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COMPUTATIONAL CHEMICAL ANALYSIS OF THE SEPARATION OF DERIVATIZED R- AND S-AMINO ACID ENANTIOMERS ON N-(tert-BUTYLAMINOCARBONYL)-(S)-VALYLAMINO-PROPYLSILICA GEL AND (R)-1-(α-NAPHTHYL)-ETHYLAMINOCARBONYL-GLYCYLAMINO-PROPYLSILICA GEL BY LIQUID CHROMATOGRAPHY

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ABSTRACT

The chiral selectivities of N-(tert.-butylaminocarbonyl)-(S)-valylaminopropylsilica gel and (R)-1-(α-naphthyl)-ethyl-aminocarbonyl-glycylaminopropylsilica gel were studied using model compounds. The differences in the final energy values of molecular interactions between the model chiral phase and derivatized (R)- and (S)-amino acids, calculated by molecular

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INTRODUCTION

Hydrogen bonds and steric effects are considered to be the major chiral recognition forces in normal-phase liquid chromatography. Indeed, analyses of chiral complexes by NMR and IR have indicated the presence of hydrogen bonds.[1] Furthermore, hydrogen bond formation has also been demonstrated by X-ray crystallography.[2] On the other hand, the development of computational chemical analysis has eased the study of molecular interaction, and some bonded phases for gas and liquid chromatography have been analyzed.[3-5] If a chiral center is defined, the energy of a chiral complex can easily be calculated and the probability of chiral separation can be estimated from the energy difference betw between chiral complexes. Several so-called Pirkle type chiral recognition phases have been synthesized and, therefore, the chiral recognition model was targeted for computational chemical analysis to understand the retention mechanism and predict enantiomer separation. The details of the conformation of model chiral phases were analyzed previously;[6-12] however, the relation between calculated energy values and the separation factor α values was not well presented. Only a limited number of such results have been reported.[13,14] The chiral recognition of N-butyrylvaline-tert-butylamide for (R)- and (S)-4-nitrobenzoyl amino acids was investigated using the CAChe™ molecular mechanics calculations, and the chiral recognition center was investigated from the geometry of the chiral phase and the energy difference between the complexes. Generation of a three-dimensional visual structure helped in studying the chiral recognition center; however, the energy values did not support the elution order.[15]

In this study, liquid chromatographic data reported by Oi[16] were analyzed using a model chiral phase and molecular mechanics calculation to understand the present limitations in both chromatographic environment and computational chemistry. The chiral phases were N-(tert.-butylamino-carbonyl)-(S)-valylaminopropylsilica gel and (R)-1-(α-naphthyl) ethylamino-carbonylglycylaminopropylsilica gel.

EXPERIMENTAL

The computer used for the calculations was a Macintosh IIfx, and the software for the computational chemical calculations was CAChe™ from Sony-Tektronix (Tokyo, Japan). The computational chemical calculation was performed without modification of the programs. The geometry of a molecule, created using CAChe™ molecular editor, was first optimized using molecular mechanics calculation. The properties used for the calculation were bond stretch, bond angle, dihedral angle, improper torsion, van der Waals,
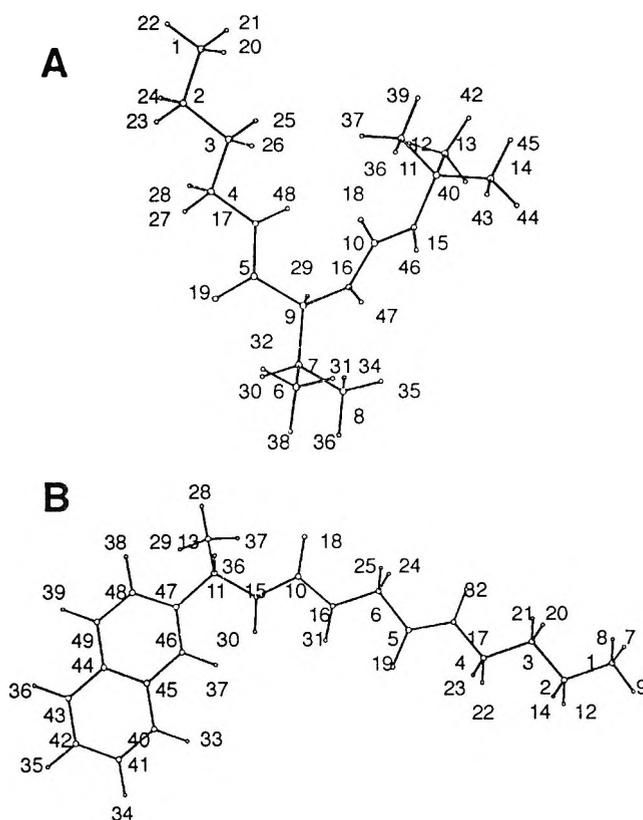
Figure 1. Structure of model compounds of Chiral Phase I (A) and II (B) with atomic numbers.   A: N-(tert.-butylaminocarbonyl)-(S)-valylaminobutane,   B: (R)-1-(α-naphtyl)ethylaminocarbonylglycylaminobutane. See the detail of atoms in Table 1.

electrostatic (MM2 bond dipoles) and hydrogen bond.   The cut-off distance for van der Waals interaction was 9Å.[17]   The electronic properties of the molecule were obtained using extended Hückel where all molecular orbitals were used for the calculation.[17]   The electron density was visualized through the CAChe™ tabulator, and the hydrogen bond center was estimated from the electron density. The optimized energy values of a complex made through hydrogen bond between a chiral phase model and an analyte were used to study the chiral selectivity.

Table 1

**Net Atomic Charge (au) of Model Chiral Phases I and II
Calculated Using Extended Huckel**

	Phase I		Phase II		NAcAl		DNBAl	
No	Atom	Charge	Atom	Charge	Atom	Charge	Atom	Charge
1	C	0.9400	C	0.0707	C	0.0351	C	0.0480
2	C	0.1250	C	0.1125	C	1.1055	C	1.0569
3	C	0.1088	C	0.0949	C	0.3493	C	0.3489
4	C	0.3898	C	0.3748	C	0.0969	C	0.1190
5	C	1.0850	C	1.1059	C	1.2298	C	1.2349
6	C	0.3302	C	0.3147	C	0.5377	C	0.5386
7	C	0.1311	H	-0.0304	N	-0.4858	N	-0.5220
8	C	0.0831	H	-0.0305	O	-1.0795	O	-1.1205
9	C	0.0862	H	-0.0330	O	-1.0241	O	-1.0283
10	C	1.2930	C	1.2922	O	-0.5986	O	-0.6061
11	C	0.4389	C	0.3847	H	-0.0119	C	0.1220
12	C	0.0687	H	-0.0451	H	-0.0120	C	0.2726
13	C	0.0729	C	0.0668	H	-0.0326	C	0.1159
14	C	0.0792	H	-0.0452	H	-0.0338	C	0.2728
15	N	-0.5629	N	-0.5832	H	-0.0392	C	0.1242
16	N	-0.5425	N	-0.5378	H	-0.0385	N	1.1000
17	N	-0.5128	N	-0.4860	H	-0.0425	O	-0.7285
18	O	-1.1307	O	-1.1346	H	-0.0507	O	-0.7284
19	O	-1.0740	O	-1.0670	H	-0.0502	N	1.1002
20	H	-0.0379	H	-0.0451	H	-0.0532	O	-0.7283
21	H	-0.0379	H	-0.0451	H	0.1982	O	-0.7293
22	H	-0.0413	H	-0.0569	-	-	H	-0.0512
23	H	-0.0520	H	-0.0569	-	-	H	-0.0380
24	H	-0.0522	H	-0.0320	-	-	H	-0.0379
25	H	-0.0530	H	-0.0320	-	-	H	-0.0394
26	H	-0.0520	H	-0.0571	-	-	H	-0.0506
27	H	-0.0582	H	-0.0258	-	-	H	-0.0509
28	H	-0.0670	H	-0.0272	-	-	H	-0.0532
29	H	-0.0479	H	-0.0290	-	-	H	0.2031
30	H	-0.0636	H	0.2101	-	-	H	-0.0438
31	H	-0.0358	H	-0.1939	-	-	H	-0.0482
32	H	-0.0400	H	0.1919	-	-	H	-0.0528
33	H	-0.0413	H	-0.0567	-	-	-	-
34	H	-0.0422	H	-0.0530	-	-	-	-

Table 1 (continued)

Net Atomic Charge (au) of Model Chiral Phases I and II
Calculated Using Extended Hückel

No	Phase I Atom	Charge	Phase II Atom	Charge	NAcAl Atom	Charge	DNBAl Atom	Charge
35	H	-0.0384	H	-0.0531	-	-	-	-
36	H	-0.0423	H	-0.0564	-	-	-	-
37	H	-0.0416	H	-0.0402	-	-	-	-
38	H	-0.0424	H	-0.0564	-	-	-	-
39	H	-0.0398	H	-0.0566	-	-	-	-
40	H	-0.0403	C	0.0169	-	-	-	-
41	H	-0.0402	C	0.0352	-	-	-	-
42	H	-0.0387	C	0.0250	-	-	-	-
43	H	-0.0440	C	0.0269	-	-	-	-
44	H	-0.0427	C	0.0756	-	-	-	-
45	H	-0.0420	C	0.0862	-	-	-	-
46	H	0.2007	C	-0.0429	-	-	-	-
47	H	0.2027	C	0.1016	-	-	-	-
48	H	0.2084	C	0.0120	-	-	-	-
49	-	-	C	0.0228	-	-	-	-

NAcAl and DNBAl are N-acetylalanine and 3,5-dinitrobenzoylalanine methyl esters, respectively; however, their structures are not given in the text. Above data indicate NH and CO groups are hydrogen bonding centers.

RESULTS AND DISCUSSION

The atomic distances within molecules and molecular shape are important for enantiomer separation. The model chiral phases of N-(tert.-butyl-aminocarbonyl)-(S)-valylaminopropylsilica gel and (R)-1-(α-naphthyl)-ethylaminocarbonylglycylaminopropyl silica gel were N-(tert.-butyl-aminocarbonyl)-L-valylaminobutane (Phase I) and (R)-1-(α-naphthyl)-ethylaminocarbonylglycylamino butane (Phase II), respectively.

These model compounds were first constructed by the molecular editor of the CAChe™ program. These chiral stationary phases were shaped very differently as shown in Figure 1; Phase I was in the V-shape and Phase II was in the L-shape. The hydrogen bonding center, the electron delocalization of
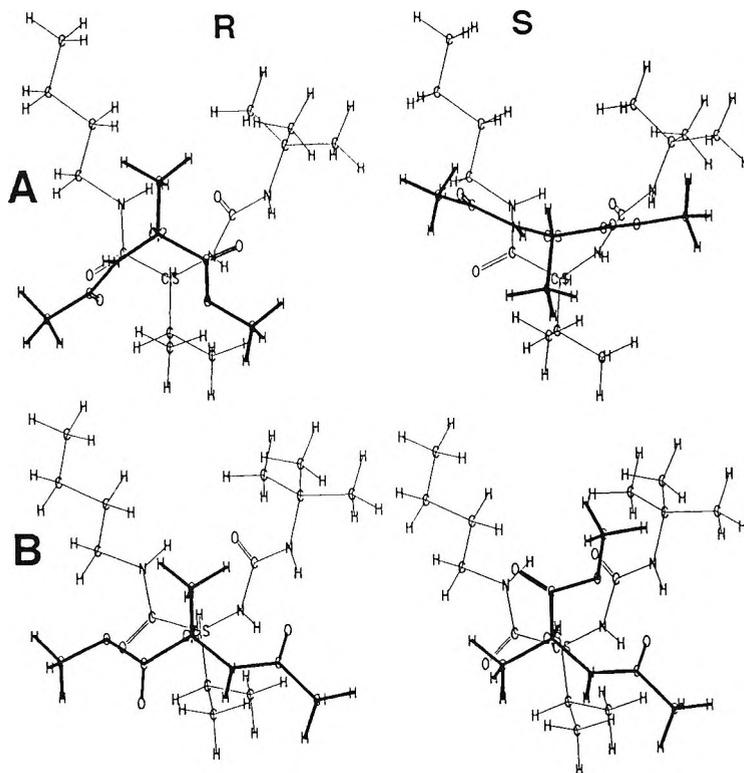
Figure 2. Chiral complex between Phase I and (R)- and (S)-N-acetylalanine methylesters. Molecules having cylinder bonds are analytes.

these phases, was studied using the Extended Huckel calculation of the CAChe™ program. The strongest electron acceptor group was the secondary amino group indicated by the atomic number 15-17 nitrogen and 46-48 hydrogen of Phase I in Fig. 1A, and the atomic number 15-17 nitrogen and 30-32 hydrogen of Phase II in Fig. 1B. The electron donor group of N-acetylamino acid methylesters was the carbonyl of the acetyl group and not that of the carboxyl group. The electron donor group of N-3,5-dinitrobenzoylamino acid methyl-esters was the carbonyl of the dinitrobenzoyl group.

The optimized complex forms of Phase I with (R)- and (S)-N-acetylalanine methyl esters are shown in Fig. 2, where the complexes of (R)- and (S)-amino acids are drawn using atomic symbols. The shadow molecule is

Table 2

Physical Properties and Separation Factor (α) of N-Acetylamino-Acid Methyl Esters Complexes with Chiral Phase I

Complex Form Compounds	α	1*[1]	e*[2]	R-form FE*[3]	HE*[4]	VE*[5]	S-form FE*[3]	HE*[4]	VE*[5]
A Alanine	1.24	R	A	-62.71	-16.83	5.35	-56.21	-15.26	5.79
Leucine	1.99	R	B	-60.40	-16.34	4.34	-54.40	-14.53	6.46
Methionine	1.48	R	A	-65.63	-16.62	3.75	-57.06	-11.67	5.15
Phenylalanine	1.66	R	B	-69.67	-16.42	3.29	-68.90	-17.22	5.26
Phenylglycine	1.39	R	B	-72.82	-18.86	6.05	-65.95	-12.91	6.01
Valine	1.73	R	B	-56.79	-15.25	6.10	-55.01	-14.52	5.80
B Alanine	1.24	R	A	-58.81	-15.52	4.16	-60.09	-13.53	4.06
Leucine	1.99	R	B	-56.84	-14.80	4.65	-58.02	-13.91	4.18
Methionine	1.48	R	A	-63.01	-14.95	2.55	-63.86	-13.64	2.70
Phenylalanine	1.66	R	B	-71.01	-14.50	3.12	-72.12	-21.98	7.34
Phenylglycine	1.39	R	B	-72.33	-16.22	5.18	-76.79	-23.03	5.40
Valine	1.73	R	B	-59.09	-15.11	4.35	-59.28	-13.80	3.79

*Notes: [1]First eluted compound;   [2]Eluent A: n-hexane / 1,2-dichloroethane / ethanol (40/10/1); Eluent B: n-hexane / 1,2-dichloroethane / ethanol (100/20/1); [3]Final energy (Kcal/mol);   [4]Hydrogen bond energy;   [5]van der Waals energy (Kcal/mol). Details of A and B: see in text.

Phase I and the clear molecules having cylinder bonds are (R)- and (S)-N-acetylalanine methyl esters. The final energy value of complexes with the (R)-N-acetylalanine methylester shown in Fig. 2A was -62.71 Kcal/mol and that with the (S)-N-acetylalanine methyl ester was -56.21 Kcal/mol. The calculated final hydrogen bonding and van der Waals energy values of other amino acid complexes are summarized in Table 2, Part A. The final energy values of all complexes with (R)-form amino acids were lower than those with (S)-form amino acids in Phase I. These complexes were constructed to form two hydrogen bonds. However, this result did not support the elution order where (R)-N-acetylalanine methylester which formed a complex with (S)-form Phase I was eluted before (S)-N-acetylalanine methylester. Therefore, we searched for an alternative location for complex formation to support the elution order. The complex form shown in Fig. 2B seemed to be an ideal complex form occurring in liquid chromatography, even though the energy value of
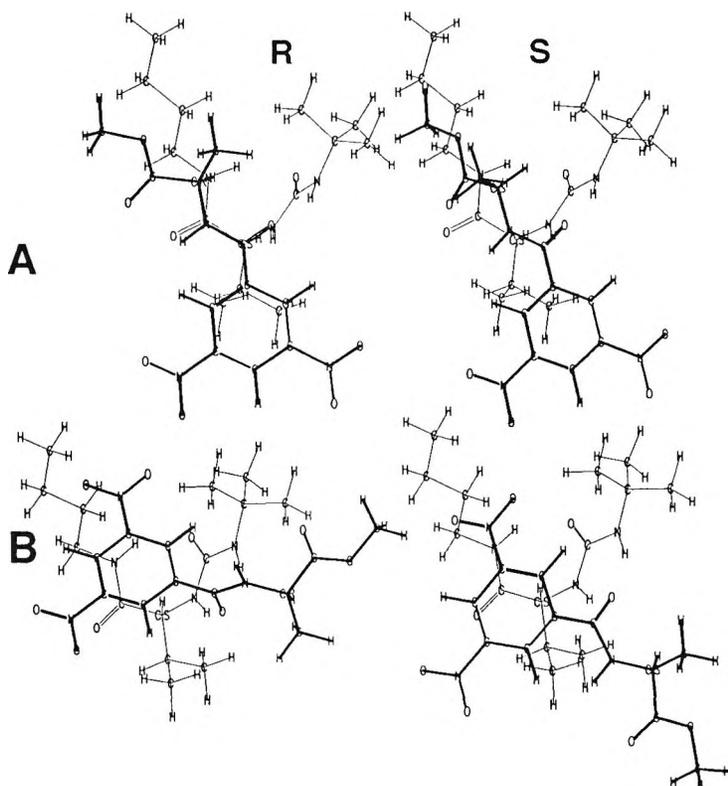
Figure 3. Chiral complex between Phase I and (R) and (S)-N-3,5-dinitrobenzoylalanine methylesters. Molecules having cylinder bonds are analytes.

(R)-N-acetylalanine methylester was 3.9 Kcal/mol higher and the hydrogen bonding energy value was 1.3 Kcal/mol lower than those of the complexes in Fig. 2A. The final energy values of complexes with the (R)- and (S)-N-acetylalanine methyl-esters were -58.81 and -60.09 Kcal/mol, respectively. The van der Waals energy values of the complex form in Fig. 2B were lower than those in Fig. 2A except (S)-N-acetylphenylalanine methylester and (R)-N-acetylleucine methylester. The calculated energy values of the complex form in Fig. 2B are summarized in Table 2, Part B. The energy values of all complex of S-form analyses were lower than those of R-form analyses. These results supported the elution order.

The optimized complex forms of Phase I with (R)- and (S)-N-3,5-dinitrobenzoylalanine methyl esters are shown in Figs. 3 and 4. The final
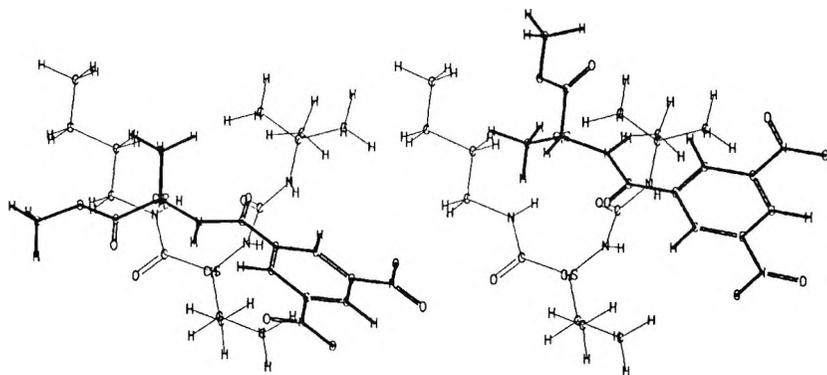
Figure 4. Chiral complex between Phase I and (R) and (S)-N-3,5-dinitrobenzoylalanine methylesters. Molecules having cylinder bonds are analytes.
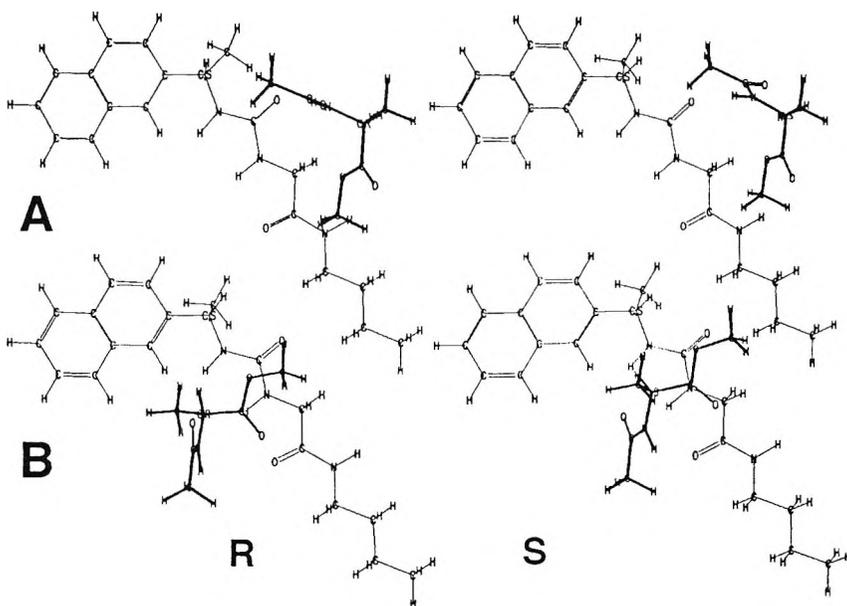
Figure 5. Chiral complex between Phase 11 and (R)- and (S)-N-acetylalanine methylesters. Molecules having cylinder bonds are analytes.

Table 3

Physical Properties and Separation Factor (α) of N-3,5-Dinitro-Benzoylamino Acid Methyl Esters Complexes with Chiral Phase I

Complex Form Compounds	α	R-form 1*[1]	e*[2]	FE*[3]	HE*[4]	VE*[5]	S-form FE*[3]	HE*[4]	VE*[5]
A Alanine	1.11	R	A	-67.43	-19.16	11.06	-66.46	-18.97	11.84
Leucine	1.16	R	B	-68.03	-18.20	10.32	-65.22	-17.99	9.46
Methionine	1.06	R	B	-73.06	-18.77	9.82	-70.84	-18.84	9.04
Phenylalanine	1.08	R	B	-76.66	-18.82	9.43	-71.42	-15.45	11.36
Phenylglycine	1.00	-	A	-83.94	-23.96	9.02	-79.51	-23.86	8.91
Valine	1.11	R	A	-68.83	-18.88	10.23	-63.63	-19.27	9.49
B Alanine	1.11	R	A	-68.28	-18.58	7.83	-67.55	-18.85	9.90
Leucine	1.16	R	B	-67.90	-19.29	8.05	-67.78	-18.58	9.29
Methionine	1.06	R	B	-73.02	-19.15	7.13	-72.11	-18.96	8.71
Phenylalanine	1.08	R	B	-78.69	-19.54	9.40	-77.68	-19.24	8.50
Phenylglycine	1.00	-	A	-84.34	-23.62	8.97	-81.19	-21.96	9.84
Valine	1.11	R	A	-68.32	-19.61	8.42	-69.19	-18.89	9.15
C Alanine	1.11	R	A	-68.79	-21.12	11.12	-68.47	-20.15	11.09
Leucine	1.16	R	B	-67.39	-19.61	11.47	-68.54	-21.58	10.93
Methionine	1.06	R	B	-72.01	-19.50	10.46	-72.75	-21.64	9.93
Phenylalanine	1.08	R	B	-78.41	-21.86	11.71	-79.30	-21.47	11.19
Phenylglycine	1.00	-	A	-81.25	-21.34	12.98	-83.26	-24.05	10.07
Valine	1.11	R	A	-66.93	-19.85	13.06	-68.27	-21.40	11.71

Symbols: See Table 2; details of A~C: see in text.

energy values of Fig. 3A calculated by MM2 were -67.43 for (R)- and -66.46 Kcal/mol for (S)-form alanine, respectively. These complexes formed two hydrogen bonds. However, these results did not support the elusion order where (R)-N-3,5-dinitrobenzoylalanine methylester which formed a complex with S-form Phase I was eluted before (S)-N-dinitrobenzoylalanine methylester. The calculated energy values of other amino acid complexes are given in Table 3A. A different location for complex formation was, therefore, searched for to support the elution order. The complex form shown in Fig. 3B seemed to be the most stable form whose van der Waals energy values were the lowest as listed in Table 3B. However, this complex form did not support the elution order, too.

Table 4

Physical Properties and Separation Factor (α) of N-Acetyl Amino Acid Methyl Esters Complexes with Chiral Phase II

Complex Form Compounds	α	1*1	e*2	R-form			S-form		
				FE*3	HE*4	VE*5	FE*3	HE*4	VE*5
A Alanine	1.00	-	A	-66.99	-15.41	14.09	-67.51	-15.47	14.00
Leucine	1.00	-	B	-64.98	-15.44	14.69	-65.28	-15.50	14.83
Methionine	1.00	-	A	-68.79	-15.82	14.91	-69.66	-15.50	14.42
Phenylalanine	1.00	-	B	-75.16	-16.04	16.21	-75.01	-16.09	16.45
Phenylglycine	1.00	-	B	-78.60	-18.02	16.92	-78.71	-17.87	16.61
Valine	1.02	R	B	-65.30	-15.51	14.39	-65.38	-15.61	14.33
B Alanine	1.00	-	A	-64.87	-16.31	13.30	-64.43	-16.25	13.55
Leucine	1.00	-	B	-66.34	-17.40	12.00	-65.63	-17.11	12.65
Methionine	1.00	-	A	-71.88	-17.02	9.85	-71.16	-17.09	10.64
Phenylalanine	1.00	-	B	-76.19	-16.73	12.13	-76.90	-16.69	11.48
Phenylglycine	1.00	-	B	-79.03	-18.64	13.93	-78.56	-18.56	14.63
Valine	1.02	R	B	-66.19	-16.75	12.18	-66.11	-16.77	11.48

Symbols: See Table 1. The details of A and B: see in text.

The complex form shown in Fig. 4 seemed to be an ideal form occurring in liquid chromatography, even though the van der Waals energy values were higher than complexes of form 3A and 3B as listed in Table 3C. The final energy difference between complexes with the (R)- and (S)- N-3,5-dinitrobenzoylamino acid methyl esters was not significant compared to the results of N-acetylamino acid methyl esters, and supported the poor separation.

The optimized complex forms of Phase II with (R)- and (S)-N-acetylalanine methyl esters are shown in Fig. 5, where the complex of (R)- and (S)-amino acids is drawn using atomic symbols. The shadow molecule is Phase II and the clear molecules having cylinder bonds are (R)- and (S)-N-acetylalanine methyl esters. The complexes of Fig. 5A formed two hydrogen bonds. The positions of (R)- and (S)-form analytes can be rotated and the calculated energy values were nearly equivalent as shown in Table 4A. The final energy value of the complex with the (R)-N-acetylalanine methyl ester shown in Fig. 5A was -66.99 Kcal/mol and that with the (S)-N-acetylalanine
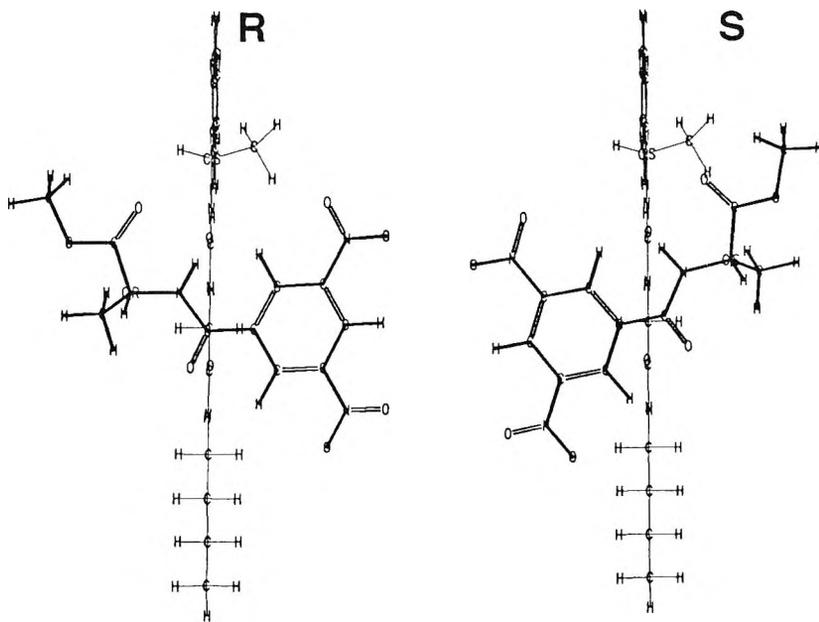
Figure 6. Chiral complex conformations (R)- and (S)-N-3,5-dinitrobenzoylalanine methyl esters with Phase II. Molecules having cylinder bonds are analytes.

methyl ester was -67.51 Kcal/mol. The complexes formed with Phase II like those shown as Fig. 5B seemed more stable than their van der Waals energy values. The final energy difference of other amino acid enantiomer complexes with Phase II was also not large. These calculated values summarized in Table 4B support the experimental results that the enantiomer separation was difficult in Phase II.

Analysis of chromatographic behavior of N-3,5-dinitrobenzoylamino acid methyl esters was not simple. The complex forms of Phase II with (R)- and (S)-form analyses are shown in Figs. 6 and 7, and the energy values of the complexes are given in Table 5. The complexes shown in Fig. 6 formed two hydrogen bonds. There was no significant difference in the final energy values of enantiomer complexes with Phase II, and the values are shown in Table 5A. However, the energy values of complexes with (R)-form methionine and phenylalanine were lower than those with (S)-forms due to the steric effect of the methyl group of the chiral center of Phase II.
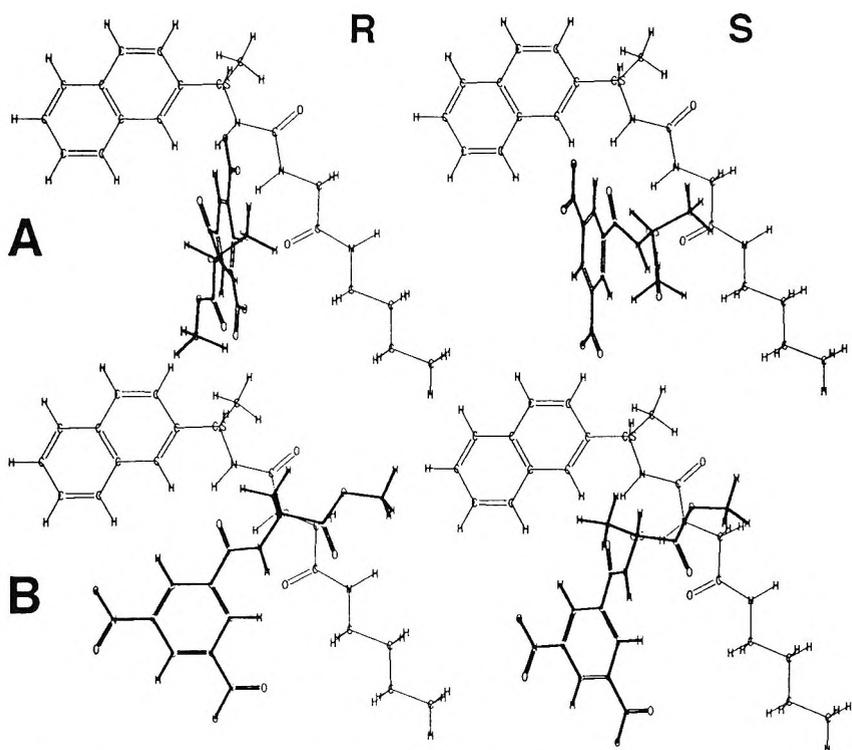
Figure 7. Chiral complex conformations (R)- and (S)-N-3,5-dinitrobenzoylalanine methyl esters with Phase II. Molecules having cylinder bonds are analytes.

The energy values were low for complexes of N-3,5dinitrobenzoylamino acid methylester when these complexes formed with Nos. 30 and 31 hydrogens in Phase II shown in Fig. 7A. The energy values were identical for the (R)- and (S)-complexes. Such large molecules could not form tight complexes with the relative small-sized Phase II, and this complex form is unlikely if the space between the chiral recognition center and the silica gel surface is estimated from Fig. 7A. The large naphthyl group of Phase II should cover the surface of the stationary phase, and the outside of the chiral phase may be opened for chiral recognition. The calculated values are summarized in Table 5B. These enantiomers were not separated in Phase II by the calculation, however they were chromatographically separable. Different complexes of N-3,5-dinitrobenzoylamino acid methyl ester with Phase II were further studied. The energy values of complex form of (R)- and (S)-N-3,5-dinitrobenzoylamino acid

Table 5

Physical Properties and Separation Factor (α) of N-3,5-Dinitro-Benoylamino Acid Methyl Esters Complexes with Chiral Phase II

Complex Form Compounds	α	1*[1]	e*[2]	R-form FE*[3]	HE*[4]	VE*[5]	S-form FE*[3]	HE*[4]	VE*[5]
A Alanine	1.69	R	B	-73.90	-20.20	19.48	-74.06	-20.62	18.95
Leucine	1.45	R	B	-76.77	-21.08	18.21	-76.02	-20.72	18.27
Methionine	1.68	R	B	-81.96	-21.32	17.08	-80.89	-21.32	17.60
Phenylalanine	1.25	R	B	-92.91	-25.21	16.84	-91.31	-23.40	16.90
Phenylglycine	1.11	S	B	-85.89	-18.82	19.91	-85.81	-18.33	20.07
Valine	1.79	R	B	-77.25	-22.21	17.19	-76.57	-21.43	17.41
B Alanine	1.69	R	B	-74.26	-25.31	23.26	-74.24	-24.84	22.95
Leucine	1.45	R	B	-75.17	-22.62	19.37	-75.29	-25.30	21.52
Methionine	1.68	R	B	-80.68	-25.71	21.67	-80.65	-25.42	21.61
Phenylalanine	1.25	R	B	-95.50	-25.97	14.65	-94.73	-24.74	14.89
Phenylglycine	1.11	S	B	-92.76	-26.69	18.48	-93.59	-27.31	16.89
Valine	1.79	R	B	-75.51	-21.84	19.35	-74.98	-24.26	21.86
C Alanine	1.69	R	B	-74.94	-20.75	17.60	-74.43	-21.46	18.22
Leucine	1.45	R	B	-77.44	-21.63	16.41	-76.86	-22.28	16.43
Methionine	1.68	R	B	-81.62	-21.53	15.31	-81.92	-22.16	14.96
Phenylalanine	1.25	R	B	-92.00	-24.40	16.01	-92.75	-24.52	15.28
Phenylglycine	1.11	S	B	-90.53	-23.54	17.28	-89.72	-23.63	18.23
Valine	1.79	R	B	-76.77	-21.52	16.48	-77.35	-22.22	16.21

Symbols: See Table 1. The details of A~C: see in the text.

methyl esters with Phase II as shown in Fig. 7B indicate stability. This seemed to be the most stable complex form due to the lowest van der Waals energy values. However, the final energy values given in Table 5C also did not support the enantiomer separation.

