JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 17 NUMBER 8 1994

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JOURNAL OF LIQUID CHROMATOGRAPHY

April 1994

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Identification Statement. *Journal of Liquid Chromatography* (ISSN: 0148-3919) is published semimonthly except monthly in May, July, October, and December for the institutional rate of $1,350.00 and the individual rate of $675.00 by Marcel Dekker, Inc., P.O. Box 5005, Monticello, NY 12701-5185. Second Class postage paid at Monticello, NY. POSTMASTER: Send address changes to *Journal of Liquid Chromatography*, P.O. Box 5005, Monticello, NY 12701-5185.

Volume	Issues	Institutional Rate	Individual Professionals and Student Rate	Foreign Postage		
				Surface	Airmail to Europe	Airmail to Asia
17	20	$1,350.00	$675.00	$75.00	$110.00	$130.00

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CODEN: JLCHD8 17(8) i-iv, 1665-1860 (1994)
ISSN: 0148-3919

Printed in the U.S.A.

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Contributions to this journal are published free of charge.

Effective with Volume 6, Number 11, this journal is printed on acid-free paper.

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1665-1677 (1994)

CHIRAL RECOGNITION OF *N*-ACYL-1-(2-FLUORENYL)-1-AMINOALKANES BY π-ACIDIC CHIRAL STATIONARY PHASES: A MECHANISTIC VIEW

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ABSTRACT

The enantiomers of *N*-acyl-1-(2-fluorenyl)-1-aminoalkanes are separable on a number of chiral stationary phases derived from *N*-(3,5-dinitrobenzoyl)amino acid esters and amides. Previously, these separations and some structure-dependent inversions in the elution order of the enantiomers of these analytes were rationalized on the basis of two competing chiral recognition mechanisms, one involving stacking of amide dipoles, the other hydrogen bonding. To rationalize data obtained using several new chiral stationary phases not available at the time of the original study, the original mechanistic proposal accounting for the origins of chiral recognition for these analytes is now modified. Two competing hydrogen bonding mechanisms, each modified by attendant intercalation effects, are thought to better account for the experimental observations.

INTRODUCTION

"The matter is a perfectly trivial one but there are points in connection with it which are not entirely devoid of interest and even of instruction." [1]

The liquid chromatographic separation of enantiomers using chiral stationary phases (CSPs) is perhaps one of the most promising yet most

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underappreciated tools currently available for the study of molecular recognition processes. In this laboratory, chromatography of homologous series of racemates on CSPs has afforded data from which structure-enantioselectivity relationships may be extracted. These relationships aid in both the understanding of chiral recognition processes and in the design of new chiral stationary phases. In several instances, an inversion in the elution order of enantiomers has been noted when one chromatographs a series of analytes in which the length of a given alkyl substituent is progressively increased [2, 3]. For example, such inversions occur when homologous series of racemic *N*-acyl-1-(2-fluorenyl)-1-aminoalkanes, shown in Figure 1, are chromatographed on a several different *N*-(3,5-dinitrobenzoyl)amino acid amide-linked CSPs. The point in the series at which the inversion occurs depends upon the length and size of the alkyl substituent on the stereogenic center (R_1) and the length of the *N*-acyl group (R_2).

Data from the original study were rationalized by suggesting that two competing opposite sense mechanisms might be operative, the contributions of each process to the overall retention of each enantiomer being influenced by the analyte's alkyl and *N*-acyl substituents. Both processes were thought to involve face-to-face π-π interaction between the selector's 3,5-dinitrobenzoyl group and the analyte's 2-fluorenyl system. Additionally, hydrogen bonding and "dipole stacking" of carboxamide groups were proposed to augment the π-π interactions [3]. Dipole-dipole interaction is well documented, and the antiparallel "stacking" of amide dipoles was suggested as possibly aiding the face-to-face approach of analyte and selector. No evidence contrary to the stacking of amide dipoles in this system has ever been presented, and the concept has been taken up (in other systems) by others [4-7]. However, additional observations and experience suggest that refined versions of the original rationale better account for the experimental data. These data, acquired using CSPs not available at the time of the original publication, along with an increased awareness of the importance of intercalative effects, lead us to modify the original rationale. The principle modification is to forego the suggestion that dipole stacking plays an essential role in the chiral recognition of these analytes and to incorporate the view that both faces of the selector's 3,5-dinitrobenzoyl system may be approached by the analyte enantiomers, although not necessarily with equal ease. Differential ease of approach to the two faces of a dinitrobenzoyl group has always been an essential feature of our chiral recognition models. However, the consequences of analyte approach to the more hindered face of the

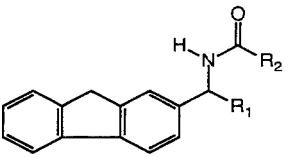


Figure 1. Structure of the analytes used in the investigation

selector have been considered explicitly only in a few instances, and then only to maintain that such approach is less likely and not apt to be the dominant contributor to chiral recognition [7, 8]. In the modified rationale, both analyte enantiomers are presumed capable of face-to-face π-π interactions with either face of the dinitrobenzoyl system. Moreover, both analyte enantiomers are thought to participate in hydrogen bonding interactions as well, although different modes of hydrogen bonding may be employed by each enantiomer. Intercalative interactions between the analyte's *N*-acyl or alkyl substituent are thought, as in the earlier rationale, to influence the relative extents to which each enantiomer binds to either face of the selector.

EXPERIMENTAL

Chromatography

Chromatography was performed with an Aspec-Bischoff model 2200 isocratic HPLC pump, a Rheodyne Model 7125 injector equipped with a 20 µl sample loop, a Milton-Roy LDC UV Monitor D™ fixed wavelength detector operating at 254 nm, and a Hewlett-Packard 3394A recording integrator. Signs of rotation were measured using an Aspec-Bischoff model 2200 isocratic HPLC pump, a Rheodyne Model 7125 injector equipped with a 20 µl sample loop, a Milton-Roy LDC UV Monitor D™ fixed wavelength detector operating at 254 nm in series with a Rudolph Autopol III digital polarimeter equipped with a 20 cm flow cell, and a Kipp and Zonen BD 41 dual channel recorder. Void volumes were determined using dodecane.

