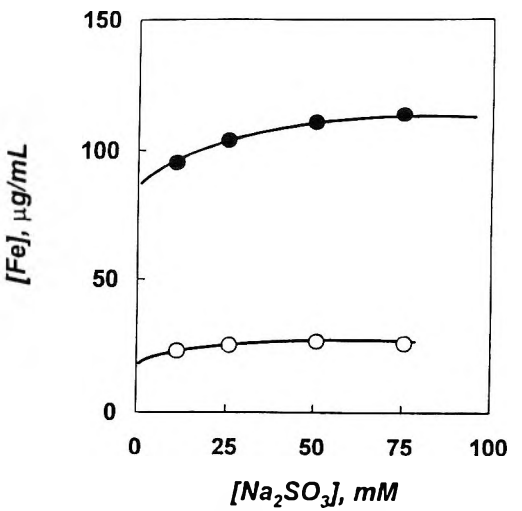

Figure 5. Plot of Fe content in the ferritin (●) and magnetoferritin (o) samples vs. [Na_2SO_3].

24.2±0.2 μg/mL to 27.0±0.5 μg/mL (10%). The plot given in Figure 5, suggests that 75 mM sodium sulfite is the optimum concentration of the reducing agent for these determinations. A higher concentration of sodium sulfite may be needed for samples containing higher concentrations of iron in the protein samples.

Three equivalents of 2,2' bipyridyl are needed for the formation of $Fe(bpy)_3^{2+}$. In the present work, the concentration of 2,2' bipyridyl was varied from 0.8 mM to 12.8 mM, which translates into the bipyridyl to iron ratio of 4 to 64 for ferritin and 16 to 256 for magnetoferritin. The iron concentration was found to be constant in the 2,2' bipyridyl concentration range studied, suggesting that >4 equivalents of bipyridyl concentration is sufficient to determine iron in these samples. Similar results were obtained from the species distribution calculations under these concentration conditions.[27]

The samples of proteins were heated at 70-80°C for iron concentration determination. The determination was also performed at room temperature. Upon addition of Na_2SO_3, 2, 2' bipyridyl, and acetic acid, a pink/red color developed slowly. The completion of color development took 10 h for ferritin and several days for magnetoferritin analysis at room temperature. The rate of the reaction of Fe^{2+} with 2,2' bipyridyl was measured by Wilkins and coworkers[28] in the pH range of 6.0 to 6.8. They calculated a second-order rate constant for the reaction of Fe^{2+} with 2,2' bipyridyl as 1.1×10^5 $M^{-1}s^{-1}$. From these data one can calculate a half-life of the reaction of the order of milliseconds. However, in the present work, acidic conditions were used where the majority of the bipyridyl is in the less reactive protonated form. The ligand protonation constant of 2,2' bipyridyl is 4.47. Krumholz[29] reported an initial rate of reaction of Fe^{2+} (1.37×10^{-5} M) with $H(bpy)^+$ (1.66×10^{-4} M) at $[H^+]$ = 1.60×10^{-3} M (-log $[H^+]$ = 2.8) as 1.85×10^{-6} M min^{-1}. A half-life of the reaction under our experimental conditions can be calculated to be less than 1 h at room temperature. Contrary to this, a longer reaction time at room temperature was observed in the present work, which could be attributed to the slow solubilization of iron core followed by its reaction with 2,2' bipyridyl under acetic acid conditions.

Recovery of the Protein

We used size-exclusion HPLC analysis of the mixture of the protein, 2,2' bipyridyl, sodium sulfite, and variable concentrations of acetic acid. In all of the cases, two broad peaks with retention times of 20 and 33-34 minutes, were observed. The first peak was identified due to $Fe(bpy)_3^{2+}$, as confirmed by an injection of preformed $Fe(bpy)_3^{2+}$. The second peak, which was observed even

at a lowest acetic acid concentration (0.175 M), was assigned to the fragments of the protein. No peak at 11 minutes was observed, suggesting that the proteins can not survive these analysis conditions.

CONCLUSION

A size-exclusion HPLC analysis of ferritin and magnetoferritin demonstrated the formation of aggregates of ferritin and magnetoferritin. Higher concentration of acetic acid is required for determination of iron in the two protein samples. The proteins can not be recovered after iron assay.

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REFERENCES

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1. For some excellent reviews see: (a) J. B. Neilands in **Advances in Experimental Medicine and Biology**, S. K. Dhar, ed. Plenum Press, New York, NY 1973; (b) E. L. Eichhorn, **Inorganic Biochemistry**, Elsevier, New York, NY 1973; (c) P. Aisen, I. Listowski, Ann. Rev. Biochem., 49, **357** (1989); (d) P. M. Harrison, R. J. Hoare, T. G. Hoy, I. G. Macara in **Iron in Biochemistry and Medicine**, A. Jacobs, M. Worwood. eds, Academic Press, London, 73 (1974); (e) C. D. Michaaelis, C. D. Coryell, S. Granick, J. Biol. Chem., **148**, 463 (1943); (f) J. W. Drysdale, T. G. Adelman, P. Arosio, F. D. Casareale, J. T. Hazard, J. T., M. Yokota, Seminar Hematol., **15**, 71 (1977); (g) P. Aisen, I. Listowski, Ann. Rev. Biochem., **49**, 357 (1980); (h) E. Chiancone, A. E. Stefanini, Adv. Rev. Cell Biol., **19**, 19 (1982); (I) G. C. Ford, P. M. Harrison, D. W. Rice, J. M. A. Smith, A. Treffry, J. L. White, J. Yariv, J. Phil. Trans. Royal Society London, **304**, 551 (1984).

2. N. Ghinea, et al., J. Submicroscopic Cytol., **18**, 647 (1986).

3. V. J. Muresan, V. J. Submicroscopic Cytol., **19**, 375 (1987).

4. S. Irie, Am. J. Anatomy, **177**, 403 (1986).

5. E. A. Sprague, Exp. Molecular Pathol., **48**, 373 (1988).

6. S. Irie, et al., Am. J. Anatomy, **177**, 403 (1986).

7. F. Antohe, et al., Endothelium Lymphatacis, **3**, 163 (1986).

8. M. Worwood, S. J. Cragg, A. M. Williams, M. Wagstaff, A. Jacobs, Blood, **60**, 827 (1982).

9. (a) S. Sirivech, Biochem. J., **143**, 311 (1974); (b) S. Granick, L. Michaelis, J. Biol. Chem., **147**, 91 (1943).

10. (a) F. C. Meldrum, V. J. Wade, D. L. Nimmo, B. Heywood, S. Mann, Nature, **349**, 684 (1991); (b) F. C. Meldrum, B. R. Heywood, S. Mann, S. Science, **257**, 522 (1992).

11. J. W. M. Bulte, T. Douglas, S. Mann, R. B. Frankel, B. M. Hoskowitz, R. A. Brooks, C. D. Baumgarner, J.Vymazal, M. -P. Strub, J. A. Frank, Mag. Res. Imag., **4**, 497 (1994).

12. R. B. Lauffer, Chem. Rev., **87**, 901 (1987).

13. D. W. Rice, G. L. Ford, J. L. White, J. M. A. Smith, P. M. Harrison, in **Advances in Inorganic Biochemistry**, ed. E. C. Theil, G. L. Eichhorn, L. Marzilli, Elsevier, New York, NY 1983, pp 39.

14. M. Heusterspreute, R. R. Crichton, FEBS Letters, **129**, 322, (1981).

15. F. Fischbach, J. W. Andregg, J. Mol. Biol., **14**, 458 (1965).

16. P. M. Harrison, T. G. Moy, I. G. Macara, R. J. Hoare, Biochem. J., **143**, 445 (1994).

17. J. F. Collawn, H. Donato, J. K. Upshur, W. W. Fish, Comp. Biochem. Biophys., **81B**, 901 (1988).

18. L. W. M. Owen, H. M. Crews, R. C. Hutton, A. Walsh, Analyst, **117**, 649 (1992).

19. F. La Torre, N. Violanate, O. Senofonte, C. D'Arpino, S. Caroli, Spectroscopy, **4**, 48.

20. J. M. A. Smith, J. R. Hellwell, M. Z. Papiz, Inorg. Chim. Acta., **106**, 193 (1985).

21. A. I. Vogel, **A Text Book of Quantitative Inorganic Analysis, Third Edition,** Longman: London, 1961.

22. J. W. Drysdale, H. N. Munro, Biochem. J., **95**, 851 (1965).

23. J. Moss, G. Mellon, Indust. Chem. Anal. Educ., **14**, 862 (1942).

24. D. E. Schmidt, J. W. Giese, D. N. Conron, B. L. Karger, Anal. Chem., **52**, 177 (1980).

25. H. Engelhardt, D. Mathes, Chromatographia, **14**, 325 (1981).

26. H. Irving, H. Mellor, J. Chem. Soc., 5222 (1962).

27. A. E. Martell, R. J. Motekaitis, **Determination and Use of Stability Constants**, VCH Publishers, 1992.

28. R. H. Holyer, C. D. Hubbard, S. F. A. Kettle, R. G. Wilkins, Inorg. Chem., **4**, 929 (1965).

29. P. Krumholz, Nature, **163**, 724 (1949).

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FUMARIC ACID IN RAT PLASMA, URINE, AND FECAL SAMPLES

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ABSTRACT

Fumaric acid, a Krebs cycle intermediate, is a potential cancer chemoprevention agent. A high performance liquid chromatographic procedure with UV detection for determination of fumaric acid in large numbers of rat plasma, urine and fecal samples was developed. Fumaric acid was extracted from

plasma, urine, and fecal samples utilizing solid phase extraction using Clean Up® Quaternary Amine 1 mL (plasma, fecal samples) and 3 mL (urine) extraction columns followed by reverse phase high performance liquid chromatography with UV detection at 215 nm. Standard curves for plasma (1 μg/mL - 200 μg/mL), urine (5 μg/mL - 200 μg/mL), and fecal material (25 μg/g - 500 μg/g) were analyzed and replicate analysis of controls were used to determine intra-day and inter-day variability. Chlorofumaric acid was used as an internal standard for plasma and fecal samples; trans-glutaconic acid for urine samples. Precision and accuracy were studied using control solutions (low and high) prepared in naive rat plasma, urine, and fecal material. Intra-day variability was determined using 3 - 6 replicates of each control solution on a single day. Coefficient of variation (CV) for 20 μg/mL control (low) in plasma was 6.11% and for 80 μg/mL (high) was 7.07%; relative accuracy (RA) was 0% and 6.01%, respectively. CV for 15 μg/mL low control in urine was 38.95% and for 150 μg/mL high control was 8.84%; RA values were -12.2% and -15.16%, respectively. For the fecal material, CV for low control (100 μg/g) was 0.92% and for the high control (400 μg/g) was 4.67%. RA values were -4.02% and -4.14%, respectively. Inter-day variability was determined over a four day period. For the 20 and 80 μg/mL plasma control solutions, CVs were 12.97% and 9.09%, respectively, and RA values were 1.1% and 4.04%, respectively. For the 15 and 150 μg/mL urine control solutions, CVs were 26.85% and 14.17%, respectively, and RA values were 33.07% and -1.24%, respectively. For the 100 and 400 μg/g fecal material controls, CVs were 2.5% and 4.39%, respectively, and RA values were -4.07% and -3.87%, respectively. The standard curves for plasma, urine, and fecal samples were linear over the range of fumaric acid assayed and the means for the regression coefficient were 0.9939, 0.9972, and 0.9956, respectively. The limits of quantitation for plasma, urine, and fecal material were 1 μg/mL, 5 μg/mL and 25 μg/g, respectively.

INTRODUCTION

Fumaric acid, a naturally occurring metabolic intermediate, is currently undergoing preclinical development by National Cancer Institute as a cancer

chemoprevention agent.[1] It has been shown to inhibit hepatocarcinogenesis in rats induced by 3'-methyl-4-(dimethylamino)azobenzene (0.06% in diet for 50 days) when given by dietary admixture (1%) and in drinking water (0.025%) for 51 weeks.[2,3] Fumaric acid also reduced the hepatocarcinogenicity of mitomycin C and aflatoxin B1.[4] Dietary administration of fumaric acid totally suppressed hepatocarcinogenesis in mice[5] and rats[6] fed thioacetamide for 40 weeks when followed by fumaric acid treatment for 48 weeks. An inhibitory effect of fumaric acid on forestomach and lung carcinogenesis induced by a 5-nitrofuran naphthydrine derivative in mice has also been evident.[7]

Fumaric acid is presently used to treat psoriasis vulgaris. This approach was initiated by the German biochemist Schweckendiek and standardized by Schäfer.[8,9] Current fumaric acid therapy consists of the daily oral intake of dimethyl fumaric acid ester or monoethyl fumaric acid ester,[8,9,10,11,12] as fumaric acid itself is poorly absorbed from the gastrointestinal tract.[8] Topical application of fumaric acid and monoethyl fumaric acid in ointment has also been used as a supporting treatment.[8] A variety of serious side effects such as lymphopenia with a decrease of T lymphocytes and nephrotoxicity (acute tubular necrosis), nausea, diarrhea, severe stomach ache, or skin irritation and contact urticaria have been reported during fumaric acid and/or fumaric acid esters therapy.[8,13]

A gas chromatographic method can be used for the determination of fumaric acid and other carboxylic acids in variety of biological matrices. Tsuda et al.[14] developed a gas chromatography method with a flame ionization detector for the determination of fumaric acid and other carboxylic acids in soft drinks and jams. All carboxylic acids were extracted using anion exchange columns. Gas chromatography - mass spectrometry methodology was described for determination of carboxylic acids including fumaric acid in rat tissues as their tert-butyldimethylsilyl derivatives.[15] In another study, concentrations of carboxylic acids as their benzyl esters were measured in human serum, urine, and rat heart ventricle using gas chromatography and capillary column with flame ionization detection.[16] High performance liquid chromatography (HPLC) has been successfully applied for determination of several carboxylic acids in food and beverages as their p-nitrobenzyl esters,[17] in wines and different beverages after derivatization,[18] in human blood,[19] anaerobic bacteria cultures,[20] and routinely in wines and champagne.[21]

The purpose of this study was to develop and evaluate a simpler, precise, accurate and fast HPLC analytical method with isocratic elution for quantitation of fumaric acid in complex biological matrices, ie. plasma, urine, and feces.

MATERIALS AND METHODS

Chemicals

Fumaric acid, rat plasma, urine, and rat fecal material were supplied by the Toxicology Research Laboratory, Department of Pharmacology, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA. Chlorofumaric acid and trans-glutaconic acid were purchased from Aldrich Chemical Company, Milwaukee, WI, USA. Acetonitrile, methanol, potassium phosphate dibasic, sodium fluoride, potassium phosphate monobasic, and o-phosphoric acid (85%) were purchased from Fisher Scientific, Itasca, IL, USA, and were HPLC grade. Physiological saline solution was acquired from Baxter Diagnostics Inc., McGaw Park, IL, USA. Clean Up® Quaternary Amine 1 mL and 3 mL extraction columns were acquired from Worldwide Monitoring, Horsham, PA, USA.

HPLC Instrumentation

Fumaric acid concentrations in plasma, fecal material, and urine were determined isocraticaly using an HPLC system. Chlorofumaric acid was used as an internal standard for plasma and fecal samples; trans-glutaconic acid was used as an internal standard for urine samples. A Waters 600E Solvent System, Waters 484 Tunable UV/VIS Detector, and a Waters 746 Data Module (Waters Chromatography Division, Milford, MA, USA) were used. The samples were introduced to the analytical column via a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA). For plasma and fecal samples, separation was achieved using a C_{18} Beckman Ultrasphere 5 μm, 250 x 4.6 mm analytical column purchased from Alltech Associates, Inc., Deerfield, IL, USA; for urine samples, a C_{18} Waters μBondapak 10 μm, 300 x 3.9 mm (Waters Chromatography Division, Milford, MA, USA) was used. The Rheodyne 7125 injector was equipped with a 20 μL sample loop and a C_{18} precolumn (Guard-Pak, Waters Chromatography Division, Milford, MA, USA) in a precolumn holder.

Quality Control

Quantitation of fumaric acid in rat plasma, urine, and fecal samples was performed using an internal standard method. The standard curves were determined by linear least squares regression analysis of the ratio of peak area of fumaric acid to peak area of the internal standard as a function of

concentration. Control solutions were prepared in naive rat urine. Concentrations of fumaric acid in naive urine were measured against standard curves prepared in 0.9% saline. Standards and controls for plasma and fecal material were prepared in spiked naive rat plasma and fecal material, respectively. Standard solutions and controls were prepared with each set of plasma, urine, and fecal samples. The concentration range of the standard curve for plasma was 1 μg/mL to 200 μg/mL; for urine it was 5 μg/mL to 200 μg/mL; and for fecal material it was 25 μg/mL to 500 μg/mL. A 1,000 μg/mL fumaric acid standard stock solution, 800, 1,000, and 2,000 μg/mL control stock solutions, and 1 mg/mL and 2 mg/mL solutions of internal standards (chlorofumaric acid and trans-glutaconic acid, respectively) were prepared in 0.9% physiological saline solution.