CONCLUSION

Phase I demonstrated the excellent chiral recognition for small molecule N-acetylamino acid methyl esters, and the final energy value difference of

complexes indicated the possibility of enantiomer separation. The hydrogen bond energy values also indicate chiral selectivity. The van der Waals energy values do not clearly indicate the chiral selectivity, but the values were useful for analyzing the fit of their chiral complexes. On the other hand, Phase II did not show chiral selectivity by the calculation, but were chromatographically separable. This contradiction may have been due to geometry. The question regarding Phase II has to be solved when it is bound using an alkyl chain longer than the propyl group. The difference is the chiral recognition space, which is unlimited   for   computational   chemical   calculation   and   limited   for chromatographic separation, is the major concern in the computational chemical analysis of molecular recognition. In this study, a one-to-one complex was used; however, this may not be suitable, especially for analyses with larger molecular size than the chiral recognition molecule. It would be better to make a large chiral phase like an amino phase where the selectivity of saccharides in liquid chromatography has been analyzed successfully.[4] However, this requires a powerful computer.

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ON THE MEASUREMENT OF DEAD TIME IN MICELLAR LIQUID CHROMATOGRAPHY

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ABSTRACT

Modelling of the retention of solutes in micellar liquid chromatography allows the optimization of the resolution of a mixture of solutes and the determination of physico-chemical retention parameters. Both tasks imply the calculation of capacity factors, which are severely affected by the value of dead time. However, the determination of the dead time is not easy when a micellar mobile phase is used owing to the wide and variable perturbations that appear at the heads of the chromatograms. Four different criteria of determination of a reference time in the chromatograms are proposed and compared. The criteria are applied to mobile phases containing a varying concentration of surfactant and modifier, in order to observe the dependence of the reference time with the mobile phase composition. The study of the influence of the errors in the dead time on the modelling of the retention indicated that an accurate dead time is necessary to calculate retention parameters, but an excellent prediction of retention times can be achieved with a wide range of values of dead time.

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INTRODUCTION

A growing interest is being paid to the use of micellar eluents in reverse phase liquid chromatography. Among the procedures reported in micellar liquid chromatography (MLC), the applications to the determination of drugs in physiological samples have a particular attraction.[1-6] Mobile phases containing a surfactant above the critical micellar concentration solubilize the proteins in these samples, avoiding thus the long previous separation steps which are often necessary in the conventional procedures. Owing to the diverse types of possible interactions (e.g., ionic, hydrophobic and steric) between solute, mobile phase and stationary phase, compounds of different character can be separated in a mixture when eluted with micellar eluents. Although in the first reports these eluents only contained a surfactant solution, most of the published procedures have used mobile phases of surfactant containing an organic modifier, usually an alcohol. The presence of the modifier is required since it usually increases the efficiency of the chromatographic peaks and allows a better control of the eluent strength.

In previous works we reported the possibility of modelling the retention behaviour of solutes in MLC.[7-10] An accurate modelling of retention is necessary in order to adequately optimize the separation of a mixture of compounds, especially when the efficiencies achieved are rather low. The retention models relate the capacity factor, k', with the composition of the eluent, thus avoiding the dependence of the model with the flow-rate.

The capacity factor is defined as:

$$k' = \frac{t_R - t_0}{t_0} \tag{1}$$

where t_R is the retention time and t_0 the dead time. The relationship between k' and the micellar concentration, [M], in the absence of any modifier, has been theoretically derived according to several approaches:[11-13]

$$k' = \frac{K_{SW}}{1 + K_{AM} [M]} \tag{2}$$

Elution of a solution in MLC depends on two partition equilibria: one between the stationary phase and water (K_{SW}), and the other inside the mobile phase between water and the micelle (K_{AM}). The constant K_{SW} is the ratio of the volume of the stationary phase to the volume of the mobile phase in the column (ϕ), multiplied by the partition coefficient between the stationary phase and water (P_{SW}). This

equation has been verified for many solutes, and the solute-micelle association constant coincided with the value obtained by other non-chromatographic techniques.[12]

When an alcohol is added to the mobile phase, the retention model grows in complexity:[10]

$$k' = \frac{K_{SW} \dfrac{1 + K_{SD}\,\varphi}{1 + K_{AD}\,\varphi}}{1 + K_{AM} \dfrac{1 + K_{MD}\,\varphi}{1 + K_{AD}\,\varphi}\,[M]} \qquad (3)$$

being φ the alcohol concentration (v/v). In this equation several new constants were introduced: K_{AD} indicates the displacement of the equilibria to release more analyte from the stationary phase and the micelle, towards the bulk water, in the presence of modifier, and K_{SD} and K_{MD} are correcting constants of K_{SW} and K_{AM}, respectively, which are necessary to take into account other interactions with the stationary phase and micelle. Equation (3) has also been checked for several groups of compounds, the errors achieved being usually below 2%.[10]

However, the capacity factor is very sensitive to errors in the value of dead time. The reliable determination of the dead time is especially difficult when the retention of a compound should be described in chromatographic systems where the nature of the mobile phase is largely modified. The change of eluent originates an important modification in the shape of the heads of the chromatograms.

In the MLC literature, the procedures described to measure the dead time made use of injection of water[14,15] salt solutions such as $NaNO_3$,[16,17] NaI and KI,[11,18] or organic solvents such as methanol[12,19] and acetonitrile.[20] The criterium applied to locate the dead time was usually the measurement of the position of the maximum of the first peak, or the measurement of the time from the injection to the first deviation from the base-line. Assignation of these times to the dead time only implies an approximation to reality, since: (i) the residence time of the mobile phase in the pre- and post-column conducts located between injector and detector is ignored, (ii) it is supposed that the injected compound used to determine the dead time does not interact with the stationary phase, and (iii) the wide and variable perturbation observed at the beginning of the chromatograms in MLC causes an uncertainty in the location of the point where the dead time should be measured. Therefore, it is questionable that the values given in the MLC literature were real dead times, although the measured times were probably close to them.

In this work, four different criteria of determination of a characteristic time in the head of the chromatograms, which will be called reference time, are proposed and compared. The criteria were applied to mobile phases containing a varying concentration of surfactant and modifier, in order to observe the dependence of the reference time with the mobile phase composition. The effect of the use of a reference time as dead time in the calculation of capacity factors to model the retention of diverse solutes is finally studied.

MATERIALS AND METHODS

Reagents

Sodium dodecyl sulphate (SDS), 1-butanol (Merck, Darmstadt, Germany), and 1-propanol (Panreac, Barcelona, Spain) were used to prepare the mobile phases and some injected solutions. The micellar mobile phases were prepared by mixing the aqueous surfactant solution with the alcohol to obtain the working concentration (v/v percentage). The mobile phases were buffered at pH 3 with citric acid monohydrate and NaOH (Panreac).

The solutes injected were diuretics: amiloride (ICI Farma, Madrid, Spain), bendroflumethiazide (Davur, Madrid), chlorthalidone (Ciba-Geigy, Barcelona), ethacrynic acid (Merck), spironolactone (Searle, Madrid), and triamterene (Sigma, Buchs, Switzerland). These compounds, except triamterene, were kindly donated by pharmaceutical laboratories located in Spain. Stock solutions were prepared in SDS micellar solutions after addition of a small volume of methanol to facilitate dissolution. Other compounds that were injected were KI (Probus, Barcelona), NaNO$_3$ (Panreac), acetonitrile (Merck) and methanol (Panreac). Barnstead nanopure, deionized water (Sybron, Boston, MA, USA) was used throughout.

Apparatus

Two Hewlett-Packard HP 1050 (Palo Alto, CA, USA) liquid chromatographs with isocratic pumps, UV-visible detectors and HP 3396A integrators were used. Most of the chromatograms were obtained with a system implemented with an autosampler. Data acquisition was performed through the PEAK-96 software (Hewlett-Packard, Avondale, PA, USA). The mobile phase flow-rate was 1 mL/min. A Spherisorb ODS-2 column (5 μm particle size, 125 mm x 4.6 mm I.D.) and precolumn (35 mm x 4.6 mm I.D.) (Scharlau, Barcelona) were used. The

mobile phase and the injected solutions were filtered through 0.45 μm and 0.22 μm Nylon membranes, respectively (Micron Separations, Westboro, MA, USA).

Data Treatment

The determination of the reference time used the following criteria:

Criterium 1: Search of the first main maximum or minimum

This procedure is the most commonly used in the MLC literature, where the appearance of the maximum or minimum of the first important perturbation in the chromatogram is searched. First, the region corresponding to the base-line (previous to the first perturbation, including time values from zero to t_b) is marked (Figure 1a), and fitted to a straight-line. Next, a second region is marked between t_{b+1} and t_p, being t_p a point following the perturbation, and the base-line is subtracted. After base-line correction, the maximum or minimum of the signal is searched in this region, by means of the bisection method. In order to improve the accuracy, a Lagrange interpolation is also applied to obtain the intermediate values.[21]

Frequently, an extra noise of different origin appears in the chromatograms, such as air bubbles, pump pressure fluctuations, modifications in the intensity of the light in the detector lamp, or even, alteration of the base-line due to previous injections. For this reason, in this criterium and in the criteria of formation of groups, a self-consistence cycle of the base-line has been included to eliminate anomalous points.

Criterium 2: Formation of groups scaling the noise

When the first alteration of the base-line appears, a simultaneous noise increase is produced. In this criterium, it is considered that the first perturbation begins when the noise exceeds a value that amounts the product of an arbitrary number, p, by the mean noise of the base-line, $\bar{n}_b$. The use of a constant value of p, previously established, may be problematic, since the noise may largely vary from one chromatogram to another. Thus, it was decided to scale the signal, by searching the highest value of the noise inside the region of interest (between t_{b+1} and t_p), n_{max}, and using as the minimum value of noise, $n_{min} = \bar{n}_b + 2.5\ \sigma_b$ (the factor 2.5 corresponds to a 98.8% probability), where σ_b is the standard deviation of the base-line noise. Finally, the noise levels were obtained by dividing the interval between n_{min} and n_{max} in a convenient number of equal divisions, N. Thus, the noise associated to level l, is given by:

$$n_l = n_{min} + \frac{1}{N} (n_{max} - n_{min}) \tag{4}$$

In this work, a value $N = 300$ has been arbitrarily taken.

The noise associated to each point in the region included between t_{b+1} and t_p was evaluated with the following expression:

$$n_i = \left| \frac{y_{i+1} + y_{i-1}}{2} - y_i \right| \tag{5}$$

where y is the value of the signal in arbitrary unities. This expression is proportional to the second derivative of the signal in the middle point of the interval. The mean noise of the base-line is obtained by:

$$n_b = \sum_{i=2}^{b} \frac{n_i}{b-1} \tag{6}$$

being b the last point taken in the region of the chromatogram considered as base-line.

Figure 1a shows a part of a chromatogram corresponding to the injection of a solution of similar composition to that of the mobile phase, which will be called blank solution. The formation of the peak is due to the unavoidable small difference in composition between the mobile phase and the injected solution, and to the presence of other reagents, such as citrate buffer.

The examination of each level always begins at time t_{b+1} . The noise in that point is measured and compared with the noise associated to the first level being established ($l = 1$ in equation 1). If the noise of the point is lower, it is rejected and the next point is taken. If the point fulfils the condition of having a noise larger than that of the examined level, the time of the point is assigned to the level, which for the first point will be t_{b+1} . The same sequence is followed with the next noise level, where the point that fulfilled the latter condition is first examined. The time of the first point that fulfils the condition of exceeding a given noise level is assigned to the level. The process is successively repeated until the maximum noise level (level 300) is reached. It is not necessary that the examination covers all the experimental points each time that a new level is studied, since a part of the chromatogram should be discarded after studying a previous noise level.
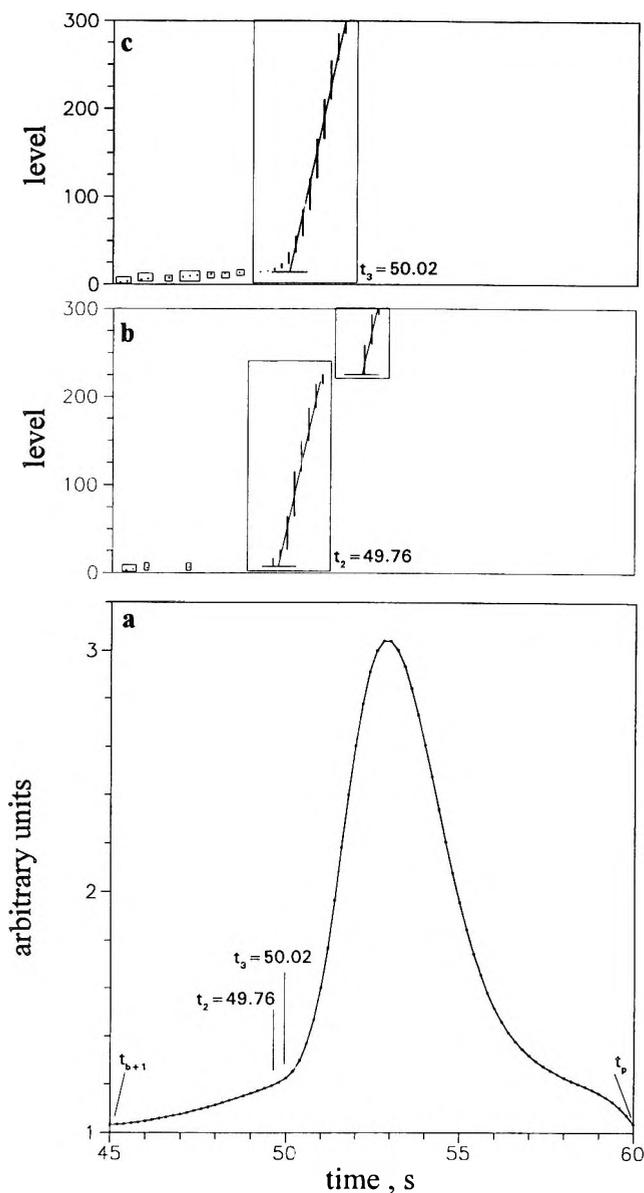
Figure 1. Methods of groups applied to the measurement of the reference time when a blank solution was injected into a 0.125 M SDS mobile phase containing 1.5% butanol. (a) Head of the chromatogram; (b) formation of groups scaling the noise; (c) formation of groups scaling the slope.

When the noise level is plotted vs. the time assigned to each level, a scaled diagram is observed (Figure 1b). Each vertical line is formed by the noise levels for which the same time has been assigned, that is, for which the same point in the chromatogram has exceeded for the first time the noise of the level. The magnitude of each vertical segment is related to the number of noise levels having the same time.

The formation of groups of lines with times corresponding to successive points in the chromatogram (separated in 0.2 s, owing to the rate of acquisition of the data by the chromatographic system) is observed in Figure 1b. The chromatogram in the figure involves five groups. The three first groups only include one or two points, whereas a large number of levels (about 220) belong to the fourth group, from 49.6 s to 51.0 s. The last group begins at 52.2 s and ends at 52.6 s, where the maximum noise level is reached.

Once the groups have been delimited, the points inside each group are fitted to a straight-line. The intersection of this line with the lowest noise level of the group will give a characteristic time. In this way, each group will be associated to a minimum value of the time, and one of these values will be taken as the reference time. The reference time should fulfil three conditions:

> (i) It should belong to the base-line or should be close to it. This implies that it should be associated to a perturbation having an initial group appearing at the beginning of the chromatogram. Those groups including a very low number of levels should be rejected.

> (ii) The importance of the perturbation is proportional to the number of levels of a group, therefore the reference time will be given by the group including more levels.

> (iii) The reference time will be associated with an abrupt raise of the noise, consequently it will correspond to that group showing the largest slope.

Therefore, the reference time will be usually associated to that group showing a large and steep increase in the noise, starting from the base-line. If the three conditions are satisfied for a given group, the associated time is taken as the reference time. This situation is frequently encountered, but sometimes some disagreements exist, and the whole available information on the different groups should be examined in detail to select a time value.
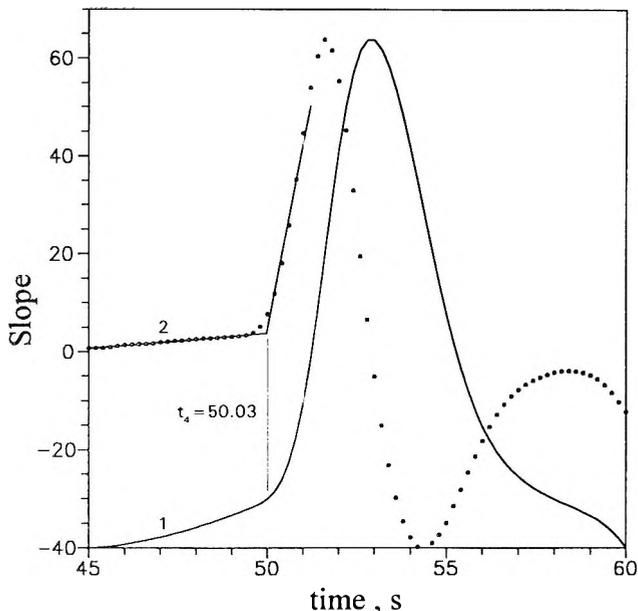
Figure 2. Method of the first derivative applied to the chromatogram shown in **Figure 1a**.

Criterium 3: Formation of groups scaling the slope

This criterium is a variant of criterium 2, where the slope around each point in the chromatogram is measured. The slope associated to a point i is measured by taking a set of five points around point i, and fitting the five points to a straight line. The slopes should be constant and with a low value in the base-line, and should change abruptly when the perturbation giving the reference time appears. To locate the reference time, the mean value and standard deviation of the slopes of the points belonging to the base-line are first obtained. Next, the slopes of the points in the marked region between t_{b+1} and t_p are measured, the largest slope is searched and the slope levels are scaled, similarly to the way indicated for the method described above to form the noise levels. Figure 1c shows the formation of eight groups, the main group being clearly the last one. This group contains the required information to measure the reference time.

Criterium 4: Method of the first derivative

This criterium is based on the detection of an important change in the slope of the base-line, when the significant perturbation is produced. The derivative of the chromatogram is calculated by measuring the slope associated to each point with a set of five points, as above (Figure 2). In this way, two regions with a well differentiated slope will appear at the beginning of the chromatogram. One of the
slopes will be nearly constant and low, and the other will increase or decrease largely. The values of slope in the proximity of the transition between both regions are plotted against time. Finally, the intersection of the two fitted straight-lines after rejecting the points in the intermediate curvature, will give the value of the reference time.

RESULTS AND DISCUSSION

Determination of the Reference Time

Figure 3 shows chromatograms corresponding to the injection of water and blank solution into a mobile phase of 0.1 M SDS/1.5% butanol, where the values of the reference time determined according to each one of the four criteria areindicated. Obviously, when the reference time was measured at the maximum of the peak (criterium 1), it was larger than the time given by the other three criteria. Criterium 1 also yielded different values for water and blank solution.

To study the nature of the signal obtained at the head of the chromatograms, several experiments were performed where water or a blank solution were injected, and the absorbance was measured at different wavelengths. It may be observed in Figure 4 that when water was injected, two well differentiated regions appeared at the beginning of the chromatogram, the first region being rather irregular and unpredictable, and the second region keeping its shape and position when the wavelength was changed. On the other hand, the signals obtained by injection of blank solution kept the position of the maximum. In this case, only the height of the signal was modified, being increased at decreasing wavelengths. The second perturbation was scarcely observed in this chromatogram. The noise increase observed with the wavelength, when water was injected, was probably associated to the state of the lamp and of the material of the detector cell. This noise was not so evident when the blank solution was injected owing to the wider range of the ordinate scale.
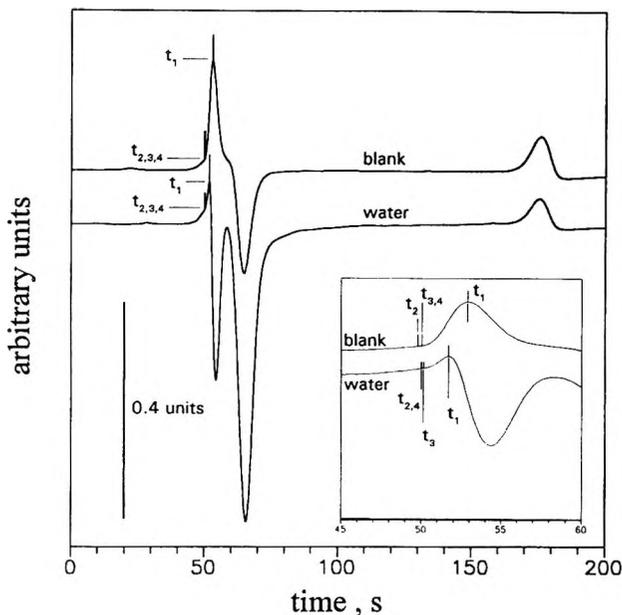
Figure 3. Heads of chromatograms corresponding to the injection of blank solution and water into a 0.125 M SDS/1.5% butanol mobile phase. The reference times indicated correspond to the maximum (t_1), formation of groups scaling the noise (t_2), formation of groups scaling the slope (t_3) and the method of the first derivative (t_4).

The first perturbation observed when water was injected had probably a refractometric origin and the second was an absorptiometric signal. The irregular shape of the first peak obtained with water was due to the large difference in composition between the injected solution and the mobile phase, which originated erratic fluctuations in the refraction index when both solutions were mixed. In contrast, when the blank solution was injected, the homogenization of the mixture was more simple. To verify this hypothesis, the concentration of SDS in the injected sample was varied in another series of experiences, from a value below the concentration of the mobile phase up to a concentration above it. In Figure 5, it may be observed that when the compositions of the injected solution and mobile phase were matched, the second peak decreased and disappeared, and further a positive signal was achieved. This behaviour confirmed the absorptiometric nature of this peak.

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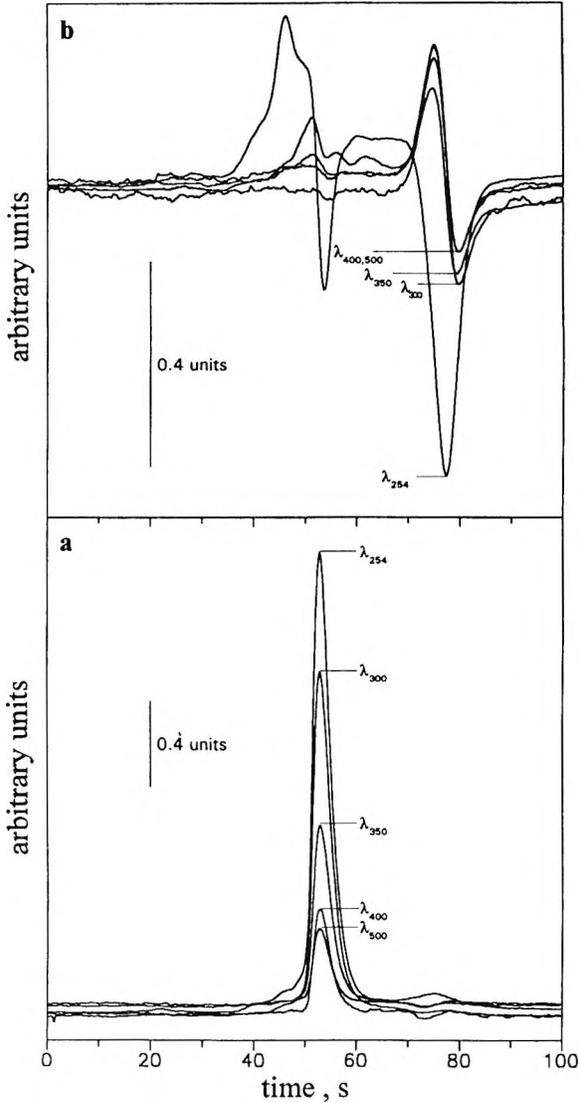
Figure 4. Influence of the wavelength in the measurement of the reference time obtained by injection of: (a) blank solution, and (b) water. A 0.05 M SDS mobile phase without modifier was used. Wavelengths are given in nm.
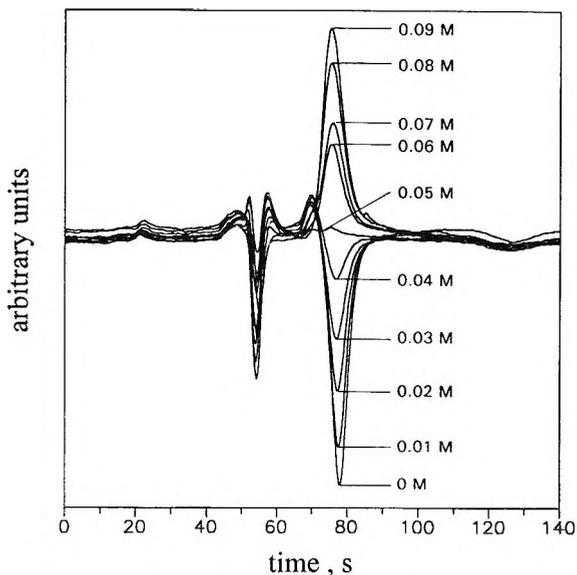
Figure 5. Influence of the concentration of SDS in the injected solution when a mobile phase of 0.05 M SDS without modifier was used

Figure 6 shows the head of four chromatograms corresponding to the injection of increasing volumes of ethacrynic acid dissolved in 0.05 M SDS into a mobile phase containing the same concentration of SDS. The start of the peak at 50 s was not modified, whereas its maximum was shifted to larger times for increasing volumes of the injected solution. This experience indicated that the measurement of a reference time is more convenient at the start of the perturbation than at its maximum.

The perturbation produced at the head of the chromatograms by injection of water, is 50 s wide, which suggested an extense diffusion of water in the mobile phase. It should be taken into account that the injected volume was 20 μL, and the flow-rate, 1 mL/min. If diffusion and retention do not take place, a narrow perturbation of about 1 s should be produced.

The values of reference time, retention time and capacity factor, k', for the injection of 14 replicates of a solution of amiloride into a mobile phase of 0.125 M SDS and 5% propanol are indicated in Table 1, according to each one of the four criteria proposed. The solution of amiloride was prepared in a medium with a composition similar to that of the mobile phase. The methods of groups led to the
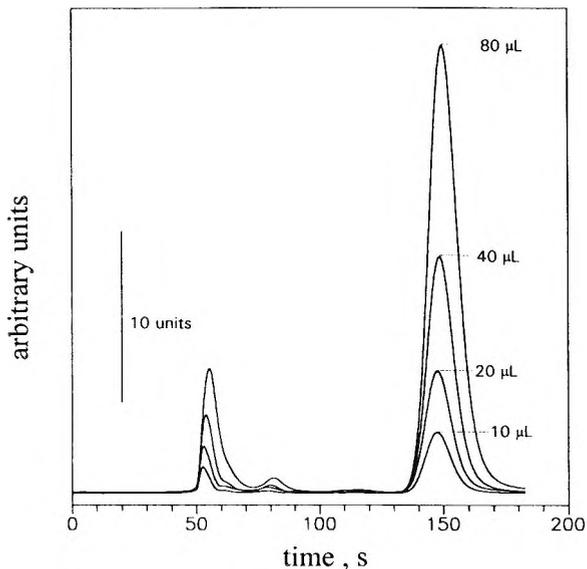
Figure 6. Influence of the volume of a solution containing 10 μg/mL of ethacrynic acid, injected into a mobile phase of 0.05 M SDS without modifier.

largest precision in the determination of the reference times and, consequently, in the calculation of capacity factors. The measurement at the maximum gave an acceptable precision, if the value of the sixth injection was eliminated (t = 66.61 ± 0.09 s after rejecting the sixth injection). It should be considered that this series of experiences represents the most favorable case to obtain reproducible data, since successive injections of aliquots of the same solution were performed into the same mobile phase, in a short time period. It should be also noticed that replicate number 6 gave good results when the other three criteria were used. The obtention of such a discrepant measurement at the peak maximum suggested that this criterium is not very reliable.

Table 2 shows the values of reference time obtained in different mobile phases of SDS and butanol, when water, blank solution and diverse solutions of diuretics were injected. The blank solution and the solutions containing the diuretics had the same matrix and therefore, the head of their chromatograms should be similar. In effect, the mean values of the reference time obtained with both solutions did not show a significant difference. The standard deviations for both types of injected solutions are not comparable, since the number of replicates was different and

Table 1

Influence of the Reference Time on the Calculation of the Capacity Factor of Amiloride in a 0.125 M SDS/5% Propanol Mobile Phase

Replicate Number	Retention Time (s)		Reference Time(s)*			Capacity Factors **			
	t_r	t_1	t_2	t_3	t_4	k'_1	k'_2	k'_3	k'_4
1	370.20	66.76	60.37	59.76	60.77	4.54	5.13	5.19	5.09
2	358.82	66.55	60.56	59.73	63.00	4.39	4.92	5.01	4.70
3	369.90	66.72	60.51	59.88	61.20	4.54	5.11	5.18	5.04
4	368.94	66.50	60.53	59.84	59.94	4.55	5.10	5.17	5.16
5	369.36	66.69	60.48	59.79	60.60	4.54	5.11	5.18	5.10
6	369.00	60.64	60.48	59.84	61.00	5.08	5.10	5.17	5.05
7	368.94	66.59	60.45	59.65	60.37	4.54	5.10	5.18	5.11
8	369.00	66.49	60.49	59.75	60.60	4.55	5.10	5.18	5.09
9	369.06	66.54	60.49	59.89	60.60	4.55	5.10	5.16	5.09
10	368.94	66.57	60.60	59.82	60.34	4.54	5.09	5.17	5.11
11	368.82	66.57	60.50	59.80	60.30	4.54	5.10	5.17	5.12
12	370.14	66.66	60.46	59.84	60.32	4.55	5.12	5.19	5.14
13	369.48	66.72	60.46	59.81	63.00	4.54	5.11	5.18	4.86
14	368.82	66.67	60.46	59.72	60.12	4.53	5.10	5.18	5.13
Mean	368.5	66.2	60.49	59.79	60.9	4.57	5.09	5.16	5.06
St. dev.	2.8	1.6	0.05	0.07	1.0	0.15	0.05	0.05	0.13

* Reference time determined according to each criterium (1. first main maximum; 2. formation of groups scaling the noise; 3. formation of groups scaling the slope; 4. method of the first derivative).
** Capacity factors calculated using the reference time obtained according to each criterium.

reduced. Therefore, only the mean time values should be compared. However, the comparison of the standard deviations inside each criteria and for each type of injected solutions is interesting.

Table 2 also shows the maximum and minimum values of reference time obtained according to each criterium and with the eleven mobile phases studied. Measurement at the peak maximum led to very disperse values of time, not only

Table 2

Variation of the Reference Time with the Mobile Phase Composition

BuOH (v/v)	Criter- ium*	SDS (M)				
		0.050	0.075	0.100	0.125	0.150
		Reference time (s)				
0	1a	52.27-***	52.67±0.29	52.04±0.34**	52.10±0.14**	51.97±0.00**
	2a	51.60-***	49.30±0.00	51.46±0.08**	49.68±0.17**	49.89±0.44**
	3a	49.16-***	49.53±0.17	49.15±0.24**	49.66±0.14**	49.49±0.32**
	4a	49.17-***	49.61±0.10	49.42±0.07**	49.34±0.03**	49.38±0.03**
	1b	52.62-***	52.97±0.08	52.74±0.26**	52.68±0.05**	52.69±0.00**
	2b	49.53-***	52.31±0.16	52.40±0.00**	49.66±0.14**	49.63±0.03**
	3b	48.71-***	49.43±0.20	49.29±0.18**	49.58±0.47**	49.19±0.26**
	4b	49.67-***	49.82±0.12	49.86±0.14**	49.64±0.07**	49.91±0.10**
	1c	52.89±0.94	52.45±0.32	52.9±2.1	53.2±1.3	53.2±1.4
	2c	49.50±0.21	49.27±0.49	50.2±1.4	49.63±0.37	49.46±0.47
	3c	49.46±0.54	49.6±1.4	49.40±0.92	49.18±0.74	49.02±0.91
	4c	49.42±0.14	49.13±0.69	49.37±0.90	49.41±0.84	49.0±1.0
0.015	1a	50.20±0.10**		50.8±1.3**		49.60-***
	2a	50.33±0.15**		50.09±0.02**		49.91-***
	3a	50.67±0.11**		50.20±0.29**		50.06-***
	4a	50.48±0.06**		50.04±0.06**		50.18-***
	1b	53.22±0.15		52.96±0.09**		51.07±0.01**
	2b	49.80±0.08		50.27±0.71**		51.01±0.06**
	3b	50.05±0.06		50.06±0.04**		51.46±0.03**
	4b	50.04±0.02		49.94±0.02**		51.21±0.04**
	1c	53.26±0.10		53.18±0.31		52.3±1.3
	2c	49.86±0.12		50.09±0.16		50.68±0.39
	3c	50.06±0.07		50.07±0.18		51.30±0.63
	4b	49.96±0.13		49.96±0.18		50.70±0.49
0.030	1a	49.82±0.09**		50.9±1.5		48.74±0.64**
	2a	50.38±0.35**		50.04±0.03		50.21±0.21**
	3a	50.46±0.00**		50.30±0.16		50.38±0.21**
	4a	50.42±0.00**		50.06±0.10		50.32±0.12**
	1b	53.33±0.01		51.9±1.2**		54.63±0.07
	2b	49.76±0.01		49.94±0.05**		49.98±0.09
	3b	50.05±0.03		50.01±0.03**		50.30±0.02
	4c	50.06±0.02		49.93±0.00**		50.24±0.01

Table 2 (continued)

Variation of the Reference Time with the Mobile Phase Composition

BuOH (v/v)	Criterium*	SDS (M)				
		0.050	0.075	0.100	0.125	0.150
				Reference time (s)		
	1c	53.40±0.06		52.97±0.48		54.58±0.03
	2c	49.31±0.06		49.96±0.11		49.95±0.11
	3c	50.10±0.13		50.02±0.16		50.20±0.16
	4c	50.06±0.14		49.95±0.13		50.05±0.21

Mean Reference Time(s) in Blanks and Compound Solutions*

	t_1	t_2	t_3	t_4
Mean	53.07±0.62	49.96±0.38	49.85±0.64	49.79±0.47
Minimum	52.07	49.50	49.05	49.17
Maximum	54.57	50.75	51.33	50.80
Range	2.52	1.26	2.28	1.63

* Numbers 1 to 4 correspond to each mathematical criterium (see Table 1), and letters a, b, c indicate the solution that has been injected: water, blank and compound solution, respectively.
** Two measurements were available.
*** Only one measurement was available.

among the replicates done with different mobile phases, but also with the same mobile phase, the variation range being 2.52 s. On the other hand, the criterium of groups scaling the noise was the most precise, with a variation range of 1.26 s, which corresponds to an uncertainty of six experimental points in the chromatograms. The errors observed with the mobile phases containing 0.075 M and 0.1 M SDS were due to an anomalous peak start. The other two criteria, formation of groups scaling the slope and the method of the first derivative are similar, but the latter criterium gave somewhat better results.