The Chiral Stationary Phases

A commercially available column (Regis Chemical Co., Morton Grove, IL) containing CSP 1 was employed. CSP 3 was prepared using a modification of the procedure used to prepare CSP 1 [9]. The preparation of CSPs 2 and 4 is reported elsewhere [10, 11].

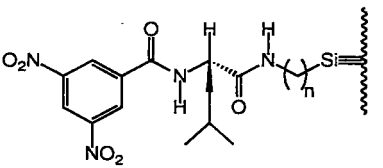
The Analytes

All *N*-acyl-1-(2-fluorenyl)-1-aminoalkanes were available from prior studies [3].

RESULTS AND DISCUSSION

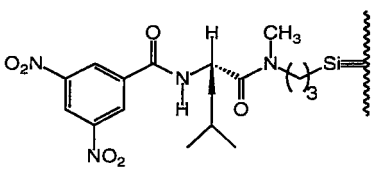
The structures of the CSPs used in the present investigation are shown in Figure 2.
CSP 2 differs from CSP 1 in that the length of the tether which anchors the phase to the silica support contains eleven methylene groups instead of three. CSP 3 differs from CSP 1 only in that the C-terminal carboxamide bears a methyl group instead of a hydrogen on nitrogen. This amide N-H is thought to be unnecessary for chiral recognition of a number of analytes. Since non-essential interactions sites are detrimental to the enantioselectivity of a CSP, this amide N-H was replaced by a less interactive methyl group. Indeed, CSP 3 typically provides decreased retention and increased enantioselectivity relative to CSP 1. CSP 4 was designed to mimic CSP 3, except that it is oriented differently with respect to the underlying silica support.

Data obtained when several series of (*R*,*S*)-*N*-acyl-1-(2-fluorenyl)-1-aminoisobutanes are chromatographed on CSPs 1, 2, 3 and 4 are shown in Table 1. As indicated by the signs of rotation, an inversion in the elution order of the enantiomers occurs on CSP 1 when the acyl group reaches three carbons in length. The (*R*)-(+)-enantiomers are more retained when the acyl group is short, and the (*S*)-(-)-enantiomers are more retained throughout the remainder of the homologous series. On CSP 2, no such inversion takes place, the (*S*)-enantiomers being the more retained throughout the series. Similar trends are observed when these analytes are chromatographed on CSPs 3 and 4, although a few early members of the series are not resolved on CSPs 3 and 4.

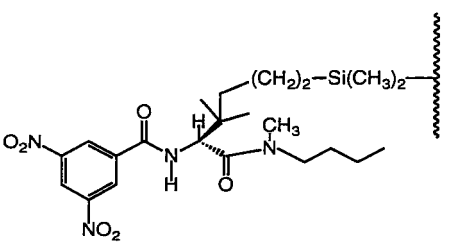


CSP 1: "n" = 3

CSP 2: "n" = 11



CSP 3



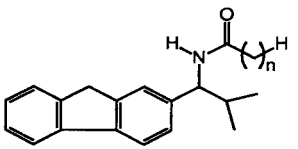
CSP 4

Figure 2. Structures of the chiral stationary phases (CSPs) used in the investigation

Data obtained when the enantiomers of various *N*-butanoyl-1-(2-fluorenyl)aminoalkanes are chromatographed on CSPs 1-4 are presented in Table 2. To a great extent, mechanistic studies have focused on the primary modes of retention of the more retained enantiomer, since the less retained enantiomer forms less stable and, in all likelihood, a greater variety of adsorbates. Situations in which some anomalous event (such as an inversion in the elution order of enantiomers) manifests itself are unusually rich in information and any mechanistic

TABLE 1

Separation of the Enantiomers of *N*-Acyl-1-(2-fluorenyl)-1-aminoisobutane on CSPs 1, 2, 3 and 4

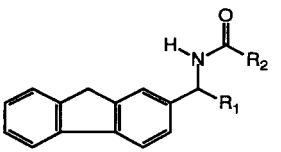


	CSP 1			CSP 2			CSP 3			CSP 4		
"n"	k'_1	α	$[\alpha]_D$ (*)	k'_1	α	$[\alpha]_D$ (*)	k'_1	α	$[\alpha]_D$ (*)	k'_1	α	$[\alpha]_D$ (*)
0	4.25	1.34	(+) (*R*)	2.35	1.17	(-) (*S*)	3.61	1.00		2.49	1.00	
1	8.82	1.30	(+) (*R*)	3.56	1.12	(-) (*S*)	5.70	1.00		2.53	1.18	(-) (*S*)
2	8.91	1.00		3.04	1.34	(-) (*S*)	3.79	1.18	(-) (*S*)	1.67	1.45	(-) (*S*)
3	7.00	1.03	(-) (*S*)	2.71	1.35	(-) (*S*)	2.95	1.20	(-) (*S*)	1.41	1.47	(-) (*S*)
4	6.05	1.15	(-) (*S*)	2.63	1.37	(-) (*S*)	2.48	1.27	(-) (*S*)	1.21	1.47	(-) (*S*)
6	4.36	1.27	(-) (*S*)	2.19	1.41	(-) (*S*)	1.86	1.33	(-) (*S*)	0.97	1.51	(-) (*S*)
7	3.86	1.37	(-) (*S*)	2.19	1.40	(-) (*S*)	1.71	1.36	(-) (*S*)	0.73	1.52	(-) (*S*)
9	3.18	1.47	(-) (*S*)	1.84	1.43	(-) (*S*)	1.51	1.38	(-) (*S*)	0.57	1.56	(-) (*S*)
13	2.41	1.58	(-) (*S*)	1.54	1.48	(-) (*S*)	1.21	1.44	(-) (*S*)	0.43	1.53	(-) (*S*)
17	1.95	1.68	(-) (*S*)	1.35	1.51	(-) (*S*)	1.03	1.47	(-) (*S*)	0.32	1.53	(-) (*S*)

On CSPs 1, 2 and 3, the mobile phase consists of 20% 2-propanol in hexane; on CSP 4 the mobile phase consists of 10% 2-propanol in hexane; "n" = number of methylene units in analyte's acyl substituent; k'_1 = capacity factor for the first eluted enantiomer; α = chromatographic separation factor; $[\alpha]_D$ = sign of rotation for the more retained enantiomer, (*) = absolute configuration of the more retained enantiomer