Two levels of control solutions were prepared for each specimen (20 and 80 μg/mL for plasma, 15 and 150 μg/mL for urine and 100 and 400 μg/g for fecal samples) and were analyzed. Precision and accuracy were determined by analyzing controls prepared in naive rat plasma, urine, and fecal material. Intra-day variability was determined using 3-6 replicates of each control solution analyzed on a single day. Inter-day variability was determined over a four day period analyzing replicates of each control solution. Relative accuracy (%) was determined employing the following equation:

RA = [(Mean Measured Conc. - Theoretical Conc.)/Theoretical Conc.] x 100%

Extraction Procedures

All plasma samples (0.2 - 0.8 mL) were diluted to 2 mL with 0.9% saline. All control solutions, standards and samples were vortex mixed, and 1 mL of 0.1 M phosphate buffer, pH 5, and 100 μL of internal standard (chlorofumaric acid) were added to each solution. All urine samples were mixed and 1 mL of each urine sample was centrifuged for 5 minutes at 13,605 *g*. A 0.25 mL volume of urine was transferred to a test tube and a 0.75 mL volume of 0.1 M potassium phosphate dibasic buffer, pH 5, was added. A 0.5 mL volume of 0.1 M potassium phosphate dibasic, pH 5, was then added to all controls and standards. A 25 μL volume of internal standard (trans-glutaconic acid) was added. All controls, standards and samples were vortex mixed.

Fecal samples were prepared as follows: each sample was weighed, placed into a mortar, and macerated with a pestle. Three mL of 0.1 M potassium phosphate monobasic buffer, pH 5, was added to each gram of fecal material. A homogenous paste was prepared and transferred to a 50 mL plastic tissue

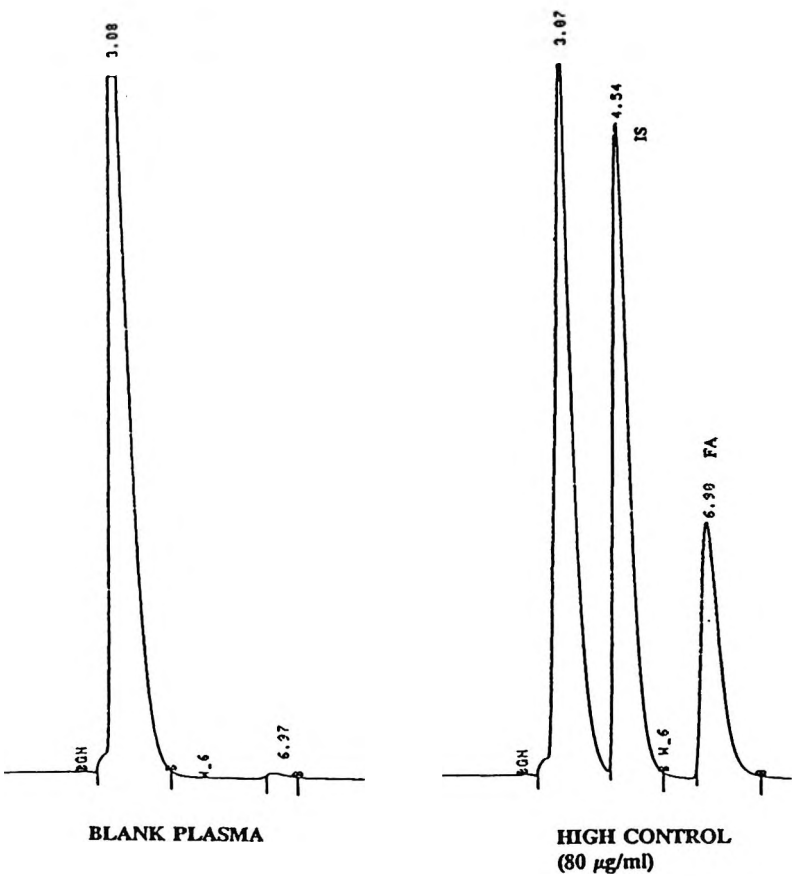

Figure 1. Chromatogram of blank rat plasma extract and extract of plasma spiked with fumaric acid (FA) and the internal standard (IS), chlorofumaric acid.

collection tube. Two hundred mg of fecal material paste was then taken from each sample for analysis. The blank rat fecal material, from untreated animals used for standards and controls, was prepared in the same way before it was spiked with fumaric acid. One mL of 0.9% saline was added to 200 mg of each sample. All controls, standards and samples were vortexed, and 2 mL of the phosphate buffer, pH 5, was added to each test tube. A 100 μL volume of internal standard (chlorofumaric acid) and 100 μL of sodium fluoride, 10 mg/mL, were added. All samples were centrifuged at 1,500 *g* for 20 minutes. The supernate was removed, placed in another glass test tube and stored at -20°C until analyzed.

All three specimens analyzed (samples, standards, and controls) were then put through a solid phase extraction procedure using quaternary amine solid phase extraction columns placed in a 12-station vacuum manifold (Varian, Harbor City, CA, USA). The procedure for extraction column preparation was as follows: the column was washed with 1 mL of 0.02 M phosphate buffer, pH 2.4, and with 1 mL of deionized water. For column conditioning, 1 mL of methanol and 1 mL of deionized water were used. A 1 mL volume of sample (plasma, fecal material, or whole urine sample) was then put through the column.

The column was next washed with 1 mL of acetonitrile and dried under maximum vacuum for 5 minutes. Samples were eluted using 1 mL of 0.02 M phosphate buffer, pH 2.4, collected into test tubes. A 20 μL of the eluent was injected onto the column. The mobile phase consisted of 0.02 M phosphate buffer, pH 2.4 adjusted with o-phosphoric acid (85%), while the flow rate was maintained at 1 mL/minute. Fumaric acid was monitored at 215 nm.

RESULTS

Typical chromatograms of naive plasma and of plasma spiked with fumaric acid and chlorofumaric acid as an internal standard are presented in Figure 1. The calibration was performed by using 5-7 concentrations for all specimens analyzed. A blank fecal material chromatogram and fecal material with fumaric and chlorofumaric acids added are presented in Figure 2. All chromatograms were recorded over a 12 minute time period. There were no contaminants eluting after that time. Under the assay conditions, reproducible retention times for fumaric acid in plasma and fecal material (Beckman C_{18} Ultrasphere 5μ column) were approximately 7 and 8 minutes, respectively, and for chlorofumaric acid, they were 5 and 7 minutes, respectively. The reproducible retention time for fumaric acid in urine using a Waters C_{18} μBondapak 10 μ analytical column was approximately 6 minutes and for trans-glutaconic acid it was approximately 9 minutes. A standard curve for urine was prepared in 0.9% saline. Calibration curves for plasma, urine (saline) and fecal material containing fumaric acid were linear over the range analyzed and had mean correlation coefficients of 0.9939, 0.9972 and 0.9956, respectively.

A representative calibration curve prepared in naive rat plasma is presented in Figure 3. Results of intra- and inter-day variability including relative accuracy and coefficients of variation for plasma, urine and fecal material are presented in Table 1.

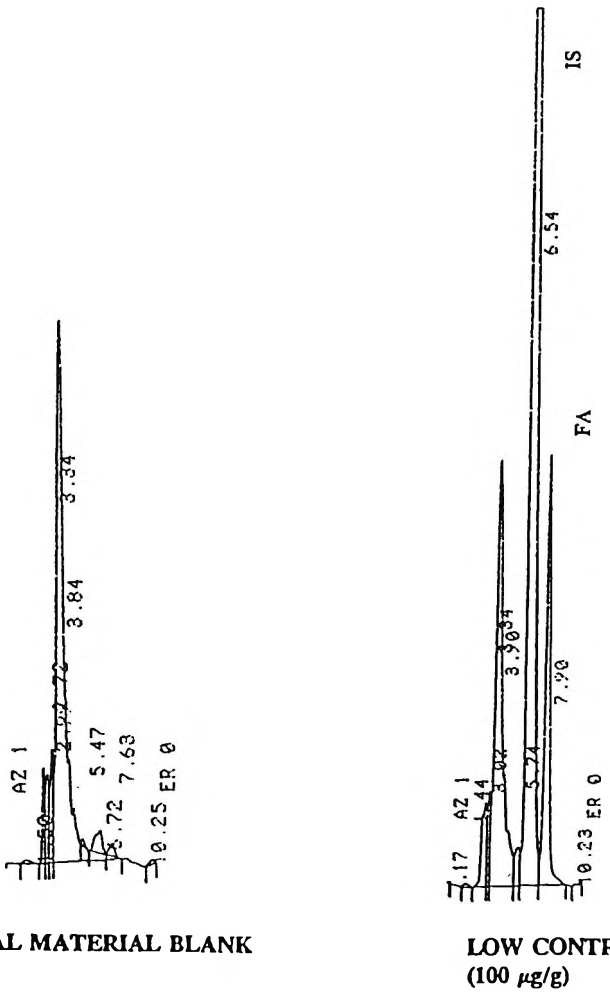

Figure 2. Chromatogram of blank rat fecal material extract spiked with fumaric acid (FA) and the internal standard (IS), chlorofumaric acid.

DISCUSSION

This paper presents an HPLC procedure for the quantitation of fumaric acid in biological matrices (rat plasma, urine, and fecal samples). Eluent monitoring at 215 nm provides adequate sensitivity, precision, and accuracy for determination of fumaric acid concentrations in all specimens studied. Standard

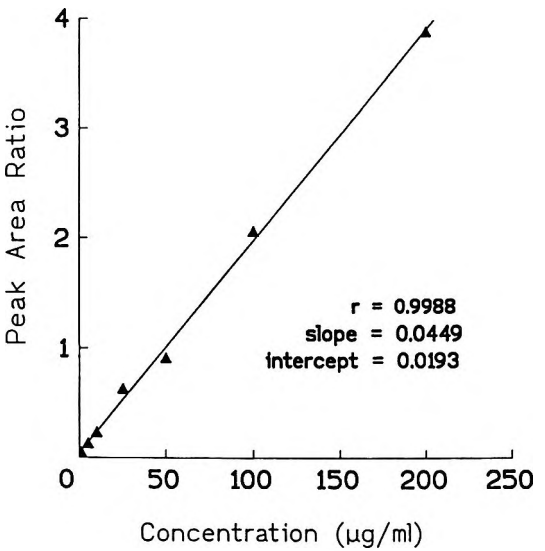

Figure 3. Fumaric acid calibration curve prepared in rat blank plasma.

curves were linear over the range of fumaric acid assayed; 1 µg/mL to 200 µg/mL for plasma, 5 µg/mL to 200 µg/mL for urine, and 25 µg/g to 500 µg/g for fecal material. The quantitation limits were achieved without time consuming derivatization. The results obtained using Ultrasphere 5 µm C_{18} (plasma and fecal samples) and µBondapak 10 µm C_{18} columns were reproducible and similar to previously described studies that utilized two Aminex HPX-87H columns for determination of several citric acid cycle intermediates including fumaric acid.[19] In that experiment, fumaric acid retention time was 26.81 ± 0.14 minutes, which is not practical when large numbers of samples and a variety of different specimens are assayed.

Extractions from rat plasma, urine, and fecal samples were performed using solid phase extraction on Clean Up^R Quaternary Amine columns which provided sufficiently clean samples to achieve good reproducibility, precision and accuracy of the analytical method described. In addition, the extraction procedure presented remains less time consuming in comparison with other published extraction procedures.[15,16,17] In summary, the method described in this paper provides several analytical advantages, e.g., simplicity; clean extracts (even for fecal material); sufficient sensitivity; reproducible retention times, precision and accuracy; low cost; and reliability for analysis of large numbers of plasma, urine, and fecal samples containing fumaric acid.

Table 1

Accuracy and Precision of Fumaric Acid Control Concentrations (μg/mL)

Intra-Day Variability

Parameter	Plasma		Urine		Feces	
	LC[1]	HC[2]	LC	HC	LC	HC
MMC[3]	20.00	84.81	13.17	127.26	95.98	383.45
(± S.D.)	(± 1.22)	(± 5.99)	(± 5.13)	(±11.25)	(±0.88)	(± 17.92)
% CV[4]	6.11	7.07	38.95	8.84	0.92	4.67
% RA[5]	0.00	6.01	-12.20	15.67	-4.02	-4.14

Inter-Day Variability

MMC	20.22	83.23	19.96	148.14	95.93	384.51
(± S.D.)	(± 2.62)	(± 7.57)	(± 5.36)	(20.99)	(± 2.40)	(±16.89)
% CV	12.97	9.09	26.85	14.17	2.50	4.39
% RA	1.10	4.04	33.07	-1.24	-4.07	-3.87

[1] Low control
[2] High control
[3] Mean measured concentration
[4] Coefficient of variation
[5] Relative accuracy

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REFERENCES

1. G. J. Kelloff, C. W. Boone, J. A. Crowell, V. E. Steele, R. Lubet, C. C. Sigman, Cancer. Epidemiol. Biomarkers Prev., **3**, 85-98 (1994).

2. K. Kuroda, M. Akao, Chem. Pharm. Bull., **37**, 1345-1346 (1989).

3. K. Kuroda, K. Terao, M. Akao, J. Natl. Cancer Ist., **71**, 855-857 (1983).

4. K. Kuroda, M. Akao, K. Terao, Jpn. J. Cancer Res., 77, 750-758 (1986).

5. M. Akao, K. Kuroda, Chem. Pharm. Bull., **38**, 2012-2014 (1990).

6. K. Kuroda, K. Terao, M. Akao, J. Natl. Cancer Inst., 79, 1047-1051 (1987).

7. K. Kuroda, M. Kanisawa, M. Akao, J. Natl. Cancer Inst., **69**, 1317-1320 (1982).

8. C. Nieboer, D. de Hoop, A. C. van Loenen, P. N. J. Langendijk, E. van Dijk, J. Am. Acad. Derm., **20**, 601-608 (1989).

9. W. M. Nugteren-Huying, J. G. van der Schroeff, J. Hermans, D. Suurmond, J. Am. Acad. Derm., **22**, 311-312 (1990).

10. A. J. Peefers, B. A. C. Dijkmans, J. D. van der Schroeff, Br. J. Rheumatol., **31**, 502-504 (1992).

11. D. N. Kolbach, C. Nieboer, J. Am. Acad. Derm., **27**, 769-771 (1992).

12. P. J. Altmeyer, U. Matthes, F. Pawlak, K. Hoffmann, P. J. Frosh, P. Ruppert, S. W. Wassilew, T. Horn, H. W. Kreysel, G. Lutz, J. Barth, I. Rietzchel, R. K. Joshi, J. Am. Acad. Derm., **30**, 977-981 (1994).

13. P. de Haan, B. M. E. von Blomberg-van der Flier, J. de Groot, C. Nieboer, D. P. Bruynzeel, Dermatology, **188**, 126-130 (1994).

14. T. Tsuda, H. Nakanishi, T. Morita, J. Takebayashi, J. Assoc. Off. Anal. Chem., **68**, 902-905 (1985).

15. T. Cronholm, C. Norsten, J. Chromatogr. Biomed. Appl., **344**, 1-9 (1985).

16. B. Schatowitz, G. Gercken, J. Chromatogr. Biomed. Appl., **425**, 257-268 (1988).

17. R. Badoud, G. Pratz, J. Chromatogr. Biomed. Appl., **360**, 119-136 (1986).

18. E. Mentasti, M. C. Gennaro, C. Sarzanini, C. Baiocchi, M. Savigliano, J. Chromatogr. Biomed. Appl., **322**, 177-189 (1985).

19. R. H. Haas, J. Breuer, M. Hammen, J. Chromatogr. Biomed. Appl., **425**, 47-57 (1988).

20. G. O. Guerrant, M. A. Lambert, C. Wayne Moss, J. Clin. Microbiol., **16**, 355-360 (1982).

21. D. Tusseau, C. Benoit, J. Chromatogr., **395**, 323-333 (1987).

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CAPILLARY ZONE ELECTROPHORETIC METHOD FOR THE QUANTITATIVE DETERMINATION OF THE β-BLOCKER ATENOLOL IN HUMAN URINE

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ABSTRACT

A simple capillary zone electrophoresis method was developed for the quantitation of the β-blocker 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy] benzeneacetamide, atenolol.

The electrophoretic separation was performed using a 78 cm × 75 μm I. D. fused silica capillary. The electrolyte consisted of a buffer Na_2HPO_4 (25mM)-$Na_2B_4O_7$ (25 mM) (50-50; V/V), pH 9.7. The introduction of the sample was made hydrostatically for 25 s and the running voltage was 20 KV at the injector end of the capillary. Photometric detection was used and a wavelength of 214 nm. The method was applied to the determination of atenolol in urine samples obtained from a hypertensive patient under medical treatment with the pharmaceutical formulation "Tenormin 100 mg" (atenolol 100 mg), another patient under treatment with the pharmaceutical combination "Tenoretic"

(atenolol 100 mg + chlorthalidone 25 mg), and also in urine samples obtained from a healthy volunteer after the ingestion of a 100 mg atenolol tablet, "Tenormin 100 mg". Using a simple solid phase extraction, a recovery of 70.69±3.27% and a very good separation from the urine matrix are achieved. A good reproducibility, linearity, and accuracy are obtained, and a quantitation limit of 0.1 μg/mL in urine, allows the method to be applied to pharmacokinetic studies of the compound.

INTRODUCTION

Atenolol, 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy] benzene acetamide, is a cardioselective β-adrenoreceptor antagonist (β-blocker), widely used in the treatment of hypertension, angina pectoris, cardiac dysrhythmias, and heart attacks. The β-blockers are also subjected to certain restrictions in sports, by the Medical Commision of the International Olympic Committee (IOC) in order to prevent their abuse in sports.[1]

Following oral administration, about 50 to 60% of an atenolol dose is absorbed with maximum plasma concentrations reached within 2 to 4 hours, less than 5% of the dose is bound to plasma proteins, and most of the absorbed atenolol is excreted unchanged in the urine.