The method of groups scaling the slope had a slight inertia with respect to the formation of groups scaling the noise, with delays of 0.2 to 0.3 s, for a flow-rate of 1 mL/min. This effect was due to the larger number of points used to calculate the slope. The inertia may be reduced by using a lower number of points, however the uncertainty in the calculation of the slope would be increased unacceptably. In spite of the slower response of the method of groups scaling the slope, this method may be preferable for very noisy chromatograms. Neither of the four criteria studied in this work was affected when the chromatograms showed an inclined base-line.

The numerous experiences performed with mobile phases of diverse composition, containing different concentrations of SDS and several alcohols, indicated that the reference time did no depend on the composition of the mobile phase. A small increase was only observed for the reference time determined at the maximum of the signal, which amounted to 0.5 and 2 s, when the concentrations of SDS or alcohol in the mobile phase were increased, from 0.05 to 0.15 M SDS, and from 0 to 3% butanol, respectively.

Figure 7 shows heads of chromatograms corresponding to the injection of salt solutions, KI and $NaNO_3$, and organic solvents, acetonitrile and methanol, and also of water and blank solution, into a 0.1 M SDS/12% propanol mobile phase. It may be observed that the shape of the perturbation is variable and more irregular with the injection of the salt solutions and organic solvents than with water or blank solution. Also, the perturbation began at a shorter time for the solutions of KI and $NaNO_3$. All the aqueous solutions injected gave a chromatogram with a maximum followed by a minimum around 68 s.

Influence of the Value Taken for the Dead Time on the Modelling of the Retention of Solutes

Equation (3) was used to study the influence of the value of the reference time in the prediction of capacity factors. The study was performed with the compounds: amiloride, bendroflumethiazide, chlorthalidone, spironolactone and triamterene. Modelling of the retention of these compounds was made with the retention data obtained in the eleven mobile phases indicated in Table 2. A non-linear fitting of the k' vs. ([M],) data to Equation (3), rewritten as:

$$k' = \frac{1 + A\varphi}{B\varphi + C[M] + D[M]\varphi + E} \tag{7}$$
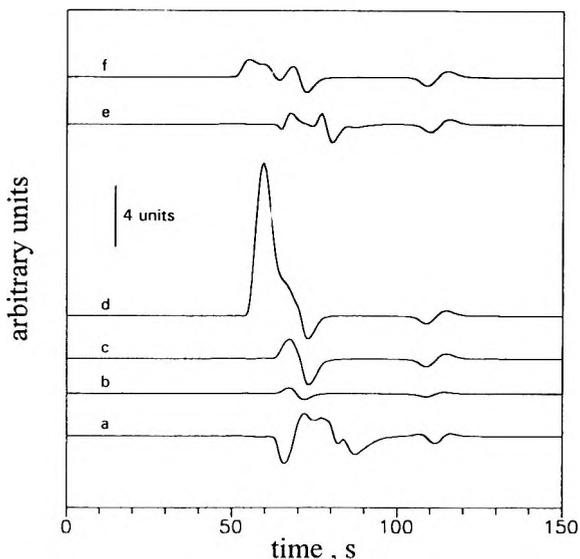
Figure 7. Heads of chromatograms obtained by injection of different solutions used to determine a reference time: (a) 10% acetonitrile/90% water; (b) blank micellar solution; (c) water; (d) potassium iodide; (e) methanol; and (f) sodium nitrate. Mobile phase: 0.1 M SDS/12% propanol. Manual injection was performed.

was made. This equation permits a more rapid convergence of the regression process. Next, the predicted and experimental capacity factors for each mobile phase were compared, and the mean relative error was calculated. Table 3 shows the errors of prediction of the capacity factors, obtained assuming different values of dead time. It is evident that the fitting of Equation (7) is scarcely affected by the errors introduced by using an incorrect dead time, at least up to a value of 80 s.

In contrast, Table 4 shows that the determination of the physico-chemical parameters K_{SW}, K_{AM}, K_{AD}, K_{MD} and K_{SD} is severely affected by the value taken for the dead time, especially for the three former constants. Changes of sign in these parameters are often observed, which indicated that the dead time used in the calculation is not correct. Large errors in the dead time can lead to ill-conditioned fittings that give non-sense negative parameters. The significant changes in the values of the parameters for the five compounds shown in Table 4, that occur between 40 and 60 s suggested that the real dead time (including the residence time in pre- and post-column conducts) belongs to this time range. Similar results were obtained for mobile phases without modifier, the physico-chemical parameters K_{SW} and K_{AM} being sensitive to the value of dead time.

Table 3

Influence of the Value of Dead Time in
the Prediction of Capacity Factors

Mean Relative Error %

Dead Time(s)	Amiloride	Bendroflu- methiazide	Chlorthali- done	Spirono- lactone	Triamterene
30	1.19	1.14	1.14	1.27	1.29
40	1.19	1.08	1.11	1.19	1.20
50	1.21	1.05	1.08	1.10	1.11
60	1.23	1.06	1.06	1.02	1.02
70	1.28	1.13	1.05	0.93	0.93
80	1.44	1.37	1.26	0.83	0.84
90	1.62	1.71	1.79	0.73	0.74
110	2.02	3.00	4.35	0.77	0.64
130	2.55	5.32	12.99	0.96	0.81

CONCLUSIONS

The determination of the real dead time is not an easy task when a micellar mobile phase is used. The observation of a large number of chromatograms showed that the shape, height and sign of the first peak is unpredictable, especially when the nature of the injected solution is different to that of the mobile phase. It was confirmed that the usual practice of taking the time measured at the maximum of the first peak, as the value of dead time, is not correct. The time measured should only be considered as a reference time. The application of the four criteria proposed in this work to the study of the first perturbation gave different values of reference time for each type of injected solution (water, blank, salt solutions and organic solvents).

In the literature, the dead time is usually determined by injection of water. However, this procedure is not very reproducible, especially when the time is measured at the maximum of the first peak, owing to the large difference in composition between water and the micellar mobile phase. The measurement of the reference time at the peak maximum is very simple, but the variability in the shape and position of the first peak leads frequently to unprecise values. In contrast, the start of the first main peak in the chromatograms is fairly reproducible and probably close to the dead time. Among the methods proposed to locate this point, the method of groups scaling the noise gave the best results.

Table 4

**Influence of the Value of Dead In the Determination
of Physico-Chemical Parameters**

Compound	Dead Time(s)	R	Physico-Chemical Parameters				
			K_{SW}	K_{AM}	K_{AD}	K_{MD}	K_{SD}
Amiloride	30	0.99992	1040	377	8.32	1.33	0.481
	40	0.99992	988	487	9.62	1.35	0.473
	50	0.99993	1092	685	11.91	1.38	0.465
	60	0.99993	1502	1152	17.23	1.41	0.457
	70	0.99993	3934	3588	44.66	1.45	0.448
	80	0.99992	-3050	-3241	-31.96	1.48	0.440
	90	0.99992	-919	-1120	-8.05	1.52	0.431
	110	0.99990	-314	-487	-0.75	1.60	0.413
	130	0.99986	-164	-312	1.43	1.69	0.394
Bendroflu-	30	0.99990	121	91	1.97	0.36	0.153
methiazide	40	0.99992	102	107	2.11	0.37	0.146
	50	0.99994	95	129	2.31	0.39	0.138
	60	0.99995	95	163	2.58	0.41	0.130
	70	0.99995	105	220	3.02	0.44	0.121
	80	0.99994	134	336	3.86	0.46	0.113
	90	0.99992	237	704	6.43	0.49	0.103
	110	0.99981	-152	-611	-2.38	0.56	0.083
	130	0.99958	-41	-216	0.50	0.66	0.061
Chlorthali-	30	0.99986	54	48	1.74	0.14	0.038
done	40	0.99989	44	55	1.85	0.16	0.026
	50	0.99992	38	63	1.97	0.18	0.013
	60	0.99994	35	75	2.12	0.21	-0.001
	70	0.99995	35	92	2.31	0.24	-0.015
	80	0.99995	37	119	2.58	0.28	-0.031
	90	0.99992	43	165	2.99	0.32	-0.047
	110	0.99976	131	715	6.66	0.44	-0.084
	130	0.99930	-43	-321	0.86	0.64	-0.124

(continued)

Table 4 (continued)

**Influence of the Value Of Dead in the Determination
of Physico-Chemical Parameters**

Compound	Dead Time(S)	R	Physico-Chemical Parameters				
			K_{SW}	K_{AM}	K_{AD}	K_{MD}	K_{SD}
Spirono	30	0.99997	2832	570	15.72	1.33	0.301
lactone	40	0.99998	3864	1053	27.45	1.34	0.297
	50	0.99998	18416	6367	156.30	1.36	0.293
	60	0.99998	-3638	-1532	-35.26	1.38	0.289
	70	0.99999	-1387	-692	-14.83	1.40	0.285
	80	0.99999	-769	-445	-8.82	1.42	0.280
	90	0.99999	-496	-328	-5.97	1.44	0.276
	110	0.99999	-257	-215	-3.18	1.49	0.268
	130	0.99999	-157	-160	-180	1.54	0.259
Triamterene	30	0.99995	300	610	16.63	1.30	0.298
	40	0.99995	2772	759	19.43	1.33	0.292
	50	0.99996	2903	1003	24.00	1.35	0.287
	60	0.99996	3529	1478	32.82	1.37	0.281
	70	0.99997	5679	2805	57.31	1.40	0.275
	80	0.99997	46948	26778	498.73	1.42	0.269
	90	0.99997	-5488	-3559	-59.70	1.45	0.263
	110	0.99998	-1349	-1092	-14.13	1.50	0.251
	130	0.99998	-662	-646	-5.75	1.57	0.238

The reference time is better measured by injecting the blank solution and the micellar solutions of the solutes, especially if the detection is performed at a low wavelength, in order to remark the differences in composition between the injected solution and mobile phase. An additional advantage is the large number of individual values of the reference time that can be easily achieved, since any injection of solutes may be used to measure the reference time. The determination of the reference time in a large number of different mobile phases showed that it did not change appreciably with the composition of the eluent. This suggested that the same value of reference time can be used to predict capacity factors of solutes eluted in a given chromatographic column, with mobile phases containing variable amounts of surfactant and alcohol.

In experimental design and in the search of the optimum mobile phase to separate a mixture of solutes, the use of an approximate value of the dead time leads to good results, since the capacity factors are only used as intermediate values in the prediction of the position of the chromatographic peaks, expressed as retention time. However, in the calculation of capacity factors, the same value of dead time must be used for all mobile phases. On the other hand, the evaluation of physico-chemical retention parameters requires the use of an accurate value of dead time. The methods that search the start of the main first perturbation on the chromatograms give satisfactory constants.

ACKNOWLEDGEMENTS

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DETERMINATION OF ALKYLATED AND SULFONATED DIPHENYL OXIDE SUR-FACTANT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Methods for the determination of the anionic surfactant Dowfax 8390 are described. Dowfax is a complex mixture of various alkylated and sulfonated diphenyl oxides. The primary component of Dowfax is monoalkylated disulfonated diphenyl oxide (MADS). This work uses ion pairing chromatography and reverse phase chromatography. Ion pairing chromatography provides a simple and fast method for quantitation of the total concentration of Dowfax surfactant in aqueous solutions. It is shown that suppressed conductivity detection and optical detection at 210 nm have comparable sensitivity. A quantitation limit of 48.4 ppm is achieved with 400 µl injection. The separation of 11 components in Dowfax is achieved using gradient reverse phase chromatography.

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INTRODUCTION

There has been considerable interest in using surfactants to enhance subsurface remediation.[1,2,3] Because surfactants can increase the solubility of hydrocarbon contaminants in groundwater systems, they could potentially greatly reduce the number of pore volumes to be pumped in a cleanup effort.[3] Dowfax, as well as other commercial surfactants, such as T-MAZ ethoxylated sorbitan fatty acid esters,[4,5] Steol CS-330 sodium laureth sulfate[6] and octylphenol polyether alcohols,[7] is a complex mixture of many isomers, homologues and oligomers.

Dowfax 8390 is a commercial blend of alkylated and sulfonated diphenyl oxides. The primary component in Dowfax 8390 is linear C_{16} monoalkylated disulfonated diphenyl oxide (MADS). Approximately 80% of the components are considered to be monoalkylated, with the entire mixture being predominately disulfonated. The other components include monoalkylated monosulfonated diphenyl oxide (MAMS) and dialkylated disulfonated diphenyl oxide (DADS). The dialkylated monosulfonated diphenyl oxide component (DAMS) is typically present at a still smaller concentration. The structural formulas of these components are shown in Figure 1. Multiple isomers exist for each component.

As indicated in previous studies,[6,8] ion pairing chromatography is ideal for the separation of ionic organic compounds. For ionic surfactant CS-330, which consists of a saturated alkyl group, ethoxyl groups and a polar head, $-OSO_3$ Na, we indicated that ion pairing chromatography was the only HPLC separation technique available.[6] However, for Dowfax, since there are two benzene rings in these molecules (Figure 1), reverse phase chromatography, as well as ion pairing chromatography, can be used to separate them.

Reverse phase chromatography with a C_{18} column has been used to separate sorbitan esters previously.[9,10] The limitations using a C_{18} column include the use of a very high concentration of organic solvent (up to 90% isopropanol), long analysis time, and the lack of separation of oligomers.[9,10] As shown in Figure 1, since molecules in Dowfax have two benzene rings and a long hydrocarbon chain which are non-polar, a less hydrophobic packing is needed. A radially compressed C_8 column offers more desirable interactions between the stationary phase, Dowfax molecules and the mobile phase than a C_{18} column. Combined with the use of a mixture of acetonitrile, methanol and sodium phosphate buffer as a mobile phase, and gradient elution, the complex mixture of Dowfax was separated. As demonstrated in the study of T-MAZ oligomers,[5] the use of a C_8 column not only significantly reduced the consumption of organic solvent, but also achieved the separation of the

Components in Dowfax 8390
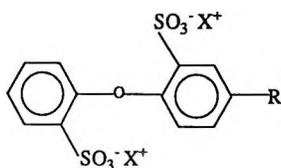
Monoalkylated Disulfonated Diphenyl Oxide (MADS)
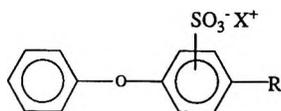
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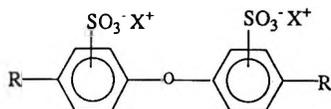
Dialkylated Disulfonated Diphenyl Oxide (DADS)
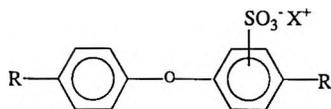
Dialkylated Monosulfonated Diphenyl Oxide (DAMS)

Figure 1. The molecular formulas of the components in Dowfax 8390.

oligomers. Recently, we have shown that the consumption of organic solvent in the analysis of TMAZ and Triton X-100 can be further reduced using a Waters Delta-Pak C_4 column.[11]

EXPERIMENTAL

Chemicals and Reagents

Dowfax 8390 and MAMS, MADS and DADS were from Dow Chemical Company (Midland, MI, USA). Dowfax is a registered trademark of the Dow Chemical Company. Tetrabutylammonium hydroxide (55% aqueous solution, TBAOH) was from Southwestern Analytical Chemicals, Inc. (Austin, TX, U.S.A.), acetonitrile and methanol from Burdick and Jackson (Baxter Healthcare Corporation, Muskegon, MI, U.S.A.). 18 MΩ water was obtained from a Millipore Milli-Q system (Marlborough, MA, U.S.A.).

Apparatus

Ion pairing chromatography

Instrumentation was from Waters (Waters Associates, Milford, MA, U.S.A.), which included a Model 996 photodiode array detector, a Model 431 conductivity detector, a Model 600E multisolvent delivery system and a Model 717 autosampler. Separations were accomplished using a Dionex (Dionex Corporation, Sunnyvale, CA, U.S.A.) IonPac NS1 column (4 mm x 250 mm) and a NG1 guard column. A Dionex anion micro membrane suppressor (AMMS-MPIC) and 25 mM sulfuric acid solution were used to suppress the background conductivity of the mobile phase.

The mobile phase was 50% acetonitrile and 3 mM TBAOH in water with a pH value of 11.8. A silica- based reverse phase column cannot be used with this strong basic eluent because it has an operating pH range of 4 to 7.5. Organic polymer packings, such as IonPac NS1 column, have a wide pH range (0 to 14) and they are ideally suited for the separation of molecules in Dowfax. The injection volume was 400 µL at an eluent flow rate of 1.0 mL/min. Data acquisition and processing was accomplished with a Waters Maxima 820 chromatography workstation, which included a system interface module and a NEC PowerMate SX/16 computer.
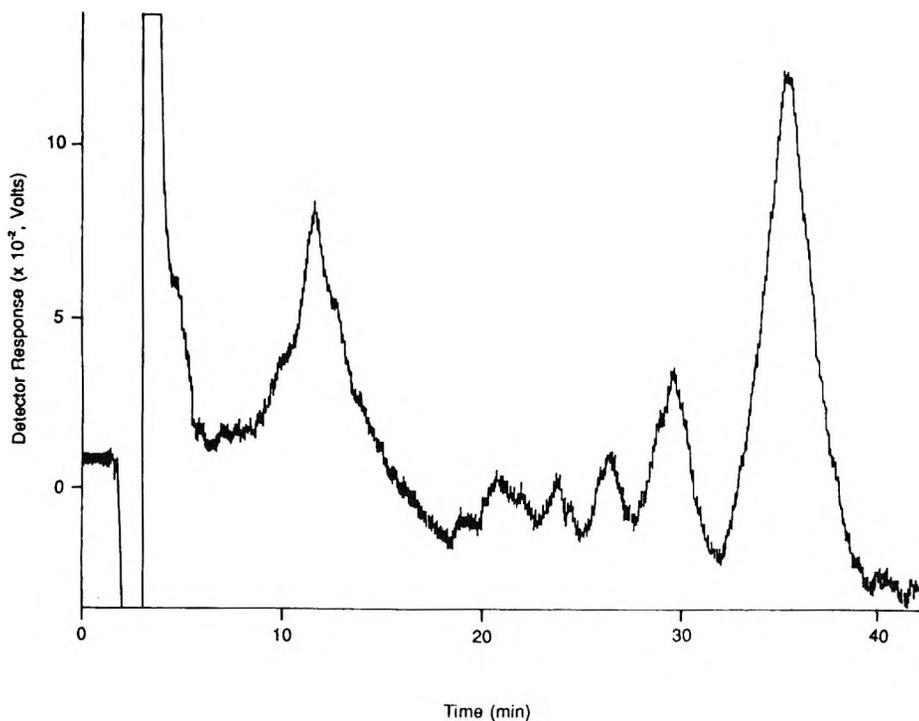
Figure 2. Ion pairing chromatogram of Dowfax. Concentration: 200 ppm.

Reverse phase chromatography

Separations were accomplished using a Waters Nova-Pak C_8 stainless steel column (3.9 x 150 mm). The injection volume was 100 μL with a flow rate of 1.0 mL/min. The linear gradient elution, which used 2 mobile phases: A: (20% acetonitrile, 10% methanol, 0.75 mM NaHCO3 and 2.2 mM Na2CO3) and B: (40% acetonitrile, 25% methanol, 0.75 mM NaHCO3 and 2.2 mM Na2CO3), started at 78% of A and 22% of B, ran to 65% of A and 35% of B at 40 minutes, to 50% of A and 50% of B at 50 minutes and held for 5 minutes, and finally to 100% of B at 60 minutes and held for 20 minutes.

Data acquisition and processing was accomplished with a Waters Millennium chromatography workstation, which used an NEC Image 466es computer. Other instrumentation used was described above.

RESULTS AND DISCUSSION

Ion Pairing Chromatography

The mobile phase, 50% of acetonitrile and 3 mM TBAOH, has a pH value of 11.6 and a conductivity background of 425 μS. With such a high conductivity background, the ratio of signal to noise with a conductivity detector is poor. The micromembrane suppressor removes cations in the column eluent after the oligomer separation is accomplished, reducing the background conductance to 82 μS.

Figure 2 shows the chromatogram of the separation of Dowfax. The concentration of Dowfax in the chromatogram was 200 ppm with 400 μL injection. Several peaks were found in the chromatogram. The peak with retention time at 35 minutes is used in the quantitation because it has the highest response. The integration was from 31.4 to 39.8 minutes. A mixture of benzene, toluene, ethylbenzene, and toluene (BTEX) is contained in the environmental samples, which elute between 5 to 10 minutes and can cause interference with the peaks with short retentions.

Dowfax was analyzed quantitatively with a good degree of precision and accuracy (Table 1). Dowfax standards, 100 and 200 ppm, were analyzed three times, and were used to determine the detection limit. The detection limits were 14.52 ppm, calculated as three times the standard deviation of the mean. From the detection limit of 14.52 ppm, a quantitation limit of 48.42 ppm was estimated, calculated as ten times the standard deviation of the mean. Standards in the concentration range from 50 to 1200 ppm were analyzed four times, and a standard from each concentration was randomly selected to generate calibration curve. As demonstrated by the relative error (Table 2), the calculated concentrations are in a good agreement with the known concentrations.

Since Dowfax molecules consist of UV-absorbing chromophores, two benzene rings, an optical detector can be used. The spectra of four principal peaks (A, B, C and D in Figure 5a) in Dowfax were given in Figure 3, showing strong UV absorption at 200 nm. Figures 4a and 4b show the chromatogram of 50 ppm Dowfax using a suppressed conductivity detector and an optical detector at 210 nm. From the comparison of the noise levels in Figures 4a and 4b, it is clear that the optical detector is as sensitive as the suppressed conductivity detector. With the peak-noise ratio of 6.4 : 1.0 and 6.9 : 1.0 in Figure 4a and 4b, the chromatograms also show that Dowfax at a concentration of 50 ppm can be quantified.

Table 1

Analytical Precision and Detection Limit with the Conductivity Detector

| Peak Group | Concentration | | Detection Limit |
	100 ppm	200 ppm	(ppm)
1	n = 3	n = 3	14.52
	x = 104.3	x = 212.4	
	SD = 4.84	SD = 3.89	
	RSD = 4.6%	RSD = 1.8%	

n: Number of standard solutions analyzed.   x: Mean solution concentration (ppm).   SD: Standard Deviation.   RSD: Percent Relative Standard Deviation (= $100 \times (SD / x)$).   Depection limite was calculated as three times the standard deviation of the mean ($3 \times SD$).

Table 2

Average Peak Area, Relative Standard Deviation, Calculated Concentrations and Relative Error with the Conductivity Detector

Conc'n (ppm)	Ave. Peak Area[a]	(RSD)[b]	Cal. Conc'n	Rel. Error (%)
50	0.7683×10^7	(5.6)	53.4	6.8
100	1.4255×10^7	(4.3)	102.4	2.4
200	2.6132×10^7	(3.2)	184.4	-7.8
400	5.6618×10^7	(4.1)	397.6	-0.6
800	11.7492×10^7	(3.4)	812.5	1.6
1200	17.2030×10^7	(2.9)	1216.9	1.4

[a] The peak areas were averaged from three experimental data.
[b] RSD: Relative Standard Deveiation = $100 \times$ (Standard Deviation/Average Peak Area
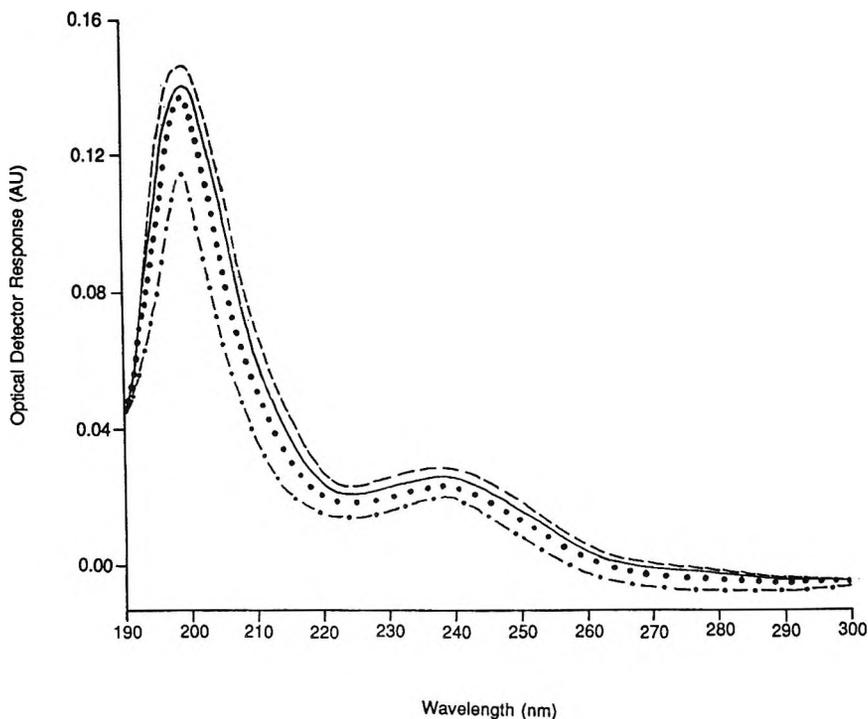
Figure 3. The spectra of 4 principal peaks (A, B, C and D in Figure 5a) in Dowfax. (-----) A, (——) B, (•••••) C and (-•-•-) D.

Reverse Phase Chromatography

Gradient elution was used to separate the components in Dowfax. Figure 5a shows the separation of 500 ppm Dowfax. The chromatographic conditions were described in the experimental section. The chromatogram demonstrates the complexity of this industrial chemical with 11 peaks found in the chromatogram. Three components, MADS, MAMS and DADS were analyzed with the identical chromatographic conditions as in Figure 5a. Figure 5b shows the chromatogram of 500 ppm MADS. The close similarity of the chromatograms in Figures 5a and 5b confirms that the primary component in Dowfax is MADS, as suggested by the Dow Chemical Company. The mobile phase used was a mixture of acetonitrile, methanol and aqueous phosphate buffer as described in the experimental section. The use of both acetonitrile and methanol in the mobile phase, rather than using only acetonitrile or methanol, can effectively separate
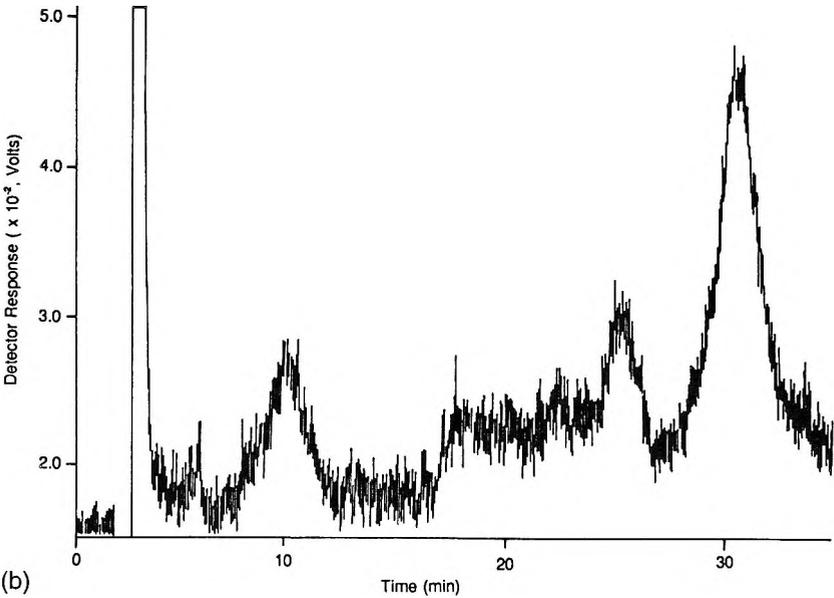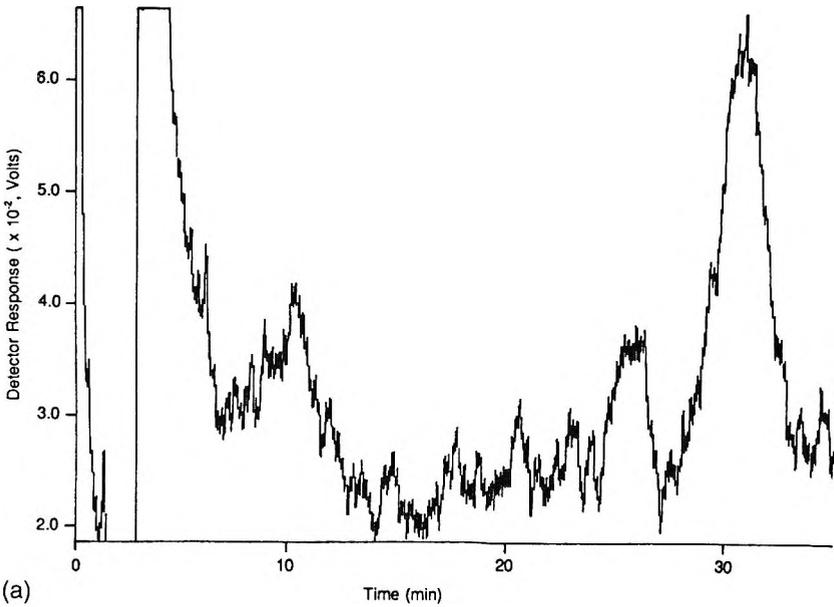
Figure 4. Ion pairing chromatograms of Dowfax. Detection: (a) suppressed conductivity; (b) UV at 210 nm.; Injection volume: 400 µL; Concentration: 50 ppm.
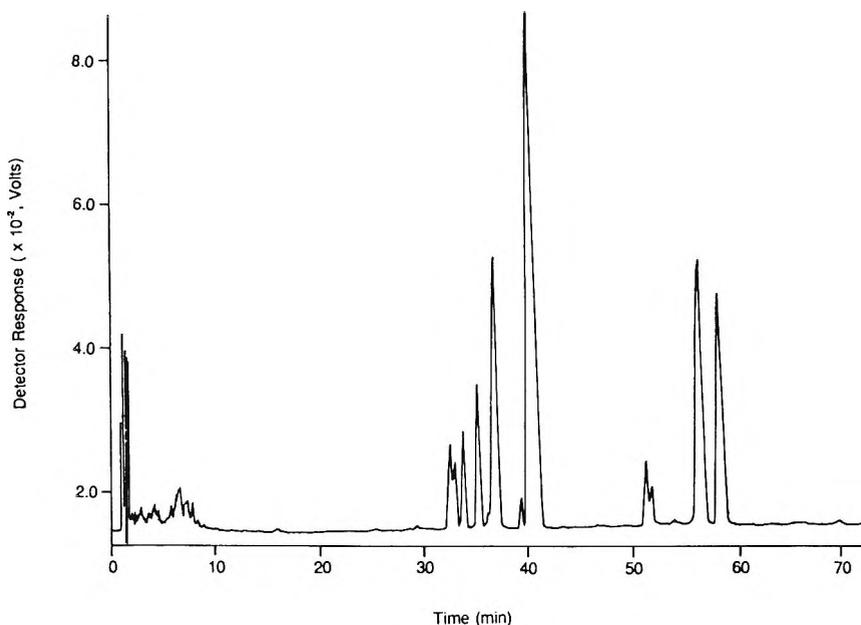
Figure 5. Reverse phase gradient elution chromatograms of Dowfax and MADS. Injection volume: 100 µl;   Concentration: 500 ppm;   Detection: UV at 239 nm.; (a) Dowfax, (b) MADS.

organic molecules with very subtle hydrophobic differences.   Phosphate was added to the mobile phase to vary solute retention and selectivity.   In reverse phase chromatography, retention of a polar compound decreases when the addition of salt increases the solubility of the solute in the mobile phase.[12]

Conversely, an increase in retention will occur when the addition of salt decreases the solubility of the solute in the aqueous mobile phase.[12]   Dowfax molecules are organic anions. Increase in ionic strength of the eluent can reduce the retention of these molecules and change the separation selectivity.

In a gradient elution, baseline drifting is very significant when the detector is set at low UV wavelength, such as 200 nm.   The optical detector was set at 239 nm, which provides a less drifting baseline, though the sensitivity is lower at 239 nm than at 200 nm.