TABLE 2

Separation of the Enantiomers of *N*-Butanoyl-1-(2-fluorenyl)-1-aminoalkanes on CSPs 1, 2, 3 and 4



		CSP 1			CSP 2			CSP 3			CSP 4		
R_1	R_2	k'_1	α	$[\alpha]_D$ (*)	k'_1	α	$[\alpha]_D$ (*)	k'_1	α	$[\alpha]_D$ (*)	k'_1	α	$[\alpha]_D$ (*)
CH_3-	C_3H_7	9.17	1.42	(-) (*S*)	3.44	1.46	(-) (*S*)	4.32	1.33	(-) (*S*)	2.91	1.43	(-) (*S*)
$(CH_3)_2CH_2$-	C_3H_7	7.00	1.03	(-) (*S*)	2.71	1.35	(-) (*S*)	2.95	1.20	(-) (*S*)	1.41	1.47	(-) (*S*)
C_6H_{13}-	C_3H_7	5.18	1.11	(+) (*R*)	2.43	1.35	(-) (*S*)	2.32	1.17	(-) (*S*)	0.95	1.54	(-) (*S*)

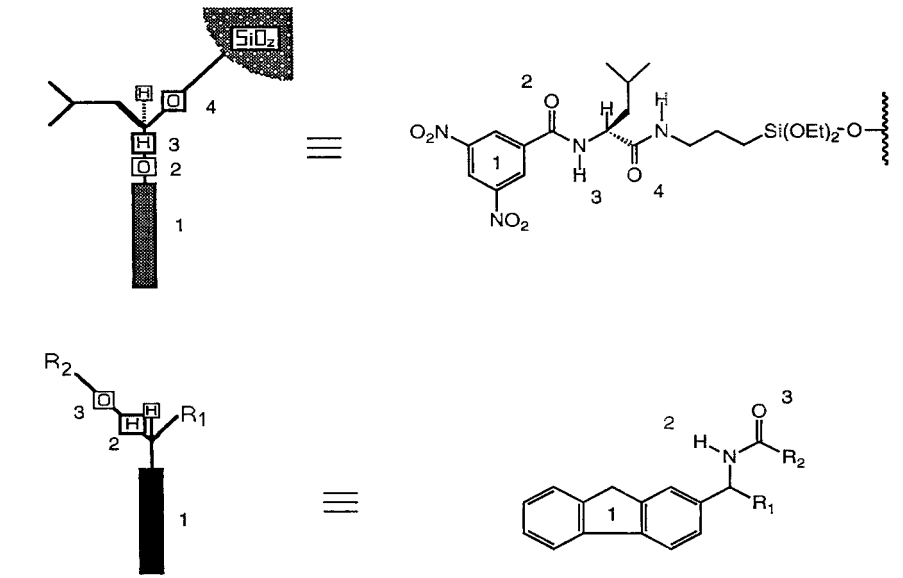
On CSPs 1, 2 and 3, the mobile phase consists of 20% 2-propanol in hexane; on CSP 4 the mobile phase consists of 10% 2-propanol in hexane; k'_1 = capacity factor for the first eluted enantiomer; α = chromatographic separation factor; $[\alpha]_D$ = sign of rotation for the more retained enantiomer, (*) = absolute configuration of the more retained enantiomer

hypothesis must account for the anomaly to be viable. Moreover, a rationale consistent with a large body of data is more compelling than one which accounts for but a few observations. In the present study, the length of the analyte's acyl substituent differentially influences the stabilities of the diastereomeric adsorbates formed with CSP 1. When the length of the acyl group is held constant and the size of the alkyl group on the stereogenic center is varied, enantioselectivity and elution order can be altered. However, on CSPs 2, 3 and 4, all of which are but slight structural variations of CSP 1, this behavior is not observed. The retention of each enantiomer is affected by contributions from the numerous processes by which the enantiomers interact with the CSP. Not all such processes are equally probable,

and it is instructive to limit discussion to the one or two modes of interaction between each enantiomer and the CSP which are thought to be the primary contributors to retention. Upon these basic models are then superimposed those subtle factors which influence the contribution of each of the competing processes.

How might the (*S*)-enantiomers of the present class of analytes interact with CSP 1? Examination of space-filling models augmented by conformational analysis suggests that these enantiomers may appoach the less hindered faces of CSPs 1, 2 and 3 (*i.e.* that face not presenting the isobutyl group) and in so doing participate in 1) a face-to-face π-π interaction between the 3,5-dinitrobenzoyl group and the π-basic fluorenyl moiety, and 2) a hydrogen bond between the 3,5-dinitrobenzamide N-H and the amide carbonyl oxygen of the analyte. This orients the methine hydrogen of the analyte toward the chiral selector and the bulky isopropyl substituent away from the selector, more or less toward the underlying silica support. The (*S*)-enantiomer's acyl group (R_2) is directed away from the tether and silica support and into the bulk mobile phase. A cartoon-like representation of the CSPs and the analytes is introduced in Figure 3, and the situation described above is represented using this convention in Figure 4.

The (*R*)-enantiomer, if it approaches the CSP from its less sterically congested face, cannot enjoy the same bonding interactions as does its antipode unless it adopts a higher energy conformation where the bulky isopropyl group on the stereogenic center must eclipse (approximately) the amide carbonyl oxygen. However, by approaching the CSPs dinitrobenzoyl group from its more hindered face (the one *syn* to the isobutyl group), this enantiomer can be oriented so as to place its (small) methine hydrogen toward the CSP's isobutyl group, undergo a face-to-face π-π interaction between the 3,5-dinitrobenzoyl group and the π-basic fluorenyl moiety, and maintain a hydrogen bond between the 3,5-dinitrobenzamide N-H and the analyte's amide carbonyl oxygen. In so doing, the (*R*)-enantiomer now enjoys the same interactions with the CSP as does the (*S*)-enantiomer, while placing the isopropyl group on the analyte's stereogenic center in a position where it undergoes little steric encumbrance. However, this also requires that the (*R*)-enantiomer direct its acyl group alongside the tether and toward the silica support. In normal phase solvents, this "intercalation" process is energetically costly and reduces the stability of that diastereomeric complex. Moreover, the extent of this reduction in stability increases with the length of the acyl group. Approach of the analyte from this face of the CSP is illustrated in Figure 5.