In adult patients with normal renal function, the elimination half-life is about 5 to 7 hours and total clearance is about 6 L/h per 1.73 m^2. However, there are wide intra and interindividual differences in the pharmacokinetic properties of atenolol.[2]

Procedures for the determination of atenolol in plasma and urine included phosphorence techniques,[3] HPLC with photometric detection,[4-12] HPLC with fluorimetric detection,[13-18] and Liquid Micellar Chromatography with laser induced fluorescence.[9] Ion Pair Chromatography,[20-21] and Gas Chromatography-Mass Spectrometry[22-23] were also used.

There are also several papers dealing with the use of Micellar Electrokinetic Capillary Chromatography,[24-25] and Capillary Zone Electrophoresis (CZE).[26-28] But none of these papers show a quantitative application of these techniques for the analysis of β-blockers in biological fluids. The aim of this paper is the application of a simple CZE system with photometric detection to the quantitation of atenolol in urine, preceded by a simple and fast solid-phase (SP) extraction procedure.

MATERIALS AND METHODS

Chemicals and Reagents

Methanol (HPLC grade) was purchased from Lab-Scan (Dublin, Ireland). Chloroform, glacial acetic acid, ammonia solution, disodium tetraborate, boric acid, and sodium hydroxide were Merck pro analysis (Bilbao, Spain). Isopropyl alcohol and disodium hydrogenphosphate were purchased from Fluka (Bilbao, Spain).

The water used was obtained by the Milli-RO and Milli-Q systems.

Reference standard of atenolol was provided by Sigma Chemical (Bilbao,Spain). A stock standard solution of the drug (1000 μg/mL) was prepared in water and stored at 4°C. Working solutions were prepared by appropiate dilution, just before use.

Bond-Elut Certify columns were supplied by Varian (Barcelona, Spain).

Apparatus and Electrophoretic Conditions

This work was performed in a 78 cm × 75 μm I.D. fused-silica capillary tube (Composite Metal Services LTD., United Kingdom). The effective separation distance was 70 cm.

The Capillary Electrophoresis system was a Waters Quanta 4000, and data were collected with the help of a PC and treated with the software Millenium 2010 (Waters Chromatography Division, Barcelona, Spain). The wavelength used for photometric detection was 214 nm. Injections were carried out hydrostatically for 25 s and the running voltage was 20 KV at the injector end of the capillary. The electrolyte consisted of a buffer Na_2HPO_4 (25 mM) - $Na_2B_4O_7$ (25 mM) (50+50 V/V), pH 9.7.

The capillary was conditioned every day with an initial wash cycle consisting of NaOH 1 M, 20 min, and deionized water, 20 min. Wash cycles before each injection: NaOH 0.1 M, 2 min, and running buffer, 3 min, in order to reduce fouling. Daily wash cycles after finishing experiments: NaOH 1M, 5 min, and deionized water, 5 min.

The system required some equilibration to ensure consistent migration behaviour (due to urine matrix effects). To this purpose, the analysis of blank urine samples was included at the beginning of an analytical sequence.

Procedure for Urine Samples

The pH of the urine samples (2.5 mL) was adjusted to 9 with borate buffer (500μl), vortex mixed for 5 s, and filtered through a 45 μm membrane filter.

Bond Elut Certify SP extraction columns were inserted into a vacuum manifold and conditioned by washing once with 2 mL methanol and 2 mL deionized water. The columns were prevented from drying. Filtered samples were poured into each column reservoir and drawn slowly through the column. The columns were washed with 2 mL deionized water, 1 mL 0.1 M acetate buffer (pH 4), and 2 mL methanol. Elution of the analyte was performed with 2 mL of a mixture of chloroform-isopropyl alcohol (80:20 v/v) containing 2% ammonia. The eluate was evaporated to dryness at 30°C under a stream of nitrogen, using a Zymark Turbo Vap LV evaporator (Barcelona, Spain). The residue was dissolved in 100 μl of electrolyte (sometimes a smaller volume of electrolyte was used) and measured under calibration conditions.

The reproducibility and efficiency of the extraction procedure was determined by extracting replicate spiked urine samples doped with 2 ppm of atenolol. The urine samples were obtained from 4 different volunteers, and as no significant diferences were observed in between these samples (in relation to matrix interferences and recoveries after the Solid-Phase extraction), 10 replicates were arbitrarily analysed for the determination of the percentage of recovery. A quantitative recovery of (mean value ± R.S.D. %) 70.69±3.27 was achieved (n=10).

RESULTS AND DISCUSSION

The primary objective of this work was the development of a validated Capillary Electrophoresis method for the quantitation of the β-blocker atenolol in human urine samples. Several parameters were optimized to ensure a reproducible and accurate method.

The samples were injected hydrostatically for 25s and the running voltage was 20kV inducing a 48μA current across the capillary. Electrokinetic injection was also assayed, and although improved, the sensitivity proved to be quite irreproducible, because a sampling bias is introduced based on the ionic

charge of the molecule. The introduction of another β-blocker as internal standard did not improve the area reproducibility, with an R.S.D around 10%, The R.S.D is calculated by the formula R.S.D = (standard deviation/mean of the peak areas)·100%.

A linear increase in peak area and height were observed with increasing hydrostatical injection times, since the amount of sample introduced into the capillary also increases linearly. For sampling times longer than 25 s no further peak height increase was observed, due to band broadening effects, therefore an optimum 25 s injection time was chosen.

The effect of temperature on the migration time of atenolol was studied. A decrease in migration times with increasing temperature was observed, as well as higher currents inside the capillary which caused baseline disturbances. The temperature was kept constant at 25°C to ensure reproducible results.

The length and diameter of the capillary were also optimized. Shorter capillaries didn't provide enough resolution between atenolol and the endogenous substances present in the urine, therefore an optimum effective length of 70 cm was chosen. Internal diameters smaller than 75 μm didn't provide enough sensitivity.

The study of the influence of pH and composition of the electrolyte gave an optimum value of pH 9.7 and a Na_2PO_4(25mM):$Na_2B_4O_7$(25mM) (50:50; V:V) ratio.

Once the optimum electrophoretic conditions had been established, a quantitative method for the determination of atenolol in urine samples was developed, (Table 1).

The relative standard deviation of the retention times is less than 1% for both intra and interday assays (intraday reproducibility determined by injecting replicate samples, n=10; and n=5 for the interday reproducibility), thus indicating high stability for the system.

The accuracy of the method was determined by the analysis of 5 control urine samples (obtained from 4 different volunteers) and spiked with 2 ppm of atenolol. Aceptable accuracy, defined as mean (found concentration/actual concentration) × 100, was achieved: 98.29 ± 2.45 %. The experimental quantitation limit, defined as the minimum atenolol concentration in urine which gives rise to a signal able to be quantified by the computer program used (after extraction of 2.5mL urine and final preconcentration to 25μl), was 0.1μg/mL.

Table 1

Determination of Atenolol*

Migration Time ± S.D. (min.)	6.54 ± 0.06
Linear Range	10-140μg/mL
Slope of Calibration Graph (area/conc.)	373.34
r^2	0.998
Area Repeatability Intraday R.S.D. (%)	0.26[a]
Area Repeatability Interday RS.D. (%)	2.24[b]
Experimental Detection Limit (μg/mL)	10

* The optimization of the electrophoretic system and estimation of the given parameters was done with spiked urine samples. (For electrophoretic conditions see the experimental section.)

[a] Five determinations at the 30ppm level.

[b] Three determinations at the 30ppm level.

Analytical Applications

The method developed was applied to the determination of atenolol in urine samples obtained from patients suffering from hypertension and under medical treatment with atenolol (Tenormin 100mg) and with a pharmaceutical combination of atenolol and chlorthalidone (Tenoretic), and urine samples obtained from a healthy volunteer after the ingestion of atenolol (Tenormin 100mg), Figure 1.

Urine was collected at different time intervals for the quantitative determination of the β-blocker: 0-2 hours, 2-4 hours, 4-8 hours and 8-24 hours. Urine samples were treated following the clean-up procedure described in the experimental section.

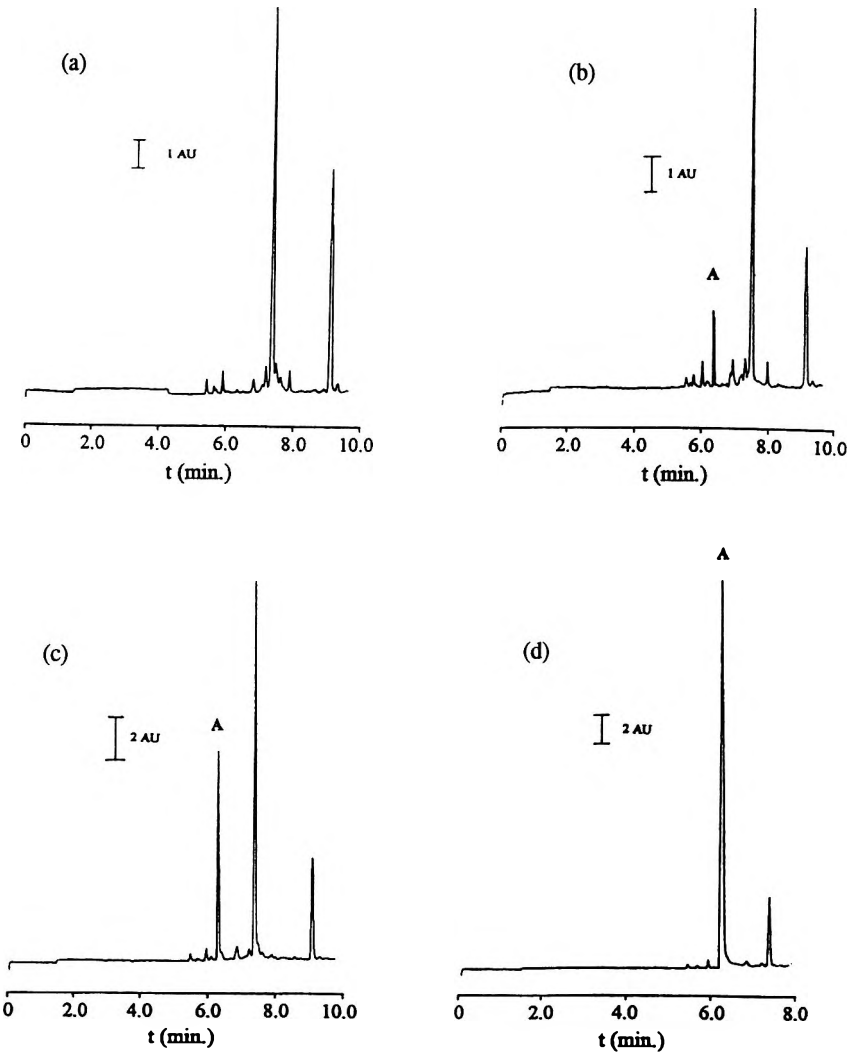

Figure 1. Electropherograms obtained from an extract of: a) Blank urine sample, b) urine sample spiked with 0.4ppm of atenolol c) Urine sample 4-8hours after the oral administration of 1 tablet of "Tenoretic" (atenolol 100mg − Chorthalidone 25mg) d) Urine sample 4-8hours after the oral administration of 1 tablet of "Tenormin 100mg" (atenolol 100mg).

Table 2

Quantitative Determination of Atenolol in Urine

Urine Samples	Time Inverval (Hours)	Atenolol (mg)	Total Atenolol Amount (mg)
Patient under	0-2	1.43	
treatment with	2-4	12.76	35.25
"Tenormin 100mg"	4-24	21.06	
Patient under	0-2	1.07	
treatment with	2-4	0.19	
"Tenoretic"*	4-8	4.55	
	0-2	1.35	
Healthy	2-4	6.07	
Volunteer	4-8	6.74	25.06
"Tenormin 100mg"	8-24	10.9	

* It was not possible to collect samples between 8 and 24 hours.

The compound was easily detected at all time intervals and the found concentrations, collected in Table 2, were in agreement with the pharmacokinetic data[2]. A considerably minor excretion was observed for the patient taking the pharmaceutical combination "Tenoretic". This decrease in the excretion was probably due to the effect of the diuretic Chlorthalidone.

DISCUSSION

The described CZE method was succesful for the determination and quantitation of atenolol in real human urine samples. The clean-up procedure is very simple and effective, and the electrophoretic separation is made in less than 8 minutes.

The assay validation was adequate in terms of reproducibility, linearity, and accuracy, showing that the CZE can also be used with quantitative purposes for the analysis of β-blockers.

The limit of quantitation of 0.1 μg/mL (with a preconcentration of a 100 factor) is sensitive enough for the determination of free atenolol in urine samples at all time intervals studied. A further preconcentration could also be possible, improving the forementioned limit of quantitation.

The poor sensitivity of the CZE-Photometric Detection method can be overcome by the preconcentration of the samples, as only a very low volume is needed for the injection.

REFERENCES

1. Medical Commission, International Olympic Committee, "List of Doping Classes and Methods," in International Olympic Charter against Doping in Sport, IOC, Lausane, Switzerland, 1990, (update May 1992).

2. A. N. Wadworth, D. Murdoch, R. N. Brogden, Drugs, **42(3)**, 468-510 (1991).

3. L. M. Cabalín, J. J. Laserna, A. Rupérez, Anal. Chim. Acta, **270**, 239-245 (1992).

4. C. Verghese, A. McLeod, D. Shand, J. Chromatogr. B, **275**, 367-375 (1983).

5. A.Tracqui, P. Kintz, J. Himber, A. A. J. Lugnier, P. Mangin, Forensic Sci. Int., **38**, 37-41 (1988).

6. M. Johansson, H. Forsmo-Bruce, J. Chromatogr., **432**, 265-272 (1988).

7. M. T. Kelly, M. R. Smyth, D. Dadgar, J. Chromatogr., **473**, 53-62 (1989).

8. M. T. Kelly, M. R. Smyth, J. Pharm. & Biomed. Anal., **7(12)**, 1757-1764 (1989).

9. M. Bogusz, M. Wu, J. Anal. Toxicol., **15**, 188-197 (1991).

10. M. S. Leloux, F. Dost, Chromatographia, **32(9/10)**, 429-435 (1991).

11. W. E. Lambert, E. Meyer, A. P. Leenheer, J. Anal. Toxicol., **19(2)**, 73-78 (1995).

12. M. T. Saarinen, H. Sirén, M. L. Riekkola, J. Chromatogr. B, **664**, 341-346 (1995).

13. K. U. Bühring, A. Garbe, J. Chromatogr. B, **382**, 215-224 (1986).

14. R. Mehvar, J. Pharm. Sci., **78(12)**, 1034-1039 (1989).

15. S. K. Chin, A. C. Hui, K. M. Giacomini, J. Chromatogr. B, **489**, 438-445 (1989).

16. T. A. Kolbah, F. Plavsi´c, A. W. Coporda, J. of Pharm. & Biomed. Anal., **7(12)**, 1777-1781 (1989).

17. A. D. Dale, S. E. Turner, J. Pharm. & Biomed. Anal., **8 (8-12)**, 1055-1058 (1990).

18. R. G. Morris, N. C. Saccoia, B. C. Sallustio, R. Zacest, Ther. Drug Monit., **13**, 345-349 (1991).

19. I. Carretero, M. Maldonado, J. J. Laserna, Química Analítica, **11(2)**, 107-119 (1992).

20. H. Siren, M. Sarinen, S. Hainari, P. Lukkari, M. L. Riekkola, J. Chromatogr., **632**, 215-227 (1993).

21. P. Lukkari, H. Sirén, J. Chromatogr. A., **717**, 211-217 (1995).

22. F. T. Delbeke, M. Debackere, N. Desmet, F. Maertens, J. Chromatogr., **426**, 194-201 (1988).

23. A. Solans, M. Carnicero, R. de la Torre, J. Segura, J. Anal. Toxicol., **19**, 104-114 (1995).

24. P. Lukkari, H. Sirén, M. Pantsar, M. L. Riekkola, J. Chromatogr., **632**, 143-148 (1993).

25. P. Lukkari, T. Nyman, M. L. Riekkola, J. Chromatogr. A, **674**, 241-246. (1994).

26. E. Gonzalez, J. J. Laserna, Electrophoresis, **15**, 240-243 (1994).

27. J. M. Vadillo, M. E. Gonzalez, I. Carretero, J. J. Laserna, Mikrochim. Acta, **118**, 273-282 (1995).

28. J. C. Hudson, M. Golin, M. Malcolm, Can. Soc. Forens. Sci., **28(2)**, 137-152 (1995).

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SUPERCRITICAL FLUID CHROMATOGRAPHIC DETECTION BY USE OF A PARALLEL FLOW RESTRICTOR

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ABSTRACT

In supercritical fluid chromatography (SFC), the role of the flow restrictor is not only to maintain supercritical conditions throughout the column but also to provide the necessary interface between the column and the detector. In the previous paper,[1] a new type of flow restrictor called parallel flow restrictor was introduced. At that time, the parallel flow restrictor was used only for the flow control. In this paper, the parallel flow restrictor was used not only for the flow control but also for the interface to the second detector. A supercritical fluid chromatographic system with a combination of two detectors based on physically different principles, flame ionization and UV absorption, was used.