DISCLAIMER

QA/QC REQUIREMENTS

All QA/QC aspects of this work were performed in accordance with the requirements of the ManTech Environmental Research Services Corporation Quality Assurance Program Plan.

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IDENTIFICATION AND DOSAGE OF 2-FURALDEHYDE AND 5-HYDROXYMETHYL-2-FURALDEHYDE IN BEVERAGES BY REVERSED PHASE CHROMATOGRAPHY WITH A MICROBORE COLUMN

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ABSTRACT

This paper reports the results of a study performed to develop a rapid and straightforward chromatographic method for the identification and dosage of 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde (FA), which are recognized indices of deteriorative changes in commercially processed food. The method employs a Supelco microbore reversed phase column (300 x 1.0 mm I.D.), eluted isocratically with a 94:6 (v/v) water/acetonitrile mixture at flow rate of 60 µL/min. Sample is detected at 280 nm in a micro flow cell of 300 nl. Peak purity and identification is assessed by comparing the UV spectra monitored at two points through the chromatographic peak in continuous flow mode in the range from 200 to 400 nm. This method is successfully applied to the identification and

quantitative determination of HMF and FA in alcoholic and non-alcoholic beverages by an internal standard method without any sample pretreatment.

INTRODUCTION

5-Hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde (FA) have been proposed as general indices of the deterioration of food quality during storage and are also useful as indicators of temperature abuse during process and storage.[1] HMF is one of the best known intermediate products in the Maillard reaction,[2] while FA is the principal product of the hydrolysis of pentoses and is widely accepted as an indicator of flavor changes.[3,4] Many different analytical techniques are available for the identification and dosage and HMF in processed food. Classical methods for the quantitative determination of these components in food are based on colorimetric measurements. These methods have the disadvantage of the instability of the coloured complex formed, the time required, the use of hazardous chemicals, and no one of the methods is specific.[5-8] Chromatographic methods have also been proposed for the determination of FA and/or HMF in different types of food matrices. These techniques include thin layer chromatography,[9] gas chromatography[10,11] and high performance liquid chromatography (HPLC).[1,12-17] The above methods differ in sensitivity and reproducibility, are not free of interferences and some of them require time consuming sample pretreatment or/and the use of relatively large volumes of organic solvents which are toxic and quite expensive.

We are interested in the development of small-scale analytical methods for substances that are involved in the deterioration reaction occurring during food processing and storage. In this respect, we have recently demonstrated that micellar electrokinetic capillary chromatography (MECC) can be successfully employed for the analysis of FA and HMF in fruit juices and honey.[18,19] Reconized advantages of using miniaturized techniques in electrophoresis and liquid chromatography are the increased mass sensitivity, the higher peak efficiency and the smaller sample volume required for analysis.[20,21] Furthermore, the lower volumes of organic solvents, when required, employed by using narrow-bore liquid chromatographic columns reduces health hazard and the cost of solvents and waste elimination

In this paper we report the results of a study aimed at developing an HPLC method for the analysis of FA and HMF in food employing a reversed phase microbore column and multi-wavelength UV detection. In order to select
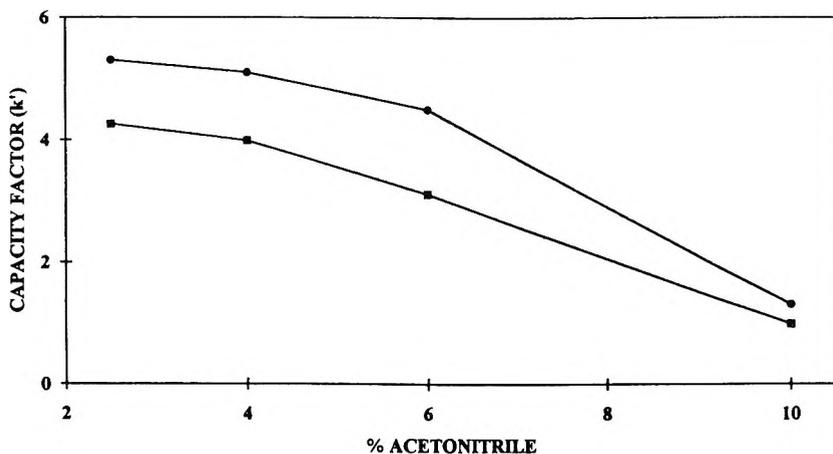
Figure 1. Dependence of the retention factor (k') of HMF (■) and FA (•) on the acetonitrile content in the mobile phase. Chromatographic conditions: column, Supelcosil LC-18 (300 x 1.0 mm I.D.); eluent, acetonitrile-water; flow rate, 60 mL/min; detection by UV absorbance at 280 nm; temperature, 25°C.

the optimum column and chromatographic conditions to perform the rapid and reproducible separations and quantification of the above furaldehydes, the effect of mobile phase composition on the retention time and selectivity was examined using a Supelcosil LC-18 microbore column having 1 mm internal diameter.

The application of this method to the identification and dosage of HMF and FA in some varieties of alcoholic beverages and soft drinks, colored with caramel, from different commercial sources is also described.

EXPERIMENTAL

Instrument and Column

The chromatographic experiments were carried out using an HPLC system equipped with a Model 421A microprocessor controller and a Model 114M single-piston reciprocating pump with the capability of delivering micro flow rates, all from Beckman Instruments, Inc.   (Fullerton, CA, USA); a 7520 Rheodyne (Cotati, CA, USA) microsample injector with a 0.5 µL sample rotor and a Model 433 variable-wavelength detector with a standard micro flow cell (300 nl), in conjunction with a Data System 450 software, both from Kontron

Instruments (Milan, Italy).   Peak purity and identification was assessed by comparing the UV spectra monitored at two points through the chromatographic peak in continuous flow mode.   The reversed phase microbore column employed in the experiments was a Supelcosil LC-18 (300 x 1.0 mm I.D., 5-μm particle size) and was supplied by Supelco (Bellefonte, PA, USA).

Chemicals

HPLC-grade water, acetonitrile and methanol were purchased from Carlo Erba (Milan, Italy). 5-hydroxymethyl-2-furaldehyde (HMF), 2-furaldehyde (FA) and 2-furyl methyl ketone (FMK) were obtained from Aldrich (Milan, Italy). All mobile phases were degassed by sparging with helium before use. All alcoholic beverages and soft drinks were purchased from a local store.

Procedure for Quantitative Analysis

A 2.0 mg/mL stock solution of HMF and FA in methanol-water (10:90 v/v) and 10 mg/mL of FMK in water as the internal standard solution were prepared daily. The stock solution was diluted to produce working standard solutions at five different concentrations within the range 0.1-50.0 μg/mL. An appropriate volume of internal standard solution was added to each solution to give a concentration of 10.0 μg/mL of FMK. Calibration graphs were plotted based on the linear regression analysis of the peak-area ratios, analyzing each working standard solution in quintuplicate.

Alcoholic beverages and soft drinks were diluted with water after the addition of the internal standard solution to give a concentration of 10 μg/mL of FMK. The soft drink samples were initially decarbonated by stirring.

RESULTS AND DISCUSSION

With the aim of developing a rapid and sensitive HPLC method for the routine analysis of the furanic aldehydes, 2-furaldehyde (FA) and 5-hydroxymethyl-2-furaldehyde (HMF) in alcoholic and non-alcoholic beverages, the systematic investigation of the effect of the mobile phase composition on the
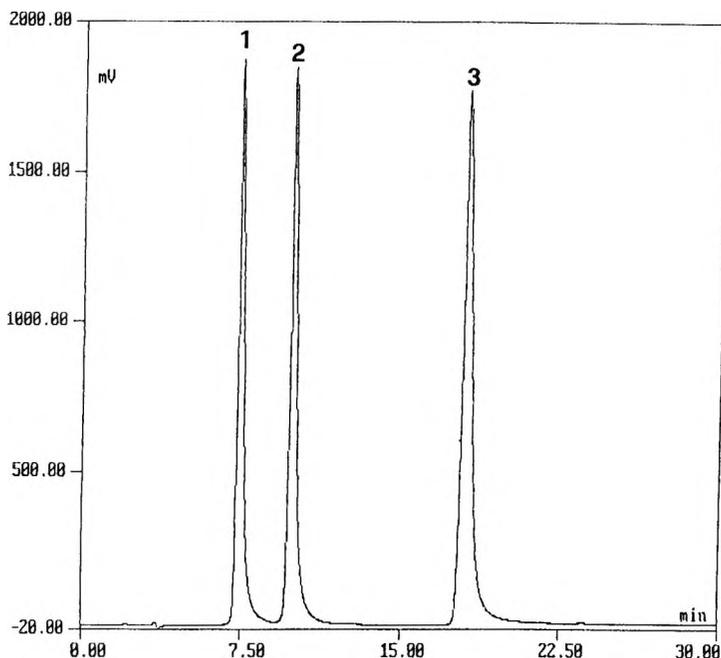
Figure 2. Separation of a standard mixture of HMF (1), FA (2) and MFK (3). Chromatographic conditions: column, Supelcosil LC 18 (300 x 1 mm I.D.); eluent, water-acetonitrile 94:6 (v/v); flow rate, 60 mL/min; detector UV set at 280 nm; temperature, 25°C

chromatographic retention of these furanic compounds in the Supelco LC 18 microbore reversed phase column was performed. The experiments were carried out by eluting under isocratic conditions both real samples and standard solutions with mobile phases containing acetonitrile in water at concentration ranging from 2.5 to 10 % (v/v). Figure 1 shows the retention behavior of the examined furanic aldehydes as a function of the content of acetronitrile in the mobile phase. As expected the retention time of HMF and FA decreased by increasing the concentration of acetonitrile in the mobile phase. Using mobile phases with acetonitrile content higher than 10% (v/v), the resolution of HMF from FA and some substances belonging to the elution front in real samples was impaired. On the other hand, by decreasing the percentage of acetonitrile

Table 1

Retention Time, Standard Deviation (S.A.) and Relative Standard Deviation (R.S.D.) of Multiple Injections of a Standard Solution of 5-Hydroxymethyl-2-furaldehyde (HMF), 2-Furaldehyde (FA) and 2-Furylmethylketone (FMK).Chromatographic Conditions as in Fig. 2.

Compound	Retention Time			Retention Time Mean	S.D.	R.S.D.
	(min)			(min)	(min)	(%)
HMF	7.39	7.31	7.21	7.25	0.079	1.09
	7.24	7.18	7.26			
	7.16	7.28	7.15			
FA	9.60	9.86	9.89	9.71	0.101	1.04
	9.69	9.82	9.72			
	9.61	9.76	9.61			
FMK	17.48	17.54	17.76	17.55	0.146	0.83
	17.72	17.74	17.48			
	17.46	17.33	17.38			

the elution times were excessively increased. On the basis of these observations, further experiments were carried out with the mobile phase containing 6% (v/v) of acetonitrile which was found to ensure sufficient resolution in a reasonable analysis time.

In order to examine the reproducibility of the retention times, the mean value, the standard deviation (S.D.) and the relative standard deviation (R.S.D.) of the retention times were calculated from the chromatograms obtained by 12 repeated injections of an equimolar solution of FA, HMF and FMK, which was employed as the internal standard in the quantitative analysis. The results are reported in Table 1 and show that the R.S.D.s were batter than 1.09 % for the three compounds.

The dosage of FA and HMF was obtained by an internal standard method. Many commercial compounds structurally related to FA and HMF were evaluated as potential internal standards before selecting 2-furyl methyl ketone (FMK). This furanic compound was selected as the internal standard as it is well resolved from both FA and HMF, is not naturally present in processed

Table 2

**Results of the Quantitative Determinations of HMF
and FA in Alcoholic and Non Alcholic Beverages**

Sample	HMF (mg/L)	R.S.D. (%)	FA (mg/L)	R.S.D. (%)
A1	47.08	2.22	1.21	5.24
A2	147.20	3.47	4.35	5.36
A3	158.87	2.02	11.11	1.65
A4	287.75	1.55	nd	----
A5	432.31	1.81	8.09	3.77
A6	109.20	0.79	nd	----
A7	79.11	1.57	nd	----
A8	291.83	1.54	nd	----
B9	5.40	1.57	nd	----
B10	9.34	0.07	nd	----
B11	343.61	0.12	nd	----
B12	347.18	1.27	1.62	3.04
B13	3.49	5.06	nd	----
C14	187.68	0.01	nd	----
C15	65.25	0.92	nd	----
C16	114.45	1.10	nd	----
C17	3.75	1.32	nd	----

A = bitter (alcoholic beverages made with herbs and/or roobs and colored with caramel); B = aperitif; C = soft drink; nd = not detected; R.S.D .= relative standard deviation.   The subscript numbers indicate different commercial sources.

food, does not interfere with the elution pf other species in the examined real samples and is eluted near the peaks of interest.   A typical chromatogram of HMF, FA and FMK is shown in Figure 2. It is observed that the peaks of HMF, FA and FMK are well resolved and eluted in less than eighteen minutes. The minimum detectable concentration of HMF and FA was 0.236 ng and 0.116 ng, respectively.   The calibration graphs for HMF and FA, obtained by
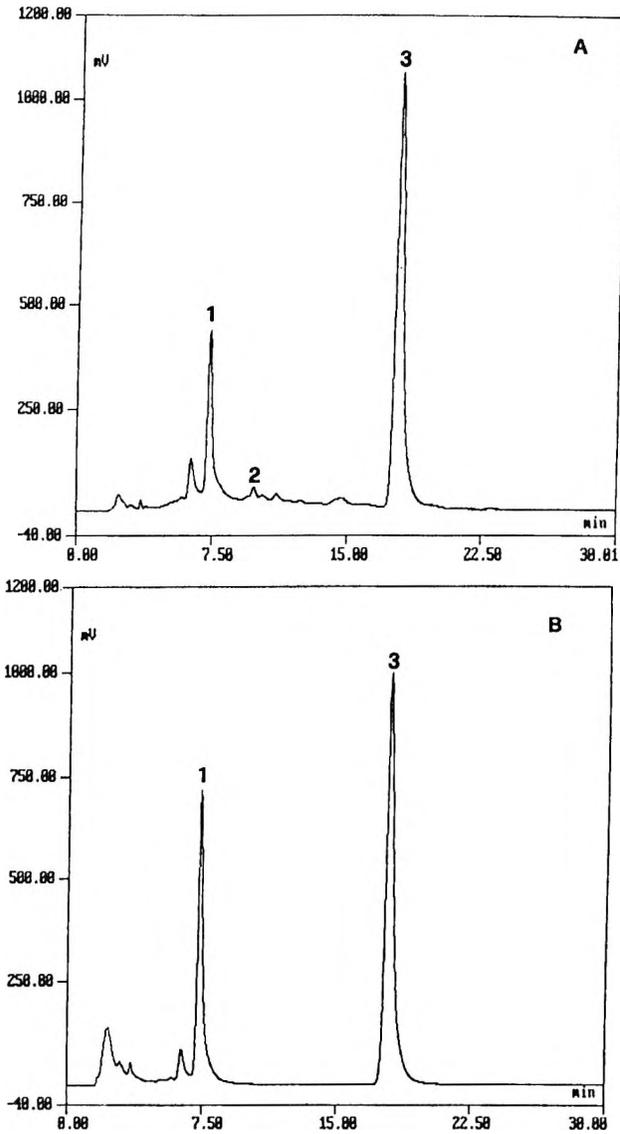
Figure 3. Typical chromatograms of an alcoholic bitter (panel A) and of a soft drink (panel B). Peak identification and chromatographic conditions as in Figure 2.
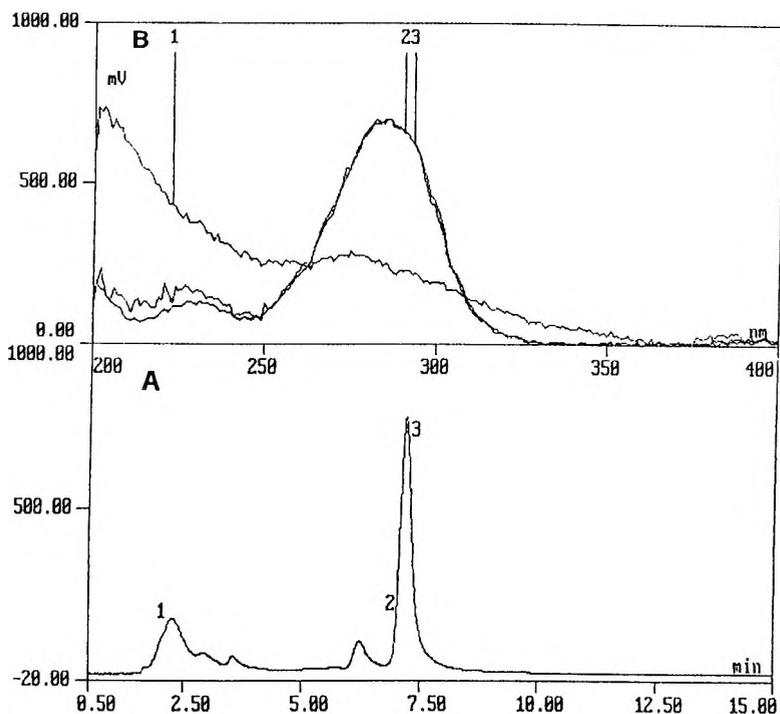
Figure 4. Chromatogram of a sample of a non-alcoholic beverage containing 11.44 mg/mL HMF, the numbers on the chromatogram indicate the points were the UV spectra were monitored (panel A). Chromatographic conditions as in Figure 2. UV spectra from 200 to 400 nm monitored at retention time of 6.99 min (spectra 2) and at retention time of 7.19 min (spectra 3), respectively (panel B).

the peak-area ratio method showed excellent linearity over the concentration range 0.1-50 µg/mL with correlation coefficients r = 0.9998 and 0.9987 respectively, and nearly passed through the origin.

The present method was employed to analyze the HMF and FA content in several varieties of alcoholic bitter beverages and soft drinks coloured with caramel. The beverages were directly injected onto the column without any sample   pretreatment,   except that they were diluted with water to a concentration range that would ensure no significant loss of resolution due to
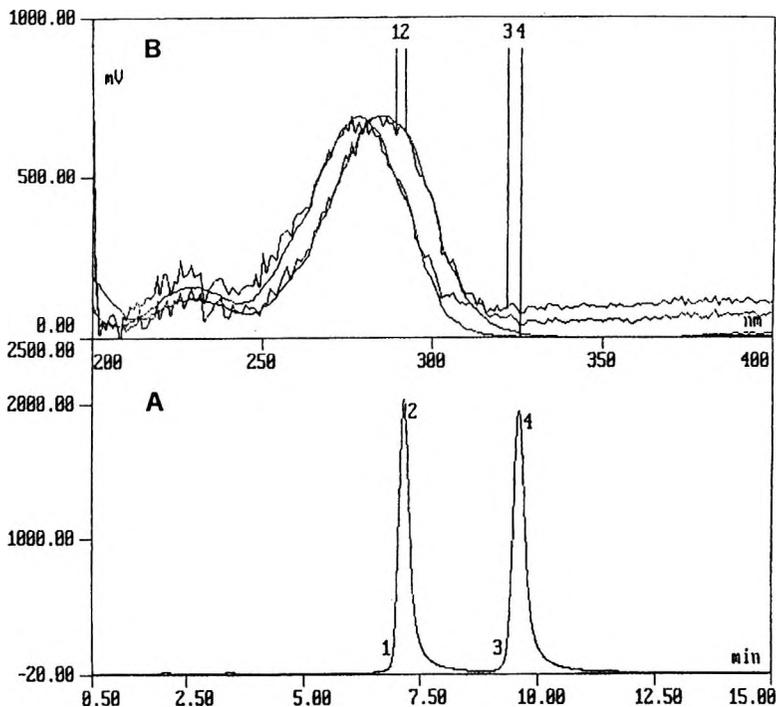
Figure 5. Separation of a standard solution of HMF and FA. Chromatographic conditions as in Figure 2, the numbers on the chromatogram indicate the points were the UV spectra were monitored (panel A). UV spectra from 200 to 400 nm monitored through the peaks at retention time of 6.89 min (spectra 1) and 7.16 min (spectra 2) for HMF and at retention time 9.30 min (spectra 3) and 9.62 min (spectra 4) for FA, respectively (panel B).

overloading of the microbore column. The diluted samples were then filtered through a 0.22 μm single use membrane filter after the addition of the internal standard solution and adjustment of the sample volume to the appropriate value. Results of three replicate determinations are summarized in Table 2 and typical chromatograms are shown in Figure 3.

Peak purity is an important consideration in quantitation. The UV variable wavelength detector used here can measure both retention times and absorption spectra in continuous flow mode, and peak components can therefore be identified by comparison of the peak spectra with those of

Table 3

Recovery of HMF and FA from an Alcoholic Bitter

Compound	Amount in the sample (mg/L)	Added (mg/L)	Found (mg/L)	Recovery (%)
HMF	15.887	2.004	17.953	100.35
	15.887	3.006	18.428	97.54
	15.887	5.010	20.339	96.87
FA	0.6650	0.2088	0.8750	101.95
	0.6650	0.4142	1.0366	97.12
	0.6650	0.8352	1.5028	101.20

Table 4

Recovery of HMF from a Soft Drink

Compound	Amount in the sample (mg/L)	Added (mg/L)	Found (mg/L)	Recovery (%)
	11.445	2.004	132.03	98.17
HFM	11.445	3.006	142.55	98.65
	11.445	5.010	164.55	100.23

standards.   Figure 4 shows the chromatogram of a soft drink in which HMF was detected at a concentration of 11.44 µg/mL. The spectra of a the component eluting with a retention time of 7.18 minutes was compared with that of a sample of standard HMF eluted under identical conditions (Figure 5).   It is observed that the spectra reported above the chromatogram in Figure 4 are almost identical to the spectra displayed in Figure 5, confirming that the peak component eluting with a retention time of 7.18 minutes was HMF.

In order to determine the accuracy of the method, recovery studies were carried out.   Known amounts of HMF and FA were added to a variety of commercial alcoholic and non-alcoholic beverages and the resulting spiked samples were subjected to the entire analytical method.   Three different amounts

of HMF and FA were added to the samples.   All samples were injected three times and an average of the response area ratio was the basis for the found concentrations. The recoveries were calculated based on the difference between the total concentration determined in the spiked samples and the concentration observed in the non-spiked samples.   Results with the relative standard deviations for all samples were of the same order as those reported in Table 3 for a commercial alcoholic bitter and in Table 4 for a soft drink.   It can be seen that the average recoveries lied between 96.87 and 101.95%, indicating that the method has an adequate degree of accuracy.

CONCLUSIONS

Reversed phase high performance liquid chromatography using the Supelco LC 18 microbore column appears to bea useful and versatile procedure for the rapid and direct determination of HMF and FA in beverages. The developed method is highly reproducible, the quantification is linear over a wide range of concentrations, and the results of the recovery studies show good accuracy. The method is simple and no sample pretreatment is required, except dilution with water to the appropriate concentration range.   Furthermore, the low volume of acetonitrile required for the analysis (about 6 µL per run), due to the microbore format of the column, drastically reduces the cost of the analysis, health hazard and the environmental impact of the waste connected to the use of this organic solvent in reversed phase chromatography.

ACKNOWLEDGEMENTS

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DIRECT INJECTION ANALYSIS OF MITOMYCIN C IN BIOLOGICAL FLUIDS BY MULTIDEMENSION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A MICELLAR MOBILE PHASE

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ABSTRACT

This paper describes the direct injection analysis of the anti-cancer drug mitomycin C in biological fluid by an HPLC column switching technique.The first chromatographic column which provides for sample extraction and clean up employs a micellar mobile phase with SDS as the modifier. The second column, coupled on-line to the first, utilizes reverse-phase conditions for analysis. UV detection is employed at 365nm. Samples from cancer patients and dogs were analyzed. The drug recoveries are 95.9 - 100.3% with a relative standard deviation of 2.76%. A sample analysis is completed within 15 minutes.

INTRODUCTION

Developments in the technique of HPLC have enabled it to become a very important method for drug studies on the metabolism of drugs. The most straightforward method of sample preparation is liquid-liquid extraction or liquid-solid distribution together with evaporation to recover and concentrate the drug and remove endogenous proteins that can be harmful to the column packings. Such procedures are laborious and time-consuming and the recovery of drug is often low. A more practicable solution to this problem is the use of a simple direct injection technique.

The use of micellar chromatography has been reported to allow such direct injection of biological fluids (serum, plasma and urine).[1-4] Micellar chromatography employs surfactants as mobile phase modifiers[5] to remove protein in untreated biological samples but gives only moderate chromatographic efficiency due to poor mass transfer. Furthermore, the sensitivity of determination is inadequate for many applications.

In recent years column switching combined with the use of micellar clean-up prior to liquid chromatography has been introduced.[6-10] Sodium dodecyl sulphate (SDS)is added to the mobile phase for loading the untreated biological samples onto a precolumn. The proteins are solubilized by the SDS and washed out whereas the analyte is retained. Next, the retained analyte is eluted with the analytical mobile phase and analyzed using conventional RP-HPLC. This method has the advantage of both micellar chromatography (direct biological samples injection, extended column life and good drug recovery) and RP—HPLC (high column efficiency).

Mitomycin C(MMC) has been widely used for cancer treatment in recent years, but its clinical applications are limited owing to inherent severe toxicity. The therapeutic efficacy of mitomycin C is closely related to the drug concentration in blood and tissues, depending on the dosage, route and frequency of administration. Therefore, an appropriate analytical assay that is simple, rapid and reliable is desired.

The determination and quantitation of MMC in biological fluids was mostly performed by means of HPLC methods. The major sample pretreatment procedure for MMC, such as liquid-liquid extraction[11] or offline liquid-solid extraction[12] are in general laborious or very time consuming for large numbers of samples. Other methods,[13-15] which use complicated equipment, have some difficulties in clinical applications.
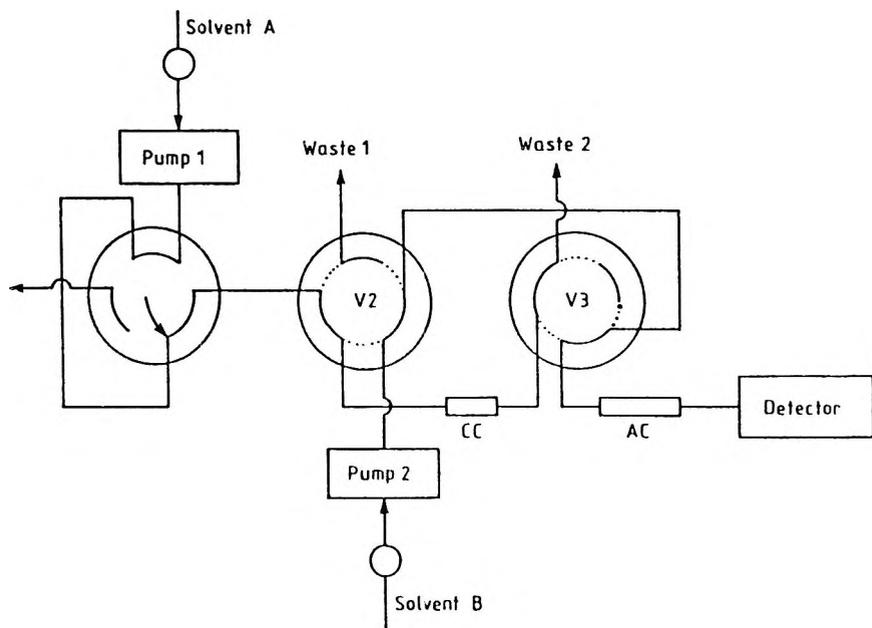
Figure 1. Schematic Diagram of Multidimensional HPLC.

In our laboratory, the column switching system is used for the direct injection analysis of MMC in biological fluids. This method is simple, rapid and reliable.

MATERIALS

Equipment

A diagram of the experimental set-up is shown in Figure 1. Solvent delivery was provided by two pumps. Pump 1 (Model YSB-2, Shanghai Science Instrument Factory, China) was used for loading the sample onto the clean-up column (CC). Pump-2 (Mode SY-5020, Beijing Analytical Instrument Factory, China) was used to deliver the chromatographic mobile phase. The switching system consisted of a Rheodyne injection valve (Type 7125) with a 10μL sample loop and two K501 injection valves (Shanghai Science Instrument Factory, China). A Mode SPD-I UV/VIS detector (Shimadzu, Kyoto, Japan) was used

for monitoring the effluent from the analytical column (AC). Data were acquired with a C-R2B chromatographic data system (Shimadzu, Kyoto, Japan).

Column

The two column cartridges used in this study were manufactured by the Beijing Analytical Instrument Factory (Beijing, China). Both the analytical column (150 x 4.6mm i.d., 5μm spherical porous particles) and the clean-up column (50 x 4.6mm i.d., 5μm spherical porous particles) were packed with RP-18 (Shanghai Chemicals Factory, China).

Chromatographic Conditions

Solvent A: the micellar clean-up eluent, methanol/water (10/90, v/v)
            containing 20mM SDS (adjusted to pH = 6.8 with 2N HCl).

Solvent B: methanol / water (30/70, v/v), adjusted to pH = 6.8 with 2N
            HCl.

Flow rate: 1.0mL/min
Detection: UV absorption (365nm)

Materials and Reagents

The standard MMC was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Biological samples from cancer patients and dogs were obtained from Gansu Province Tumours Hospital. Sodium dodecyl sulfonate (SDS) was the chemical pure reagent of Xian Chemical Reagent Plant (Xian, China), recystallized twice with alcohol before use. Blood plasma was obtained from Gansu Province Tumers Hospital.

Assay Procedure

When mobile phase was delivered through the solid lines shown in Figure 1, solvent A (a weak eluent) was carried into CC by pump 1, the dissolved proteins and other weakly retained interfering biological components were rinsed out from CC to waste while MMC was still retained in CC. After eliminating proteins, valves 2 and 3 were switched into the transfer position and the mobile

phase was pumped through the line shown dotted in Figure 1. MMC was thus transferred from CC to AC by solvent B (strong eluent). Then, valves 2 and 3 were rotated back and MMC was eluted and separated by solvent B on AC. Finally, MMC in the biological samples were determined by UV detection.

Optimization of Mobile Phase and Switch Time

As described above, we know that it was necessary to rinse the cleanup column with a weak solvent to wash out the proteins and other interfering biological components whilst the MMC was retained in CC. We found that when 10% aqueous methanol containing 20mM SDS was used as the weak solvent to flush CC at a flow rate of 1mL/min, the protein and interfering components were eluted within 5 min, but MMC was still in the pre-column.

If 30% aqueous methanol was used, the MMC was eluted within 2 min. Therefore, 10% aqueous methanol containing 20mM and 5 min were chosen as solvent A and switching time 1, respectively. Between 5 min and 9 min the whole of the MMC was delivered into AC. After 9 min, valves 2 and 3 were rotated back. AC was eluted by solvent B while CC was again equilibrated by solvent A. This operating method not only minimizes analytical time, but also avoids prolonged high back-pressure in the chromatographic system.

RESULTS AND DISCUSSION

Chromatographic Separation and Calibration Curve

Chromatograms of MMC in biological sample are shown in Figure 2. Blank plasma of patient gave some system peaks (Fig. 2a), but did not interfere with the determination. Figure 2b is the chromatogram of blank plasma of patient with added MMC. Figure 2c and 2d are chromatograms of patient's and dog's plasma, respectively. The results show this method to be suitable for the separation of both synthetic and real samples.

Using the chromatographic condition of Figure 2 the peak height was plotted against the concentration of MMC to give a calibration curve (Fig. 3), with a correlation coefficient of 0.9992 and whose regression is: $Y= 1.17x-0.23$. The linear range of MMC is 1 to 400mg /L.
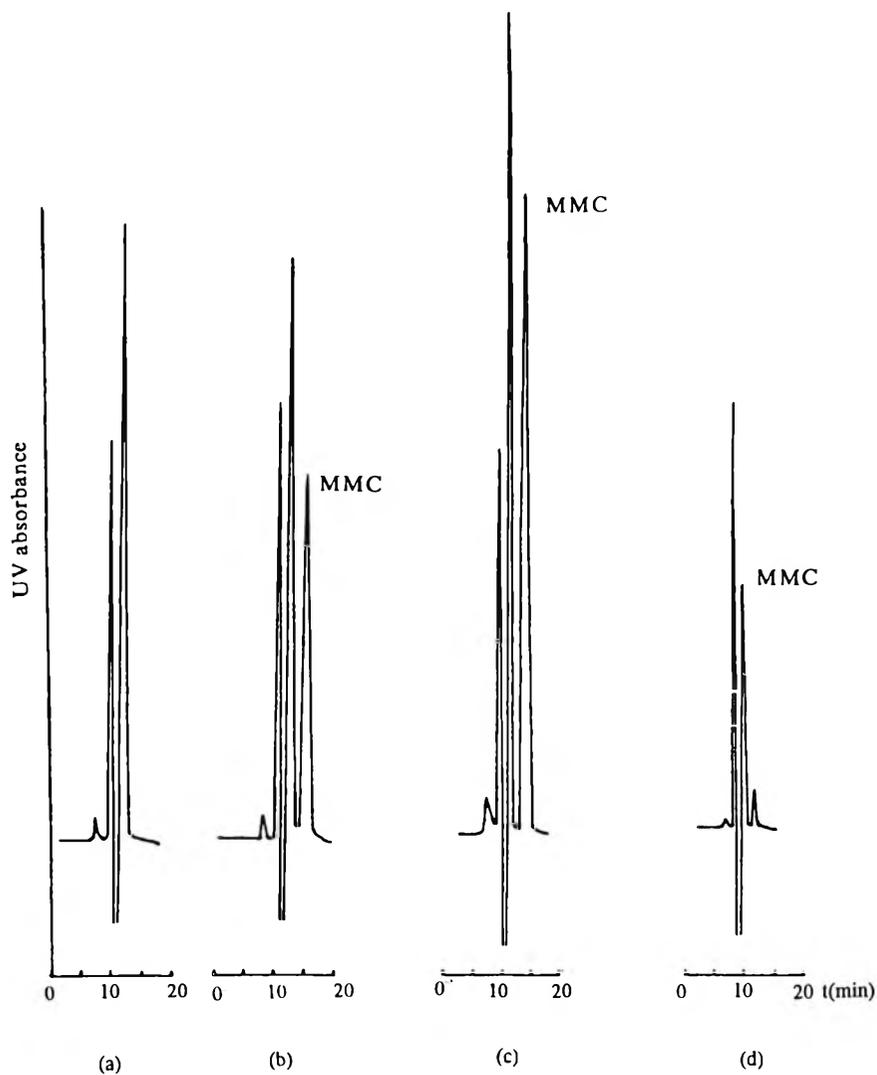
Figure 2. Chromatogram of MMC.   (a) Blank Plasma;   (b) Blank Plasma with Added MMC;   (c) Patient's Plasma Sample;   (d) Dog's Plasma Sample.
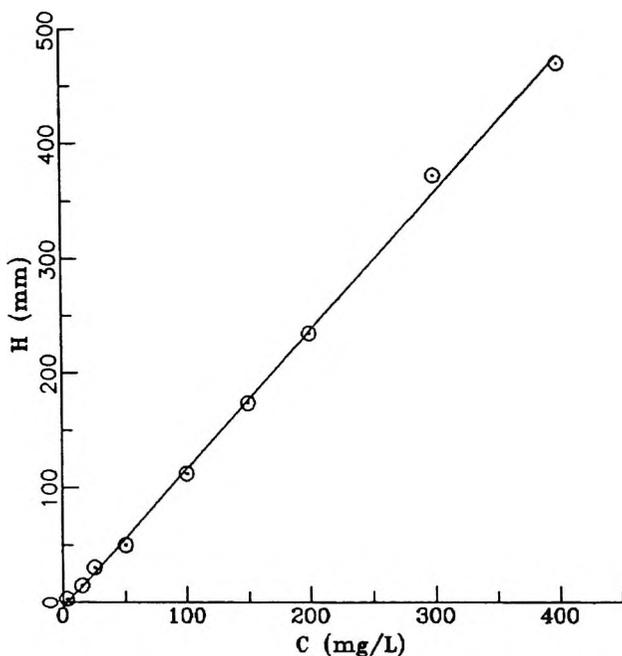
Figure 3. Calibration Curve of MMC.