Group is oriented toward the viewer

Group is oriented away from the viewer

Figure 3. Legend to accompany Figures 4 and 5.

If one accepts the foregoing, it follows that Hydrogen Bonding Scheme B (Figure 5) is more exergonic than Hydrogen Bonding Scheme A (Figure 4) when both the alkyl group and the acyl group of the analyte are short, since the (*R*)-enantiomer is the more retained on CSP 1. As the acyl group (R_2) of the (*R*)-enantiomer becomes longer, the complex is rendered less stable by intercalative effects, the retention of the (*R*)-enantiomers is reduced relative to their antipodes, and, eventually, an inversion of the elution order occurs.

The data in Table 2 are consistent with intercalation of the alkyl group (R_1) on the stereogenic center of the (*S*)-enantiomers of these analytes. Progressive

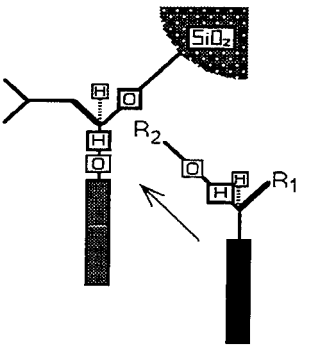


Figure 4. Hydrogen Bonding Scheme "A": The (*S*)-enantiomers of the analytes may approach the CSP from the less hindered face. The essential bonding interactions are described in the text.

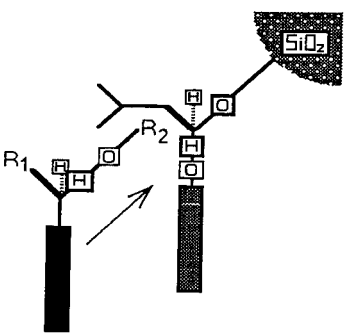


Figure 5. Hydrogen Bonding Scheme "B": The (*R*)-enantiomers of the analytes may approach the CSP from the more hindered face, causing intercalation of R_2. The essential bonding interactions are described in the text.

lengthening of this group likewise produces an inversion in the elution order of the enantiomers of (*R*,*S*)-*N*-butanoyl-1-(2-fluorenyl)-1-aminoalkanes. This, in effect, restores the original elution order as the effects of intercalation of the longer alkyl substituents of the (*S*)-enantiomers more than compensate for the intercalation of the butanoyl group of the (*R*)-enantiomers. The (*S*)-enantiomers, however, are the more retained throughout the series on CSPs 2, 3 and 4.

Thus, the data in Tables 1 and 2, when taken collectively, imply that CSPs 2, 3 and 4, by virtue of their structural dissimilarities to CSP 1, relieve the energetic consequences of intercalation of R_1 during Hydrogen Bonding Scheme A and the energetic consequences of intercalation of R_2 during Hydrogen Bonding Scheme B. How might this occur?

Owing to the long tether utilized to anchor CSP 2 to the silica support, the effect of intercalation of either R_1 or R_2 is never severe enough to produce an inversion in elution order. The complexes formed between the (*S*)-enantiomers of the analytes and CSP 2 are more stable than those involving the (*R*)-enantiomers, regardless of the length of R_1 or R_2. On CSPs 1 and 3, the short three-carbon tether causes the energetic consequences of intercalation to be greater, thereby more profoundly influencing retentions. However, the effects of intercalation are not so evident on CSP 3 as they are on CSP 1. This difference is suspected to stem from the fact that the C-terminal amide of CSP 1 has a very strong preference for the "Z"-amide rotamer whereas CSP 3 presumably has no great preference for either rotamer. Examination of the ^1H NMR spectrum of the silane precursor to CSP 3 in $CDCl_3$ indicates that the Z : E ratio for this amide is approximately 1:1. Furthermore, this ratio depends upon solvent and temperature and may be altered by complexation with an analyte. It appears likely, based on the inspection of space-filling models, that the intercalation problem introduced by Hydrogen Bonding Scheme A is less severe in the E-rotamer than in the Z-rotamer. For this reason, separation of the analyte enantiomers on CSP 3 occurs only when the R_2 groups on the (*R*)-enantiomers are long enough so that their intercalation has significant energetic consequences. As a consequence of this, no elution order inversions are noted on CSP 3 and enantioselectivity increases steadily throughout the homologous series. On CSP 4, contributions to retention from Hydrogen Bonding Scheme B should be minor, since access to the more hindered face of the CSP should be effectively blocked by the surface of the silica itself. Again, no inversion in the elution order of the enantiomers is detected as the lengths of the R

substituents are increased. Note that the retention of both enantiomers drops rather rapidly as the lengths of the acyl groups (R_2) are increased. No dependence of elution order of the enantiomers on the length of R_1 is noted with CSP 4, since the orientation of this CSP relative to the silica support precludes any intercalative effects by this group.

CONCLUSION

It is important to understand those factors which are capable of altering the elution order of enantiomers from chiral stationary phases if absolute configurations are to be assigned from observed elution orders. Moreover, such understanding is crucial to the rational design of new chiral stationary phases. It is herein demonstrated that relatively small structural changes in a CSP can sometimes influence elution order of enantiomers from that CSP and that the elution orders of analytes in a homologous series may depend upon the influence of subtle factors possibly in delicate balance. In such instances, enantioselectivities are apt to be modest. While one would perhaps wish to avoid such circumstances, this can only be done if one recognizes that the CSP being used is a poor choice for the analytes of interest. A clear understanding of the origins of chiral recognition is a great aid in selecting an appropriate CSP. As our studies progress, ideas on the origins of chiral recognition evolve and become more sophisticated. The present modification of the early rationale is felt to be a closer approximation of reality and to better accommodate a body of data.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support from the NSF, Eli Lilly and Company, and Glaxo Research Laboratories. Solvents employed for the chromatography were generously supplied by EM Science, a Division of EM Merck.

REFERENCES

1. A.C. Doyle, *The Blue Carbuncle,* first published in 1891, republished in Sherlock Holmes: The Complete Novels and Stories, Volume I, Bantam Books, Inc., New York, 1986. Sherlock Holmes says this to his friend and colleague, Dr. Watson.