INTRODUCTION

Klesper et al.[2] first reported the use of a supercritical fluid as a chromatographic mobile phase in 1962. Although the work of this group[2,3] and later that of Giddings and co-workers[4,5] and Sie and Rijnders[6,7], with both dense gases and supercritical fluids, helped to develop supercritical fluid chromatography (SFC) both experimentally and theoretically, the technique did not become popular until some 20 years later.

In 1981, a re-emergence of the technique was initiated following a report by Novotny et al.[8] describing separations performed using open-tubular capillary columns and supercritical pentane as the mobile phase. Since then, hundreds of papers have appeared describing SFC instrumentation, coupling of SFC with various detectors, and numerous applications of the technique.

In SFC, various types of detectors including the UV absorption detector,[9] flame ionization detector (FID),[10] fluorescence,[11] refractive index,[12] mass spectrometer,[13] etc., have been used. With supercritical CO_2 and several other SFC mobile phases, it is possible to use a FID. This provides the ideal combination of a solvating mobile phase and sensitive and universal detection. With GC, both volatility and thermal stability are required for a successful analysis. Although these are not required for HPLC, sensitive, universal detection is very difficult today with liquid mobile phases. The combination of SFC and FID is certainly a convenient sensitive capability for determining low volatility or thermally labile analytes that do not strongly absorb light. For these reasons, FID is the most popular detector in capillary SFC. UV detectors have been the most preferred in packed column SFC.[14]

For fractionation, a non-destructive type detector is favourable because it is not necessary to split and waste the effluent containing sample solutes. For this reason, a UV detector is the most feasible among the detectors, which are compatible with supercritical fluids. The UV detector generally offers a stable baseline, high sensitivity, and wide linear dynamic range even with supercritical fluids. In addition , supercritical carbon dioxide is transparent even at 190nm, which is the short wavelength limit of most of the commercial variable wavelength UV detectors.

The use of a supercritical fluid as a mobile phase requires that a flow restrictor be provided at the outlet of the column in order to maintain the mobile phase above the critical pressures throughout the column. In the coupling of 50-100 μm i.d. open-tubular SFC columns with gas chromatography (GC)-type detectors such as the flame-ionization detector, the role of the flow restrictors is not only to maintain supercritical conditions

throughout the column but also to provide the necessary sample introduction interface between the column and the detector. Three types of flow restrictors are frequently used in capillary SFC: linear restrictors,[15] tapered restrictors,[16] and integral restrictors.[17]

Recently, we developed two different types of temperature programmable restrictors, i.e., a two-stage restrictor[18] and a parallel flow restrictor.[1] Among them, a parallel flow restrictor consists of two separate restrictors in parallel. In this paper, the successful coupling of capillary SFC to both flame ionization (FID) and UV absorption detection by use of a parallel flow restrictor is described.

Recently, there has been a good deal of new interest in SFC using packed capillary columns.[19] Advantages of packed column SFC include fast analysis speeds, great sample capacities, and high plate numbers per unit length. Advantages of open tubular column in SFC include small pressure drop per unit column length which allows the use of long column to generate high efficiencies for the separation of complex mixtures. It was known that packed capillary columns can take advantages of both colunm types, i.e., packed and capillary.[20]

Due to these many anvantages, packed capillary columns have been used extensively in this work. For the present work, we used a fused silica capillary column (dimensions: 200μm i.d. × 60cm) packed with 5μm ODS particles.

EXPERIMENTAL

The chromatographic system comprised a Model 600 SFC pump (Dionex, Sunnyvale, CA, USA) for pressure control of the carbon dioxide mobile phase and the Model 600 GC / SFC oven (Dionex). The column used was a 60cm × 200μm i.d. fused silica capillary packed with 5μm ODS bonded silica.[19] The packing material was obtained from Phenomenex (Rancho, Palos Verdes, CA, USA). The packed capillary column was connected directly to the injection valve, and the UV detection of solute occurred immediately after the bed support using the 200μm i.d. column as the flow cell. A model 203 UV/ Vis detector (Linear, Reno, NV, USA) was used for UV detection.

The system was equipped with a C14W loop injector (valco Instruments, Houston, TX, USA) and a flame ionization detector. SFC grade carbon dioxide (Scott Specialty Gases, Plumsteadville, PA, USA) was used as the mobile phase.

The effluent from the column was split between the flame ionization detector and UV absorption detector with the parrallel flow restrictor. The splitter was a Valco 1/16 union in which the column was connected to one end and the FID and UVD to the other end. The parallel flow restrictor is comprised of two linear restrictors (14μm i.d. × 10cm).

Flow rates were measured using an Alltech Flowmeter (7445). This flowmeter monitors the mass flow rate of the gas in the range of 0 ~ 50 standard cubic centimeters per minute (SCCM). Accuracy is 20% of full scale over a wide temperature and pressure range, and time response is 2 seconds.

RESULTS AND DISCUSSION

In the previous paper,[1] we found a series of correlations between the column flow rates and temperatures of the restrictor at various pressures to develop a temperature programmed restrictor. This was accomplished using the Alltech model 7445 flow meter. The mobile phase flow velocities, as a function of the temperature of the restrictor and the pressure of the column, were measured using different length of 14μm fused silica tubes as restrictors. It was also demonstrated that if the temperature and the length of the 14μm i.d. fused silica tubing is varied, the flow rate could be varied, and could also be maintained near the optimum value of a Van Deemter curve. For example, it was seen that when the temperature of the restrictor was increased, the flow rates were decreased to a noticeable extent. It was also seen that if the length of the restrictor was doubled, the flow rates were halved.

These phenomena can be explained theoretically by Poiseuille's equation. In this equation, for an incompressible fluid, the volume of flow(ΔV) through a tube (radius r, length l, viscosity η) in some time interval(Δt) is:

$$\frac{\Delta V}{\Delta t} = \frac{\pi r^4 \Delta P}{8 \eta l} \qquad (1)$$

where ΔP is the pressure drop between the ends. If the fluid is a compressible ideal gas, the rate of flow is

$$\frac{\Delta V}{\Delta t} = \frac{\pi r^4}{16 \eta l} \left(\frac{P_i^2 - P_o^2}{P_m} \right) \qquad (2)$$

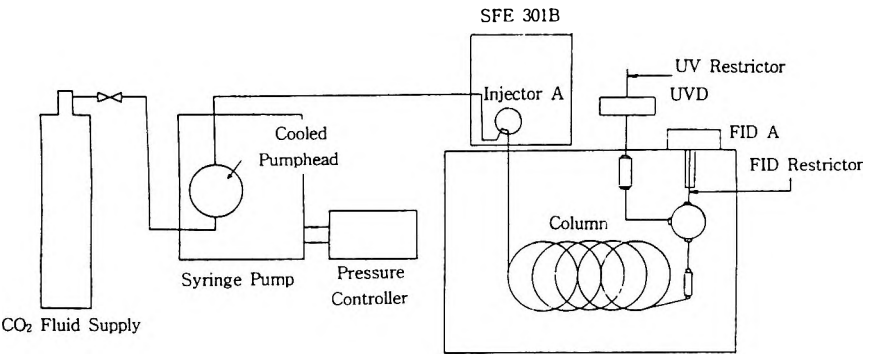

Figure 1. Schematic diagram of the parallel flow restrictor.

where P_i is the inlet pressure, P_o is the outlet pressure, and P_m is the pressure at which the volume of the gas was measured. As a supercritical fluid is not a gas and not a liquid, it is difficult to apply one of these equations to SFC restrictors, but as far as the viscosity and the length are concerned, both of these equations agree that the mass flow rate through the tube is inversely proportional to the viscosity of the fluid and the length of the tube. It is obvious that when the temperature of the tube is increased, the viscosity of the fluid passing through it also increases. For these reasons, in SFC, as the temperature of the restrictor is increased, the flow rate is decreased.

In this paper, the successful coupling of packed capillary SFC to both flame ionization (FID) and UV absorption detection by use of a parallel flow restrictor is described. Since a parallel flow restrictor consists of two linear restrictors in parallel, it is not difficult to install two detectors in parallel on a column of supercritical fluid chromatographic system(see Figure 1).

Figure 2 and 3 show chromatograms of four polyaromatic hydrocabons detected by both FID and UVD. As expected, the relative ratio of flame ionization detector response and UV absorption detector response was dependent on the temperatures of two restrictors. Individual compounds also showed different detector responses. Anthracene and Chrysene showed much greater detector response than FID, Pyrene showed similar detector responses for both FID and UV. First of all, the temperature of a restrictor connected to FID(it will be called FID restrictor afterwards) was maintained at 320°C, and the temperature of a restrictor connected to UV detector was varied from 50°C to 200°C. The relative detector response ratios for Pyrene were obtained and

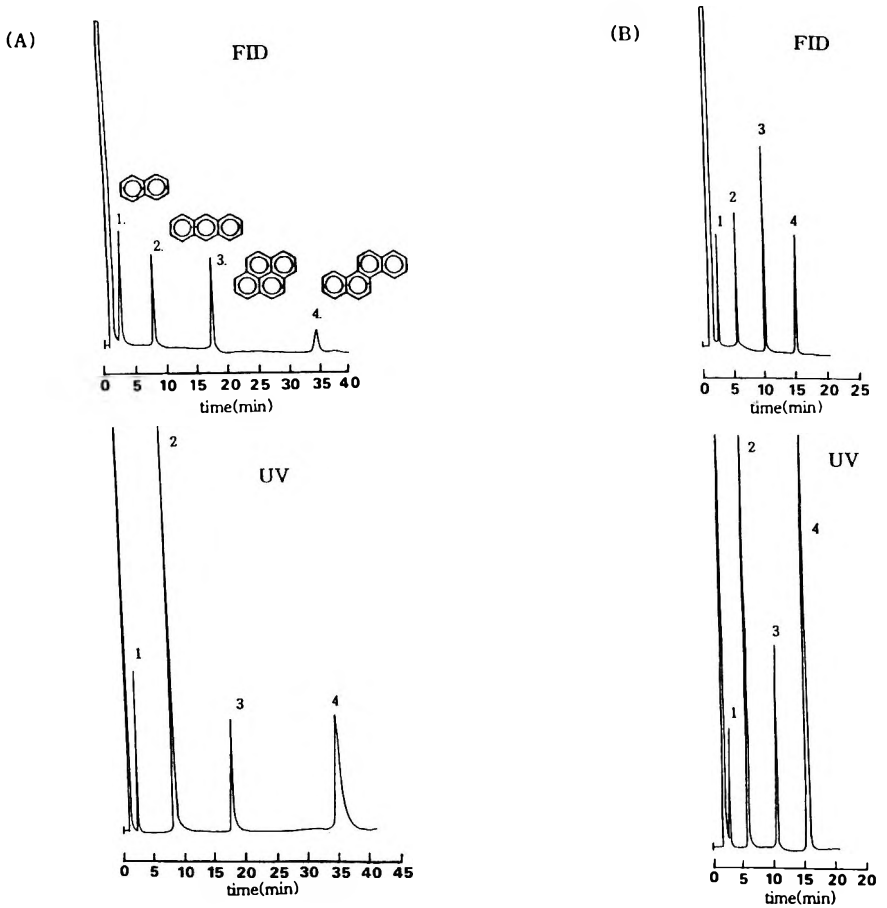

Figure 2. Supercritical fluid chromatograms of mixture of polyaromatic hydrocarbons at the UV restrictor temperature of (A) 150°C and (B) 60°C conditions: 60 cm × 200 μm i.d. fused silica capillary packed column, 5μm ODS silica, CO_2 mobile phase at 100°C; pressure programmed.

shown in Table 1. Figure 2 shows the supercritical fluid chromatograms of mixture of polyaromatic hydrocarbons at different UV restrictor temperatures maintaining the temperature of FID restrictor at 320°C. As the temperature of the restrictor connected to UV detector increases, the relative detector response (FID signal / UV signal) decreases.

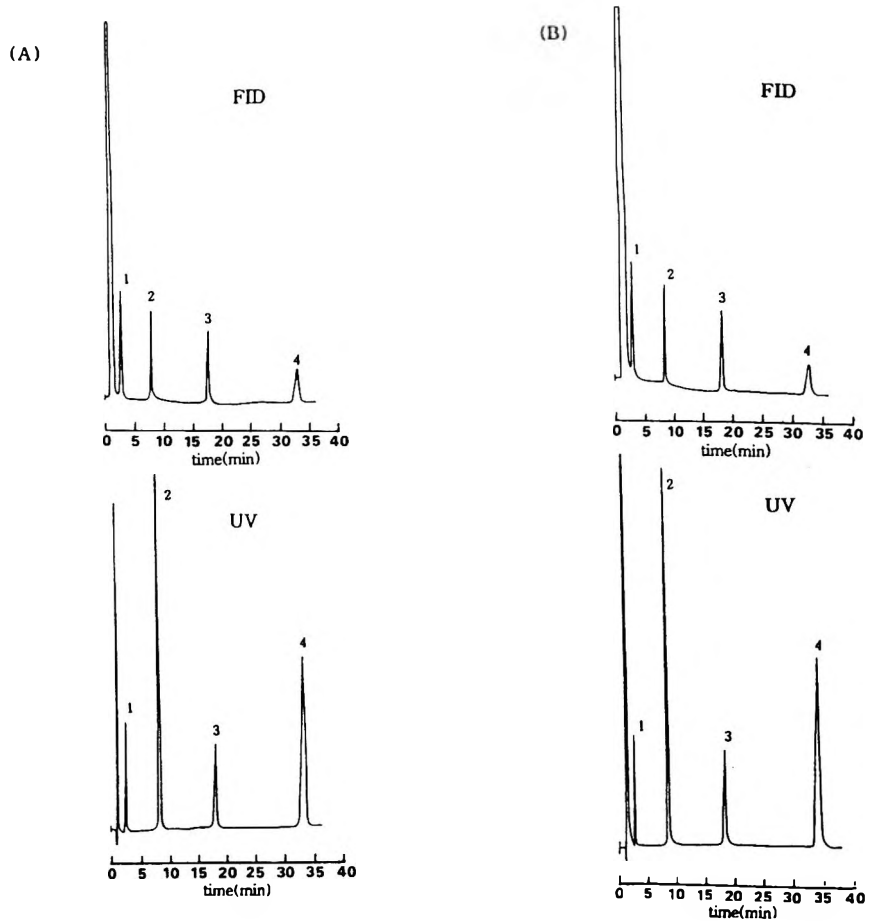

Figure 3. Supercritical fluid chromatograms of mixtures of polyaromatic hydrocarbons at the FID.

This phenomena, the decrease of the UV detector signal with increasing the temperature of the restrictor connected to UV detector, can be understood that, as the temperature of the restrictor increases, the volume of the fluid passing through it also increases, changing the extent of restriction of the restrictor connected to UV detector.

Other experiments, in which the temperature of the UV restrictor is maintained at 100°C, the temperature of the FID restrictor is varied, have been made.

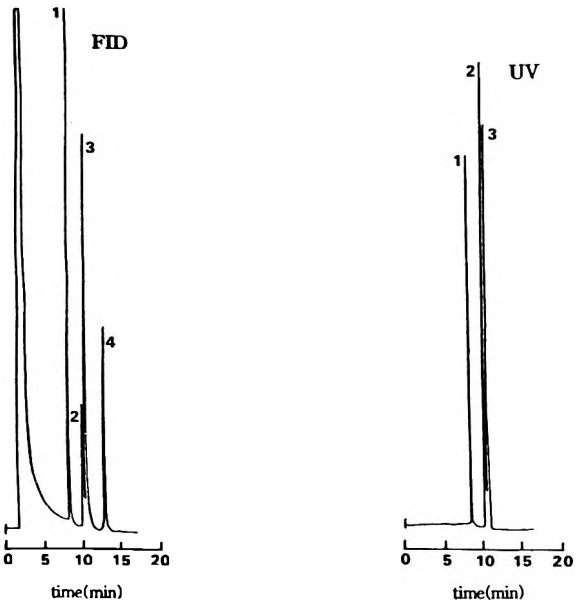

Figure 4. Supercritical fluid chromatograms of mixture of pesticides conditions: 60 cm × 200 μm i.d. fused silica capillary packed column, 5μm ODS silica, CO_2 mobile phase at 100°C; pressure programmed from 100atm to 300 atm at 4atm/min., FID restrictor temperature at 300°C, UV restrictor temperasture at 100°C, 280nm UV detection. Peak identification: 1. Carbofuran 2. Chlorpyrifos 3. Naphthol 4. Lindane.

Table 1

The Relative Ratios of FID Signal and UVD Signal at Different UV Restrictor Temperatures Maintaining the Temperatures of FID Restrictor at 320°C

Temp. of UV Restrictor	FID Signal / UVD Signal
50°C	2.02
100°C	1.78
150°C	1.53
200°C	1.42

Table 2

The Relative Ratios of FID Signal and UVD Signal at Different UV Restrictor Temperatures Maintaining the Temperatures of FID Restrictor at 100°C

Temp. of UV Restrictor	FID Signal / UVD Signal
360°C	0.174
340°C	0.171
320°C	0.175
280°C	0.173

The relative ratios of FID signal and UVD signal were obtained (Table 2). Figure 3 shows the chromatograms when the temperature of FID restrictor was varied from 360° to 320° maintaining the temperature of UVD restrictor at 100°C, the relative detector response ratios for Pyrene were almost same. It is well known that when the temperature of FID block is increased, the FID detector response is also increased. In this case, this effect was compensated for the decreasing of FID signal owing to the elevation of the temperature of FID restrictor.