Recovery and Reproducibility of MMC

Different quantities of MMC was spiked to patients' and dog's plasma samples and the recoveries were calculated according to the following equation:

Recovery = $[(S_m-B) / S \times 100\%]$

where; S = the peak height given by a standard solution of MMC; B = the peak height of MMC in patients' or dog's plasma; S_m = the total peak height after MMC was added to the patients' or dog's plasma.

The recoveries were 95.9 - 100.3%, as shown in Table 1. The reproducibility was determined by an arbitrary plasma sample. The Relative Standard Deviation (R.S.D.)is 2.76% (n= 5) as shown in Table 2.

Table 1

Recovery of MMC

Sample	B	S	S_m	Recovery (%)
1	0.0	15.4	15.9	100.3
2	6.7	5.75	12.3	97.8
3	3.05	12.17	15.2	99.8
4	6.45	18.45	24.65	95.9

1. The blank plasma of patient
2. The human plasma of locaiton
3. The human plasma of all body
4. The plasma of dog
B, S, S_m : Concentrations in terms of peak height (mm)

Table 2

The Reproducibility of MMC

Peak Height	Relative Error (%)	R. S. D. (%)
113.4	2.84	
112.0	1.44	
112.0	1.44	2.76
115.6	4.96	
111.0	0.56	

Determination of MMC in Patients's / Dog's Plasma

The direct local administration on tumour will give higher drug concentration and lower toxicity than those of general administration. Patients' and dog's plasma samples after administration were analyzed in our study. Patients and dogs were given MMC at a dosage of 10mg and 6mg, respectively. Figure 4 and Figure 5 show the data of the analysis. We can observe higher MMC concentration of local administration than that of general administration at same dosage.
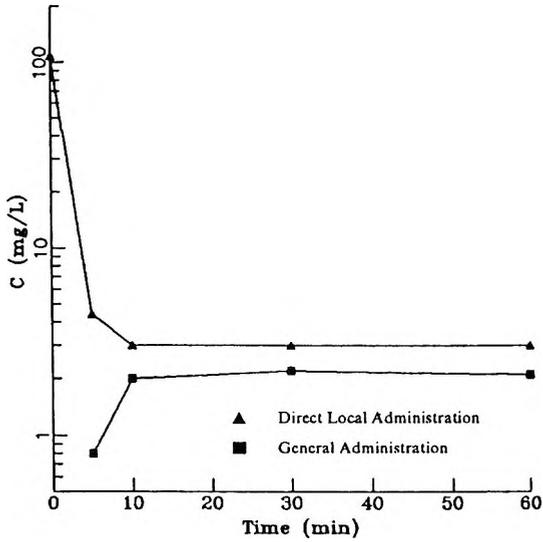
Figure 4. Elimination Curve of MMC in Patient's Plasma.

Figure 5. Eliminiation Curve of MMC in Dog's Plasma.

CONCLUSION

We have developed a convenient direct injection HPLC method for the determination of MMC in biological fluids utilizing a column switching technique. Human and dog plasma samples were tested. The results show that this method is simple, rapid and accurate.

ACKNOWLEDGMENTS

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SIMULTANEOUS DETERMINATION OF DEXTROMETHORPHAN AND DEXTRORPHAN IN HUMAN PLASMA, URINE AND CEREBROSPINAL FLUID BY HPLC WITH FLUORESCENCE DETECTION

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ABSTRACT

Dextromethorphan is a non-narcotic antitussive drug that recently attracted interest because of its anticonvulsant and neuroprotective properties. Dextromethorphan undergoes polymorphic oxidation and 5-10% of human subjects are characterised as poor metabolizers on the basis of the formation rate of its O-demethylated metabolite, dextrorphan catalysed by isozyme CYP2D6.

A sensitive, selective and reliable high performance liquid chromatographic (HPLC) method for the simultaneous determination of dextromethorphan and dextrorphan in human

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plasma, urine, and cerebrospinal fluid, is described, using levallorphan as the internal standard. The analytes were extracted from biological fluids by a liquid-liquid extraction procedure. The chromatographic separation was performed on a 5-μm cyano analytical column (220 X 4.6 mm) using a mixture of acetonitrile/distilled water/n-octylamine (19/80.95/0.05, v/v) pH 2.8 as the mobile phase with a flow rate of 1.0 mL/min at 40^0C and the chromatographic peaks were measured by fluorescence detection.

The calibration curves were linear with a correlation coefficient of 0.999 or better from 1-100, 5-500, and 1-100 ng/mL for both drugs in plasma, urine, and cerebrospinal fluid, respectively. The method was not interfered with by other endogenous components or concurrent antiepileptic drugs. The within-day precision of the method was evaluated for three concentrations and was found to be less than 6%, and the accuracy was nearly 100%.

INTRODUCTION

Dextromethorphan is a widely used and effective non-narcotic antitussive drug. It is a centrally acting opioid congener and its potency is nearly equal to that of codeine and unlike the latter, it does not cause any addiction or respiratory depression.[1,2] Recently dextromethorphan has attracted interest because of its anticonvulsant and neuroprotective properties.[3,4]

Dextromethorphan is O-demethylated to dextrorphan, its main active metabolite, which is mainly in conjugated form as the glucuronide. The O-demethylation of dextromethorphan in man is catalysed by a specific isozyme (CYP2D6) and it is under genetic control, cosegregating with the well-known polymorphic debrisoquine 4-hydroxylation.[5,6] This particular isozyme is also responsible for the metabolism of other commonly used drugs.[7,8] CYP2D6 may be absent or deficient in a certain percentage of people. Most subjects are characterised as "extensive metabolizers", whereas the rest (5-10%) as "poor metabolizers". Dextromethorphan is used as a marker for the CYP2D6 isozyme and the ratio of parent drug to its metabolite dextrorphan defines a subject as an "extensive" or "poor" metabolizer.[9,10]

This paper describes a selective, sensitive, precise, and accurate high performance liquid chromatographic assay for the simultaneous determination of

dextromethorphan and its major active metabolite dextrorphan, in human plasma, urine, and cerebrospinal fluid, with advantages over previously published HPLC methods.[5,9,11-17] This method has been applied to the pharmacokinetic study in epileptic patients receiving dextromethorphan in combination with other antiepileptic drugs and for determining phenotype status for the CYP2D6 isozyme.[18]

MATERIALS AND METHODS

Chemicals

HPLC-grade acetonitrile and hexane and analytical-reagent grade 85% phosphoric acid, sodium carbonate and n-octylamine were obtained from Merck (Darmstadt, Germany). Water was deionized and distilled from glass apparatus. All solvents and solutions for HPLC analysis were filtered through a 0.45 μm pore size nylon filter (Alltech, Deerfield, IL, USA) and vacuum degassed by sonication before use. Dextromethorphan hydrobromide, dextrorphan tartrate and levallorphan tartrate (internal standard) were kindly supplied by Hoffman-La Roche (Basle, Switzerland).

Apparatus

A model 2510 liquid chromatographic system (Varian, Palo Alto, CA, USA), equipped with a 7125 Rheodyne manual injection valve with a fixed 100 μL loop (Cotati, CA, USA), a model 502 variable fluorescence detector (Scientific Systems Inc., State College, PE, USA) coupled with a 4290 Varian integrator was used. Separation was performed using a Brownlee Spheri-5 cyano analytical column (5 μm, 220 X 4.6 mm, ID) (Applied Biosystems, Foster City, CA, USA) proceeded by a cyano guard column (5 μm, 10 X 4.6 mm, ID).

Chromatographic Conditions

The mobile phase consisted of acetonitrile/distilled water/n-octylamine (19/80.95/0.05, v/v), adjusted to pH 2.8 with phosphoric acid. The chromatographic system was operated isocratically at a flow rate of 1.0 mL/min at 40^0C, resulting in an inlet pressure of approximately 1600 psi, and the chromatographic peaks were measured by fluorescence detection at 230 and 330 nm for λ_{ex} and λ_{em}, respectively.

Sample Preparation

Venous blood samples were collected in heparinized glass tubes and centrifuged at 2000 g for 10 min, and the plasma fractions were removed. Urine samples were collected via spontaneous voiding, the total volume of urine was recorded and an aliquot was kept. Cerebrospinal fluid samples were obtained using a standard lumbar puncture technique. All plasma, urine, and cerebrospinal fluid samples were stored at -70^0C and thawed and vortex mixed before analysis.

To a 15-mL stoppered silanized glass centrifuge tube containing 1 mL of plasma, urine (diluted 3:1 or more with distilled water), or cerebrospinal fluid, were added 100 μL of internal standard solution (0.1 μg/mL). After vortexing for a few seconds, 0.5 mL of saturated sodium carbonate solution was added, the sample briefly mixed and 5 mL of hexane containing 0.1% n-octylamine was added. After mixing on a vortex mixer for 60 sec, the sample was centrifuged at 2000 g for 10 min and the organic phase was transferred into a clean centrifuge tube. The aqueous phase was re-extracted with 5 mL of hexane containing 0.1% n-octylamine and the combined hexane extracts were evaporated to dryness under nitrogen in a 50^0C water bath. The residue was reconstituted with 150 μL of 0.1 M HCl and a 100-μL aliquot was injected into the HPLC system.

Working standard solutions of dextromethorphan, dextrorphan and internal standard, levallorphan were prepared in distilled water (1 μg/mL, as base). The procedure was standardized by analysing drug-free plasma, urine, or cerebrospinal fluid samples, spiked with internal standard solution and appropriate volumes of working standard solutions to formulate dextromethorphan and dextrorphan concentrations of 1-100 ng/mL, 5-500 ng/mL, and 1-100 ng/mL for plasma, urine, and cerebrospinal fluid, respectively, as in the sample preparation procedure.

The concentrations of dextromethorphan and dextrorphan in plasma, urine, and cerebrospinal fluid samples, were calculated by interpolation from the linear least-squares regression line of the standard curve plot of peak height ratios of dextromethorphan/internal standard and dextrorphan/internal standard, versus dextromethorphan and dextrorphan concentrations in the calibration standards.

RESULTS AND DISCUSSION

The peaks of the studied analytes were well separated from each other giving retention times of 5.3, 7.4 and 10.7 min for dextrorphan, levallorphan
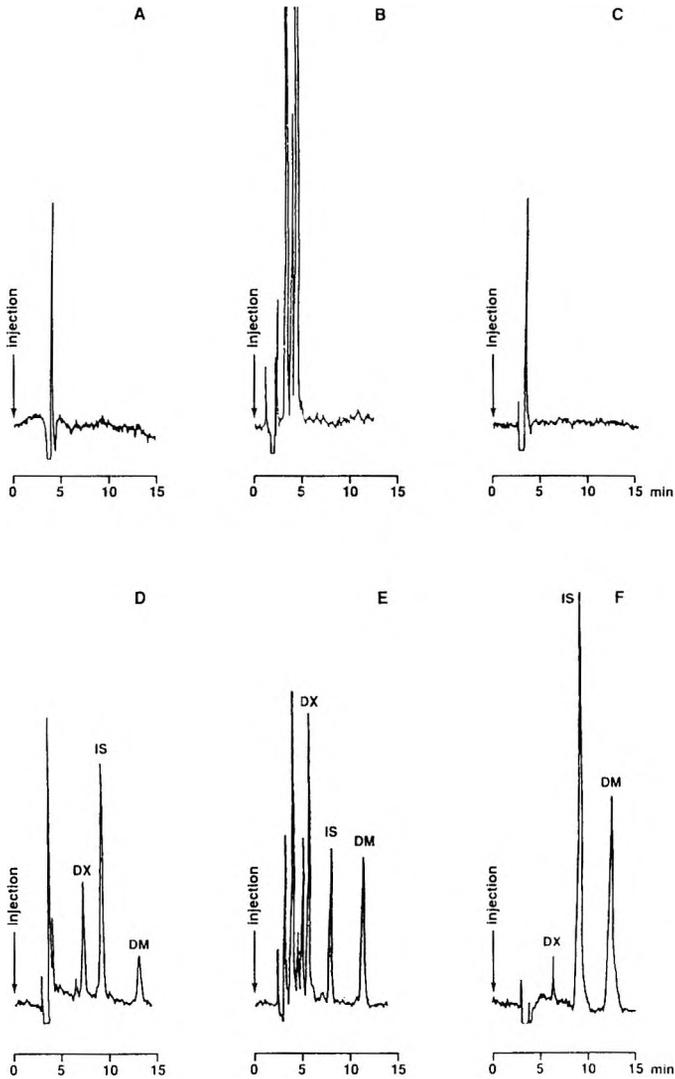
Figure 1. Chromatograms of (A) drug-free plasma; (B) drug-free urine; (C) drug-free cerebrospinal fluid; (D) plasma sample from an epileptic patient after the last steady-state oral dose of 50mg/6h of dextromethorphan hydrobromide; (E) urine sample from the same patient; (F) cerebrospinal fluid sample from the same patient. Peaks: DX=dextrorphan (retention time=5.3 min); IS=internal standard levallorphan (retention time=7.4 min); DM=dextromethorphan (retention time 10.7 min).

Table 1

**Within-day Precision and Accuracy Data of the Determination
of Dextromethorphan and Dextrorphan in Human Plasma,
Urine and Cerebrospinal Fluid**

Concentration Added (ng/mL)	Concentration Found (ng/mL)	Precision CV (%) (n=6)	Accuracy Concentration Found/Added (%)
Plasma			
Dextromethorphan			
5.0	5.2	5.4	104
50.0	48.3	3.3	97
100.0	98.8	2.6	99
Dextrorphan			
5.0	4.7	5.8	94
50.0	51.4	3.5	103
100.0	101.7	2.8	102
Urine			
Dextromethorphan			
10.0	9.8	5.6	98
200.0	203.1	2.9	102
400.0	395.6	2.6	99
Dextrorphan			
10.0	10.3	5.9	103
200.0	197.2	3.1	99
400.0	403.8	2.9	101
Cerebrospinal Fluid			
Dextromethorphan			
5.0	4.8	5.3	96
50.0	51.5	3.6	103
100.0	102.1	2.9	102
Dextrorphan			
5.0	5.3	5.9	106
50.0	48.6	3.8	97
100.0	99.3	3.1	99

(internal standard), and dextromethorphan, respectively. The retention times were found to be reproducible and the coefficients of variation were less than 0.5%. Fig. 1 shows representative chromatograms for dextrorphan, levallorphan, and dextromethorphan in human plasma, urine, and cerebrospinal fluid samples. Fig. 1A, 1B, and 1C show the chromatograms from the analysis of drug-free human plasma, urine, and cerebrospinal fluid, respectively.

Fig. 1D, 1E, and 1F show typical chromatograms of the plasma, urine, and cerebrospinal fluid samples (at designated intervals) from an epileptic patient, receiving concurrent antiepileptic drugs (phenytoin and carbamazepine), after the last steady-state oral dose of 50mg/6h of dextromethorphan hydrobromide. In these chromatograms there were no interfering peaks due to endogenous substances or antiepileptic drugs at the retention times of dextrorphan, levallorphan, and dextromethorphan.

A least-squares linear regression analysis was used to calculate the equation relating the peak height ratio of dextromethorphan and dextrorphan to internal standard (Y) versus drug concentration (ng/mL) in spiked plasma, urine or cerebrospinal fluid samples (X). The calibration curves were linear with a correlation coefficient of 0.999 or better for concentrations ranging from 1 to 100 ng/mL, 5 to 500 ng/mL, and from 1-100 ng/mL for both analytes in plasma, urine, and cerebrospinal fluid, respectively. The detection limit calculated for a signal-to-noise ratio of 4 was *ca.* 1 ng/mL and 0.5 ng/mL for dextrorphan and dextromethorphan, respectively.

The within-day precision and accuracy data of the method were evaluated for three concentrations of each drug in spiked plasma, urine, and cerebrospinal fluid samples. The results, expressed as the mean of six determinations are presented in Table 1. The within-day precision of the assay, expressed as the coefficients of variation for the determined concentrations of both analytes in human plasma, urine, and cerebrospinal fluid was less than 6% and the accuracy, assessed as the percent of the estimated concentration divided by the nominal concentration was nearly 100%.

In conclusion, the HPLC method we developed for the simultaneous quantitation of dextromethorphan and its main active metabolite, dextrorphan, in human plasma, urine, and cerebrospinal fluid, is precise, accurate, selective, and sufficiently sensitive and seems well suited to characterize dextromethorphan and dextrorphan pharmacokinetics, as well as for determining "extensive" and "poor" metabolizers of the debrisoquine/sparteine oxidative polymorphism involving CYP2D6 isozyme.

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STUDIES OF THE STABILITY OF A SUBSTITUTED PHOSPHOROTHIOATE USING AN IMPROVED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY COUPLED WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A sensitive, reproducible and rapid HPLC assay was developed for preformulation and stability studies of a potential radioprotector, S-2-(3-methylaminopropylamino) ethyl-phosphorothioate, WR-3689. This method is based on the elution of WR3689 by high performance ion-pair reverse phase liquid

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chromatography using an electrochemical detector with an oxidation potential set at + 0.19 v. The detector response was found to be linear over the concentration range of 0.02 - 20 μg/mL with an absolute limit of quantitation of 170 pg. The coefficient of variation ranged from 0.75 - 6.52% for the interday reproducibility. This HPLC assay was employed to determine the stability of WR-3689 at various pH (2, 4, 7, 10 and 12) and temperatures (22 °C, 35 °C, 43 °C and 100 °C). Results of the stability studies indicated that WR-3689 is more stable in basic than in acidic aqueous solutions. For example, the half-life of WR-3689 at 45 °C was 13.8 days at pH 12 as compared to 12.7 h at pH 4. With regard to the effect of temperature, the half-life of this compound increased from 19.2 h at 43 °C to 41.8 days at 22 °C. In conclusion, the excellent reproducibility and stability indicating capacity of this assay makes it a useful method for formulation studies. The high sensitivity and specificity of this assay make it useful for pharmacokinetic studies.

INTRODUCTION

Chemical radiation protectors are receiving considerable attention as cancer treatment agents. Several sulfhydryl (-SH) containing compounds, particularly aminothiols and phosphorothioates have been found to exhibit chemical radioprotection through three general mechanisms, i.e., radical scavenging, hydrogen atom donation and oxygen depletion.[1-4] Thus far, WR-2721, S-2-(3-aminopropylamino) ethylphosphorothioate is the most widely studied phosphorothioate compound.[5-6] It is currently being studied in clinical trials involving patients undergoing radiotherapy and chemotherapy treatment.[7,8] However, at present, WR-3689 (S-2-(3-methylaminopropylamino) ethylphosphorothioate) is gaining attention due to the recent findings that it is a better radioprotector than WR-2721 when given orally.[3,9,10] In addition, WR-3689 was found to be a more potent radioprotector than WR-2721 at a low dose that produced no behavioral side effects.[4] Hence, collaborative drug development studies involving WR-3689 are being conducted.

During drug development, it is important to assess the stability of drugs of interest. One of the major drawbacks in studying the stability of certain drugs is the scarcity of simple and efficient analytical methods for such purposes. The fact that among phosphorothioate compounds, WR-2721 and its metabolites are the most widely studied compound is due to the availability of various assays. Among these are high performance liquid chromatography

$$CH_3NHCH_2CH_2CH_2NHCH_2CH_2SPO_3H_2$$

A

$$CH_3NHCH_2CH_2CH_2CH_2CH_2NHCH_2CH_2SPO_3H_2$$

B
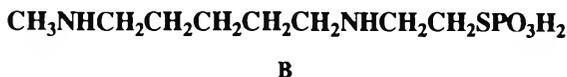
Figure 1. Chemical Formula of (A) WR-3689 and (B) WR149846.

(HPLC) assays involving the use of fluorescence[11-13] and electrochemical detection.[14-17] For WR-3689, a compound that differs from WR-2721 by one methyl (-CH3) unit (Fig.1), only one method for its measurement in plasma has been published.[18] This method, however, has few shortcomings which when improved will result in a very powerful HPLC assay for the analysis of WR-3689 and related compounds. As will be discussed later, our method provides several advantages without affecting or sacrificing the high sensitivity and efficiency which was exhibited by the previously published method. Indeed, the development of this simple, efficient and fast HPLC assay for WR-3689 is important not only for stability studies but also for preformulation as well as pharmacokinetic studies.

MATERIALS AND METHODS

Instrumentation

The chromatographic system used in this study consisted of a Waters Model 590 programmable solvent delivery module and Waters Intelligent Sample Processor (WISP) Model 712 (Milford, MA). A Bioanalytical Systems (BAS) Model LC-4C electrochemical detector (W. Lafayette, IN) equipped with a Hg/Au electrode was used. This assembly was connected to a Maxima 820 chromatography station.

Reagents

Reagent grade chloroacetic acid and 1-sodium heptane sulfonate (SHS) and sodium octyl sulfate were purchased from Sigma Chemical Company (St. Louis, MO). HPLC grade acetonitrile was from Fisher (Fair Lawn, NJ), sodium hydroxide was from Mallinckrodt, Inc. (St. Louis, MO), glacial acetic acid

and concentrated hydrochloric acid were from J.T. Baker (Phillipsburg, NJ). Both WR-3689 and WR-149846 were supplied by the U.S. Army (Walter Reed Army Medical Center, Bethesda, MD).

Chromatographic Conditions

The mobile phase consisted of 5 mM SHS and 5% acetonitrile in 0.1 M chloroacetic acid at pH 3.0. The column used was a C_{18} (Spheri-5 ODS, 5 μm, 250 x 4.6 mm I.D.) from Bioanalytical Systems (West Lafayette, IN). The flow rate was 1.2 mL/min and the detector (Hg, Au electrode) oxidation potential was set at +0.19 v. The injection volume was 20 μL.

Sample Preparation

For each test conducted, a freshly prepared aqueous stock solutions of WR-3689 (2.5 mg/mL) and WR-149846 (1.0 mg/mL) were prepared. Dilution of these aqueous stock solutions were made in order to prepare the desired standard solutions. In the case of the internal standard (WR-149846), a stock solution of 1.0 mg/mL was prepared.

RESULTS AND DISCUSSION

The degradation of WR-3689 in aqueous solution was followed by using ion-pair reverse phase chromatography coupled to amperometric detection (Hg/Au). This type of detection offers high selectivity and sensitivity, hence, requires a minimum amount of sample (μL level) to give a quantifiable response even at very low concentrations of the analyte (μg/mL level). The detector reaction is quite indirect and is based on the oxidation of mercury in the presence of certain species such as thiols. The sulfhydryl (-SH) group of WR-3689 forms a complex with the mercury (Hg). It is actually the sulfhydryl-Hg complex which undergoes an oxidation reaction at a given potential.[19] This is best described by the following reaction:

$$2RSH + Hg \text{ --------- } Hg(SR)_2 + 2H^+ + 2e$$
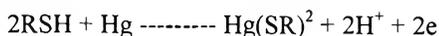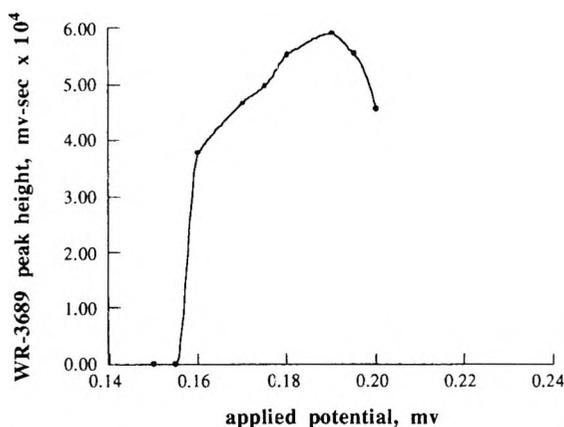
Figure 2. Plot of the measured peak heights of WR-3689 at various applied potentials. An electrochemical detector with Hg/Au working electrode set at 0.1 μA.

In order to obtain the highest response, the best applied potential was determined by measuring the peak heights of WR-3689 (25 μg/mL) at various oxidation potentials under the conditions described by Han and Lin.[18]   The results obtained illustrate that + 0.19 v is the most appropriate applied oxidation potential for WR-3689 detection (Fig. 2).

As mentioned earlier, for the quantitative determination of WR-3689 by HPLC, only one published method exists.[18] Although this method proved to be very sensitive and efficient, there are two major shortcomings which are very apparent: a.) the relatively long total analysis time and, b.) the use of a relatively high organic solvent concentration (20 % acetonitrile).

Because WR compounds are known to be unstable at room temperature, making the analysis time as short as possible is very essential especially when analyzing several samples. On the other hand, the use of a lower concentration of organic solvent in the mobile phase is more cost effective and reduces the problems usually associated with organic waste disposal. Most importantly, it is necessary to reduce the amount of organic solvent in the mobile phase since its presence in high concentration may lead to the leaching of the mercury from the working electrode, hence, decreasing the sensitivity of the assay over time.
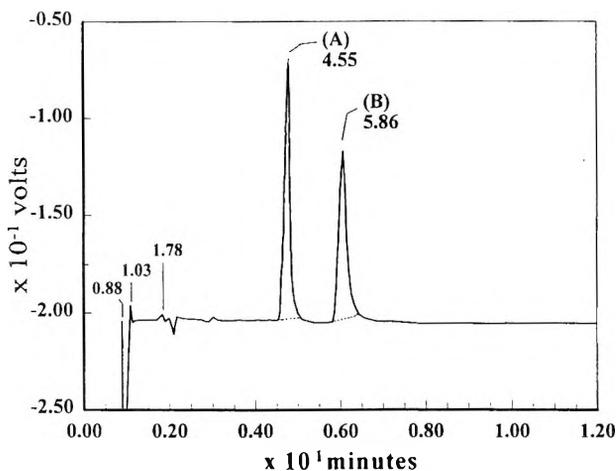
Figure 3. A typical chromatogram showing the elution and separation of the analyte WR-3689 (A) and the internal standard W R- 149846 (B).

To improve the shortcomings of the only available HPLC assay for the quantitative determination of WR-3689, several chromatographic conditions were investigated. Among the conditions evaluated, the ion-pair reverse phase chromatography using an octadecyl column (C_{18}) and a mobile phase consisting of 5.0 mM sodium heptane sulfonate and 5% acetonitrile in 0.1 M chloroacetic acid at pH 3.0 was found to be the best in terms of selectivity, peak shape and analysis time (Fig 3).

The above-described HPLC assay offers several advantages over the previously published method. With this method, shorter analysis time was obtained as indicated by the lowering of the retention times of both WR-3689 and its internal standard, WR-149846, by about 47 % (i.e., from 8.5 min to 4.5 min and from 11.1 min to about 5.8 min for WR-3689 and WR-149846, respectively). As expected, the use of the weaker ion pairing agent, sodium heptane sulfonate, instead of the commonly used stronger ion pairing agent, sodium octane sulfonate, resulted in an adequate resolution at lower retention times for both WR-3689 and WR-149846, hence leading to a shorter analysis time.

Under these conditions, efficient separation was obtained even with 75 % reduction in acetonitrile concentration. As discussed earlier, the use of a lower acetonitrile concentration provides several benefits.

Table 1

Interday and Intraday Reproducibility of the HPLC Assay

	Conc. µg/mL	Coefficient of Variation (%)
Interday[a]	12.5	0.75 - 6.14
	125	2.17 - 6.52
Intraday[b]	12.5	2.91
	125	2.16

[a] Results were taken from five determinations each for five days.

[b] Results were taken from five determinations on the same day.

Reproducibility of the HPLC Assay

The inter- and intraday reproducibility of the HPLC assay was evaluated by chromatographically analyzing 12.5 µg/mL and 125.0 µg/mL WR-3689 solutions. The intraday reproducibility was measured by calculating the coefficient of variations between the peak height ratios obtained from five determinations within the same day. For interday reproducibility measurements, the correlation coefficients between peak height ratios of five replicates for five days were calculated. The results of this study are summarized in Table 1. The interday coefficient of variation ranged from 0.75-6.14% and 2.17-6.52% for 12.5 µg/mL and 125 µg/mL WR-3689, respectively. For intraday reproducibility, the coefficient of variation was found to be 2.91% and 2.16% for the low and high concentrations of WR-3689, respectively.

Linearity

To 5 µL of WR-3689 solutions (0.4, 4, 40, 200 and 400 µg/mL in water), 5.0 µL of the internal standard and 100µL of deionized water were added. The peak height ratios of the WR-3689/internal standard were plotted against the

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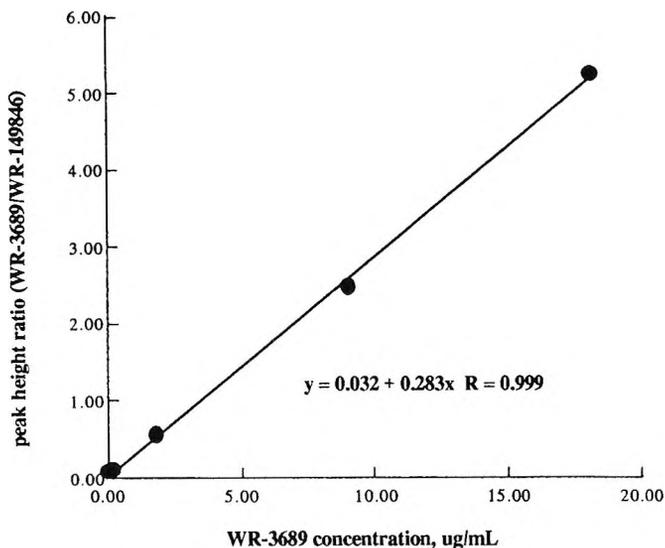
Figure 4. Linearity of the HPLC assay at various WR-3689 concentrations spiked with a constant concentration of WR-149846 (internal standard). The results were obtained from the means of five replicates at each concentration.

Table 2

Summary of the Observed Degradation Rate Constants (k) and Half-Lives ($t_{1/2}$) of WR-3689 at Various pH Conditions at 45 °C

pH	k (day^{-1})	$t_{1/2}$
2	49.6	28.8 min
4	1.30	12.7 h
10	0.70	23.5 h
12	0.05	13.83 days

corresponding WR-3689 concentration (Fig. 4).   Linear regression analysis resulted in a best fit line with a y-intercept of 0.032 and a slope of 0.283 and a correlation coefficient (r^2) of 0.999, hence, indicating an excellent linearity over a concentration range of about 0.02 -20 μg/mL.

Absolute Limit of Quantitation

To determine the sensitivity of the assay, the absolute limit of quantitation was measured. Based on the 3 signal/noise ratio (3 S/V), the limit of quantitation was found to be 170 pg.

Stability Studies of WR-3689

Stability studies are necessary in order to determine the conditions and length of time in which the drug is effective without getting converted into inactive or toxic products. In this report, the stability of WR-3689 was studied under different temperature and pH conditions.

Effect of pH

The effect of pH on the stability of WR-3689 at a constant temperature was investigated. Initially, attempts were made to determine the effects of pH at 100 °C. Under this condition, WR-3689 was found to completely degrade (as indicated by the complete disappearance of the WR-3689 peak) after 5-10 minutes at pH 2 and 4 and after 2-3 hours at pH 10 and 12. Since the decomposition rates of WR-3689 at 100 °C were too fast to follow, solutions at various pH were kept at a lower temperature, i.e., 45 °C.

The plots of the logarithm of percent WR-3689 concentration *versus* time display the dependence of WR-3689 stability on pH at a constant temperature. The stability of WR-3689 was found to increase with pH (Fig. 5 and Table 2). Table 2 shows that the half-life ($t_{1/2}$) of WR-3689 at 45 °C increased from 28.8 min at pH 2 to 13.8 days at pH 12. On the other hand, the degradation rate constants (k) decreased with increasing pH, i.e., from 49.6/day at pH 2 to 0.05/day at pH 12. This observation is consistent with the earlier studies on the stability of a very similar phosphorothiote, WR-2721.[11,20] This drug was found to exhibit an increasing non-enzymatic hydrolysis with decreasing pH. At 37 °C, WR-2721 was found to have half lives of 0.4 h to 8.0 h over the pH range of 1-3.