2. W.H. Pirkle, M.H. Hyun, and B. Bank, J. Chromatogr. 316 585-604 (1984).

3. W.H. Pirkle and R. Dappen, J. Chromatogr. 404 107-115 (1987).

4. I.W. Wainer and T.D. Doyle, J. Chromatogr. 284 117 (1984).

5. D.A. Nicoll-Griffith, J. Chromatogr. 402 179-187 (1987).

6. R. Dernoncour and R. Azerad, J. Chromatogr. 410 355-361 (1987).

7. G. Uccello-Barretta, C. Rosini, D. Pini and P. Salvadori, J. Am. Chem. Soc. 112 2707-2710 (1990).

8. K.B. Lipkowitz and R. Zegarra, J. Comput. Chem., 10(5) 718-732 (1989).

9. W.H. Pirkle, A.Tsipouras and T.J. Sowin, J. Chromatogr. 319 392-395 (1985).

10. C.J. Welch, Ph.D. Thesis, University of Illinois, 1992.

11. W.H. Pirkle, P.G. Murray and J.A. Burke, J. Chromatogr., 641 21-29 (1993).

Received: January 6, 1994
Accepted: January 14, 1994

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1679-1694 (1994)

SEPARATION OF MONOACYLGLYCEROL ENANTIOMERS AS URETHANE DERIVATIVES BY CHIRAL-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High Performance Liquid Chromatographic (HPLC) separation of monoacylglycerol enantiomers, as [di-]3,5-dinitrophenyl isocyanate (urethane) derivatives, was carried out on a chiral column containing N-(R)-1-(α–naphthyl)ethylaminocarbonyl-(S)-valine chemically bound to γ-aminopropyl silanized silica as stationary phase.In addition to the separation of commercial rac-, sn-2- and sn-3-monoacylglycerols with saturated and unsaturated acyl groups, the analysis of natural source mixtures of sn-1(3)-monoacylglycerols obtained by partial chemical and enzymatic deacylation, was reported. These natural source monoacylglycerols, originated from peanut and cottonseed oil triacylglycerols isolated by combination of argentation-TLC and reversed-phaseHPLC. They were first fractionated by HPLC according to chain length, unsaturation and partially according to positional isomerism (sn-2- separated from sn-1(3)-monoacylglycerol) and then derivatized. An isocratic elution at ambient temperature with mixtures of hexane- ethylene dichloride- ethanol was used for the separation of the monoacylglycerol-isomers, which were detected by their refractive indices or their UV absorption. The sn-1- and sn-3-isomers of a racemic mixture were very well separated, so as the sn-2- and sn-3-isomers. On the other hand, the separation between the sn-2- and sn-3-isomers was generally incomplete. The observed elution order of the three

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isomers was sn-1- followed by sn-2- and finally sn-3-monoacylglycerol. In complex mixtures, the separation of the isomers differing by two acyl carbon number or one double bond was very poor. The elution order indicates that for each isomer the retention time increases with decreasing chain length and increasing unsaturation of the constituent fatty acid. The relationships between the logarithmic retention volumes against the partition number in each homologous series of isomers were practically linear. The enantiomer composition of racemic mixtures, calculated from peak areas was very close to the composition expected, indicating that chiral-phase HPLC analysis of monoacylglycerol enantiomers could be used to study the stereospecific distribution of fatty acids in natural oil triacylglycerols.

INTRODUCTION

Theoretically the best method for studying the stereospecific distribution of fatty acids in the triacylglycerol molecules would be to determine the fatty acid profiles of the three sn-1, sn-2 and sn-3 monoacylglycerol stereoisomers unspecifically formed by an appropriate deacylation procedure.

Natural triacylglycerols generally generate mixtures of monoacylglycerol isomers too complex to be analyzed in one step. Analysis by reversed-phase HPLC allows separations according to chain length, unsaturation and partially to position isomerism since sn-2-isomers can be separated from the sn-1 and sn-3 groups together eluting [1-3]. The different monoacylglycerol isomers are sufficiently well separated to be collected with minor contamination.

The group of sn-1(3)-isomers need further analysis for the two enantiomers to be separated. Ôi et al. [4-6] have developed different chiral stationary phases for high-performance liquid chromatography (HPLC) which show characteristic enantioselectivities for enantiomer derivatives of amino-acids, carboxylic acids and alcohols. Two of these phases present as chiral selectors, N-(S)-2-(4-chlorophenyl)isovaleroyl D-phenyl glycine and N-(R)-1-(α–naphthyl)ethylaminocarbonyl-(S)-Valine respectively. Both have been used by Takagi and coworkers [7-10] to separate monoalkyl- and monoacylglycerol enantiomers previously derivatized with 3,5-dinitrophenyl isocyanate (urethane derivatives)

Regarding results obtained by these authors, we have worked out separations of monoacylglycerol optical isomers as urethane derivatives by chiral phase HPLC. The

method was more particularly applied to mixtures obtained after partial chemical deacylation of natural triacylglycerols. Results show that urethane derivatives of monoacylglycerol enantiomers can be accurately analyzed by this method, both qualitatively and quantitatively.

EXPERIMENTAL

Samples

Monoacylglycerols were from commercial and natural sources :

i) Optically active sn-3-monopalmitoylglycerol (sn-3-16:0) was from Fluka AG (Buchs, Switzerland). Rac-1-monopalmitoylglycerol (rac-1-16:0), rac-1-monostearoylglycerol (rac-1-18:0), rac-1-monooleoylglycerol (rac-1-18:1), rac-1-monolinoleoylglycerol (rac-1-18:2) were purchased from Serdary (London, Ontario, Canada), as were optically inactive sn-2-isomers, namely sn-2-monopalmitoylglycerol (sn-2-16:0) and sn-2-monooleoylglycerol (sn-2-18:1). These monoacylglycerols were used without prior purification.