Finally, two detectors in parallel with parallel flow restrictors were used for the SFC separations of pesticides. Figure 4 shows the chromatograms of mixtures of three pesticides using two detectors, i.e. FID and UVD and two restrictors. The temperatures of FID restrictor and UVD restrictor were 300°C and 100°C each. Although carbofuran, chlorpyrifos, and naphthol were detected by both detectors, lindane was detected only by FID.

ACKNOWLEDGMENT

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REFERENCES

1. D. Pyo, Chromatographia, **37**, 635 (1993).

2. E. Klesper, A. M. Corwin, D. A. Turner, J. Org. Chem., **27**, 700 (1962).

3. E. Klesper, Angew. Chem. Int. Ed. Engl., **17**, 738 (1987).

4. M. N. Myers, J. C. Giddings, Separation Sci., **1**, 761 (1966).

5. L. Mclaren, M. N. Myers, J. C. Giddings, Science, **159**, 197 (1968).

6. S. T. Sie, G. W. A. Rijnders, Separation Sci., **2**, 729 (1967).

7. S. T. Sie, G. W. A. Rijnders, Separation Sci., **2**, 755 (1967).

8. M. Novotny, S. R. Springston, P. A. Peaden, J. C. Fjeldsted, M. L. Lee, Anal.Chem., **53**, 407 (1981).

9. R. E. Jentoft, T. H. Goww, Anal. Chem., **48**, 2195 (1976).

10. B. E. Richter, D. J. Bornhop, J. T. Swanson, J. Chromatogr. Sci., **27**, 303 (1989).

11. P. A. Peaden, J. C. Fieldsted, M. L. Lee, S. R. Springston, M. Novotny, Anal. Chem., **54**, 1090 (1982).

12. W. Asche, Chromatographia, **11**, 411 (1978).

13. S. B. Hawthorne, D. J. Miller, Fres. Z. Anal. Chem., **330**, 235 (1988).

14. Y. Hirata, J. Chromatogr., **315**, 39 (1984).

15. J. Kohler, A Rose, G. Schomburg, J. High Resolut. Chromatogr., **11**, 191 (1988).

16. T. A. Berger, Anal. Chem., **61**, 359 (1989).

17. S. B. Hawthorne, D. J. Miller, J. Chromatogr., **403**, 63 (1987).

18. D. Pyo, Analyst, **119**, 1315 (1994).

19. A. Malik, W. Li, M. L. Lee, J. Microcol. Sep., **5**, 361 (1993).

20. V. G. Berezkin, V. S. Gavrichev, A. Malik, J. Liq. Chromatogr., **10**, 1707 (1987).

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DEVELOPMENT OF A SENSITIVE INDIRECT CHROMATOGRAPHIC METHOD TO CHARACTERIZE IODINE MONOCHLORIDE

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ABSTRACT

A highly specific indirect method for the analysis of iodinemonochloride (ICl) has been developed to assess the potential for minor chlorination side reactions when using ICl to iodinate an aniline derivative. The technique offers great advantages over the non-specific thiosulfate titrimetric method commonly used to characterize ICl and differentiates samples previously thought to be identical. The method described in this paper uses a simple and rapid "bench top" analytical laboratory version of the formal iodination reaction as a pre-HPLC sample

derivatization, followed by detection of the chlorinated aniline analogues. The derivatization method mimics the ability of the formal reaction to predict the chlorination potential of ICl samples with varying levels of excess chlorine. The principles used for this specific application should be suitable for related iodination reactions as long as a suitable laboratory derivatization reaction can be developed.

INTRODUCTION

Many quantitative methods are available for the determination of iodine species. Atomic spectroscopy and indirect titrations based on iodometric process which liberate iodine are well documented common techniques.[1, 2] Numerous reports of the use of ion chromatography to determine halogen anions, including iodide, in environmental / biological matrices have been published recently, with various detection schemes employed such as amperometry,[3] post-column reaction,[4] coulometry,[5] and indirect photometric detection[6] to name a few. However, far fewer reports of chromatographic or titrimetric means of determining iodine have been published. A microtitration based on the fluorescence quenching of binaphthyl amphiphiles for trace iodine has been proposed.[7] After derivatization of iodine to 4-iodo-2,6-methylphenol, gas chromatography has been coupled with mass spectrometry (selected-ion monitoring) to determine iodine in drinking water.[8] Iodine was extracted from incinerated food samples and chromatographed on an ion-exchange resin with electrochemical detection.[9] Trace iodine species were determined spectrophotometrically after separation as iodide on a reversed phase column and post-column reaction.[10] In this approach, iodine catalyzes the redox reaction between cerium (IV) in the post column reagent stream and arsenic (III) in the mobile phase. Quantitation of iodine was made indirectly by measuring the fluorescence of the cerium (III) product at 350 nm.

Iodinemonochloride (ICl) has been widely used as an iodinating reagent for aromatic compounds for decades. It is formed via the reaction of iodine and chlorine and is commerically available as a neat liquid or as a solution in aqueous or organic solvents, such as dichloromethane. Aqueous ICl reagent is prepared in the presence of chloride ions (NaCl or HCl) as a solution stabilizer.

$$I_2 + Cl_2 \longleftrightarrow 2\,I^+Cl^- \qquad (1)$$

Reports of iodination procedures which incorporate ICl have been published for phenols,[11-13] (poly)methylbenzenes and (poly)methoxy-

benzenes,[14-17] haloaromatics,[15,18] polyaromatics,[17,19] cyclic tryptophans,[20] and anilines.[14, 21-22] Often, polar solvents are used to promote a reaction mechanism of heterolytic cleavage of ICl, followed by subsequent electrophilic attack of the resulting iodine cations on the aromatic ring system.[12,23] Based upon electronegativities, iodination is the predominant reaction anticipated with an aromatic ring. However, there have been published reports of ring chlorination, especially in solvents of lower dielectric constant such as carbon tetrachloride (CCl_4).[14,24] The chlorination reaction has been attributed to electrophilic substitution by chlorine cations which are generated via homolytic cleavage of ICl to form the trichloroiodine interhalogen compound [$ICl_2^-Cl^+$].[12,18,23]

$$ICl + Cl_2 \longleftrightarrow ICl_2^-Cl^+ \qquad (2)$$

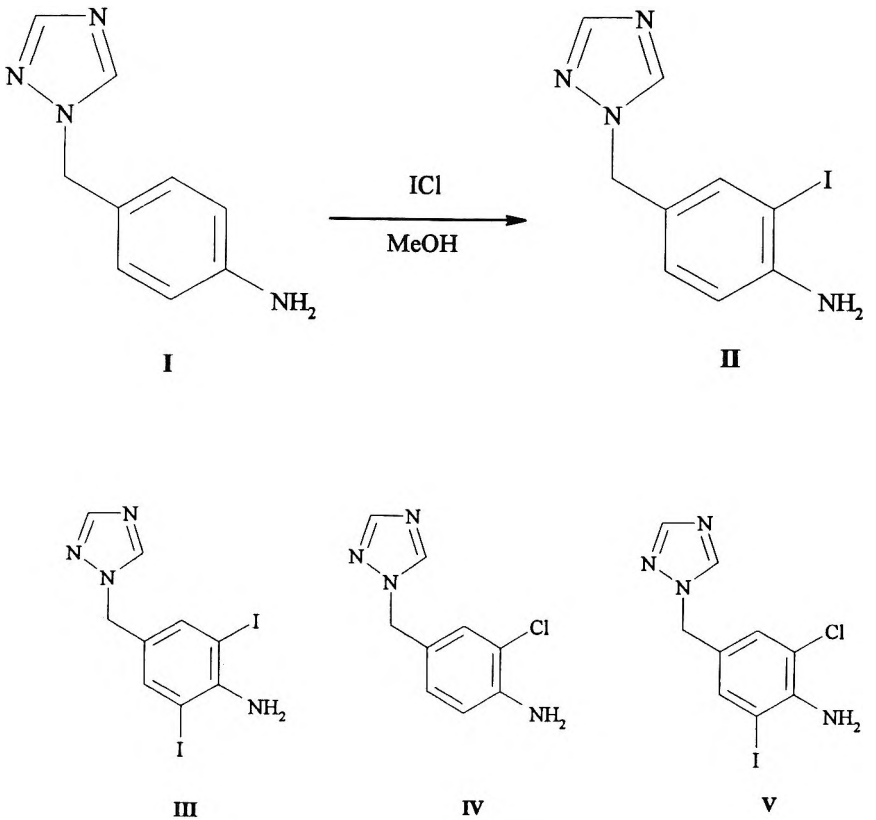
Such an interhalogen iodine compound may also result from a defICIt of iodine relative to chlorine during the manufacturing process.

ICl is soluble in aqueous solutions of NaCl and HCl at concentrations up to approximately 5.5 M; excess iodine levels will result in the precipitation of solid iodine. Aqueous ICl solutions are a deep bromine red color at this concentration, but appear as orange solutions at lower molarities (approximately 3.5 M).[25] Decomposition in aqueous media will produce insoluble I_2 and I_2O_5, which will float on the solution surface:

$$10\ ICl + 5H_2O \longrightarrow 10\ HCl + 4\ I_2\ (s) + I_2O_5\ (s) \qquad (3)$$

Aqueous 5.0 - 5.5 M ICl stabilized by NaCl and HCl (and identified in this paper as ICl) was used to selectively iodinate an aromatic intermediate **I** at the 3-position of the aniline ring system to form intermediate **II** as seen in Scheme 1.[26-27] Compound **III** is an impurity which results from an unavoidable side reaction with the aromatic ring, which proceeds with an initial rate constant that is approximately 3.5 times slower than that of the main reaction.[27]

An effort was made to identify the cause of chlorination in this reaction system to minimize the formation of the undesired chloroderivatives **IV** and **V**. It is apparent from the literature, as described above, that chlorination of **I** should proceed in a solvent of low dielectric constant primarily through the presence of excess chlorine (Equation 2). It, therefore, follows that the quality of the ICl reagent, especially with respect to excess chlorine (iodine undercharge), is the key parameter which controls formation of **IV** and **V** in polar solvent.



Scheme 1. Formal reaction of intermediate I and ICl to form intermediate II, as well as potential impurities III, IV, and V.

Common wet chemical and qualitative analytical techniques for ICl analysis were found to be inadequately selective and sensitive for the determination of undercharge of iodine (or excess chlorine) at the levels required in this application. This paper describes the development of a new indirect technique for ICl analysis in which a large excess of **I** was rapidly derivatized in a polar solvent with ICl (simulating the reaction selectivity) and the product was analyzed by reversed phase HPLC for chlorination products. This approach provides a tool for iodine undercharge (chlorine excess) analysis in ICl with the selectivity and sensitivity of chromatography, and as such, serves as an accurate predictor of the reaction performance of ICl. While the technique is illustrated for this application, it is believed to be applicable to the iodination of substituted anilines in general.

EXPERIMENTAL

Materials Used

Samples of ICl reagent were obtained for testing from Deepwater Iodides (Woodward, OK; 5.1 M; sample 1), Ajay Chemicals (Powder Springs, Georgia; 5.5 M; samples 2 and 3) and DSM Andeno (Baton Rouge, LA; 3.6 M; sample 4). The Ajay and Deepwater solutions were reported to contain 2 wt% HCl and 10 wt% sodium chloride, while the DSM material was reported to contain 13 wt% HCl and 0 wt% NaCl by the respective manufacturers. Sodium thiosulfate pentahydrate (0.1N standard solution), 1% (w/v) starch solution, and citric acid were purchased from Aldrich (Milwaukee, WI).

Development of an HPLC Procedure

An HPLC system consisting of Shimadzu components was used, which included an SIL-10A autoinjector, SCL-10A system controller, LC-10AS gradient pumps, and SPD-10AV UV/VIS detector. The reversed phase HPLC procedure developed to monitor the reaction that forms **II** was also used to evaluate the results of the ICl derivatization procedure. A Zorbax RX C8 column (25 cm x 4.6mm i.d., 5 μm particles) was used with a 5 mM aqueous KH_2PO_4 / acetonitrile mobile phase gradient from 20 to 80% acetonitrile over 15 minutes at ambient column temperature. Detection was performed with a ultraviolet detector at 226 nm. Method flow rate was set at 1.0 mL/min.

Common Techniques for Analysis of ICl

Samples of ICl were characterized by the following common wet chemical and qualitative tests: solution appearance (color and presence of particulates), the USP test for the presence of chloride anion, solution pH to confirm the presence of HCl solution stabilizer, and thiosulfate titration to verify titer and the presence of iodine. The titration is performed as follows. A sample of aqueous ICl is diluted 100 fold in 0.5 N HCl , then diluted 5 fold more in deionized water. To this solution, 1 g of KI is added with vigorous stirring until the KI is completely dissolved. Titration with standardized thiosulfate is immediately commenced until the ICl solution turned pale yellow. At this point, 1 mL of 1% (w/v) starch solution is added to produce an intense dark purple color. Dropwise, thiosulfate addition is continued until the ICl solution returns to a clear color and remains clear for approximately thirty seconds.

Icl Treatment Procedures

Two procedures were used in this work to effect the formation and/or disappearance of free chlorine in samples of ICl reagent as a means of demonstrating the sensitivity of the new analytical test for ICl in detecting slight changes in the ICl. The changes brought about by both of these procedures was found to produce ICl of similar quality as that received from the vendors listed in this paper. Excess chlorine gas was bubbled through ICl sample 2, producing an ICl sample containing 1 wt% chlorine. This sample was termed ICl sample 5. It is known that suffIClent exposure of ICl to solid iodine eliminates the free chlorine in ICl. Studies were performed to determine that the addition of 2 wt% iodine to ICl at room temperature, followed by at least 4 hrs of agitation and then filtration, was adequate to remove the excess chlorine in sample 3 ICl (as measured by the detection of **IV**) to undetectable levels by HPLC. Addition of 8 wt% iodine was necessary to remove excess chlorine from sample 4 ICl.

Development of a Lab Derivatization Procedure for the HPLC Analysis of ICl

A simple and rapid laboratory derivatization procedure was developed to mimic the reaction which forms **II** (Scheme 1). In the formal reaction, **I** is nearly consumed upon completion of the ICl addition at sub-ambient temperature.[27] However, the derivatization procedure was developed with two significant changes to further ensure that the reaction would instantaneously be driven to **II**. First, an approximate 30% molar excess of **I** relative to ICl was used to accelerate the reaction kinetics (the reaction is pseudo-first order in **I**) and second, the procedure was performed at ambient temperature. The excess of **I** used results in a significant amount of residual **I** in the derivatization reaction solution which is analyzed by HPLC. For this reason, the chromatographic peak for **I** is not included in the computation of results for any of the derivatization experiments.

The derivatization procedure developed is as follows: approximately 500 mg of **I** and 100 mg of citric acid (pH buffer) were combined in a 50 mL Erlenmeyer flask and dissolved in 20 mL of HPLC grade methanol by gently swirling the flask and its contents. A 200 mL aliquot of ICl was quickly added to the flask and the solution was swirled for 2 min to ensure the reaction was completed. The reaction was then quenched with 100 mL of 40 wt% sodium thiosulfate solution while briefly swirling the reaction mixture. Finally, the reaction mixture is diluted 75x in HPLC grade methanol and analyzed by HPLC.

Table 1

Results of Common Tests for the Analysis of ICl

Test	ICl Sample 1	ICl Sample 2	ICl Sample 3	ICl Sample 4
Identity				
Iodine Chloride	Positive Positive	Positive Positive	Positive Positive	Positive Positive
Thiosulfate Titration	4.04 M	5.51 M	5.46 M	3.60 M
pH	≈0	≈0	≈0	≈0
Appearance	Deep black liquid, free of particulates	Deep black liquid, free of particulates	Deep black liquid, free of particulates	Deep black liquid, free of particulates

RESULTS AND DISCUSSION

Summary of Results from the Analysis of ICl Using Common Techniques

Analytical results using the common techniques for the different samples of ICl used in this work are shown in Table 1. The table includes data for the ICl samples as they were received from the vendor. There is no discernable difference between samples 1 through 3 based upon this set of tests; sample 4 was orange in color and of much lower molarity as per manufacturer's specifications.

However, if the performance of ICl in the formal reaction in question (Scheme 1) is used to compare these samples, significant differences are noticed in the HPLC chromatogram of isolated **II**. Figure 1 shows the impurity profile of compound **II** made using ICl samples 2, 3 and 4.