Effect of temperature

In order to evaluate the influence of temperature on WR-3689 stability, solutions at pH 7 were kept at 22 °C, 35 °C and 43 °C. Table 3 summarizes the observed degradation rate constants as well as the half-lives of WR-3689 at
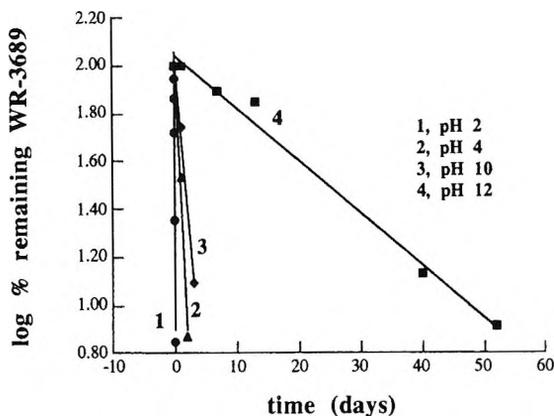
Figure 5.   Effect of pH on the stability of WR-3689 at 45°C.

Table 3

**Summary of the Observed Degradation Rate Constants (k) and
Half-Lives (t$_{1/2}$) of WR-3689 at Various Temperatures at pH 7**

Temperature (°C)	k (day^{-1})	t$_{1/2}$
22	0.02	41.75 days
35	0.13	5.41 days
43	0.86	19. 2 hours

various temperatures. The degradation rate constant (k) of WR-3689 was found
to increase with increasing temperature at a constant pH.   It increased from
0.02/day at 22 °C to 0.86/day at 43 °C. Conversely, the half life decreased with
increasing temperature, i.e., from 41.8 h at 22 °C to 19.2 h at 43 °C.   These
results were found to be parallel with those obtained with WR-2721.   For
example, the t$_{1/2}$ values for the hydrolysis of WR-2721 were 107 h and 87 min at
0°C and 25 °C, respectively.

An Arrhenius plot of the log of the observed degradation rate constant as a
function of the reciprocal of the absolute temperature was linear with a
correlation coefficient of 0.998 (Fig. 6).   The activation energy (Ea) for the
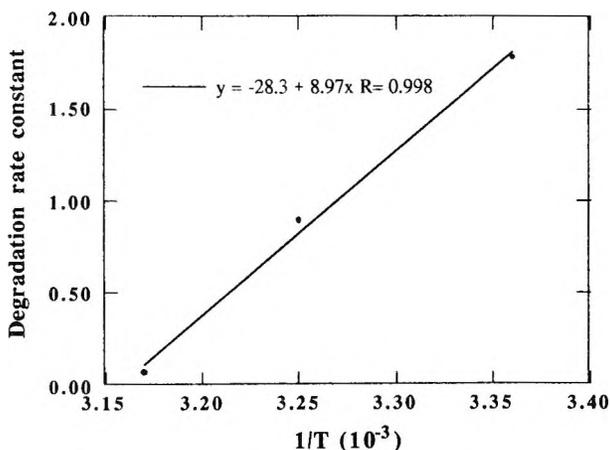
Figure 6. Arrhenius plot of the degradation of WR-3689.

decomposition of WR-3689 at pH 7.0 over the temperature range studied (22 °C- 43 °C) was 41 kcal/mol. The fairly low E_a obtained indicates that WR-3689 is fairly stable in aqueous solution at pH 7.0.

The finding that WR-3689 degrades by about 1% in one day at 25 °C indicates that the loss of WR-3689 is insignificant during the experimental process where it is exposed for at least five minutes at 25 °C at pH 7.0.

CONCLUSION

The HPLC assay that we have developed proved to be very simple, efficient, reproducible and capable of analyzing a large number of samples in a short period of time. This method offers several advantages over the currently available HPLC assay for WR-3689. This assay was employed for the stability studies of WR-3689 in aqueous solutions at various pH and temperature. Results of the study showed that the stability of WR-3689 is greatly influenced by both temperature and pH.

This HPLC assay is being validated for the stability studies of WR-3689 in biological fluids. In future, it is anticipated that this assay will become a very useful tool in the pharmacokinetic studies of WR-3689 in biological fluids.

ACKNOWLEDGMENT

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COMPARABILITY OF RP-HPLC RETENTION INDICES OF DRUGS IN THREE DATABASES

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ABSTRACT

Three databases of HPLC retention indices in the 1-nitroalkane scale were recently established (IFM= Institute of Forensic Medicine, MCL = Microchemistry Laboratory, MTSS= Merck Tox Screening System). In two of them (IFM and MTSS) virtually identical chromatographic conditions were applied but different RP-columns. In the third system (MCL) a different column and different elution conditions were used. Among a total of 1149 drugs examined in all three labs, 258 drugs were common for IFM and MTSS, 179 were common for MCL and MTSS, 162 were common for IFM and MCL, and 135 drugs were common for all three databases.

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Good agreement of retention index values in IFM and MTSS was demonstrated ($r^2 = 0.9778$). The correlations between IFM and MCL and MTSS and MCL data were less strong ($r^2 = 0.9245$ and 0.9339, respectively), due to some deviating results. These deviations concerned mainly substances with pKa values between 1 - 4 and were probably caused by the differences in the pH of the two mobile phases. The standardization of chromatographic conditions is a prerequisite of interlaboratory reproducibility of retention indices.

INTRODUCTION

An unequivocal identification of toxic substances in biological samples in the case of acute poisoning is of critical importance in clinical and forensic toxicology. The multitude of potentially toxic substances in the human environment dictates a need for such methods of identification, which allow for the examination of the largest possible number of relevant compounds in one analytical run. For this reason, chromatography is a method of choice in general toxicological screening.

Among various chromatographic techniques, thin layer chromatography (TLC) and gas chromatography (GC) have found broad application for identification of toxic substances. Both techniques have been standardized and respective databases, comprising retention parameters for over 6.000 toxicologically relevant compounds have been established.[1-3] The application of hyphenated techniques like e.g. GC/MS[4] or TLC/UV[5] have resulted in particularly high identification power.

High pressure liquid chromatography (HPLC) may be regarded as a very attractive alternative in systematic toxicological screening. This method is more sensitive and selective than TLC, and more universal than GC, enabling direct examination of non-volatile, polar and thermolabile drugs. The standardization of HPLC results with retention index (RI) scales made it possible to establish databases of relevant compounds. Among the numerous retention index systems tested in HPLC, three have been widely applied. They are the alkan-2-ones, alkyl aryl ketones and 1-nitroalkanes.[6,7] The latter scale[8] has been proven useful for identification of various acidic, neutral and basic drugs in reverse phase HPLC and was applied for general toxicological screening.[9] It was observed, that the differences in selectivities of various brands of reverse phase packings may result in very different RI values of the same substances when analyzed on commercially different HPLC columns. This problem was circumvented to some

extent by the application of selected drugs as secondary retention index standards.[10] However, the elution conditions should be carefully standardized in order to obtain reproducible results.[11-12]

The HPLC identification system was developed under standardized conditions, using the 1-nitroalkane retention index system, selected drugs as secondary RI standards and a diode array detector (DAD). In this system a database of 225 compounds was established.[13] This database was expanded to about 400 toxicologically relevant drugs and endogenous compounds at the Institute of Forensic Medicine in Aachen (IFM).[14] The RI values were not affected by co-extracted biological matrices.[15] The overall identification power of combined HPLC-RI and UV data was comparable with the identification power of combined GC retention indices and UV spectral data.[16]

Recently, Hill and Kind from the Microchemistry Laboratory in Storrs, CT (MCL) published a RI library comprising data for 469 drugs.[12] Also, the RI library of Merck Tox Screening System (MTSS) with data for some 900 substances has become commercially available.[17] In both databases the 1-nitroalkane RI scale was used. The existence of these three libraries, containing a number of common substances, made it possible to assess the ruggedness of the HPLC retention index system based on the 1-nitroalkane scale.

Two out of three libraries (IFM and MTSS) were developed in virtually identical elution conditions, but different columns were used. This made it possible to compare the RI-values of drugs examined under standardized conditions. On the other hand, in the third lab (MCL) a different column and different gradient elution conditions were applied. This could help to examine, to what extent the RI-values of drugs may be affected by these differences, and to identify particularly sensitive compounds.

An ultimate purpose was to inspect the possibility of the interlaboratory use and exchange of retention data.

MATERIALS AND METHODS

Among a total of 1149 drugs examined, the following number of common drugs was observed:
    258 drugs were common for IFM and MTSS databases,
    179 drugs were common for MCL and MTSS databases,
    162 drugs were common for IFM and MCL databases.
    135 drugs were examined in all three laboratories.

Table 1

HPLC Conditions Applied in Three Laboratories Establishing HPLC/RI Databases

Library	Column	Mobile Phase	Gradient Profile	Flow Rate
IFM (n = 404)	Superspher 100 RPI8EC 125 x 4 mm	A:TEAP buffer pH 3.0 B: CH_3CN	0 to 70% B in 30 min 5 min 70% B pH 3.0 - 4.2	1 mL/min temp. ambient*
MCL (n= 469)	Zorbax RXC_8 250 x 4.6 mm	A:$0.15M\ H_3PO_4$ 0.05M TEA in H_2O B: $0.15M\ H_3PO_4$ 0.05M TEA 20% H_2O in CH_3CN	2.2 min 100% A 0 to 100% B in 30 min 5 min 100%B overall pH 2.2	2 mL/min temp. 30 °C
MTSS (n=875)	Lichrospher 60 RP Select B 125 x 4 mm	A: TEAP buffer pH 3.0 B: CH_3N	0 to 70% B in 30 min 5 min 70%B pH 3.0 - 4.2	1 mL/min temp. 25 °C

The RI values of common drugs were subjected to comparison directly or after correction, using the method described elsewhere.[9,12,13] Separate secondary RI standards were used for acidic drugs (paracetamol, barbital, brallobarbital, pentobarbital, secobarbital, clobazam, indomethacine) and for basic drugs (morphine, chloroquine, benzoylecgonine, cocaine, diphenhydramine, haloperidol, amitriptyline, thioridazine and meclozine). Only the RI-values of drugs eluting inside the elution range of secondary standards were corrected.

The primary and secondary RI data were subsequently subjected to comparative analysis. Table 1 shows the HPLC conditions applied in three labs developing RI libraries.

RESULTS AND DISCUSSION

All applied HPLC systems (IFM, MTSS and MCL) assured good selectivity for acidic, neutral and basic drugs throughout the whole elution range (Fig. 1). All three columns exhibited low base absorptivity due to base-deactivation (Superspher or Lichrospher Select B) or the use of type B silica (Zorbax RX). It was demonstrated by several authors, that these column packings show some silanol activity which may be effectively suppressed by addition of an
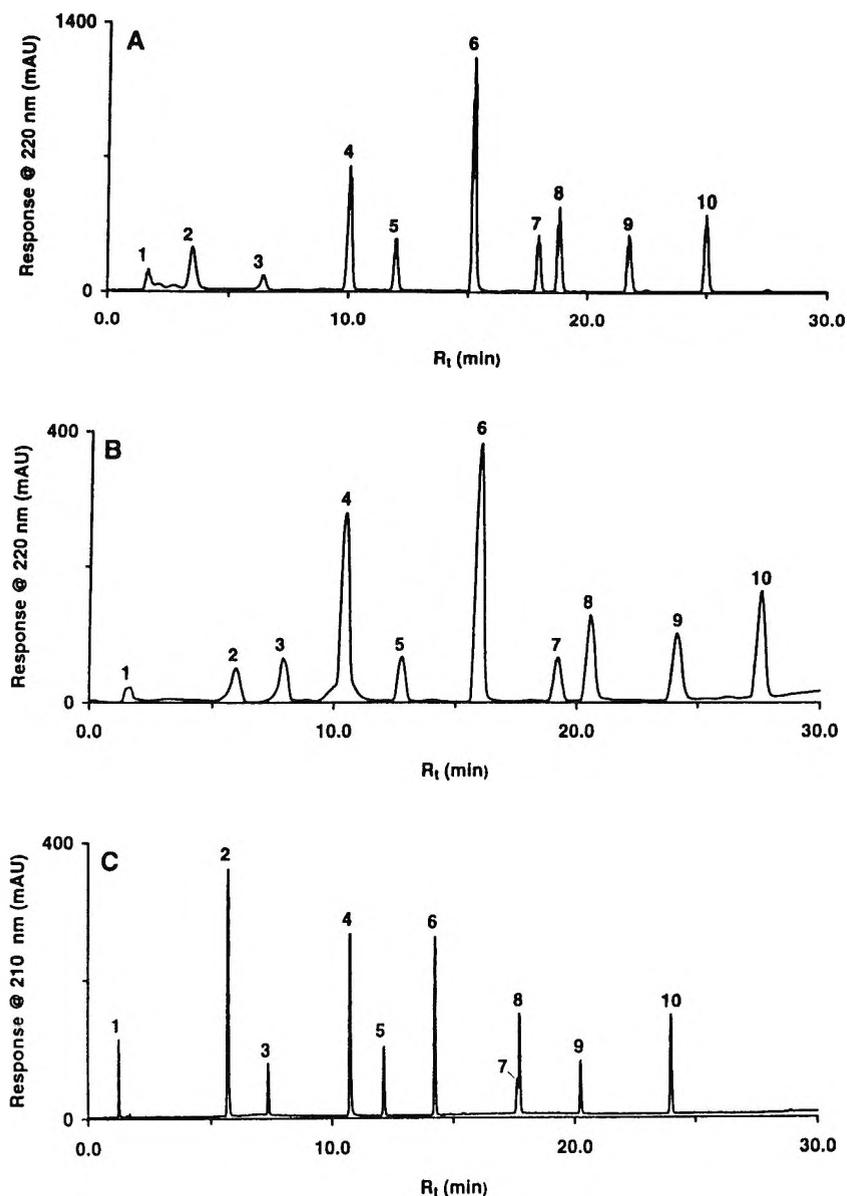
Figure 1: Chromatograms of representative drugs analyzed on the (A) MTSS, (B) IFM and (C) MCL HPLC systems. (1) nicotine, (2) morphine, (3) atenolol, (4) caffeine, (5) primidone, (6) noscapine, (7) pentobarbital, (8) imipramine, (9) thioridazine, (10) indomethacin.

IFM DATA vs. MTSS DATA

——— Linear regression
· · · · 95% Confidence Interval

CORRECTED MTSS DATA vs. IFM DATA

——— Linear regression
· · · · 95% Confidence Interval
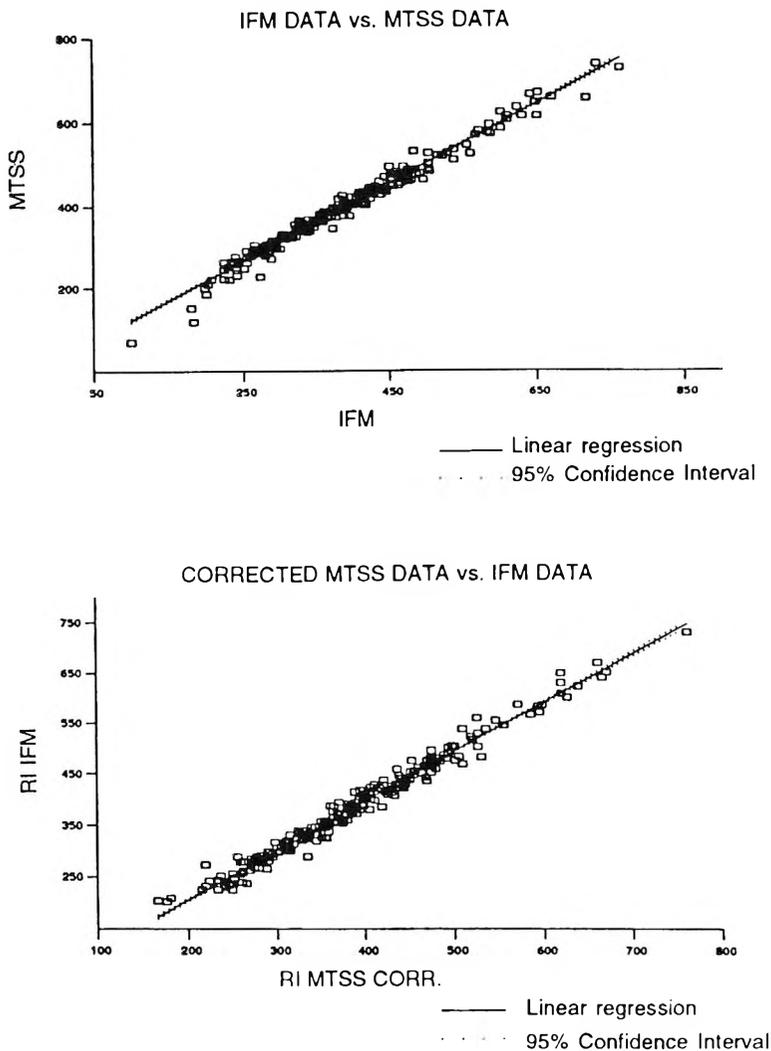
Figure 2: Comparison of RI-values (uncorrected and corrected data) for common drugs in IFM and MTSS databases.

amine to the mobile phase.[18-21] The gradient elution the in acidic mobile phase with addition of an amine modifier was proved superior to isocratic procedures and has been applied for screening purposes in toxicology.[12,13,15,22,23]

Table 2

**Retention Indices, Available pKa Values and Scale
Applied for Correction of Data in Three Databases**

	Substance	MCL	IFM	MTSS	Scale	Pk$_a$
1	Acebutolol		311	325	BAS	9l.4
2	Acecarbromal		422	429	ACN	0.0
3	Acepromazine	385	399		BAS	9.3
4	Acetanilide	328	316		ACN	0.6
5	Acetophenazine	345		395	BAS	
6	Allobarbital		340	346	ACN	7.8
7	Alprazolam		443	470	ACN	2.4
8	Amiloride		233	257	BAS	8.7
9	Aminophenazone		243	262	ACN	5.0
10	Amiodarone		762	731	BAS	5.6
11	Amitriptyline	430	446	440	BAS	9.4
12	Amobarbital	410	415	404	ACN	7.9
13	Amoxapine	336	372	398	BAS	0.0
14	Amphetamine	242	241	244	BAS	9.9
15	Apomorphine	282		348	BAS	
16	Aprobarbital	355	347	357	ACN	8.0
17	Aspirin	348	326	350	ACN	3.5
18	Atenolol	235	224	243	BAS	9.6
19	Atropine	297	287	306	BAS	9.9
20	Barbital	298	287	308	ACN	8.0
21	Benperidol		371	393	BAS	
22	Benzocaine	374	380	404	BAS	2.8
23	Benzoic Acid	354		360	ACN	4.2
24	Benzoylecgonine	304	295	295	BAS	
25	Benztropine	444	461		BAS	10.0
26	Bisacodyl		483	531	ACN	
27	Brallobarbital	359	359	371	ACN	
28	Bromazepam	348	378	397	ACN	2.9
29	Bromisoval		354	365	ACN	10.8
30	Brompheniramine	279	355		BAS	3.9
31	Brucine	303	288	312	BAS	2.3
32	Buflomedil		324	347	BAS	
33	Bupivacine		355	366	BAS	8.1
34	Bupranolol		373	391	BAS	
35	Buprenorphine	384	386	397	BAS	8.5

Table 2 (continued)

Substance		MCL	IFM	MTSS	Scale	Pk$_a$
36	Buspirone	350	353	369	BAS	
37	Butacaine	366		392	BAS	
38	Butalbital	373	380	394	ACN	7.6
39	Butaperazine		496	464	BAS	
40	Butobarbital		365	384	ACN	8.0
41	Caffeine	295	265	305	BAS	1.0 14.0
42	Camazepam	549	566		ACN	
43	Carazolol		354	381	BAS	
44	Carbamazepine	399	380	418	BAS	
45	Carbromal	398	400	410	ACN	
46	Chloramphenicol	368		390	ACN	
47	Chlordiazepoxide	338	357	363	ACN	4.6
48	Chloroquine	273	265	282	BAS	8.4
49	Chlorotheophylline 8	300	295		BAS	
50	Chlorpromazine	438	466	456	BAS	9.3
51	Chlorprothixene		476	459	BAS	8.8
52	Chlorthalidone		359	367	ACN	9.4
53	Cimetidine	236	229	251	BAS	6.8
54	Cinchonidine	379		396	BAS	4.2 8.4
55	Clenbuterol	316	311	326	BAS	
56	Clobazan		484	488	ACN	
57	Clomethiazole		469	495	BAS	3.2
58	Cloimipramine		471	462	BAS	
59	Clonazepam	443	451	465	ACN	1.5
60	Clonidine		237	258	BAS	8.2
61	Clopamide		356	377	ACN	
62	Clopenthixol		456	448	BAS	
63	Chlorazepate	376	464	475	ACN	3.5
64	Clozapine		338	368	BAS	
65	Cocaine	327	336	348	BAS	8.6
66	Codeine	258	243	266	BAS	8.2
67	Colchicine	379	357	382	BAS	1.7
68	Coumarin	383	368		ACN	
69	Cyclobarbital		374	384	ACN	7.6
70	Cyclopentobarbital		374	391	ACN	
71	Demoxepam		388	416	ACN	4.5
72	Desipramine	414	421		BAS	10.2
73	Dextromethorphan	367	370	377	BAS	8.3

Table 2 (continued)

	Substance	MCL	IFM	MTSS	Scale	Pk$_a$
74	Dextromoramide		440	440	BAS	
75	Dextropropoxyphene	409	438	442	BAS	6.3
76	Diamorphine	328	327	340	BAS	7.6
77	Diazepam	458	529	528	ACN	3.3
78	Diazoxide		345	368	ACN	8.5
79	Dibenzepine		349	361	BAS	
80	Diclofenac	614	630	616	ACN	4.5
81	Digoxin	387	389		BAS	
82	Dihydrocodeine		237	261	BAS	8.8
83	Dilthiazen	393	392		BAS	
84	Dimethindene		334	338	BAS	
85	Dimethoxymethylamphe	353		371	BAS	
86	Diphenhydramine	372	385	393	BAS	9.0
87	Diprophylline	264		275	BAS	
88	Dipyridamole		387	393	BAS	6.4
89	Disopyramide		372	345	BAS	8.4
90	Disulfiram	731	730	741	ACN	
91	Dothiepine		428	428	BAS	
92	Doxepin	379	401	404	BAS	9.0
93	Droperidol		369	385	BAS	
94	Ephedrine	231	224	221	BAS	9.6
95	Estrazolam		424	445	ACN	
96	Estrone	554		544	ACN	
97	Ethacrynic Acid	564	516	521	ACN	3.5
98	Ethomidate		466	475	ACN	4.2
99	Ethonitazene	361		406	ACN	
100	Ethorphine	328		344	BAS	
101	Ethosuximide	297	284	301	ACN	9.5
102	Ethylephrine		183	118	BAS	9.0
103	Ethylmorphine	289	283	291	BAS	
104	Ethyltolilmalonamide	328		338	ACN	
105	Famotidine	228		233	BAS	6.7
106	Fenbufen		522	520	ACN	4.5
107	Fencamfamine	345		354	BAS	
108	Fenetylline		325	366	BAS	
109	Fenfluramine		364	371	BAS	9.1
110	Fenoprofen	565	587	574	ACN	4.5
111	Fentanyl	377	377	373	BAS	9.0

Table 2 (continued)

	Substance	MCL	IFM	MTSS	Scale	Pk$_a$
112	Flecainide		410	419	BAS	
113	Fluanisone		398	423	BAS	
114	Flubendazole	392		454	BAS	
115	Flufenamic Acid	687		671	ACN	3.9
116	Flumazenil		362	387	ACN	
117	Flunarizine		571	581	BAS	
118	Flunitrazepam	480	459	483	ACN	1.8
119	Fluorouracil	135		70	ACN	8.0 13.0
120	Flupenthixol		487	475	BAS	
121	Fluphenazine	391	480	462	BAS	3.9
122	Flurazepam		392	397	ACN	1.9
123	Flurosemide	422	414	435	ACN	3.9
124	Glibenclamide		623	637	ACN	5.3
125	Gliclazide		538	536	ACN	5.8
126	Glipizide	493	464	478	ACN	0.0
127	Gluthetimide	437	430	436	ACN	9.2
128	Guaifenesine		307	328	ACN	0.0
129	Haloperidol	403	409	421	BAS	8.3
130	Harmane		301	319	BAS	
131	Heptabarbital		404	416	ACN	0.0
132	Hexobarbital	416	404	419	ACN	8.2
133	Hexylresorcinol	561		579	ACN	
134	Homatropine	260		272	BAS	9.7
135	Hydrochlorothiazide	289	275	294	ACN	7.0
136	Hydrocodone	268	262	286	BAS	8.3
137	Hydromorphone	234	231	220	BAS	8.2
138	Hydroxyzine		428	437	BAS	2.1
139	Ibuprofen	620	650	616	ACN	4.4
140	Imipramine	424	434	437	BAS	9.5
141	Indomethacin	612	610	607	ACN	4.5
142	Isoniazid	67	132		ACN	1.8
143	Ketamine	293	294	311	BAS	7.5
144	Ketazolam		583	577	BAS	
145	Ketoprofen	500	501	495	ACN	4.5
146	Ketotifene		365	373	BAS	
147	Labetalol		350	365	BAS	7.4
148	Levomepromazine		446	435	BAS	9.2
149	Levorphanol	303	308		BAS	8.2

Table 2 (continued)

	Substance	MCL	IFM	MTSS	Scale	Pk$_a$
150	Lidocaine	282	285	288	BAS	7.9
151	Lorazepam	423	422	444	ACN	1.3
152	Lormetazepam	484	474	487	ACN	
153	Loxapine	355	399	407	BAS	6.6
154	LSD	342	358	362	BAS	7.5
155	Lysergic-D Acid	285	269		ACN	3.4
156	Maprotiline		440	438	BAS	
157	Mazindol	347		357	ACN	
158	MDA	253	261	278	BAS	
159	MDE	291	244		BAS	
160	MDMA	274	280	278	BAS	
161	Mebendazole	382		438	BAS	
162	Meclozine	602	601	587	BAS	3.1
163	Medazepam	355	395	405	ACN	6.2
164	Mefenamic Acid	695	670	661	ACN	4.2
165	Melperone		327	360	BAS	
166	Mepacrine	355	331	345	BAS	7.7
167	Mephenesine	358	349	364	BAS	
168	Mephenytoin	395	382		ACN	8.1
169	Mepivacaine	288	300	296	BAS	7.7
170	Mescaline	258	255	272	BAS	
171	Mesuximide	422	458		ACN	
172	Metamizol		289	316	ACN	
173	Metapirylene	234		342	BAS	3.7 8.9
174	Methadon	435	443	440	BAS	8.3
175	Methamphetamine	259	255	262	BAS	10.0
176	Methaqualone	435	455	449	BAS	2.5
177	Methohexital		503	503	ACN	8.3
178	Methoxyamphetamine	263		274	BAS	
179	Methyl Phenidate	311	316	328	BAS	8.8
180	Methyldopamine	166	166		BAS	10.6 2.2 9.2 12.0
181	Methylphenobarbital		420	435	ACN	7.8
182	Methyltestosteron	619	580		ACN	
183	Methyprylon	338		347	ACN	
184	Metoclopramide		308	324	BAS	9.0
185	Metoprolol	313	317	326	BAS	9.7
186	Metronidazol		236	257	ACN	2.5

Table 2 (continued)

Substance	MCL	IFM	MTSS	Scale	Pk$_a$
187 Mianserine		390	391	BAS	
188 Midazolam		386	399	ACN	6.2
189 Mondacetylmorphine-6	274	276		BAS	
190 Moperon		387	406	BAS	
191 Morphine	212	198	200	BAS	8.0
192 Morphine-3-Glucur.	206	167		BAS	
193 Nadolol	274	271	288	BAS	
194 Nalorphine	243	237	260	BAS	7.8
195 Naloxone	258	251		BAS	7.9
196 Naproxene	492	476	488	BAS	4.2
197 Nefopam	355	344		BAS	9.2
198 Nicotine	64	100	69	BAS	3.2
199 Nifedipine	531	503	527	ACN	
200 Niflumic Acid	561	586	595	ACN	0.0
201 Nikethamide		280	304	ACN	3.5
202 Nimodipine		641	668	ACN	
203 Nitrofurantoin		307	319	ACN	7.2
204 Nitrazepam	404	430	448	ACN	3.2
205 Nitrendipine		601	625	ACN	
206 Nomifensine		346	349	BAS	
207 Nordiazepam	377	464	470	ACN	3.5
208 Nortriptyline	419	418	430	BAS	9.7
209 Noscapine	339	354	368	BAS	6.2
210 Opiramol		387	377	BAS	3.8
211 Orciprenaline		180	151	BAS	9.0
212 Orphenadrine		416	418	BAS	8.4
213 Oxazepam	422	441	441	ACN	1.7
214 Oxazolam		340	339	ACN	
215 Oxprenolol		332	354	BAS	9.5
216 Oxycodone	262	260	277	BAS	8.9
217 Oxymorphone	222		217	BAS	
218 Oxyphenbutazone	516	503	501	ACN	4.7
219 Oxytetracycline	278		299	ACN	7.3 9.1 3.3
220 Papaverine	335	346	363	BAS	6.4
221 Paracetamol	257	234	264	ACN	9.5
222 Paroxetin		385	426	BAS	
223 Pecazine		440	443	BAS	9.7
224 Pemoline	292	281	307	BAS	10.5

Table 2 (continued)

	Substance	MCL	IFM	MTSS	Scale	Pk$_a$
225	Penfluridol		716	659	BAS	
226	Pentazocine	358	357	372	BAS	8.5
227	Pentobarbital	409	405	424	ACN	8.0
228	Pentoxiphylline		320	355	BAS	
229	Perazine		418	403	BAS	
230	Periciazine		405	410	BAS	
231	Perphenazine		438	428	BAS	7.8
232	Pethidine		334	345	BAS	8.7
233	Phenacetin	371	356	377	ACN	2.2
234	Phenazocine	386		409	BAS	
235	Phenazone		303	333	ACN	1.5
236	Phencyclidine	355	356	375	BAS	8.5
237	Phenelzine	193	200	184	BAS	
238	Pheniramine	216	279	283	BAS	4.2
239	Phenobarbital	366	357	379	ACN	7.4
240	Phenothiazine	668		665	BAS	
241	Phenprocoumon		609	616	ACN	
242	Phentermine	269	277		BAS	10.1
243	Phenylbutazone	687	651	672	ACN	4.4
244	Phenylcyclohexylamin	329		323	BAS	
245	Phenylephrine	101		80	BAS	9.8 8.8
246	Phenytoin		415	431	ACN	8.3
247	Physostigmine	277	267	296	BAS	1.8
248	Pindolol		277	300	BAS	9.7
249	Pipamperone		286	299	BAS	
250	Piribedil		301	328	BAS	
251	Piritramid		395	377	BAS	
252	Piroxicam		425	431	BAS	
253	Prazepam	572	648	648	ACN	2.7
254	Primidone	320	308	322	ACN	
255	Probenecid	541	560	526	ACN	3.4
256	Procainamid		202	208	ACN	9.2
257	Procaine		236	264	BAS	9.0
258	Prochlorperazine		462	450	BAS	8.1
259	Progesteron	797		672	ACN	
260	Promazine		418	407	BAS	9.4
261	Promethazine		411	409	BAS	9.1
262	Propafenone		408	433	BAS	

(continued)

Table 2 (continued)

	Substance	MCL	IFM	MTSS	Scale	Pk$_a$
263	Propiomazine	394		440	BAS	6.6
264	Propranolol	351	370	377	BAS	9.5
265	Propyhenazone		422	441	ACN	
266	Protriptyline		424	418	BAS	
267	Quinidine	261	316	322	BAS	4.2
268	Quinine	276	308	327	BAS	4.1
269	Ranitidine	229	240		BAS	2.3
270	Reserpine	437	473	467	BAS	6.6
271	Resorcinol	348		243	ACN	6.2
272	Saccharin	295	268	291	BAS	1.6
273	Salbutamol	225	207	220	ACN	9.3
274	Salicylamide	319	305	327	ACN	8.2
275	Salicylic Acid	375	331	359	ACN	3.0
276	Scopolamine	256	288	270	BAS	7.6
277	Secbutabarbital		365	377	ACN	
278	Secobarbital	434	437	437	ACN	7.9
279	Sotalol		273	226	BAS	8.3
280	Spironolactone		504	502	ACN	
281	Strychnine	296	292	302	BAS	2.3
282	Sulfadiazine	271	260		ACN	6.5
283	Sulfanilamide	125	142		ACN	10.4
284	Sulpiride		240	250	BAS	8.9
285	Sulthiam		321	344	ACN	10.0
286	Temazepam	473	466	472	ACN	1.6
287	Terfenadine		567	571	BAS	
288	Testosterone	573		534	ACN	
289	Tetracaine	354	381	389	BAS	8.5
290	Tetracycline	292		314	ACN	7.7 9.5 3.3
291	Tetramizole	259		261	ACN	
292	Tetrazepam		538	511	ACN	
293	Thebaine	316	324	340	BAS	8.2
294	Theobromine	259	224	262	BAS	1.0 14.0
295	Theophylline	270	239	276	BAS	1.0 14.0
296	Thiabendazole	266	283		ACN	
297	Thiamylal	509		516	ACN	7.3
298	Thiopental		481	485	ACN	7.6
299	Thioridazine	454	504	490	BAS	9.5
300	Tiaprofenic Acid		475	484	ACN	3.0

Table 2 (continued)

Substance	MCL	IFM	MTSS	Scale	Pk$_a$
301 Timolol		297	317	BAS	
302 Tocainide		251	247	BAS	7.8
303 Tolazamide	479		452	ACN	5.7
304 Tolazoline	217		225	BAS	10.3
305 Tolbutamide	474	470	477	ACN	5.3
306 Tolmetine	468		470	BAS	3.5
307 Trancylpromine	232	241	230	BAS	8.2
308 Trazodone		258	278	BAS	
309 Triamcinolone	366		348	ACN	
310 Triamterene		291	298	BAS	6.2
311 Triazolam		452	476	ACN	
312 Trichlormethiazide	385	377		ACN	8.5
313 Trifluoperazine	396	491	480	BAS	8.1
314 Trifluperidol		436	459	BAS	
315 Triflupromazine		505	484	BAS	9.4
316 Trihexyphenidyl	416	410	429	BAS	
317 Trimethoprim	286	289	299	ACN	7.2
318 Trimethoxyamphetamin	269		290	BAS	
319 Trimipramin		451	454	BAS	
320 Tripelenamine	237	337	336	BAS	3.9
321 Tripolidine	276	360	388	BAS	6.5
322 Tropacocaine	319		332	BAS	9.7
323 Tryamine	109	124		BAS	9.5
324 Verapamil	437	454	447	BAS	
325 Viloxazine		321	325	BAS	
326 Vinylbital		410	424	ACN	
327 Warfarin	538	555	546	ACN	5.0
328 Yohimbine	318	333	340	BAS	
329 Zopiclone		314	331	BAS	

Table 2 shows the uncorrected RI values for all common drugs, as well as the available pKa values and the ionization character of each compound.