ii) Natural source monoacylglycerol samples were prepared by partial chemical deacylation [11, 12] and enzymatic deacylation [13] of oil triacylglycerols. Those were isolated from peanut oil and cottonseed oil by a combination of argentation-TLC and reversed-phase HPLC [14-16]. They comprised palmitoyldioleoylglycerol (16:0 18:1 18:1), trioleoylglycerol (18:1 18:1 18:1), palmitoyloleoyllinoleoylglycerol (16:0 18:1 18:2), dioleoyllinoleoylglycerol (18:1 18:1 18:2), oleoyldilinoleoylglycerol (18:1 18:2 18:2) isolated from peanut oil. Palmitoyloleoyllinoleoylglycerol was also isolated from cottonseed oil for comparison with peanut oil and with results previously obtained [17]. Monoacylglycerols were separated from the other hydrolysis products by TLC on silicagel G (Merck, Darmstadt, Germany) impregnated with 5 % (w/w) boric acid [11, 18]. The plates were developed with petroleum ether / diethyl ether (50 / 50 ,v/v). The monoacylglycerol mixtures were fractionated by reversed-phase HPLC according to chain length, to unsaturation and partially according to positional isomerism, i.e. the sn-2 isomers were separated from the group of sn-1(3)-isomers, as previously described [3]. The sn-1(3)-monoacylglycerols were collected at the outlet of the detector and derivatized.

Preparation of urethane derivatives

Monoacylglycerol 3,5-dinitrophenyl urethane derivatives were prepared according to the procedure described by Ôi and Kitahara for derivatization of chiral alcohols [19] and adapted to monoacylglycerols by Takagi and Itabashi [7, 9, 10].

Twenty µmol (ca 7 mg) of monoacylglycerols were dissolved in 450 µl of dry toluene in a 0.5 ml glass vial with teflon linked screw cap. To this solution were added 45 µl (ca 10 mg) of 3,5-dinitrophenyl isocyanate powder (Sumitomo, Osaka, Japan) and 45 µl of dry pyridine. The mixture was heated at 70 °C for 1 h in an oven (or left for 3 h without heating) with occasional shaking. At the end of the reaction, the solution was cooled and the solvent was removed under nitrogen. The resulting urethane derivatives were dissolved in 0.2 ml of chloroform and purified by TLC on silicagel 60 F 254 precoated plates (20 cm x 20 cm, 0.25 mm thick) from Merck. The plates containing a fluorescence indicator were previously activated for 1 h at 110 - 120 °C in an oven. They were developed using a mixture of hexane / ethylene dichloride (or dichloromethane) / ethanol (40/10/3, v/v/v). The plates were dried under nitrogen. The monoacylglycerol derivatives were revealed under UV light (254 nm), delineated and the corresponding silicagel was scraped off the plate. The urethane derivatives were extracted from the adsorbent with diethyl ether.

Alternatively the crude urethane derivatives of monoacylglycerols were purified by reversed-phase HPLC instead of TLC. In this case, at the end of the derivatization reaction, the mixture of urethane derivatives, remaining reagent, toluene and pyridine was left to decant. The limpid upper phase was filtered through hyperfine glasswool. The solvent was evaporated under nitrogen and the monoacylglycerol derivatives were dissolved in chloroform for storage or in acetonitrile (or the mixture corresponding to the HPLC mobile phase) for fractionation.

Liquid chromatography

The sn-1- and sn-3-isomers were separated on a chiral column, 250 mm L x 4 mm I.D., packed with 5 µm particles of N-(R)-1-(α-naphthyl)ethylaminocarbonyl-(S)-valine chemically bound to γ-aminopropyl silanized silica, OA-4100 (Sumitomo, Osaka, Japan). It was installed on a liquid chromatograph Waters (Milford, MA, USA) comprising a model 6000 A solvent delivery system and a Model 450 variable wavelength UV detector. A Lichrocart 4-4 guard column filled with Lichrosorb Si 60 (Merck) was attached at the column inlet.

The analyses were carried out isocratically using hexane/ ethylene dichloride (or dichloromethane) / ethanol (40/12/3 ,v/v/v) at a constant flow rate of 1.0 ml min^{-1} and at ambient temperature. Peak areas were measured by means of an Enica 21 calculator-integrator (Delsi, Suresnes, France).

Definitions

Separation of monoacylglycerol derivatives were characterized by several parameters :

i) The retention time (min) and the retention volume (ml) corrected from the column void volume.

ii) The separation factor [20] between two peaks expressed as the ratio of their retention times (or volumes).

iii) The resolution factor [20] calculated from the formula :

$$R_s = 2 (t_{R2} - t_{R1}) / (w_2 + w_1)$$

in which t_R is the retention time of two peaks 1 and 2 (eluting in that order) and w the peak width at baseline.

Monoacylglycerol molecules were characterized by their equivalent carbon number (ECN) calculated from the formula :

$$ECN = CN - 2\,DB \quad [21]$$

where CN is the acyl carbon number and DB the acyl double-bond number.

RESULTS AND DISCUSSION

Derivatives

As previously stated [7, 9, 10], the monoacylglycerols react very rapidly to completion with the derivatization 3,5-dinitrophenyl isocyanate reagent provided they were completely dissolved in the solvent (dry toluene) in the presence of pyridine. Moreover this reaction does not induce isomerization. However the urethane derivatives of unsaturated monoacylglycerol are more liable to be degraded than the underivatized molecules, so that they should be analyzed shortly after they have been synthetized.

Separation

Figure 1 shows the chromatograms registered in the analysis of rac-monopalmitoylglycerol (rac-16:0 or sn-1(3)-16:0) (panel A), of the single enantiomer sn-3-monopalmitoylglycerol (sn-3-16:0) (panel B) and of the sn-2-monopalmitoylglycerol (sn-2-16:0) (panel C). The chromatograms show separation of sharp and symmetrical peaks within moderate retention times (less than 20 min). The chromatogram in panel A shows that the rac-16:0, which was eluted as a single peak by reversed-phase HPLC [3] was now separated into two well-separated peaks in the presence of the chiral phase OA-4100. The retention time of sn-3-16:0 (panel B) corresponded to that of the second peak, indicating that the elution order of the two enantiomers was sn-1 and sn-3. The separation of the two antipodes brought about by this chiral phase was very high since the observed separation and resolution factors were 1.35 and 4.31 respectively. It was much better than the separation obtained by Itabashi and Takagi [7-9] in the presence of another chiral phase OA-2100, since the separation and resolution factors were only 1.19 and 2.58 respectively. However these authors recently achieved the same good separation of sn-1 and sn-3 monoacylglycerols [10] as we did on the same chiral phase. This proves that the chiral selector enantioselectivity of N-(R)-1-(α-naphthyl)ethylaminocarbonyl-(S)-valine (OA-4100 from Sumitomo) towards monoacylglycerols is higher than of N-(S)-2-(4-chlorophenyl)isovaleroyl-D-phenylglycine (OA-2100 from Sumitomo).