Under identical reaction conditions, all of the samples listed in Table 1 produce < 3 A% compound **III**. Production of **III** is minimized in the reaction via equimolar charges of compound **I** and ICl, as well as careful control of reaction pH.[26]

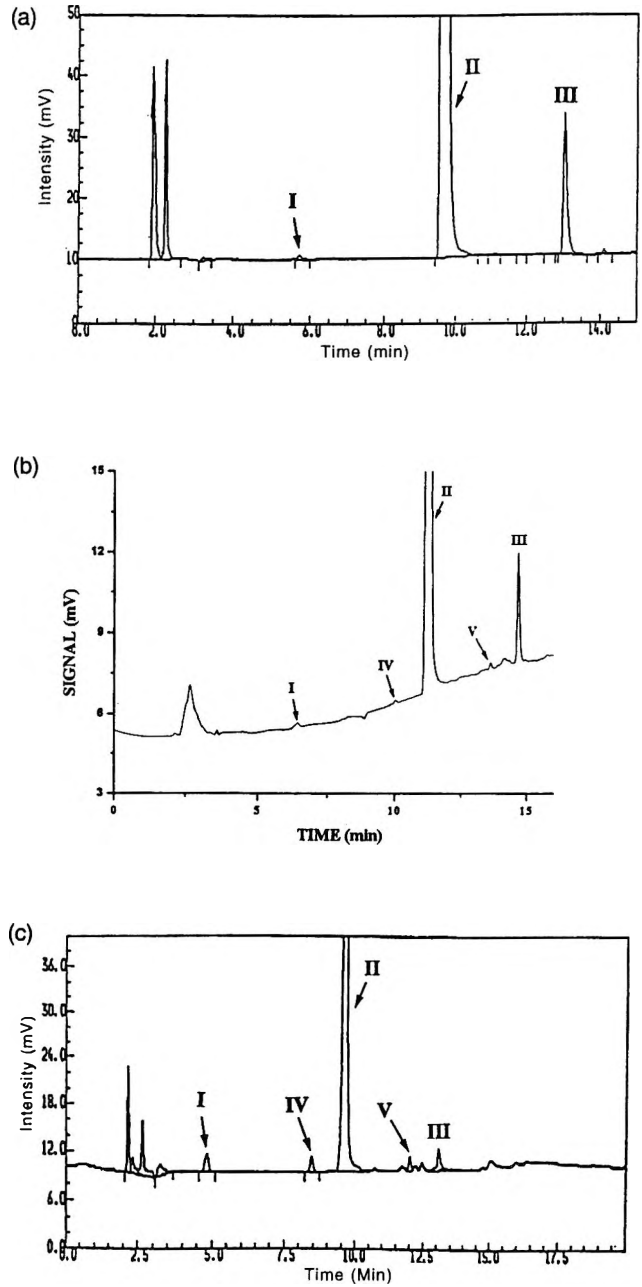


Figure 1. Comparison of impurity profiles of II made via formal reaction with a) ICl sample 2; b) ICl sample 3; and c) ICl sample 4.

While all of the reactions in Table 1 produce some **III**, only samples 3 and 4 produce **IV** and **V** at detectable levels (both impurities were < 0.1% for ICl sample 3 and ≥ 0.5 % for ICl sample 4). The identities of **IV** and **V** were verified by spiking authentic samples of **IV** and **V** into the solutions of **II** used for the chromatographic analysis.

It was postulated that excess chlorine (iodine defICl) in ICl samples 3 and 4 was responsible for the appearance of **IV** and **V** in the reactions performed. To confirm this, ICl sample 5 (containing 1 wt% chlorine) was used in the reaction described in Scheme 1. It was noted that ICl sample 5 was a dark orange color, similar in appearance to ICl sample 4 (Table 1).[28] Isolated **II** from this reaction was found to contain ≥ 0.3 A% each of both **IV** and **V**, simulating the HPLC results of the reaction with ICl sample 4. This confirms that excess chlorine in the ICl raw material is responsible for the unwanted chlorination observed with samples 3 and 4 ICl. Further, it follows that sample 3 ICl contains less excess chlorine than ICl samples 4 and 5 from the ICl solution appearance and reaction performance. However, solution appearance is not a very sensitive analytical technique and only shows utility for samples with significant chlorine excess, such as ICl samples 4 and 5. For ICl samples with lower chlorine content (smaller iodine defICl) such as sample 3 ICl, a more sensitive technique for evaluating ICl raw material and predicting reaction performance prior to use had to be developed.

Derivatization Procedure for the Analysis of ICl

The only available method that was adequately sensitive and specific enough to predict the reaction performance of all types of ICl was a formal reaction of the ICl and **I**, followed by HPLC analysis of the reaction product **II**. While this procedure worked, it was very cumbersome for an analytical raw materials laboratory to perform as a routine evaluation of a raw material. Based on this, a simple and rapid "bench-top" version of the formal reaction was developed as described in the experimental section for analyzing ICl.

With this "bench top" procedure, which includes the derivatization of **I** with ICl as a means of analyzing the ICl for chlorination potential, the sensitivity and selectivity of the full formal reaction is maintained while making the test amenable to routine testing in a raw materials evaluation laboratory. In the following discussion, this test is demonstrated with (i) samples of ICl as received from the vendor and (ii) samples of ICl that have been altered via exposure to solid iodine.

Table 2

Comparitive HPLC Results from Samples of II Produced by Formal Reactions and by the Derivatization Procedure

	Formal Reaction		Derivatization Solution	
	A% of IV	A% of V	A% of IV	A% of V
ICl Sample 2	n.d.	n.d.	n.d.	n.d.
ICl Sample 3	0.01	0.03	0.14	0.04
ICl Sample with added iodine	n.d.	n.d.	n.d.	n.d.
ICl Sample 4	0.65	0.50	0.70	0.14
ICl Sample 4 with added iodine	n.d.	n.d.	n.d.	n.d.

Both the derivatization procedure and formal reaction, followed by HPLC analysis, were completed on ICl samples 2, 3, and 4 and the results are summarized in Table 2. The results of both experiments agree in terms of detectability of impurities **IV** and **V** for all ICl samples considered, indicating that the derivatization procedure mimics the results of the formal reaction in this respect. However, due to differences in the reaction and isolation conditions used for the two sample preparation techniques, the levels of **IV** and **V** differ for ICl samples 3 and 4. Therefore, the derivatization test will be used to identify ICl samples with excess chlorine, but not to quantitate the actual amount of excess chlorine via **IV** or **V** that one would expect from a formal reaction. Further, ICl samples 3 and 4 were treated with solid iodine in the manner described in the experimental section to quench the excess chlorine, then were derivatized and chromatographed and the results are also summarized in Table 2.

Once again, the results from the derivatization samples agree with those obtained from the formal reaction samples. This indicates that the derivatization procedure is adequately sensitive to accurately differentiate ICl samples which have chlorine excesses that generate $\leq 0.1\%$ of **IV** and **V**. This represents an improvement over the thiosulfate titration, which was found to have a method precision of 0.1% RSD at the 5 M level, or the same order of magnitude as the estimated excess of chlorine (iodine deficit) in ICl sample 3.[31] Representative chromatograms of the derivatization reaction solution of ICl sample 3 before and after iodine addition are found in Figure 2.

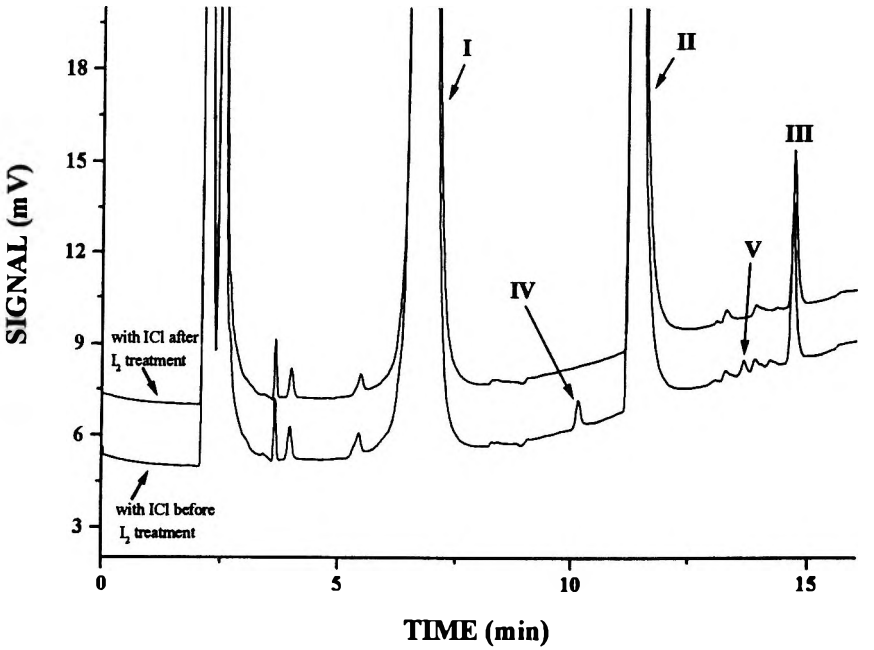


Figure 2. Comparison of impurity profiles of the derivatization solution made with ICl sample 3 before and after iodine treatment.

It is anticipated from the kinetics of the main reaction that **IV** is the predominant chlorination side product of the derivatization reaction, since formation of **II** is greatly favored over **III**. Further, **IV** elutes in a portion of the chromatogram (Figure 2) in which there are no chromatographic interferences from the sample matrix, while **V** elutes near several matrix components. This facilitates routine and unambiguous detection of **IV** by HPLC retention time. For these reasons, detectability of **IV** in the derivatized solution was selected as the marker for ICl quality evaluation and **V** will no longer be discussed.

Validation of the HPLC and Derivatization Methods

The capabilities of the derivatization procedure / HPLC method combination were validated to a level appropriate for the application in accordance with International Conference on Harmonization (ICH) guidelines.[29,30] In addition to the demonstrated method specificity for the compounds of interest (**I**, **II**, **III**, **IV**, and **V**), the chromatographic method was

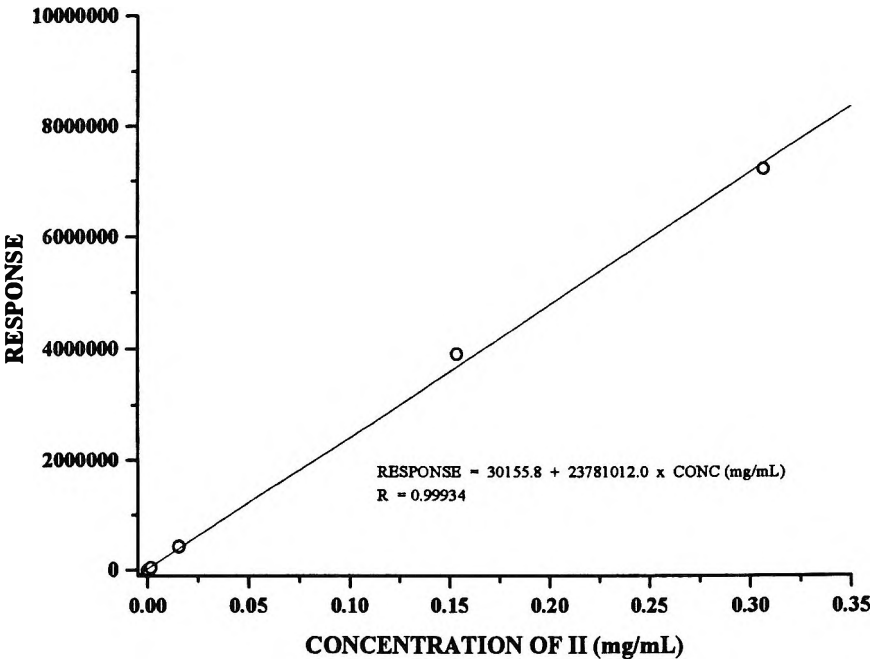


Figure 3. Linearity of the chromatographic method for compound II.

determined to be linear for serial dilutions of **II** in methanol over the concentration range of 0.3066 mg/mL to 3.066E-5 mg/mL, or 200% to 0.02% of the method target concentration of 0.15 mg/mL (R = 0.99934) (Figure 3). The injection precision of **II** was determined from six replicate injections of a single 0.15 mg/mL preparation to be 0.4% RSD.

The method reproducibility for multiple preparations of the derivatization reaction using samples 3 and 2 ICl are provided in Tables 3 and 4, respectively. These experiments represent the worst case scenario for evaluating ICl - the chlorine excess in ICl is small enough to produce levels of **IV** near the method LOD / LOQ. The results for the experiments in Table 3 are all interpreted as "unacceptable ICl" since impurity **IV** was consistently detected. The reproducibilities of quantitation of impurity **IV** for preparations 1 and 2 of **II** were 28.9 % and < 0.1 % RSD, respectively, at the 0.1 % level and the overall reproducibility was 26.1% RSD.

Table 3

Derivatization/HPLC Procedure Reproducibility for Sample 3 ICl

	II	III	IV	Evaluation of ICl
Sample 3 - Preparation 1	99.40	0.48	0.08	unacceptable ICl
Analyst 1	99.34	0.48	0.14	unacceptable ICl
	99.34	0.48	0.14	unacceptable ICl
% RSD - Preparation 1	<0.1%	<0.1%	28.9	
Sample 3 - Preparation 2	99.63	0.19	0.09	unacceptable ICl
Analyst 2	99.62	0.21	0.09	unacceptable ICl
	99.61	0.21	0.09	unacceptable ICl
% RSD - Preparation 2	<0.1%	5.7 %	<0.1%	
% RSD-Overall	0.1%	44.4%	26.1%	

Table 4

Derivatization/HPLC Procedure Reproducibility for Sample 2 ICl

	II	III	IV	Evaluation of ICl
Sample 2 - Preparation 1	99.40	0.60	n.d.	acceptable ICl
Analyst 1	99.38	0.62	n.d.	acceptable ICl
	99.39	0.61	n.d.	acceptable ICl
% RSD - Preparation 1	<0.1%	1.6%	n/a	
Sample 2 - Preparation 2	99.66	0.34	n.d.	acceptable ICl
Analyst 1	99.71	0.29	n.d.	acceptable ICl
	99.71	0.29	n.d.	acceptable ICl
% RSD - Preparation 2	<0.1%	9.4%	n/a	
% RSD - Overall	0.2%	36.4%	n/a	

These results from two different analysts demonstrate that ICl sample 3 will consistently generate both detectable and quantitatable levels of **IV**, suggesting that both the derivatization procedure and HPLC analysis are acceptably robust.

The results in Table 4 obtained from experiments with ICl sample 2 once again demonstrate the reproducibility and robustness of the method for two preparations of an "acceptable ICl" sample. The experimental variability observed in the levels of **III** for the derivatization samples is similar to that observed for numerous formal reactions to **II**, and is therefore not due to errors associated with the HPLC or derivatization methods.[31]

The HPLC method limit of detection (LOD) for **IV** was determined as the lowest concentration for which the signal to noise ratio $\geq$ 3:1, which occurred at 0.01% for a formal reaction sample in Table 2 and 0.08% for an injection of the derivatization solution sample in Table 3. No additional chromatographic interferences with **IV** were observed for derivatization samples vs. formal reaction samples (Figures 1 and 2), hence it is believed 0.01% **IV** would also be detectable in derivatization solutions. The limit of quantitation (LOQ) for **IV** was estimated as 3.3 x LOD or 0.03%. The concentration range of **II** in the derivatization preparation over which **IV** remained detectable (method range) was 300 % to 38% of that described in the experimental section. System suitability requirements include baseline resolution ($R \geq 2.0$) of **II**, **III**, and **IV** and detection of a 0.1% spike of **IV** into a methanolic solution of **II**.

CONCLUSIONS

Trace levels of excess chlorine (iodine deficit) in ICl produce minor, but undesired, chlorination side products **IV** and **V** during the iodination of aromatic aniline compound **II**. Direct means of analysis of ICl raw material are not capable of reproducibly detecting either trace levels of chlorine excess or trace iodine deficits, and as such can not be used as a predictive tool for evaluating ICl raw material prior to use in this type of reaction. To better characterize ICl, an indirect means of assessing the quality of the material has been developed to differentiate ICl samples.

The method described in this paper uses a "bench top" laboratory version of the synthetic reaction in question, followed by HPLC. This new technique was demonstrated to be both adequately specific and sensitive for the application described, while remaining simple and rapid enough to serve as a routine derivatization procedure for an analytical raw materials testing laboratory. The derivatization method was demonstrated to reproducibly identify ICl samples with levels of excess chlorine (iodine deficit) which produce as little as 0.1% of **IV**. The ability to determine the chlorination potential of ICl as a raw materials screening test gives one the option to remediate the raw material before use, thus avoiding removal of various

chlorinated impurities downstream which may be less desirable. The principles used for this specific application should be suitable for related iodination reactions so as long as a suitable laboratory derivatization reaction can be developed.

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REFERENCES

1. S. D. Ingle, S. R. Crouch, **Spectrochemical Analysis**, Prentice Hall, Englewood Cliffs, NJ, 1988, Chapter 8.

2. D. G. Peters, J. M. Hayes, G. M. Hieftje, **Chemical Separations and Measurements: Theory and Practice of Analytical Chemistry**, W.B. Saunders Company, Philadelphia, PA, 1974, Chapter 10.

3. G. A. Sacchetto, G. Favaro, P. Pastore, M. Fiorani, Anal. Chim. Acta, **294 (3)**, 251-260 (1994).

4. A. C. M. Brandao, W. W. Buchberger, E. C. V. Bulter, P. A. Fagan, P. R. Haddad, J. Chromaogr. A, **706 (1-2)**, 271-275 (1995).

5. F. Moussa, M. C. Rauxdemay, F. Veinberg, F. DePasse, R. Gharbi, J. Y. Hautem, J. Chromatogr. B, **667 (1)**, 69-74 (1995).