The comparison of IFM and MTSS data showed a high agreement of RI-values (Fig.2). More than 90% of all results showed deviations in the range of ± 20 RI units.
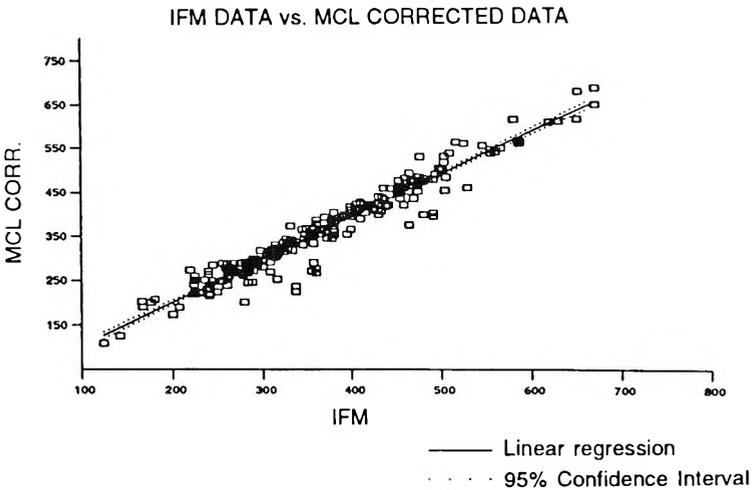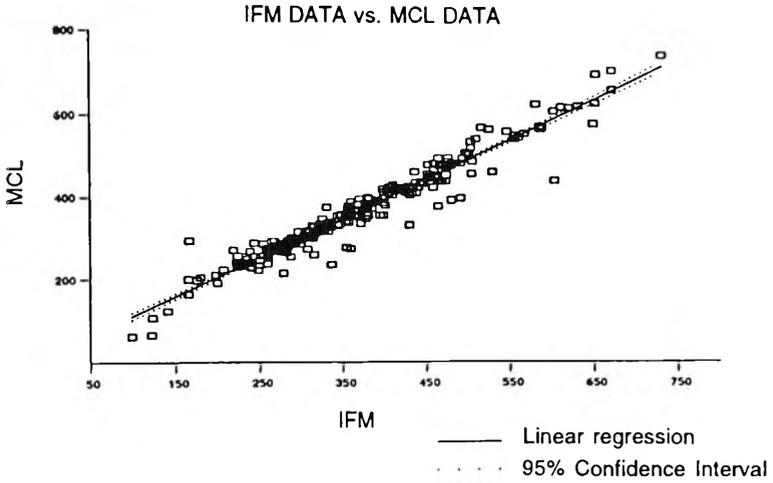
Figure 3. Comparison of RI-values (uncorrected and corrected data) for common drugs in IFM and MCL databases.
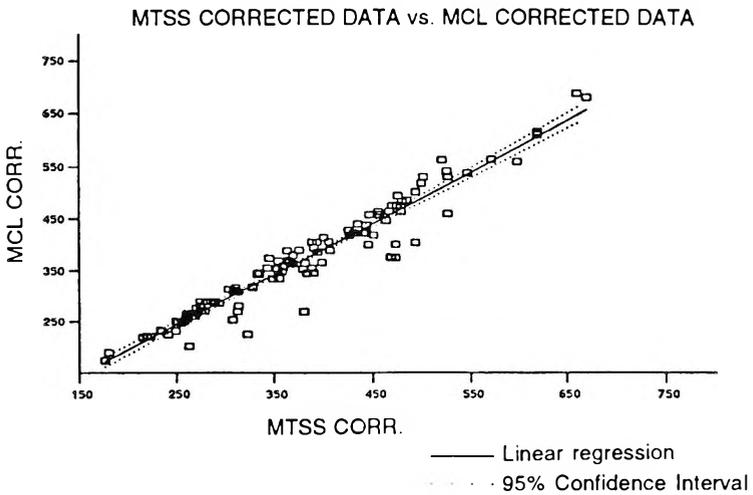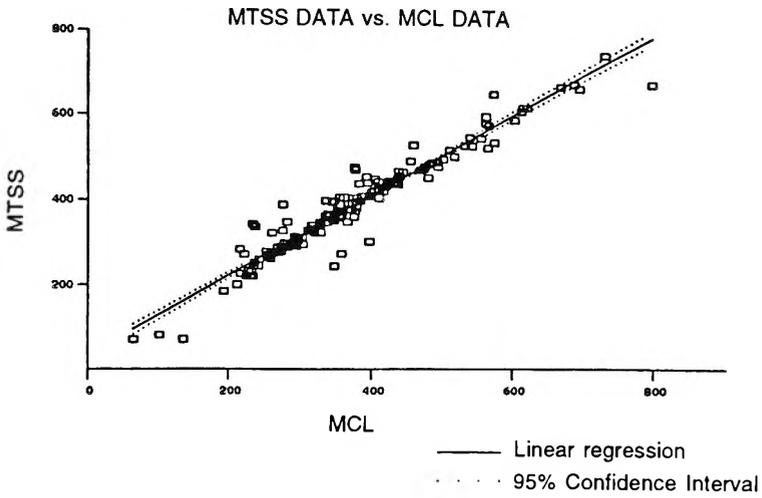
Figure 4. Comparison of RI-values (uncorrected and corrected data) for common drugs in MCL and MTSS databases.
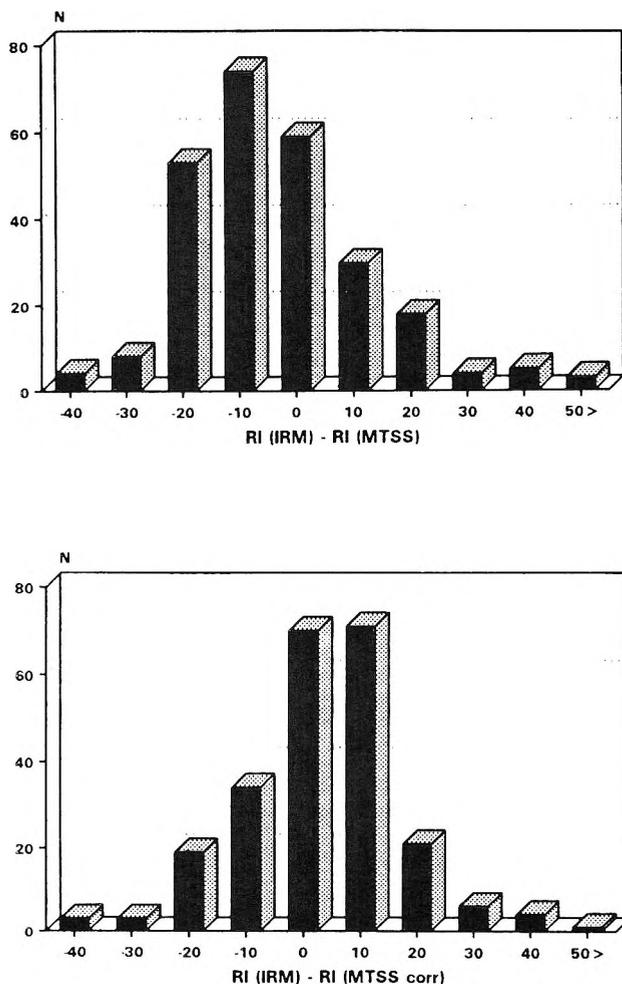
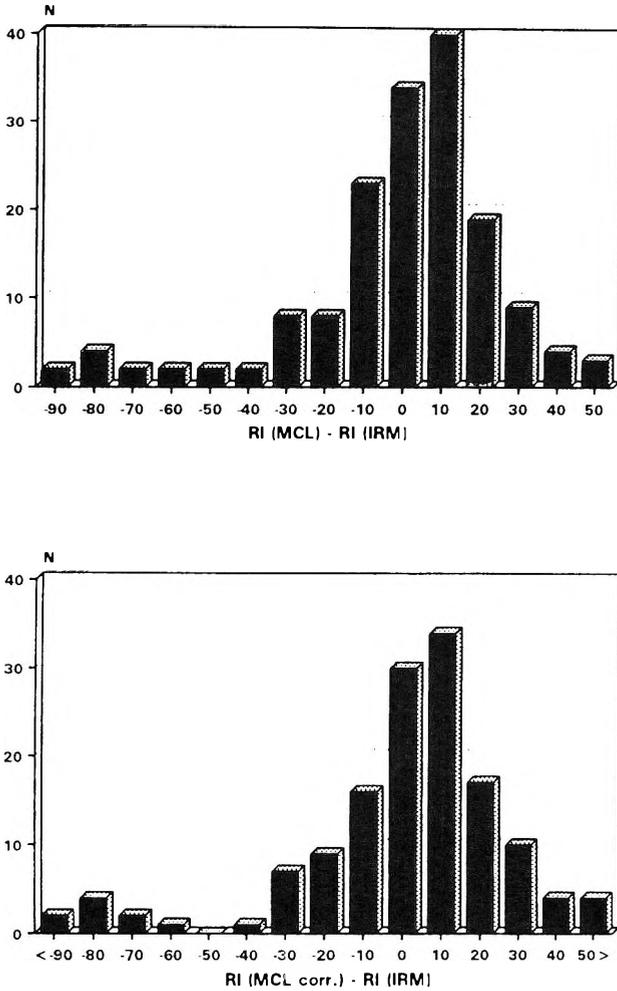**Figure 5**. Differences in RI-values of common drugs (uncorrected and corrected data) for common drugs in IFM and MTSS databases

Retention indices obtained in MCL also showed good agreement with IFM and MTSS data in most of cases. However, several distinct deviations were observed, which have influenced the correlation (Figs.3,4). The correction procedure resulted in only a slight improvement of the correlation values. These results are similar to the comparative data published recently[12] (Table 3).

Figure 6. Differences in RI-values of common drugs (uncorrected and corrected data) for common drugs in IFM and MCL databases.

Figs. 5-7 show the distribution of differences in RI-values between particular databases. Considering these data, the search window of 30 RI units may be recommended when the retention data obtained are compared with a database

Table 3

**Correlation Coefficients of RI-Values of Common Drugs
Examined in Three Databases**

Library	r^2 Value Uncorrected Data	r^3 Value Corrected Data
MCL/IFM*	0.9059	0.9132
IFM/MTSS	0.9778	0.9805
IFM/MCL	0.9245	0.9267
MCL/MTSS	0.9339	0.9368

* comparison of data published by Bogusz and Wu[12] with data of Hill and Kind[16] - 90 common drugs

established in standardized conditions in another laboratory. The search window in intralaboratory use of the user-created library practically never needed to exceed 10 RI units. The same experience was shared by the users of the MTSS system, who apply exactly the same conditions and the same column (Lichrosorb Select B, supplied together with the system) in various laboratories.

Evaluation of the data obtained in two laboratories using virtually the same conditions but different columns (IFM and MTSS) disclosed some groups of drugs showing particularly high variability, i.e.:

-xanthine derivatives (caffeine, theophylline, theobromine, fenetylline and
 pentoxiphylline),
-some benzodiazepine derivatives (alprazolam, demoxepam, triazolam,
 flunitrazepam),.
-pyrazolone derivatives (phenazone, metamizol),
-salicylates (salicylic acid, asperin).

A common feature of these compounds, as well as some others showing distinct differences in RI-values (physostigmine, brucine, ibuprofen, probenecid) was the presence of functional groups with low pKa values (1 - 4.5). It might be possible that the chromatographic mobilities of these drugs are more susceptible to the small differences in apparent pH values of the mobile phase due to the different degree of ionization. The analysis of RI-data obtained in MCL in comparison with data obtained in IFM or MTSS also showed some regularities.
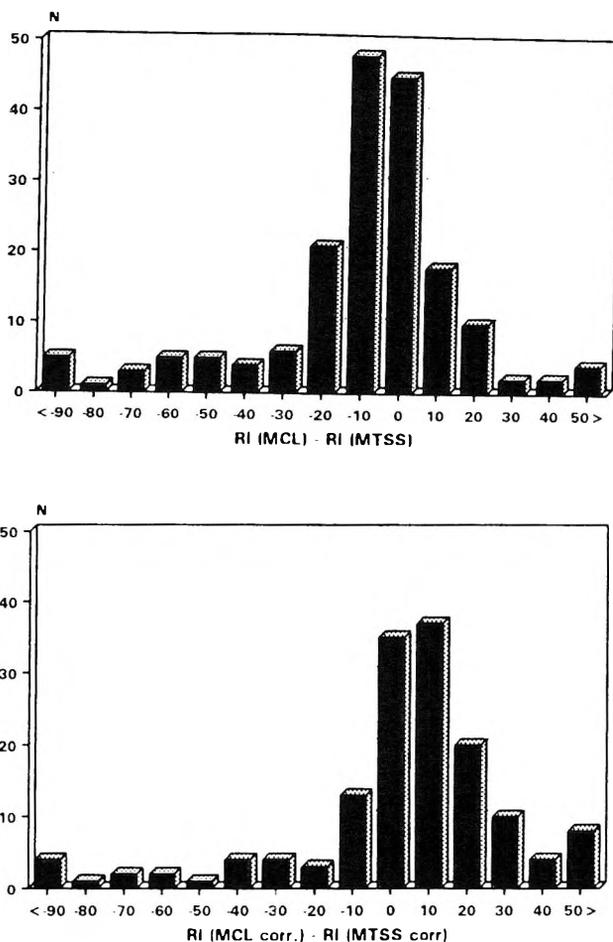
Figure 7. Differences in RI-values of common drugs (uncorrected and corrected data) for common drugs in MCL and MTSS databases

Drugs showing distinctly lower RI-values in the MCL library were some phenothiazine derivatives and basic drugs with pKa values below 4 (e.g., tripenelamine, fluphenazine, diazepam, nordiazepam, prazepam). Acidc drugs with low pKa values showed higher RI-values than in IFM or MTSS libraries. The comparison of HPLC conditions shows that the pH of the system used in the MCL was distinctly lower than in the IFM or MTSS. Moreover, the elution system applied in the MCL assured the same pH value of 2.2 throughout
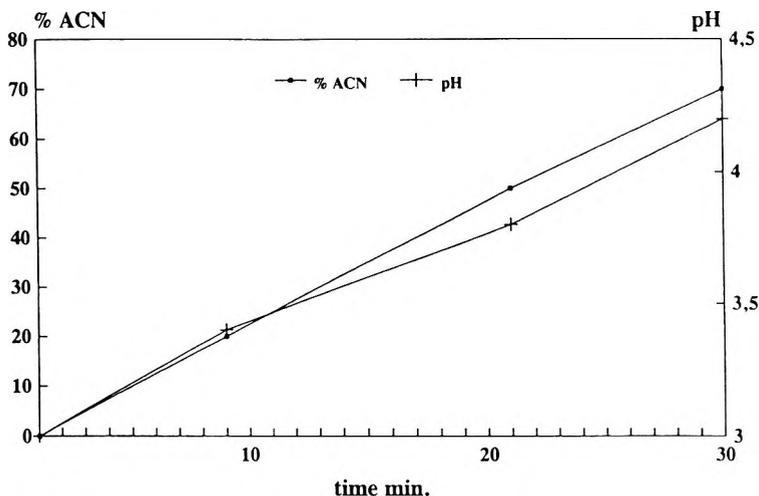
mob.phase: ACN-TEAP buffer pH 3.0
gradient: 0 - 70% ACN in 30 min

Figure 8. Changes in apparent pH of mobile phase during gradient elution in IFM/MTSS system.

the whole elution range. This was not the fact for the system used in the IFM and MTSS. Measurements of the pH of the mobile phase during elution showed a steady rise from pH 3.0 at the beginning to 4.2 at the end of gradient profile (Fig. 8). This means that the difference in pH of the mobile phase between MCL and IRM/MTSS systems increased with the time of analysis. The lower pH value of the mobile phase in the MCL system most probably caused higher ionization and higher mobility of basic compounds having pKa values in critical range[1-4] and an opposite effect on acidic drugs. This in consequence may have caused the differences in observed relative retention values (RI's).

The pKa values showed in Table 2 were taken from several sources.[24-30] Unfortunately, not all pKa were available to us, which makes the interpretation of some outlying data more difficult.

The structures of most of the compounds that we have analyzed indicate the presence of more than one ionizable functional groups. Moreover, it should be stressed that the pKa values listed in the literature are for the drugs in aqueous solutions. In the presence of organic solvents these values may be

substantially shifted. This was demonstrated for benzodiazepine derivatives, which have pKa values of the base ionizable group in acetonitrile 7 to 8 units higher in acetonitrile than in water,[31] or for diclofenac, showing linear increase in its pKa value from 4.0 to 6.8 with increasing concentrations of ethanol.[25]

Another factor that may affect the chromatographic mobility of some compounds is the possible difference in concentrations of metal ions in different stationary phases. This may cause different chelation of solutes and in consequence a shift in RI values. This could be the cause in the variable RI results obtained for salicylic acid.

The interaction of drugs with active silanol groups on the silica surface may be another factor causing variability of retention. The theoretical pKa value of silanol groups is 6.8 ± 0.5 (32) or 7.1 ± 0.5, but practically it varies in the range of 1.5 to 10.[33,34] This means that in applied conditions most, but probably not all, silanol groups may be suppressed. This possibility should be regarded, however, as very remote, since in applied conditions (acidic mobile phase containing amine modifier) most of the silanol effects were eliminated.[12,19,21]

CONCLUSIONS

1. RI data obtained in two labs in standardized conditions showed generally satisfactory reproducibility, allowing interlaboratory use and exchange of data. A search window of ± 30 RI units is recommended for the use of data obtained with a different column in identical conditions. Within one laboratory or exactly the same system the search window of 10 RI units is justified.

2. The use of different HPLC conditions, especially a different pH of the mobile phase, may influence the RI values of some compounds, particularly those with pKa values around the pH of the mobile phase. Therefore, the importance of an exact standardization of HPLC conditions for interlaboratory exchange of RI-data was confirmed. However, the majority of retention data from all three libraries showed good agreement. The RI data from RI-libraries obtained in similar, but not exactly identical conditions may be used with caution.

ACKNOWLEDGEMENTS

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DETERMINATION OF PROGUANIL, CYCLOGUANIL AND 4-CHLOROPHENYL-BIGUANIDE IN SALIVA AND PLASMA BY ION-PAIRING COLUMN SWITCHING HPLC

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ABSTRACT

The application of a column switching HPLC system to the analysis of the antimalarial proguanil and its two metabolites in plasma and saliva is described. The assay is based on a previously developed hydrophobic ion-pairing separation using sodium dodecylsulphate on ODS Hypersil. The use of a precolumn to concentrate and allow pre-treatment of these drugs in the two matrices is shown. This is followed by the transfer and subsequent separation of the biguanides by the analytical phase containing a high concentration of hydrophobic pairing ion. It is

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found that such on-line sample pre-treatment can yield comparable analytical characteristics to off-line solid phase extraction procedures if the precolumn is conditioned between sample injections. The columns can be used for large numbers of saliva assays but when plasma is the biological matrix components of the plasma cause rapid deterioration of the precolumn.

The column switching assay described is used to determine the steady state concentrations of proguanil, cycloguanil and 4-chlorophenylbiguanide in plasma and saliva. The concentrations of the biguanides in saliva are small. This would limit the information on compliance with a prophylactic regimen and the potential efficacy of the antimalarial which could be obtained from analysis of patient saliva.

INTRODUCTION

In a previous publication[1] the effect of both analytical and precolumn dimensions in column switching systems designed to pretreat and concentrate analytes from both aqueous and biological fluid matrices was described. The analytes used as model compounds were non-ionisable neutral species so that adsorption and desorption on to and from the precolumn could be effected by simple alteration of organic modifier concentration. In addition, the flushing of the precolumn with the analytical mobile phase during the transfer of the solutes from the precolumn to the analytical column did not produce any slowly reversible alteration to the precolumn surface. This is not the case when ion pairing systems incorporating high concentrations of hydrophobic pairing ions are required for resolution of the analytes.

The use of ion pairing has been reported in column switching techniques in several ways. In some reports[2,3] the hydrophobic pairing ion is incorporated into the loading solvent only. The rationale for this is that the retention on the precolumn is enhanced by the ion pairing agent and that the absence of this in the analytical mobile phase allows peak compression to take place on the analytical column. Alternatively, the hydrophobic pairing ion may be used as a micelle forming surfactant to avoid precipitation of protein. In other methods[4,5] ion pairing agent has been included in both the load phase and in the analytical mobile phase and the required peak compression obtained on the analytical column by alteration of organic modifier. Few assay methods, using column switching, have been located in the literature, in which ion pairing agents have been included in the analytical mobile phase only. Those which do[6] use pairing

ions in this way use low concentrations of tetra alkyl ammonium salts which appear to be acting to mask the effect of the residual silanol groups.

The purpose of the present paper is to report the use of column switching in separations where a relatively high concentration of hydrophobic pairing ion is required to obtain resolution among basic compounds such as the biguanides proguanil and metabolites. The determination of these in biological fluids requires that the sample be applied to the precolumn of the column switching system in aqueous solution. In such cases the nature of the stationary phases in both the analytical and, more importantly the precolumn will be affected by the adsorption and desorption[7] of the hydrophobic pairing ion with consequent alteration in retention properties. The analytical parameters of the assay method resulting from the use of ion-pairing agents in this way coupled with column switching for preconcentration and pre-treatment of biological matrices will be reported.

In addition, with evidence of proguanil excretion in human saliva[8], the assay will be applied to a study of the relationship between plasma and saliva concentrations of proguanil and metabolites in a group of human volunteers at steady state following consumption of 4 daily doses of 200 mg proguanil. This will allow assessment of the feasibility of monitoring compliance with malaria prophylaxis and the concentration of the active metabolite, cycloguanil, by analysis of saliva samples.

EQUIPMENT AND MATERIALS

The column switching system previously described[1] was used. This consisted of Varian 2510 and Shimadzu LC5A pumps coupled with a Jasco 875-UV variable wavelength detector. The analytical column was 100 x 2 mm slurry packed in the laboratory with 3 μm ODS Hypersil. The precolumn was 10 x 4 mm fabricated as described previously [1] and dry packed with 40 μm C_8 or C_{18} bonded silica. Data were recorded on a Hewlett-Packard 3395 integrator and peak height or area measurements were recorded. Sample injection was by Rheodyne 7125 valve fitted with a 1 mL loop and the column switching valve was a Rheodyne 7010. Organic solvents methanol and acetonitrile were supplied by Rathburn Chemicals and water was purified after distillation using a Millipore Milli-Q System. The biguanides proguanil (P), cycloguanil (C), 4-chlorophenylbiguanide (CPB) and chlorproguanil (IS) were kindly donated by ICI (Zeneca). Sodium dodecylsulphate (SDS) was obtained from Fisons and sodium phosphate from Sigma.

PROCEDURES AND RESULTS

The assay method is based on the biguanide separation previously reported[9] in which a mobile phase of acetonitrile - 10 mM aqueous phosphate buffer (pH 2.0) (50:50) containing 200 mM SDS with a 3 μm C_{18} stationary phase resulted in complete resolution among the three biguanides and from endogenous peaks remaining after off-line solid phase extraction.

Loading Procedure

It was found that loading the analytes in water produced maximum recovery. Peak heights were measured after loading 1mL of appropriate concentrations in water on to the precolumn, flushing with 4 mL of water and transferring to the analytical column with the mobile phase used for the separation. The analytical mobile phase used for this study was acetonitrile: 20 mM aqueous phosphate buffer (pH 2.5) (40 : 60) containing 200mM SDS. These peak heights were compared with the corresponding values obtained when identical masses of analyte were injected directly into the analytical column. The percentage recoveries were calculated as (C)-99.2, (CPB)-98.7 and (P)-101.3. It was not found possible to load sample or to wash the precolumn after loading with water containing low concentrations of organic solvents without reducing the recoveries dramatically. It was also found necessary to flush the precolumn with methanol and recondition with water after successive injections. Omission of this washing to remove adsorbed SDS resulted in significantly lower recoveries being obtained on subsequent injections. Analytical column efficiency was not affected by the inclusion of the precolumn when samples were in purely aqueous solution. When the biguanides were loaded from a saliva matrix some peak broadening occurred. To determine the recovery of the biguanides from saliva, therefore, peak areas were measured and compared with those obtained using pure water. Both C_8 and C_{18} stationary phases were evaluated in the precolumn. No significant differences were observed in the percentage recoveries recorded. For the C_{18} phase which was used in all subsequent studies the percentage recoveries from saliva were (C)-93.6 ± 2.9, (CPB)- 93.0 ± 2.8, (P)-96.0 ± 2.8. These recoveries could not be improved by dilution of the sample with water. As reported previously for a set of steroids[1] the recoveries determined from a plasma matrix were lower than from saliva and also varied with the extent to which the plasma was diluted with water. The percentage recoveries of the biguanides obtained from plasma diluted by the addition of one third its volume of water were (C)- 88.5, (CPB) - 80.3, (P)- 90.8. For subsequent determinations of the biguanides in plasma samples were diluted as above before loading.

As recommended in the literature,[10] saliva and plasma samples were centrifuged prior to injection in order to minimise analytical and precolumn deterioration. Saliva and plasma samples were centrifuged at 13000g for 10 minutes. It was found that about 70 mL saliva could be injected on to the system before replacement of the analytical column was required. With saliva the precolumn did not require regular replacement. Plasma samples had a more adverse effect upon column life requiring that the precolumn be renewed after application of approximately 6 mL of plasma and the analytical column after 40 mL of sample.

Table 1

**Showing Typical Regression Calibration Equations for
C, CPB and P in Saliva and Plasma**

Matrix	Analyte	Regression Equation	r^2	RSD%
Saliva	C	Ratio = 0.02040 A - 0.00315	0.9997	1.04
	CPB	Ratio = 0.04956A - 0.00381	0.9987	2.05
	P	Ratio = 0.04756A - 0.03941	0.9988	2.01
Plasma	C	Ratio = 0.00155A + 0.00695	0.9992	1.39
	CPB	Ratio = 0.00366A + 0.00104	0.9995	1.06
	P	Ratio = 0.00298A - 0.0105	0.9980	2.25

Calibration

To determine the linearity of response of the system to the three analytes 6 standard solutions in saliva and 7 in plasma were prepared. These were designed to cover the anticipated ranges of concentration expected to be obtained in samples from human volunteers after consumption of 200 mg proguanil. For saliva the concentration ranges of the respective analytes were - (C) 2.5 - 26, (CPB) 2.4 - 10, (P) 5 - 50 ngmL^{-1}. For plasma the concentration ranges were - (C) 15.6 - 166, (CPB) 5.0 - 70, (P) 50 - 404 ngmL^{-1}. These standards were subjected to centrifugation as described, and chlorproguanil as internal standard at concentrations of 50 ngmL^{-1} for saliva and 400 ngmL^{-1} for plasma was added. Standard solutions were applied to the precolumn via the 1mL loop. For both saliva and plasma standard calibrations were determined on 4 successive days. Typical regression equations are shown in Table 1.

Table 2

Within Day and Day to Day Accuracy and Precision
of the Assay in Saliva and Plasma

Analyte	C		CPB		P	
	Within Day	Day to Day	Within Day	Day to Day	Within Day	Day to Day
Saliva						
Spiked conc. /ngmL^{-1}	4.16	4.16	3.20	3.20	8.08	8.08
Mean conc. found /ngmL^{-1}	4.14	4.31	3.30	3.36	7.87	8.23
Accuracy%	99.5	103.6	103.1	105.0	97.4	101.9
RSD%	3.85	3.80	4.80	5.72	2.68	3.32
Plasma						
Spiked conc. /ngmL^{-1}	62.4	62.4	17.2	17.2	222.2	222.2
Mean conc. found /ngmL^{-1}	61.1	61.5	17.6	16.8	229.2	226.8
Accuracy%	97.9	98.6	102.3	97.7	103.2	102.1
RSD%	1.95	4.54	2.59	2.85	1.51	2.66

The day to day RSD% in the slopes of the calibration lines in saliva were (C)- 18.4, (CPB) - 23.2, (P) - 9.4 and in plasma (C) - 12.1, (CPB) - 12.9, (P) - 3.2. Thus while individual calibration lines for all analytes showed good linearity there was considerable day to day variation in the response of the system which perhaps reflects the deleterious effects of matrix components.

Accuracy And Precision

To assess the accuracy and precision of the assay for the three analytes test solutions were prepared in saliva and plasma matrices. The within day accuracy and precision were determined by assaying the test solution 9 times using   a single   calibration.   The   day   to   day   accuracy   and   precision   were

determined by assaying the test solution 9 times over 4 days calibrating on each day with standard solutions in the appropriate matrix. Accuracy is reported as the percentage of the spiked value found by analysis. The results are shown in Table 2.

Limits Of Detection And Quantification

The limits of detection for the analytes were determined by subjecting low concentrations of the analytes to the sample pre-treatment and subsequent chromatography in the appropriate matrices and estimating the limit of detection as the concentration resulting in a signal to noise ratio of three. The limits of quantification taken as twice this value in saliva were estimated to be (C)- 2.6, (CPB)- 3.2, (P)- 2.6 ngmL^{-1}. These are close to the lowest standard concentrations used in the saliva calibration. In plasma the limits of quantification were estimated as (C)- 9.0, (CPB)- 5.4, (P)- 5.0 ngmL^{-1}.

Determination Of Proguanil And Metabolites In Saliva And Plasma

Ethical approval was obtained for the trial and ten volunteers were given two 100 mg proguanil tablets daily for four days to establish steady state conditions. On the fourth day 10 mL of venous blood and a saliva sample were collected from each subject. Samples were centrifuged and plasma and saliva frozen until analysed. Where there was enough sample the determinations were carried out in duplicate. Samples were diluted with an appropriate solution of the internal standard and 1 mL samples were injected into the 1 mL sample loop. The sample was transferred to the precolumn, flushed with 4 mL of water and transferred to the analytical column by diverting the analytical mobile phase through the precolumn. The peak area ratios of the three analytes to that of the internal standard were determined from the resulting chromatogram and the concentrations calculated from the regression equation of the calibration line obtained on the same day. Specimen chromatograms obtained from one subject is shown in Figure 1.

The concentrations of each of the biguanides for the various volunteers in both saliva and plasma are shown in Table 3. All values are the mean of two separate determinations other than those marked with an asterisk (*) for which there was only enough saliva for a single determination.
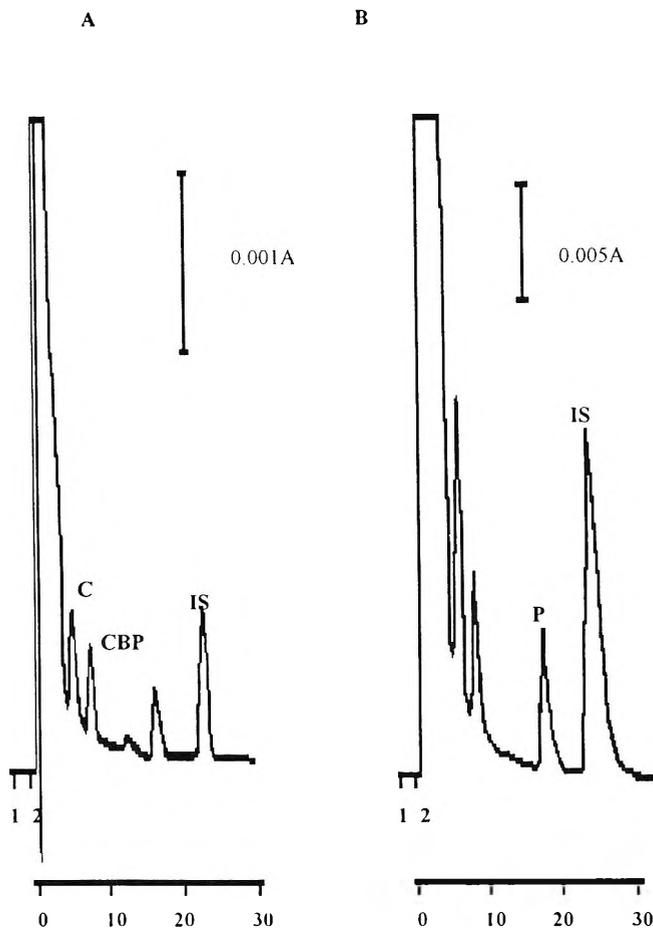
Figure 1. Specimen chromatograms obtained from Subject No. 2 after 4 days dosing with 200 mg Proguanil.
(A) Saliva sample (B) Plasma sample. Numbers 1 and 2 represent sample injection and column switching respectively. Compound identification and chromatographic conditions as in the text.