Analysis of the sn-2-monopalmitoylglycerol (Panel C) showed that this isomer was eluted just after the sn-1-isomer on the chiral phase. The presence of traces of the sn-1- and sn-3-isomers in this sample confirms that the elution sequence of sn-1, sn-2, sn-3 was in that order. It also shows that separation of the sn-1- and sn-2-monopalmitoylglycerols was incomplete. This is more evident in Fig. 2 illustrating the separation of a mixture of the 3 monopalmitoylglycerol isomers. In comparison the chiral-phase OA-2100 allowed a more clearcut separation of the 3 isomers [9].

The more or less pronounced retention of the two antipodes sn-1- and sn-3-monopalmitoylglycerols is governed by their diastereoisomeric interactions with the chiral phase. The interaction was higher with the sn-3-isomer on both phases but the difference was more pronounced with the OA-4100 chiral column. Retention of the sn-2-isomer seems more related to the lesser polarity of the molecule versus the polarity of the chiral phase silica support, as can be observed in TLC on silicagel [22]. This could suggest that between the two chiral phases, OA-4100 is less polar than OA-2100.

Resolution between the sn-1- and the sn-2-isomers could be improved by decreasing the analysis temperature or decreasing the mobile phase polarity (lesser proportion of

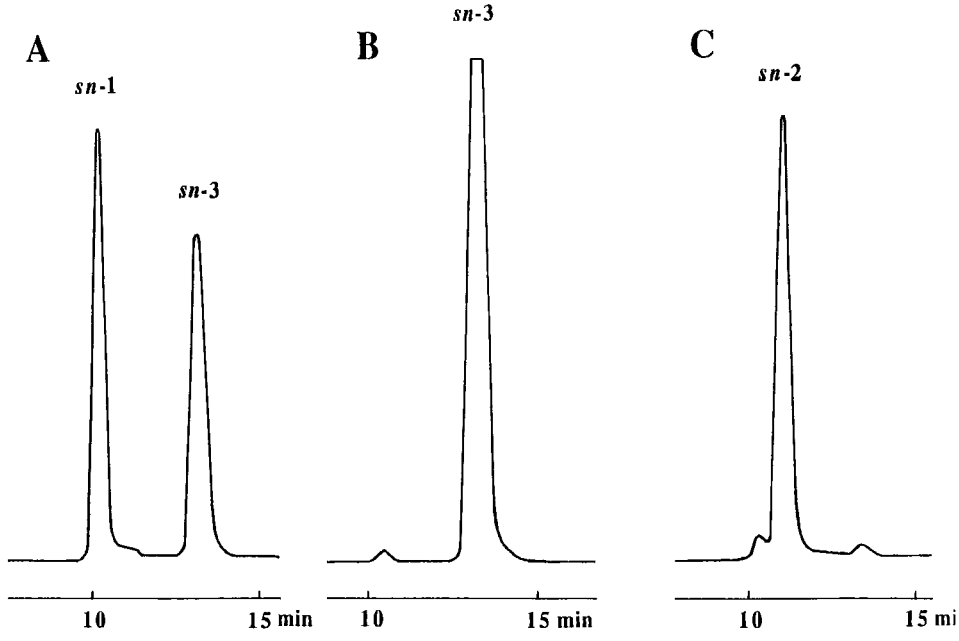

Figure 1. HPLC isocratic analysis of monopalmitoylglycerol isomers as [di-]3,5-dinitrophenyl isocyanate (dinitrophenyl urethane = DNPU) derivatives at ambient temperature and with UV absorption detection (254 nm), on stainless-steel (250 mm x 4 mm i.d.) chiral OA-4100 column. The column was packed with 5 μm particles of N-(R)-1-(α-naphthyl)ethylaminocarbonyl-(S)-valine chemically bonded to γ-aminopropyl silanized silica (Sumipax OA-4100); mobile phase was hexane- ethylene dichloride-ethanol (40:12:3, v/v/v) at 1 ml min^{-1}.

A = *rac*-16:0; B = *sn*-3-16:0; C = *sn*-2-16:0.

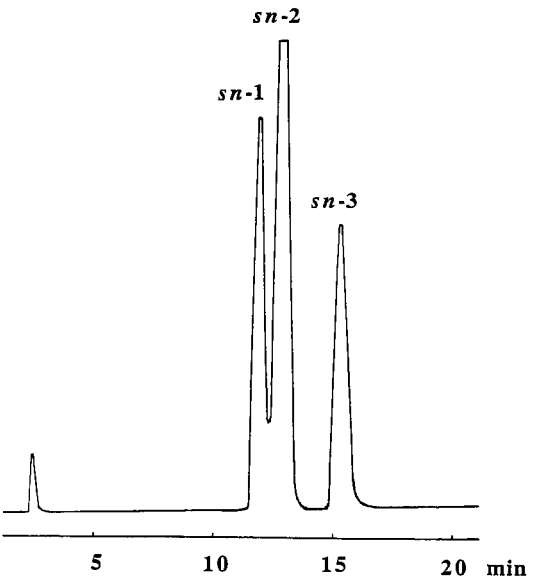

Figure 2. Chiral-phase HPLC separation at ambient temperature ot a synthetic mixture of the 3 monopalmitoylglycerol isomers as [di-]3,5-DNPU derivatives on Sumipax OA-4100. Mobile phase was hexane- ethylene dichloride- ethanol (40:12:3, v/v/v) at a flow rate of 1 ml mn^{-1}.

ethanol or / and higher proportion of hexane), but it would be to the detriment of the retention times which would be increased.