6. S. Wilson, E. Yeung, Anal. Chim. Acta, **157**, 53-60 (1984).

7. B. Juskowiak, E. Galezowska, Chemia Analityczna, **40 (6)**, 971-977 (1995).

8. H. S. Shin, Y. S. O. Shin, J. H. Kim, J. K. Ryu, J. Chromatogr. A, **732**, 327-333 (1996).

9. H. Kuwahira, Y. Asai, J. Food Hygienic Society of Japan, **35 (3)**, 253-257 (1994).

10. H. Yamada, S. Kajiyama, K. Yonebayashi, Bunseki Kagaku, **44 (12)**, 1027-1032, (1995).

11. G. H. Woolet, W. W. Johnson, **Organic Syntheses (Coll. Vol. 2)**, Wiley, New York, New York, 1943, page 343.

12. F. W. Bennett, A. G. Sharpe, J. Chem. Soc., **284**, 1383-1384 (1950).

13. D. Papa, H. F. Ginsberg, I. Lederman, V. DeCamp, J. Am. Chem. Soc., **75**, 1107 (1953).

14. L. J. Lambourne, P. W. Robertson, J. Chem. Soc., **217**, 1167-1168 (1947).

15. R. M. Keefer, L. J. Andrews, J. Chem. Soc., **72**, 5170 (1950).

16. L. J. Andrews, R. M. Keefer, J. Chem. Soc., **79**, 1412 (1957).

17. B. Jones and E.N. Richardson, J. Chem. Soc., **146**, 713-715 (1953).

18. G. Olah, A. Pavlath, G. Varsanyi, J. Chem. Soc., **79**, 1823 (1957).

19. D. E. Turner, R. F. O'Malley, D. J. Sardella, L. S. Barinelli, P. Kaul, J. Org. Chem., **59**, 7335-7340 (1994).

20. D. E. Zembower, M. M. Ames, Synthesis, **12,** 1433-1436 (1994).

21. R. B. Sandin, W. V. Drake, F. Leger, **Organic Syntheses (Coll. Vol. 2)**, Wiley, New York, New York, 1943, page 196.

22. E. Berliner, J. Am. Chem. Soc., **78**, 3632-3637 (1956).

23. C. W. McCleland, J. S. Pizey editor, **Synthetic Reagents (Vol. 5)**, Wiley, New York, New York, 1983, pages 86-164.

24. R. M. Keefer, L. J. Andrews, J. Org. Chem., **31**, 541-544 (1966).

25. Laboratory observation made of a 3.6 M iodinemonochloride in aqueous HCl solution purchased from DSM Andeno.

26. C. Y. Chen, D. R. Lieberman, R. D. Larsen, R. A. Reamer, T. R. Verhoeven, P. J. Reider, I. F. Cottrell, G. Houghton, Tet. Lett., **35 (38)**, 6981-6984 (1994).

27. Merck and Co., Department of Chemical Engineering, 1997, Unpublished results.

28. Merck and Co., Developmental Laboratories, 1997, Unpublished results.

29. **ICH Harmonized Tripartite Guideline: Text on Validation of Analytical Procedures**, Guidance Q2A, March 1995.

30. **ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures: Methodology**, Guidance Q2B, November 1996.

31. Merck and Co., Department of Analytical Research, 1997, Unpublished results.

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SIMULTANEOUS DETERMINATION OF SUGARS, URIC AND OROTIC ACIDS IN INFANT FORMULAE BY HPLC-UV/RI

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ABSTRACT

In a simple, rapid isocratic HPLC method sugars (glucose, galactose, saccarose, maltose, lactose), uric and orotic acids were separated on a Spherisorb NH2, 5 μm Chromatographic column and detected using refractive index and ultraviolet (λ = 280 nm) detectors in series. The identification was made by comparison of the retention times with those of the corresponding standards; xilose and melizitose were used as internal standards. The determinations were performed in the linear range of 0.5-30.0 g/L for sugars, 1.0-18.0 mg/L and 0.5-20.0 mg/L for uric acid and orotic acid, respectively. The detection limits were 0.20 g/L for xilose, glucose, galactose, and lactose and 0 10 g/L, 0.35 g/L, 0.40 g/L for saccarose, maltose, and melizitose, respectively. For uric and orotic acids the detection limits were 0.5 mg/L and 0.1 mg/L, respectively. The validity of the method was verified. For recovery studies of internal standards (I.S.), several determinations were conducted, using the standard addition method at three specific concentrations (1.0, 5.0, and 10.0 g/l). The recoveries ranged from 95 to 101%.

The precision of the method was also evaluated, the %CV being 1.01, 0.51, 0.45, 0.73, 0.82, 0.82, and 0.74 for xilose, saccarose, maltose, lactose, melizitose, uric acid, and orotic acid, respectively. The sample pre-treatment was simple with a single extraction. The good precision and accuracy obtained proved that this method is suitable for routine analysis.

INTRODUCTION

The infant formulae industry tries to obtain powdered formulae that upon reconstitution have a composition similar to that of the human milk, thus, their composition relatively to some constituents, namely the sugars, is not the same as the cow's milk, from which they are originally prepared. Regulation establishes which sugars can be added and their limits.[1] Other constituents such as uric and orotic acids are endogenous in milk, consequently, they appear naturally in infant formulae. Uric acid is an undesirable constituent as opposed to orotic acid whose presence is important, however the suplementation of the infant formulae with orotic acid is still a controversial subject.[2]

The determination of sugars (glucose, galactose, saccarose, maltose, lactose), uric, and orotic acids on infant formulae takes on considerable importance with respect to the quality control of those products, for both labelling and nutritional reasons. To this end, a precise, reproducible, rapid, and economic analytical procedure is required for routine quality control in infant formulae industry. Owing to the good characteristics of separation, identification, and evaluation of different components in complex matrices showed by the HPLC methodology, alied to a good precision and accuracy, HPLC methods have been employed to analyze uric and orotic acids[3-4] and sugars[5-6] in dairy products. An attempt for simultaneous determination of some sugars and organic acids on cheese,[7] using an ion-exchange column has been reported.

The objective of our research was the development of a rapid, simple, and specific method for simultaneous determination of sugars, uric, and orotic acids in infant formulae by HPLC - UV/RI. Xilose and melizitose were used as internal standards. With the HPLC chromatographic column used (Spherisorb NH_2) and according to the literature,[8] the separation between glucose and galactose is difficult, but the separation of saccarose, maltose, lactose, and melizitose is good. The ion-exchange columns are considered preferential on the separation of monossacarides, but they present difficulties on the separation of some disaccharides.[6,9]

The separation of uric and orotic acids on ion exchange columns[4] has proved to be well reproducible, but owing to the column characteristics and slow elution, the peaks were broad and the sensitivity of the method correspondingly low. In order to retain the selectivity of ion-exchange chromatography, but improve the sensitivity, we attempted to use ion-pair formation of uric and orotic acids and separation on a Spherisorb NH_2 column.

The objective of our research was the development of a rapid, simple, and specific method for simultaneous determination of sugars, uric, and orotic acids in infant formulae by HPLC - UV/RI. Xilose and melizitose were used as internal standards.

MATERIALS AND METHODS

Reagents and Solutions

All reagents used were (p.a) from Merck. The standards for the orotic and uric acids were from Sigma Chemicals Co. The water used for chromatography analysis had a resistance greater than 15 MΩ. This was filtered through a membrane of 0.45 μm porosity and subsequently degassed. The acetonitrile (Lichrosolv) used was, Merck "gradient grade". The mobile phase used was acetonitrile/HCL 0.01M (84:16).

Apparatus

The chromatographic analyses were carried out in a Gilson, high performance liquid chromatograph equipped with a type 305 pump and a type 7125 Rheodyne Injector with a 20 μl loop. A Gilson variable wavelength UV/VIS detector, a 132 RI detector and a Gilson 712 HPLC System Controller Software were also used.

The detectors were connected in series. The UV detector, set at 280 nm, was used for quantification of uric and orotic acids. The RI detector was used for quantification of sugars. The chromatographic separation was achieved with a Spherisorb NH_2 chromatography column, 5 μm, 250 mm x 4.6 mm i.d.. The analyses were performed isocratically at a flow rate of 1.0 mL/min and at room temperature.

Calibration and Calculations

Single standard solutions of sugars and acids were prepared to establish elution times. Quantification was based on the internal standard method, using xilose and melizitose as internal standards (I.S.). Five aqueous mixed standards for sugars (xilose, glucose, galactose, saccarose, maltose, lactose, melizitose), uric, and orotic acids covering a broad concentration range were prepared to establish calibration curves. Resulting peak heights were determined for duplicate 20 μl injections. Standard curves for each component were prepared by linear regression of peak height vs concentration. Afterwards, individual standard solutions were added one by one to infant formulae samples to verify correct identification of peaks using elution times.

Sample Preparation

Approximately 1.5 g of previously homogenized infant formulae powder was dissolved in 2 mL of warm distilled water. 0.5 mL of 5% oxalic acid (w/v) and 5 mL of 95% ethanol were then added. The mixture was homogenizeded for 5 min. The volume was adjusted to 20 mL with deionized water and allowed to stand for 10 min. The supernant was injected into the 20 μL loop with a 5 mL syringe fitted with a syringe filter holder containing 0.2 μm membranes filters.

Precision of the Method

Complete duplicate analyses were performed on all samples to enable calculation of average deviations which were useful as a mesure of the extraction and chromatographic reproducibility. The precision of the analytical method was evaluated by measuring the peak height 10 times on the same sample.

Recovery Study

The recovery of internal standards added to infant formulae was evaluated to determine the accuracy of the test. Infant formulae were analyzed in duplicate before and after the addition of known amounts of I.S. Three different concentration levels of internal standards were analyzed, complete duplicate analyses were performed.

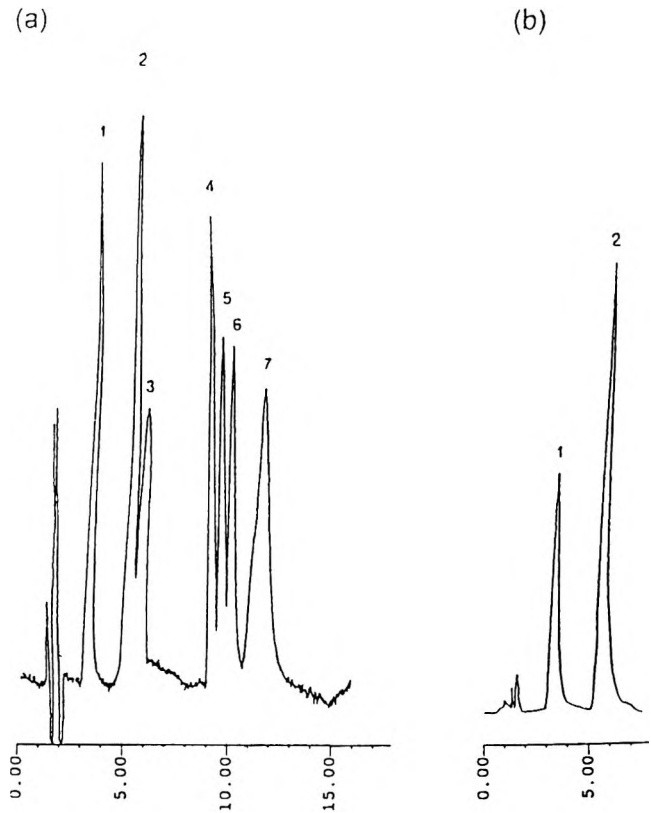

Figure 1. Typical chromatograms of aqueous standard solutions. A - sugars from RI detector. Sugar standards: (1) xilose, (2) glucose, (3) galactose, (4) saccarose, (5) maltose, (6) lactose, (7) melizitose. B - Acids from UV detector at 280 nm. Acid standards: (1) uric, (2) orotic.

RESULTS AND DISCUSSION

Standard Calibration Curves

Simultaneous determination of sugars, uric, and orotic acids in infant formulae samples was made possible by using the UV and RI detectors connected in series. Typical chromatograms depicting separation of aqueous standard solutions of sugars and organic acids are shown in Fig.1.

Table 1

Sugars, Uric andOrotic Acids Composition of Infant Formulae Samples[a]

Samples	Glucose (g/100g)	Galactose (g/100g)	Saccarose (g/100g)	Maltose (g/100g)	Lactose (g/100g)	Uric Acid (mg/100g)	Orotic Acid (mg/100g)
1	N.D.	N.D.	N.D.	N.D.	54.2 ± 2.3	6.80 ± 0.11	26.7 ± 1.0
2	N.D.	N.D.	N.D.	N.D.	58.2 ± 1.1	8.02 ± 0.07	25.1 ± 0.9
3	N.D.	N.D.	N.D.	N.D.	45.6 ± 2.2	6.10 ± 0.09	21.8 ± 1.1
4	N.D.	N.D.	N.D.	N.D.	54.4 ± 0.8	10.8 ± 0.10	37.6 ± 1.7
5	N.D.	N.D.	N.D.	N.D.	55.8 ± 0.9	5.12 ± 0.09	15.1 ± 0.8
6	0.51 ± 0.03	<0.3	7.12 ± 0.24	3.40 ± 0.10	47.8 ± 1.2	8.67 ± 0.13	23.1 ± 0.3
7	N.D.	N.D.	5.18 ± 0.71	N.D.	46.7 ± 1.3	4.17 ± 0.09	14.2 ± 0.7
8	0.53 ± 0.4	N.D.	N.D.	0.61 ± 0.08	26.7 ± 0.5	10.9 ± 0.20	34.1 ± 1.1

[a] Values are expressed as mean ± standard deviation of two determinations.
N.D. - not detected.

Under the assay conditions described, a linear relationship between the concentration of sugars and the refractive index was obtained. The same happened between the concentration of uric and orotic acids and the UV absorbance at 280 nm. This linearity was maintained over the concentration range of 0.5-30.0 g/L for sugars, 1.0-18.0 mg/L for uric acid, and 0.5-20.0 mg/L for orotic acid.

The correlation coefficient for each standard curve invariably exceeded 0.99 for all compounds. The calibration curves for sugars, uric, and orotic acids were obtained by duplicate determinations of each of the calibration standards and the peak height values (arbitrary units) were plotted as average values.

The relative percent average deviations of duplicates were less than 2 % in all cases. The average regression equation for xilose, glucose, galactose, saccarose, maltose, lactose, melizitose, uric, and orotic acids were $y = 147.77 x + 0.981$; $y = 135.64 x + 0.331$; $y = 82.704 x + 0.355$; $y = 81.393 x + 0.859$; $y = 187.51 x + 5.10$; $y = 31.761 x + 1.75$; $y = 20.592 x + 2.106$; $y = 25.331 x + 7.1$; $y = 18.852 x + 5.1$, respectively.

The detection limit was calculated as the concentration corresponding to three times the SD of the background noise, obtained with 10 determinations. The detection limits were 0.20 g/L for xilose, glucose, galactose, and lactose and 0.10 g/L, 0.35 g/L, 0.40 g/L for saccarose, maltose, and melizitose, respectively. For uric and orotic acids the detection limits were 0.5 mg/L and 0.1 mg/L, respectively.

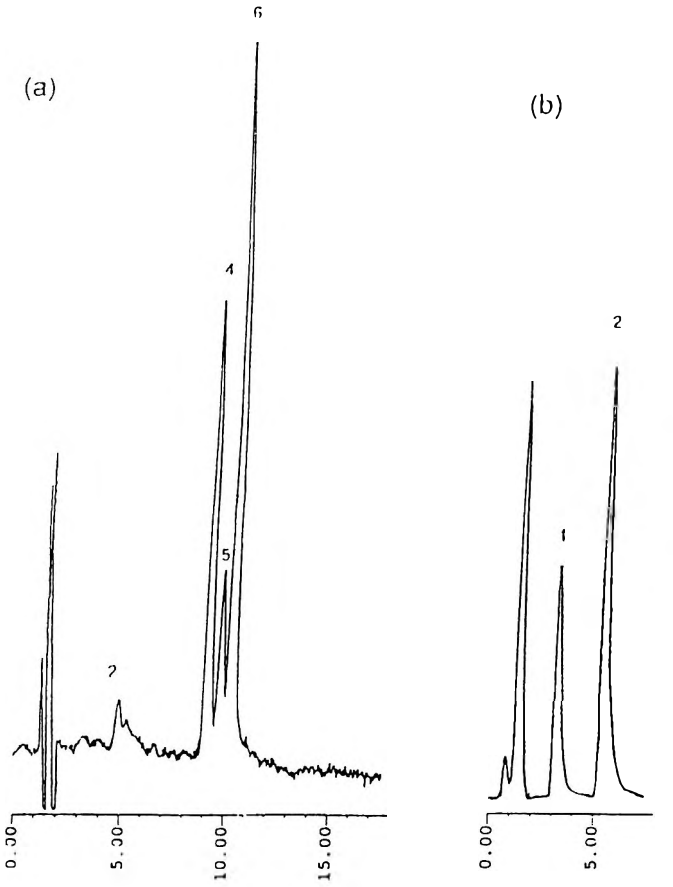

Figure 2. Typical chromatograms of infant formulae (chromatographic conditions described in the text). The letters and the numbers correspond to the letters and numbers in Fig. 1 with respect to peak identification.

Typical Analytical Results and Chromatograms for Infant Formulae

Typical results and average deviations of duplicate determinations for a variety of commercial infant formulae are presented in Table 1. Figure 2 (a and b) shows the typical chromatograms for infant formulae. Figure 3 (a and b) shows the chromatograms for infant formulae with internal standards addition, after having been subjected to identical treatment.