DISCUSSION

The results presented show that column switching can be used in situations where the separation on the analytical column depends upon the presence of hydrophobic ion-pairing agent such as sodium dodecylsulphate adsorbed on to

Table 3

**Concentration of Biguanides Determined in Saliva and Plasma
After 4 Days Daily Dosing With 200 mg Proguanil**

| Subject No. | Mean Concentration/ngmL^{-1} | | | | | |
| | (C) | | (CPB) | | (P) | |
	Saliva	Plasma	Saliva	Plasma	Saliva	Plasma
1	7.36*	86.4	4.81*	28.0	11.66*	263.1
2	8.44	116.2	3.10	38.4	5.56	80.0
3	6.44	307.1	4.17	22.6	16.15	224.4
4	7.06*	76.9	2.55*	24.3	10.62*	238.6
5	2.17	32.5	1.57	8.0	19.43	194.5
6	2.71*	62.9	1.89*	23.2	7.69*	212.8
7	3.71	67.7	2.11	15.8	11.49	154.8
8	5.41	41.7	2.85	8.2	10.39	209.3
9	9.35	145.7	7.55	32.0	14.75	214.1
10	7.58	112.6	5.20	46.7	6.40	136.9

*See text

precolumn in a chromatographically weak solvent from which ion-pairing agent is absent. Also, to maintain recovery, any ion-pairing agent which may be adsorbed on to the C_{18} surface of the precolumn due to contact with the analytical mobile phase must be removed before subsequent injections. If these precautions are taken the column switching approach yields comparable assay characteristics to those obtained using off-line solid phase extraction with the added advantage of fewer separate manipulations and a separate preconcentration stage. The main disadvantage is the lack of robustness of the chromatographic system when dealing with plasma samples.

Our findings, both in this work and in the previous report concerning the determination of steroids in plasma, indicate that components of plasma are irreversibly adsorbed or mechanically trapped in the stationary phase of the precolumn. This leads to the need for frequent replacement as indicated in the text above. This is not the general impression given in the literature[11-12] where reports have indicated that such precolumns used in column switching systems can be used for large numbers of injections of plasma with no deleterious effects. Examination of the literature indicates that in these reports either small samples are being applied to the column switching system or that additional pre-treatment stages such as liquid-liquid extraction are being incorporated prior to the use of

column switching methods. Both of these techniques, to a degree, render the use of column switching redundant. The present report, however, shows that, within the constraints of column longevity, column switching can be substituted for off-line preconcentration with no additional analytical steps. The analytical characteristics for the plasma matrix in terms of the accuracy and precision are comparable with the off-line pre-treatment method and adequate resolution is maintained for separation of proguanil from its two metabolites. All three biguanides can be separated from residual endogenous components remaining after the on-line pre-treatment. This is in contrast to the few studies which measure levels of chlorophenylbiguanide in plasma samples[13].

In the present study, the steady state plasma concentrations of the parent proguanil show considerable inter-subject variation. The mean and standard deviation value for the parent drug, proguanil, of 192.8 ± 54.2 ngmL^{-1} found for this group of subjects is consistent with previously published work[14]. Likewise the ratio of parent drug to active metabolite, cycloguanil, concentration is variable as has been reported previously[15]. This ratio is important as poor metabolisers develop a sub-therapeutic concentration of the active cycloguanil leading to an increased risk of malaria despite compliant chemoprophylaxis[14]. The cycloguanil concentration found for Subject No. 3 was determined to be outside the calibrated range and was determined using standards of increased concentration.

In comparison with the plasma samples, the concentrations determined in saliva were relatively small with saliva / plasma ratios of respectively (C) - 0.070 $\pm$ 42.0%, (CPB) - 0.165 $\pm$ 50.2% and (P) - 0.061 $\pm$ 32.2%. The concentrations of proguanil determined in saliva covered a range from 5.56 - 19.43 ngmL^{-1}. These values are considerably lower than those reported in an earlier publication[8] in which only proguanil was determined and in which no specificity with respect to the metabolic products was established. Detection of proguanil in saliva may provide compliance data. However, the low concentrations of cycloguanil obtained for the subject group examined suggest that monitoring of saliva to determine the potential efficacy of the antimalarial would not be advisable. Possible reasons for the presently reported low saliva / plasma ratios and the correlation between saliva and plasma concentrations are discussed in a separate publication[16].

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HPLC DETERMINATION OF MORPHINE-ONDANSETRON AND MEPERIDINE-ONDANSETRON MIXTURES IN 0.9% SODIUM CHLORIDE INJECTION

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ABSTRACT

High performance liquid chromatography procedures have been developed for the assay of morphine-ondansetron and meperidine-ondansetron mixtures in 0.9% sodium chloride injection. The separation and quantitation of morphine-ondansetron were performed on an underviatized silica column at ambient temperature using a mobile phase of 60:40 v/v 0.01 $\underline{M}$ monobasic potassium phosphate pH 4.0 - methanol at a flow rate of 1.0 mL/min with the detection set at 233 nm. The separation was achieved within 20 min. Morphine and ondansetron were linear in the 134 - 536 and 68 - 271 µg/mL ranges, respectivly. Accuracy and precision were in the range 0.04 - 4 and 0.2 - 1%, respectively, for the two analytes and the limits of detection for morphine and ondansetron were 210 and 110 ng/mL, respectively, based on a signal to noise ratio of 3 and a 20 µL injection. The separation and quantitation of the meperidine-ondansetron mixture were also achieved on an underivatized silica column at ambient temperature using a mobile

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phase of 60:40 v/v 0.01 $\underline{M}$ monobasic potassium phosphate pH 4.0 - methanol at a flow rate of 1.0 mL/min with detection of the two analytes at 254 nm. The separation was achieved within 20 min. Meperidine and ondansetron were linear in the 556 - 3331 and 89 - 536 μg/mL ranges, respectively. Accuracy and precision were in the range 0.7 - 5.1 and 0.1 - 0.6%, respectively, for the two analytes and the limits of detection for meperidine and ondansetron were 1.73 μg/mL and 47 ng/mL, respectively, based on a signal to noise ratio of 3 and a 20 μL injection.

INTRODUCTION

Mixtures of morphine-ondansetron (Mixture A) and meperidine-ondansetron (Mixture B) are administered as perioperative injections in U.S. hospitals. Interest in our laboratories in the stability and compatibility of each drug mixture over time in 0.9% sodium chloride injection required the development of HPLC methods. A search of the literature indicated that HPLC methods were not available to assay each analyte in mixture A or mixture B concurrently in a single injection.

Morphine has been analysed by HPLC with electrochemical (EDC) or UV detection.[1-3] The HPLC methods are not as sensitive as radioimmunoassay procedures, but are more specific and often used in the analysis of the compound. The HPLC-ECD method involved separation of morphine on an octylsilane column using a mobile phase of 15:85 v/v absolute methanol-50 mM dibasic sodium phosphate pH 3.5 containing 3 mM octanesulphonic acid.[1] The electrode potential was set at +600 mV vs Ag/AgCl. Morphine has also been analysed using UV detection at 210 nm with an HPLC system consisting of an octadecylsilane column operating at a flow rate of 0.8 mL/min and 26.5:73.5 v/v acetonitrile - 0.8m$\underline{M}$ sodium dodecyl sulphate in 10 m$\underline{M}$ monobasic phosphate buffer mobile phase.[3] The official USP 23 assay for morphine utilizes an HPLC separation on an octadecylsilane column with detection at 284 nm.[4]

Meperidine has been assayed by a variety of analytical methods. Gas chromatography using mass spectrometry has been reported.[5,6] Spectrophotometry, colorimetry and derivative spectroscopy have also been utilized by various investigators.[7-9] The use of an ion-selective electrode was reported by one laboratory.[10] The official USP 23 assays for drug substance and a syrup dosage form utilize a reverse-phase HPLC separation and non-aqueous titrimetry, respectively.[11,12]

Ondansetron has been assayed by high performance thin layer chromatography. (HPTLC), HPLC methods and radioimmunoassay methods. The HPTLC method was developed especially for plasma samples, but the sample throughput was low and the equipment is not generally available in most laboratories.[13] The HPLC assays used either a silica column with an aqueous-organic mobile phase or a cyanopropyl column operated in the reverse phase mode.[14,15] Detection of the analyte was either by UV at 305 nm or radiochemical detection. The radioimmunoassay was combined with sample cleanup using a cyanopropyl solid phase extraction cartridge to provide a subnanogram per mL determination of ondansetron.[16]

In this paper, isocratic HPLC assays are presented for the simultaneous analysis of morphine and ondansetron (Mixture A) and meperidine and ondansetron (Mixture B) in 0.9% sodium chloride injection. Both mixtures were separated on an underivatized silica column using a buffered aqueous-methanol eluent. Each separation was achieved within 20 min with sensitivity in the μg/mL range for meperidine and ng/mL for morphine and ondansetron.

EXPERIMENTAL

Reagents and Chemicals

The structural formulae of the compounds studied are shown in Figure 1. Morphine sulfate and meperidine hydrochloride were purchased as their respective salts from The United States Pharmacopeia (Rockville, MD 20852, Lots H-1 and G-1, respectively). Ondansetron hydrochloride (Lot No. AWS332A) was a gift from Glaxo, Inc. (Research Triangle Park, NC 27709). Methanol (J.T. Baker, Phillipsburg, NJ 08865) was HPLC grade and water was purified by a cartridge system (Continental Water Systems, Roswell GA 30076). Monobasic potassium phosphate, potassium hydroxide and concentrated phosphoric acid were Baker analysed reagents.

Instrumentation

The chromatographic separations were performed on an HPLC system consisting of a Waters Model 501 pump (Milford, MA 01757), an Alcott Model 728 autosampler (Norcross, Ga 30093) equipped with a 20 μL loop, a Beckman Model 163 variable wavelength UV-VIS detector (Fullerton, CA 92634) and a

MORPHINE   MEPERIDINE

ONDANSETRON
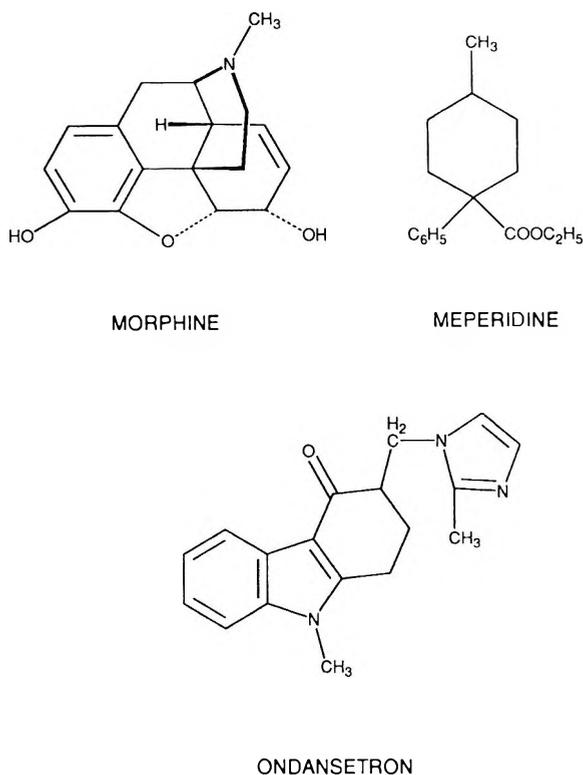
Figure 1. Chemical stuctures of compounds studied.

Hewlett Packard Model 3395 integrator (Palo Alto, CA). Separation of Mixture A was achieved on a 22 cm underivatized silica column (4.6 mm i.d., 5 μm particle size, Brownlee Silica Applied Biosystems, Inc. San Jose, CA 95134). The mobile phase consisted of 60:40 v/v 0.01 $\underline{M}$ aqueous monobasic potassium phosphate, pH 4.0 (adjusted with 10% phosphoric acid)-methanol. The separation of Mixture B was also accomplished on a 22 cm underivatized silica column. The mobile phase consisted of 60:40 v/v 0.01 $\underline{M}$ aqueous monobasic potassium phosphate pH 4.0 (adjusted with 10% phosphoric acid)-methanol. The mobile phases were filtered through a 0.45 μm nylon-66 filter (MSI, Westborough, MA 01581) and degassed by sonication prior to use. The flow rate was set at 1.0 mL/min for both mixtures and the detector was set at 233 nm for mixture A and 254 nm for mixture B.
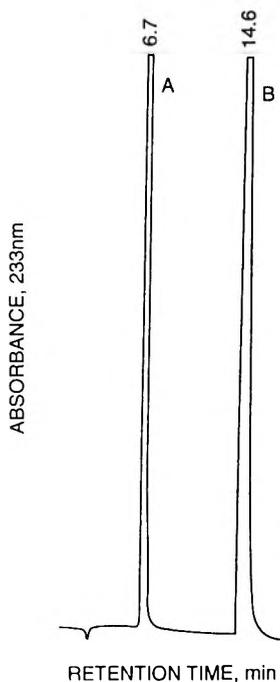
Figure 2. Typical HPLC chromatogram of Morphine (A) and Ondansetron (B) on a silica column with 60:40 v/v 0.01 M phosphate buffer pH 4.0 - methanol. See Experimental Section for assay conditions.

Preparation of Standard Solutions

A combined standard solution containing morphine and ondansetron was prepared by accurately weighing 35.5 mg of morphine sulfate and 15.0 mg ondansetron hydrochloride in a 10 mL volumetric flask. Another standard solution containing meperidine and ondansetron was prepared by accurately weighing 95.6 mg of meperidine hydrochloride and 15.0 mg ondansetron hydrochloride in a 10 mL volumetric flask. Sodium chloride injection was added to each mixture and the flasks were shaken vigorously for 2 minutes followed by addition of 0.9% sodium chloride to volume.   Dilutions 1:5, 1:7.5 and 1:20 of the combined morphine-ondansetron standard solution and 1:2.5, 1:7.5 and 1:15 dilutions of the combined meperidine-ondansetron standard solution gave solutions in the 134-536 µg/mL and 68 - 271 µg/mL range for morphine-ondansetron, respectively, expressed as free base concentrations and 555-3331 µg/mL and 89-536 µg/mL range for

Table 1

**Analytical Figures of Merit for Morphine-Ondansetron
and Meperidine-Ondansetron Mixtures**

Mixture	r^{2a}	System Suitability[b]	LOD ng/ml[c]	k^1	Theoretical Plates[d]	Tailing Factor[e]	Rs
A							
Morphine	0.9994	0.57	210	1.6	3000	1.1	
Ondansetron	0.9991	0.79	110	4.7	3779	1.2	11.5
B							
Meperidine	0.9969	0.98	1730	2.4	2969	2.5	
Ondansetron	0.9998	1.04	47	4.6	5978	2.0	8.0

[a] Range examined from 134-536µg/mL morphine (n = 9) and 68-271 µg/mL ondansetron for Mixture A at 233 nm and 555-331 µg/mL meperidine and 89-536 µg/mL Ondansetron for Mixture B at 254 nm.
[b] Mean RSD% of 6 replicate injections at 268 µg/mL morphine and 136 µg/mL ondansetron for Mixture A at 233 nm and 1110 µg/mL meperidine and 179 µg/mL ondansetron for Mixture B at 254 nm.
[c] Limit of Detection, S/N = 2.
[d] Calculated as $N = 16 (tr/w)^2$.
[e] Calculated at 5% peak height.

meperidine-ondansetron, respectively, expressed as free base concentrations. Dilutions (1:10 and 1:15 for Mixture A standard solutions and 1:5 and 1:10 for Mixture B standard solutions) were prepared in 0.9% sodium chloride injection to serve as spiked samples for each analyte to determine accuracy and precision. Quantitation was based on linear regression analysis of analyte peak height versus analyte concentration in µg/mL.

RESULTS AND DISCUSSION

There were no reports in the scientific literature describing separations of morphine-ondansetron and meperidine-ondansetron mixtures. Initial studies to develop HPLC methods for each mixture using isocratic conditions involved the use of underivatized silica, phenyl, octyl, deactivated octyl and octadecyl columns with various mobile phases containing methanol-aqueous phosphate buffers
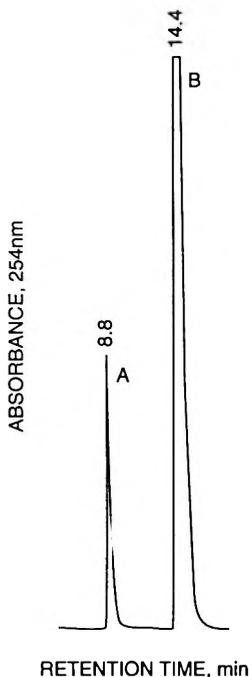
Figure 3. Typical HPLC chromatogram of Meperidine (A) and Ondansetron (B) on a silica column with 60:40 v/v 0.01 $\underline{M}$ phosphate buffer pH 4.0 - methanol. See Experimental Section for assay conditions.

and/or acetonitrile-aqueous phosphate buffers at 1 mL/min. The best resolution of the analytes in both Mixtures A and B was obtained on an underivatized silica column using a 60:40 v/v phosphate buffer pH 4-methanol mobile phase with total run times of 15-20 min. The column also allowed the separation of methylparaben (preservative found in commercial injections) from the analytes (R_t of 3 min). Typical chromatograms showing the separation of each mixture are shown in Figures 2 and 3.

From an earlier study in this lab, it was shown that morphine and ondansetron absorbed strongly at 233 nm in a methanol-phosphate buffer solvent system. It was also determined that meperidine and ondansetron absorb around 254 nm in the same solvent system.   Therefore, 233 nm and 254 nm were selected as the

Table 2

Accuracy and Precision Using Samples with Added Drug

Mixture	Concn Added μg/mL	Concn Found μg/mL	Percent Error	RSD (%)
A				
Morphine	179	171.9 ± 1.7	4.0	1.0
	358	359.6 ± 0.6	0.4	0.2
Onansetron	91	88.6 ± 0.9	2.6	1.0
	181	187.6 ± 1.0	3.6	0.5
B				
Meperidine	833	845.9 ± 0.8	1.5	0.1
	1665	1750.8 ± 6.0	5.1	0.3
Ondansetron	134	134.9 ± 0.8	0.7	0.6
	268	278.0 ± 1.3	3.7	0.5

[a] Mean ± standard deviation based on n = 3.

detection wavelengths for morphine-ondansetron and meperidine-ondansetron mixtures, respectively, since they provided good accuracy and precision data for the two component mixtures.

The HPLC method for Mixture A showed concentration versus absorbance linearity for morphine-ondansetron in the 134-536 and 68 - 271 μg/mL ranges, respectively, at 233 nm. Table 1 gives the analytical figures of merit for each of the analytes in Mixture A. The HPLC method for Mixture B showed concentration versus absorbance linearity for meperidine-ondansetron in the 555-3331 and 89-536 μg/mL ranges, respectively, at 254 nm. Table 1 also gives the analytical figures of merit for each of the analytes in Mixture B. A photodiode array detector (Model 990, Waters Associates, Milford, MA 01757) was used to verity that none of the degradation products of the analytes in either Mixture A or B (analysed under their respective analytical conditions) interfered with the quantitation of each drug at 233 or 254 nm. These experiments were performed on solutions of each drug in 0.9% sodium chloride injection after they had been degraded for 6 hr at 80°C in both 1.0 N hydrochloric acid and 1.0 N sodium hydroxide.

Percent error and precision of the methods were evaluated using spiked samples containing each analyte. The results for mixtures A and B are shown in Table 2. The results indicate that the procedures give acceptable accuracy and precision for the analytes in both mixtures.

Intra-day variabilities for morphine and ondansetron (Mixture A) expressed as % RSD were 0.57 and 0.79% (n=6), respectively. Inter-day variabilities of the assay for morphine and ondansetron were in the 0.45-0.79 and 0.51-1.03% (n=18 over 3 days) ranges, respectively. Intra-day variabilities for meperidine and ondansetron (Mixture B) expressed as % RSD were 0.98 and 1.04% (n=6), respectively. Inter-day variabilities of the assay for meperidine-ondansetron were in the 0.98-1.49 and 0.48-1.92% (n=18 over 3 days) ranges, respectively.

In summary, an underivatized silica column with an aqueous 0.01 $\underline{M}$ pH 4.0 buffer-methanol mobile phase was shown to be suitable for the separation and quantitation of a morphine-ondansetron mixture (A) and a meperidine-ondansetron mixture (B) in 0.9% sodium chloride injection. This study suggests that the above listed HPLC methods can be used to investigate the chemical stability of the analytes in either mixture.

ACKNOWLEDGEMENTS

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DETERMINATION OF CATECHOLAMINES IN HUMAN PLASMA BY HPLC WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

An assay procedure is described to quantitate low picogram concentrations of catecholamines in human plasma using reversed phase ion-pair HPLC with electrochemical detection. Optimization of chromatographic conditions allows use of samples as small as 0.25 mL. The overall recovery of 78% offers high precision and accuracy at low concentrations. The lower limit of detection is 0.87 pg/mL for epinephrine, 3.5 pg/mL for norepinephrine and 8.3 pg/mL for dopamine.

INTRODUCTION

The exact relationship between dose of intravenously administered dopamine (DA) and hemodynamic effect produced in children is unclear.[1-5] Pharmacokinetic and pharmacodynamic studies on DA and its metabolites epinephrine (E) and norepinephrine (NE) in this area are thus necessary. Such studies in the pediatric population, however, are often difficult to perform due to the limited sample volume available. Low basal concentrations of catecholamines (CA) coupled with small sample size require an assay having high sensitivity and selectivity. Current HPLC assays for CA generally require 1 to 4 mL of sample, limiting their use in pediatric studies.

Radioenzymatic assays offer the ability to measure CA in sample volumes less than 1 mL, but the complexity and poor reproducibility of this technique prevent its widespread use.[6] The combination of electrochemical detection (ECD) with HPLC for the analysis of CA was introduced by Kissinger et al.[7] Hallman et al.[8] applied this technique to cation exchange chromatography for the determination of plasma CA. Introduction of reversed phase ion-pair separations improved the efficiency, sensitivity and versatility of the technique.[9] Various methods of sample enrichment have been developed. Boronyl compounds have been used for sample preparation. Extraction of CA onto boronic acid gel[10] or extraction into organic solvent using diphenylborate as a complexing agent has been reported.[13] Dihydroxyborylsilica has been used for on-line sample preparation.[14] Extraction with organic solvents or adsorption of CA onto cationic exchange columns followed by extraction into organic solvent has been reported.[11] The micro-batch alumina absorption method originally used by Anton and Sayre[12] remains the most simple, economical and widely used technique. According to a recent review,[15] the analytical recovery by this method has been less than 50% for published assays.

We present a method for the determination of CA using alumina extraction. Improved recovery allows sample volumes as small as 0.25 mL.

MATERIAL

Norepinephrine bitartrate, epinephrine bitartrate, dopamine hydrochloride, 3,4-dihydroxybenzylamine hydrobromide (DHBA), Trizma® base (tris[hydroxymethyl]aminomethane), sodium metabisulfite, 1-octane sodium sulfonic acid and Na_2EDTA were obtained from Sigma Chemical Co. (St. Louis, Missouri). Hydrochloric acid, perchloric acid and phosphoric acid were purchased from Fisher Scientific Co. (Fair Lawn, New Jersey).

Acid-washed aluminum oxide (AAO) was purchased from Bioanalytical Systems (West Lafayette, Indiana). Microfilters were from Rainin Instruments (Woburn, Massachusetts).

The HPLC system consisted of a Model 6000A Solvent Delivery System (Waters Associates, Milford, Massachusetts), a Model 7125 injection valve fitted with a 100 μL sample injection loop (Rheodyne Inc., Cotati, California) and a Resolve™ C_{18} (150 x 3.9 mm) column packed with 5 μm spherical particles (Waters Associates, Milford. MA). Column eluant was monitored by an LC-4 amperometric detector equipped with a TL-5 glassy carbon electrode assembly (Bioanalytical Systems, West Lafayette, Indiana). Detector signal was monitored by an HP-3390A electronic integrator (Hewlett-Packard, Corvallis, Oregon).

The flow rate through the column was 1.0 mL/min. The detector electrode was set at 0.65 V vs Ag/AgCl. The noise filter was set at 0.3 Hz. The mobile phase consisted of buffer mixed with methanol. The buffer contained 1 mM Na_2EDTA, 1 mM 1-octane sulfonic acid and 75 mM NaH_2PO_4, adjusted to pH 3.1 using 85% phosphoric acid. The buffer was filtered through a 0.22 μm nylon filter. Methanol was added (4% v/v) and the mobile phase was degassed by sonication under vacuum. The methanol concentration was adjusted to give stable retention times as the column aged. After equilibration, the mobile phase was recycled through the system. This practice gave a more stable baseline and conserved mobile phase. A single batch of mobile phase (4 L) could be used for about one month before being replaced.

All CA stock solutions were prepared in 0.1 N HCl containing 0.1 mM Na_2EDTA. These solutions were stored at 4° C protected from light. They were stable for approximately two months under these conditions. Standard solutions were prepared from the stock solutions daily.

Internal standard stock solution (DHBA, 1.0 mg/mL) was prepared and stored under the same conditions as the CA stock solutions. This solution was stable for approximately 6 months. The working internal standard solution was prepared weekly from the stock.

Tris buffer (1.5 M) was prepared by dissolving 45 g Trizma® base and 5 g Na_2EDTA in 0.250 L filtered deionized water. The pH was adjusted to 8.7 with 3 N HCl. The solution was stored at 4° C until use. Sodium metabisulfite solution (1 mg/mL) was prepared daily.

METHODS

Blood samples (0.5-1 mL) were withdrawn in the supine position via an indwelling catheter into heparinized tubes containing about 3 mg Na_2EDTA per tube. The sample tubes were stored at $4°$ C until centrifugation within one hour of collection. Centrifugation was performed at room temperature for 15 min at 2500 rpm. The separated plasma was transferred into tubes containing sodium metabisulfite crystals (approximately 3 mg/mL plasma). The tubes were stored at $-70°$ C until assayed.

Samples were protected from light during the extraction procedure. Aluminum oxide (AAO 10 mg) was placed into a 5 mL Reactivial®. Tris buffer containing Na_2EDTA (1 mL) was added and the vials were vortex mixed for 30 seconds. Sodium metabisulfite solution (0.1 mL) was added, followed by 40 μL internal standard and 0.25 mL thawed plasma or standard solutions. The vials were mixed on a rotary mixer (Ernest, Model SA7-2424, Lester, Pennsylvania) for 15 minutes.

The AAO was separated from the supernatant by centrifugation at 2500 rpm for 15 minutes. The supernatant was discarded and the AAO was washed twice with cold water on the rotary mixer and separated from the supernatant as above. The water was discarded after each wash. The AAO was then transferred to a microfilter and dried under vacuum.

Perchloric acid (120 μL, 0.1 M) containing 40 μg/mL sodium metabisulfite was added to the AAO with mixing and the samples were incubated at room temperature for 5 minutes. The filters were capped and vortex mixed for 30 seconds. They were centrifuged at 3500 rpm for 7 minutes at $4°$ C. The filtrate was transferred again to the AAO and centrifuged a second time. The collected filtrate was stored on ice until analysis. Sample injection volume was 40 μL. Retention times for NE, E, DA and I.S. were 2.6, 4.6, 9.3 and 5.7 minutes respectively.

RESULTS

The total run time for the determination of CA in plasma was 11 minutes. The reproducibility of the HPLC step was determined by injecting a standard mixture containing NE, E and DA repeatedly for 15 days. The coefficient of variation for these samples was 2.5% for NE at 1.0 ng/mL, 8% for E at 0.5 ng/mL and 5.8% for DA at 2.4 ng/mL.

Table 1

**Reproducibility of Catecholamine Assay Using HPLC-ECD
From Plasma Spiked With Known Amounts of Catecholamines**

Catecholamine	Concentration (pg/mL)	Within-run C.V. (n = 3)	Between-day C.V. (n = 7)
NE	1000	2.4	0.8
	500	3.6	1.3
	100	4.2	1.8
	20	5.4	2.1
	10	6.0	2.9
E	500	7.0	5.0
	200	7.6	5.5
	50	8.2	5.8
	10	9.1	6.1
	2.5	9.7	6.3
DA	2400	4.0	5.4
	1200	4.8	5.7
	400	6.9	6.0
	100	8.0	6.5
	25	9.0	7.2

Within-run and between-day variation were determined by analyzing plasma samples to which a known amount of CA was added. Five concentrations of each CA were analyzed in triplicate over 7 days. Within-run variation ranged from 2.4 to 9.7% and between-day variation was from 0.8 to 7.2%. The results for the individual analytes are presented in Table 1.

Calibration curves constructed for each compound were linear, unbiased and reproducible. Correlation coefficients were all greater than 0.99. The linearity of the detector response was confirmed by injection of standard solutions of known concentration. Response for each compound was linear over the range 0-10 ng/mL for NE, 0-5 ng/mL for E and 0-72 ng/mL for DA. Sensitivity of the assay determined as signal-to-noise ratio of 5 was 1.3 pg/mL NE, 0.3 pg/mL E and 3 ng/mL DA.

Recovery of the extraction procedure was determined by comparing the yields from a series of extracted samples to a solution containing known amounts of CA. Individual recoveries of 77% (NE), 78% (E), 88% (DHBA) and 71% (DA) were achieved. This degree of recovery has not been previously reported for micro-batch alumina extraction.

DISCUSSION

Pharmacokinetic and pharmacodynamic studies of dopamine in children require sensitive assays using small sample volumes. Present methods do not meet these criteria. Those assays using small sample volumes are not sensitive enough to measure baseline levels of circulating DA.[16-18]

HPLC coupled with electrochemical detection is the most commonly used technique for the determination of CA. The method is relatively simple and does not require special instrumentation or extensive chemical modification of the CA structure as do other techniques.

Non-chromatographic techniques such as radioenzymatic assay[6] and ELISA[19] have been employed for the determination of CA. These techniques require a separate assay for each CA of interest.

Microbore HPLC with electrochemical detection is becoming more popular because of both increased sensitivity and reduced sample volume.[20] It has also been combined with microdialysis for the preparation of plasma samples.[21] The current sample preparation method to increase sensitivity is compatible with this technique.

Separation of CA by HPLC followed by detection with post-column derivatization and detection by chemiluminescence[22,23] or fluorescence[24] has also been reported. These procedures introduce more variables into the determination of CA.

Gas chromatography with mass spectrometric detection has been reported,[25] but application of this technique is limited due to the small number of laboratories with this instrumentation available.

Oxidation of the catechols results in poor sensitivity of most assays for the determination of CA due to low recovery of analytes. In the current assay, sodium metabisulfite reduces oxidation of CA and increases analytical recovery from about 50% to almost 80%, resulting in increased sensitivity. Glutathione
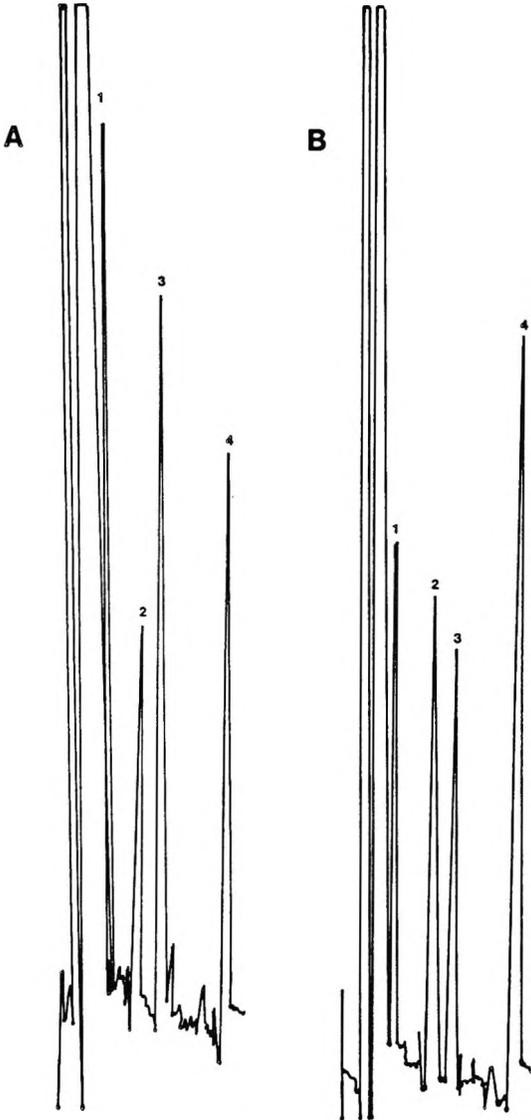
Figure 1. Chromatograms showing the extraction of catecholamines from human serum using AAO: A) with the addition of sodium metabisulfite and B) without the addition of sodium metabisulfite. Peaks: 1) NE; 2) E; 3) I.S., DHBA; 4) DA.

has also been reported to reduce oxidation of catechols during sample preparation.[26] Keeping samples cold during work-up by washing with cold water and storing extracted samples on ice while awaiting injection also helps slow oxidation. Figure 1 presents chromatograms of extracted plasma samples with and without the addition of sodium metabisulfite during the extraction procedure.

Goldstein and Feuerstein[27] suggest useful precautions to improve ECD performance in order to lower the detection limit of epinephrine using 1mL of plasma and injection of total elute. Among these are use of sodium metabisulfite during sample preparation and frequent cleaning or replacement of electrodes. They report a lower limit of 20 pg for E. In the present method, the lower limit of detection for E is 0.87 pg and uses only one third of the eluant from the AAO.

The improvements achieved in reduction of CA loss during the assay offers the opportunity of replicate injections or smaller sample size. The latter factor is especially important when dealing with pediatric patients.

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LIQUID CHROMATOGRAPHY CALENDAR

1996

MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JULY 1 - 3: International Symposium on Polymer Analysis and Characterization, Keble College, Oxford University, U.K. Contact: Prof. J. V. Dawkins, Dept. of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire, LE11 3TU, U.K.

JULY 14 - 18: 5th World Congress of Chemical Engineering, Marriott Hotel, San Diego, California. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 9 - 14: 31st Intersociety Energy Conversion Engineering Conference (co-sponsored with IEEE), Omni Shoreham Hotel, Washington, DC. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 17 - 20: 31st National Heat Transfer Conference, Westin Galleria, Houston, Texas. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization," Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

SPETEMBER 7: Field-Flow Fractionation Workshop VIII, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy.

SEPTEMBER 9 - 11:   Sixth International Symposium on Field-Flow Fractionation, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy.

SEPTEMBER 9 - 12:   Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

SEPTEMBER 15 - 20: 21st International Symposium on Chromatography, Stuttgart, Germany. Contact: Dr. L. Kiessling, Geselleschaft Deutscher Chemiker, Postfach 900440, D-60444 Frankfurt/Main, Germany.

OCTOBER 16 - 19:   52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

APRIL 14 - 19: Genes and Gen Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada.
Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899,
USA.

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

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact:
ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact:
ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact:
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SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact:
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Following are acceptable reference formats:

Journal:

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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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