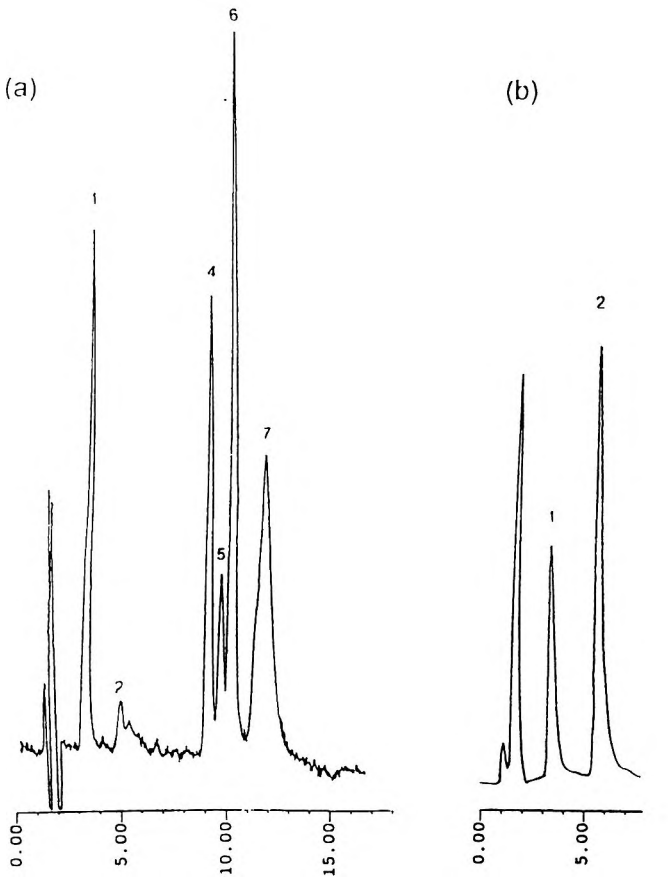

Figure 3. Typical chromatograms of infant formulae spiked with I.S. (chromatographic conditions described in the text). The letters and the numbers correspond to the letters and numbers in Fig. 1 with respect to peak identification.

The initial solvent peaks for all chromatograms occur between 1 and 2 min and result from water and other unretained components. The precision of the method was also evaluated by measuring the peak height of each compound, 10 times on the same sample. The %CV being 1.01, 0.51, 0.45, 0.73, 0.82, 0.82, and 0.74 for xilose, glucose, galactose, saccarose, maltose, lactose, melizitose, uric acid, and orotic acid, respectively (concentration of saccarose, maltose, lactose, uric, and orotic acids in infant formula were 7.12 g/100 g, 3.40 g/100g, 47.8 g/100g, 8.67 mg/100g, and 23.1 mg/100g respectively).

Table 2

Recovery Study of Internal Standards Added to Homogenized Infant Formulae Powder

Xilose

Added (g/L)	Found (g/L)	Standard	CV%	Recovery
1.00	0.953	0.006	0.63	95.3
5.00	4.95	0.087	1.75	99.0
10.0	9.51	0.006	0.063	95.1

Mean value 96.5 ± 2.2%

Melizitose

Added (g/L)	Found (g/L)	Standard	CV%	Recovery
1.00	0.953	0.006	0.63	95.3
5.00	4.92	0.029	0.59	98.4
10.0	9.51	0.012	0.13	95.1

Mean value 96.2 ± 1.9%

Recovery Study

As can be seen from Figure 2 (b), there is no chromatographic indication of interfering endogenous compounds during the retention time relating to xilose and melizitose. Table 2 presents the results obtained from the study of internal standards recovery. Internal standard recovery percentages ranged between 95 and 101% for the three concentrations studied, the average percent recovery was 96.5 ± 2.2 % for xilose and 96.2 ± 1.9 % for melizitose. These two findings highlight the appropriateness of the chosen internal standards and can be taken as a guarantee of the accuracy of the method developed.

CONCLUSIONS

The described procedure seems to fulfill the criteria of selectivity, sensitivity, reproducibility, and convenience of a method suited for routine assay of various sugars, uric acid, and orotic acid in infant formulae. The main

advantages of this method are simple sample preparation (a single extraction) and the use of a single NH_2 HPLC column which does not need to be regenerated and has a lower cost compared with ion-exchange columns. Furthermore, the method is rapid and easy to carry out. The method, herein, presented, possesses the demonstrable simplicity of allowing simultaneous analysis of five sugars and two organic acids in less than 15 minutes.

The results obtained allow us to foresee that the developed methodology could be extended to monitoring the levels of these compounds in other types of milk produts. The merging of all these qualities makes the method extremely useful for routine analysis and, especially so, after its automation.

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REFERENCES

1. 91/5/EC Directive, relating with infant formulae, J.O.E.C., # L 175/35, 4.7.91.

2. **Nutrient and Energy Intakes for the European Community**, Thirty-first series, Reports of the Scientific Commitee for Food, Commision of the European Community, 1993, p. 224.

3. R. T. Marsili, H. Ostapenko, R. E. Simmons, D. E. Green, J. Food Sci., **46**, 52-57 (1981).

4. K. P. Navder, R. F. S. Huang, E. B. Fryer, H. C. Fryer, J. Food Sci., **55(2)**, 585-586, (1990).

5. M. R. P. Ferreira, R. Leitão, M. Ferreira, Rev. Port. Farm., **XLV(2)**, 75-78 (1995).

6. B. Herbreteau, Analusis, **20**, 355-374 (1992).

7. J. Bouzas, C. A. Kantt, F. Bodfelf, J. A. Torres, J. Food Sci., **56(1)**, 276-278 (1991).

8. J. F. Pirisino, J. Food Sci., **48**, 742-754 (1983).

9. M. Nouri, Alim. Equipos. Tecnol., Jan-Feb, 69-74 (1993).

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J. LIQ. CHROM. & REL. TECHNOL., 20(20), 3431–3434 (1997)

THE BOOK CORNER

RELIABLE DESIGN OF MEDICAL DEVICES, Richard C. Fries, Marcel Dekker, Inc., New York, 1997, 703 pages, $195.00, ISBN: 0-8247-9843-0.

This book has been written with the intention of collecting, in one place, the current state of affairs pertaining to the design of medical devices. The author points out how the design and functional complexity of medical devices has increased during the recent past, and how this affects the nature of the devices required for current use. His particular concern is that, as device functionality becomes more intricate, concerns arise regarding efficacy, safety, and reliability, which play an ever-increasing role.

These ever-increasing requirements are counter-balanced by the concerns of the commercial side of the business, which seeks to have the devices produced for the lowest cost in the shortest time possible. Medical device development is seen as a complex process that requires the careful integration of diverse disciplines, technical activities, standards, regulatory requirements, and administrative project controls. The need for systematic approaches to product development and maintenance is necessary to ensure a safe and effective device for the user and patient, an economical and competitive success for the manufacturer, and a reliable, cost-effective, investment for the user.

For his approach, the author has chosen the Reliability Assurance program. In this manner, one can provide the theoretical and practical tools by which the probability and capability of systems and their components to perform required functions can be specified, predicted, designed, tested, and demonstrated. His primary goal is to acquaint the developer of medical devices, as well as the purchaser of medical equipment, with the basic concepts and major issues of medical device reliability, to describe current product development processes and techniques, and to provide a basis for evaluating new technologies. He seeks to provide a practical approach to the formation and operation of a reliable assurance program, emphasizing a practical approach.

The book has been organized so as to follow the typical product development process. The first section introduces the reader to the concept of medical devices as being compromised of hardware and software. The concept of hardware/software failure is discussed as a prelude to the discussion of Reliability Assurance, and the section concludes with reviews of current device safety, and of economics ideas and issues.

Section 2 deals with the forest of device standards and regulations as these exist on both the domestic and the international scene. Domestically, the role of FDA in the regulation of medical devices is discussed, along with current guidelines and regulations, including the pending guideline on Human Factors. Internationally, the Medical Device Directives, and their impact on the product development process, are discussed. Other hardware regulations and standards are then reviewed, including ISO, IEEE, and military standards.

Section 3 deals with the specification and design of a medical device, beginnning with the definition of the product. As part of developing the specification of the device, risk management and human factors are considered, as well as the use of metrics. The hardware discussion addresses design alternatives, selecting components that will meet the intended use of the device, establishing a safety margin, establishing load protection, developing a Requirements Traceability Matrix, and selecting among various design technologies. The software discussion addresses design alternatives, selecting the software architecture, choice of methodology, selection of language, developing a Requirements Traceability Matrix, and selecting among various design techniques. Finally, the coding of the software concludes the design portion of the life cycle.

Section 4 discusses system verification and validation, whereby one proves that the design meets its specifications. The basics and various types of testing are reviewed, followed by the various types of testing which can be conducted. Verification and validation are discussed for both hardware and software, with examples being taken from actual product development activity. The section concludes with an analysis of test data that has been accumulated, and the calculation of reliability parameters.

Section 5 deals with manufacturing as a continuation of the product development process. A device may be reliably designed, but if it is not reliably manufactured, it will not be a success. The current Good Manufacturing Practices regulations are discussed. The possible outsourcing of manufacturing is discussed, and critically compared to the analogous in-house process.

Configuration management and its many implications are also reviewed. Finally, techniques for the analysis of field data are discussed in relation to the building of an efficient database for all product development personnel.

This book represents a comprehensive and encompassing view of the entire procedure which accompanies the design, development, and eventual manufacture of a medical device. Written by an author who has clearly lived the process (and survived), it is amply evident to this reviewer that one need acquire no other book if one was to undertake a similar mission. Those seeking to succeed in the area of medical device manufacture must acquire and read this book cover-to-cover if they wish to maintain a competitive advantage. Readers should note that this volume makes a very appropriate companion to that written by S. C. Gad, *Safety Evaluation of Medical Devices*, which was recently issued by the same publisher and is reviewed below.

SAFETY EVALUATION OF MEDICAL DEVICES, Shayne Cox Gad, Marcel Dekker, Inc., 1997, 388 pages, $165.00, ISBN: 0-8247-9827-9.

This volume has been written with the sole objective of providing a practical guide for those who are responsible for, or concerned with, ensuring the safety of medical devices for patients, health care providers, and those involved in their manufacture. As such, the basic aspects of device regulation and materials utilized in devices have been addressed. In areas where it was deemed appropriate, the history and underlying science have also been presented to allow the reader to make an informed decision.

The first chapter provides a solid introduction, covering the scope of medical devices and their markets, a brief history of medical devices, the regulatory basis of safety evaluation, and toxicity testing as it applies to devices. The second chapter is one of the most useful in the book, in that it lays out a road map for developing a test program. This chapter is followed by a short outline of sampling and sample preparation.

At this point, the author catalogs the diverse range of possible testing in a series of chapters which are replete with illustrative examples. Covered in this sequence are the testing programs related to cytotoxicity, blood compatibility, irritation and pyrogenicity, immunotoxicology, implantation studies, genotoxicity, subchronic and chronic toxicity, carcinogenicity, reproductive, and developmental toxicity.

The volume ends with several special topics chapters, which cover important topics not treated in the earlier chapters. The science of dealing with sterility and heavy metals is touched upon, and a solid coverage of clinical studies for medical devices follows. A number of special studies and special cases are treated in the following chapter. However, the author saves the best for last, discussing a number of highly relevant case histories and providing the history as to how these problems were resolved.

The author has attempted to present a program for the safety evaluation of devices and device materials, as would be implemented during the overall development of new products. The approach appears to be sound, and the quantity of practical information (a lot of which has been drawn from the author's experience) makes this book very worthwhile for workers in the field. In fact, this volume makes a very appropriate companion to that written by R. C. Fries, *Reliable Design of Medical Devices*, which was recently issued by the same publisher and is reviewed above.

Reviewed by
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88 Courter Avenue
Maplewood, NJ 07040

J. LIQ. CHROM. & REL. TECHNOL., 20(20), 3435 (1997)

EDUCATION ANNOUNCEMENT

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The instructor, Dr. Jack Cazes, is founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Series Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on GPC, and has taught at Rutgers University. He is currently Visiting Scholar at Florida Atlantic University.

Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097. Tel.: (954) 570-9446; E-Mail: cazes@worldnet.att.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(20), 3437–3442 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1998

FEBRUARY 26 - 28: Sample Handling and Analysis of Organic Pollutants, Archamps, France. Contact: ACTIVE Assoc. Office, c/o Pharmapeptides, CUR Business Park, 74166 Archamps, France.

FEBRUARY 28 - MARCH 1: 28th Annual Spring Prog. in Polymers: Degradation & Stabilization of Polymers, Hilton Head Is., South Carolina. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561. Email: ims@mhv.net.

MARCH 1 - 5: 28th International Symposium on Environmental Analytical Chemistry (ISEAC 28), University of Geneva, Switzerland. Contact: ACTIVE Assoc. Office, c/o Pharmapeptides, CUR Business Park, 74166 Archamps, France.

MARCH 1 - 6: PittCon '98, New Orleans, Louisiana. Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 8 - 12: AIChE Spring National Meeting, Sheraton, New Orleans. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

MARCH 15 - 18: 14th Rocky Mountain Regional Meeting, Tucson, Arizona. Contact: R. Pott, Univ of Arizona, Tucson, AZ 85721.

MARCH 23 - 25: 5th Meeting on Supercritical Fluids: Materials and Natural Products Processing, Nice, France. Contact: F. Brionne, Secretariat, ISASF, E.N.S.I.C., 1 rue Grandville, B.P. 451, F-54001 Nancy Cedex, France. Email: brionne@ensic.u-nancy.fr.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

MAY 3 - 8: HPLC'98 – 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques, Regal Waterfront Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 20 - 22: 32nd Middle Atlantic ACS Regional Meeting, Wilmington, Delaware. Contact: G. Trainor, DuPont Merck, P. O. Box 80353, Wilmington, DE 19880-0353. Email: reglmtgs@acs.org.

MAY 26 - 29: VHIth International Symposium on Luminescence Spectrometry in Biomedical and Environmental Analysis: Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Las Palmas de Gran Canaria (Canary Islands), Spain. Contact: Dr. Jose Juan Dantana Rodriguez, University of Las Palmas of G. C., Chem. Dept., Faculty of Marine Sci., 35017 Las Palmas de G. C., Spain. Tel: 34 (9) 28 45.29.15 / 45.29.00; FAX: 34 (9) 28 45. 29.22; Email: josejuan.santana@quimica.ulpgc.es.

MAY 27 - 29: 30th Central Regional ACS Meeting, Ann Arbor, Michigan. Contact: D. W. Ewing, J. Carroll Univ., Chem. Dept., Cleveland, OH 44118. Tel: (216) 397-4241. Email: ewing@jevaxa.jeu.edu.

JUNE 1 - 3: 31st Great Lakes Regional ACS Meeting, Milwaukee, Wisconsin. Contact: A. Hill, Univ. of Wisc., Chem. Dept., Milwaukee, WI 53201, USA. Tel: (414) 229-4256; Email: reglmtgs@acs.org.

JUNE 2 - 5: Third International Symposium on Hormone and Veterinary Drug Residue Anbalysis, Congres Centre Oud Sint-Jan, Bruges, Belgium. Contact: C. Van Peteghem, Dept. of Food Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel: (32) 9/264.81.34.

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483.

JULY 12 - 16: SFC/SFE'98: 8th International Symposium & Exhibit on Supercritical Fluid Chromatography, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS, 1155 16th Street, NW, Washington, DC 20036, USA.

AUGUST 31 - SEPTEMBER 3: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Charleston Place, Charleston, South Carolina. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

SEPTEMBER 13 - 18: 22nd International Symposium on Chromatography, ISC'98, Rome, Italy. Contact: F. Dondi, Chem. Dept., Universita di Ferrara, Via L. Borsari, 46, I-44100 Ferrara, Italy. Tel: 39 (532) 291154; FAX: 39 (532) 240707; Email: mo5@dns.Unife.it.

SEPTEMBER 23 - 25: International Symposium on Preparative and Industrial Chromatography and Allied Techniques (SPICA '98), Strasbourg, France. Contact: Mlle. Francoise Brionne, E.N.S.I.C., 1, rue Grandville, B.P. 451, F-54001 Nancy Cedex, France.

SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania. Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

NOVEMBER 15 - 20: AIChE Annual Meeting, Fontainbleu/Eden Roc, Miami Beach, Florida. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036. Email: natlmtgs@acs.org.

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations, Seattle, Washington. Contact: Barr Enterprises, P.O.B. 279, Walkersville, MD 21793, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036. Email: natlmtgs@acs.org.

AUGUST 25 - 31: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

2003

MARCH 23 - 28: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

SEPTEMBER 7 - 12: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 2: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC.

AUGUST 22 - 27: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 18: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 2: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2006

MARCH 26 - 31: 231st ACS National Meeting, Atlanta, GA. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 10 - 15: 232nd ACS National Meeting, San Francisco, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2007

MARCH 25 - 30: 233rd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 24: 234th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs @acs.org.

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Following are acceptable reference formats:

Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

Book:

1. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York, 1979.

Chapter in a Book:

1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page.

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used. **Hand-drawn characters are not acceptable.**

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5. **Figures** (drawings, graphs, etc.) should be professionally drawn in black India ink on separate sheets of white paper, and should be placed at the end of the text. They should not be inserted into the body of the text. They should not be reduced to a small size. Preferred size for figures is from 5 inches x 7 inches (12.7 cm x 17.8 cm) to 8½ inches by 11 inches (21.6 cm x 27.9 cm). Photographs should be professionally prepared, black and white, *glossy* prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn.

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SUPERCRITICAL FLUID CHROMATOGRAPHY WITH PACKED COLUMNS

TECHNIQUES AND APPLICATIONS

(Chromatographic Sciences Series/75)

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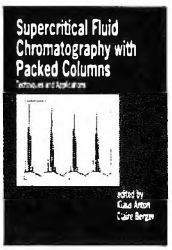

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