In our work, the problem of the separation of the sn-1- and sn-2-isomers does not raise since the sn-2-isomers are well separated by reversed-phase HPLC from the sn-1(3)-isomers [3]. Only the latter group, collected at the HPLC column outlet, will be further analyzed by chiral-phase HPLC for compositional determination of the two enantiomers in the mixture. This is probably also the reason why Takagi and Ando [10] only reported enantiomer separation in their last publication, excluding the sn-2-isomers.

Table I reports the separation factors calculated from the chromatograms obtained in the separation of different mixtures of saturated (16:0 and 18:0) and unsaturated (18:1 and 18:2) rac-1- and sn-2-monoacylglycerols.. For each type of monoacylglycerol, the racemic mixture and the sn-2-isomer were first analyzed separately and then mixed. If

TABLE I.

SEPARATION OF MONOACYLGLYCEROL STEREOISOMERS AS URETHANE DERIVATIVES BY CHIRAL-PHASE HPLC

STEREO-ISOMERS[a]	Retention time (min)[b]	Separation factor[c]	Constituent fatty acid[d]	STEREO-ISOMERS	Separation factor[e]	STEREO-ISOMERS	Separation factor[f]
sn-1-18:0	9.25			*sn*-1		*sn*-1	
sn-1-16:0	9.75	1.05	18:0	*sn*-2	1.10	*sn*-3	1.35
sn-1-18:1	9.79	1.00		*sn*-3	1.22		
sn-2-18:0	10.20	1.04		*sn*-1		*sn*-1	
sn-1-18:2	10.36	1.02	16:0	*sn*-2	1.09	*sn*-3	1.35
sn-2-16:0	10.64	1.03		*sn*-3	1.24		
sn-2-18:1	10.71	1.00		*sn*-1		*sn*-1	
sn-2-18:2	11.37	1.06	18:1	*sn*-2	1.09	*sn*-3	1.36
sn-3-18:0	12.48	1.10		*sn*-3	1.24		
sn-3-16:0	13.18	1.06		*sn*-1		*sn*-1	
sn-3-18:1	13.30	1.01	18:2	*sn*-2	1.10	*sn*-3	1.35
sn-3-18:2	13.98	1.05		*sn*-3	1.23		

a) Listed according to elution order.
b) Retention times corrected from column void volume.
c) Ratio of the retention time of a peak to the retention time of the preceding one.
d) Constituent fatty acid of the monoacylglycerol isomers.
e) Separation factor between the two isomer couples (*sn*-2, *sn*-1) and (*sn*-3, *sn*-2).
f) Separation factor between the two *sn*-1 and *sn*-3 enantiomers.

separation of two peaks is considered to be achieved when the separation factor (3 rd column) is at least equal to 1.10 (retention times differing from 10 %) data in theTable show that only one pair of isomers would be completely separated, namely sn-2-18:2 and sn-3-18:0. The other isomers would be only partially separated except sn-1-18:2 and sn-2- 18:0 which eluted practically together, as did the monopalmitoyl- and monooleoylglycerol isomers. Figure 3 illustrates the partial separations observed between

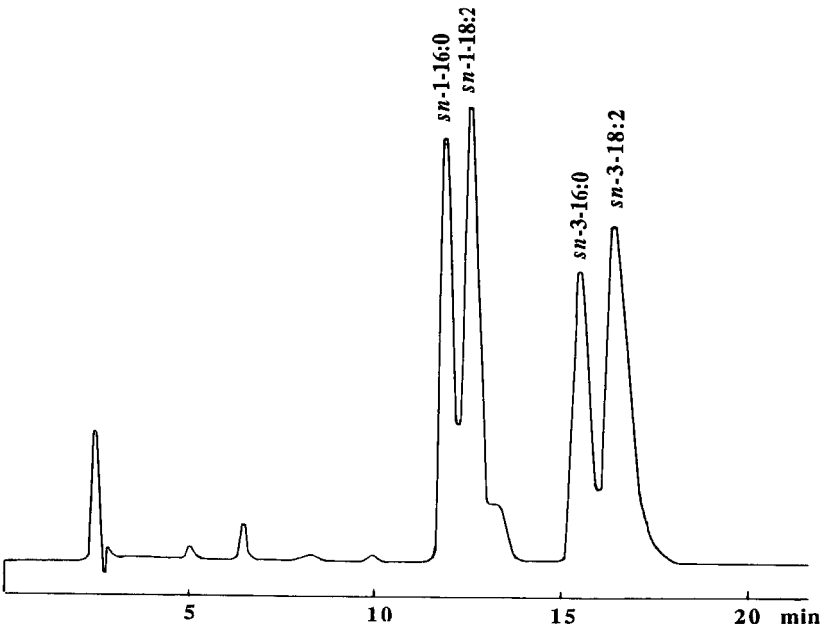

Figure 3. Carbon number separation of a mixture of monopalmitoyl (*sn*-1-16:0, *sn*-3-16:0) and monolinoleoylglycerol (*sn*-1-18:2, *sn*-3-18:2) enantiomers, as [di-]3,5-DNPU derivatives on Sumipax OA-4100. The minor satellite peak emerging after *sn*-1-18:2 represents *sn*-2-18:2. Mobile phase was hexane- ethylene dichloride- ethanol (40:12:3, v/v/v) at a flow rate of 1 ml min^{-1}.

the two sn-1 and sn-3 isomers series of monopalmitoyl-, and monolinoleoylglycerols. The chromatogram shows that monoacylglycerols were eluted into two distinct groups corresponding to the two isomers. In each group the elution order was the same and was according to increasing degree of unsaturation.

With simpler mixtures comprising the 3 isomers of only one monoacylglycerol, separation of the 3 isomers was generally complete, except for monopalmitoyl- and monooleoylglycerols as was also illustrated in Figure 2 for monopalmitoylglycerols. Between the two sn-1 and sn-3 antipodes of a same monoacylglycerol the separation was always observed very good (last column of Table I) and did not vary with the constituent fatty acid in the four studied monoacylglycerols.

In our work on stereospecific analysis of triacylglycerols, samples to be analyzed by chiral-phase HPLC only comprise the two sn-1- and sn-3-isomers of the same monoacylglycerol after fractionation by reversed-phase HPLC [3]. Their very complete resolution allow peak areas to be accurately determined, without any cross-contamination between peaks.

Separation between the sn-1 and sn-2-isomers neither varied with chain length nor unsaturation, remaining partially incomplete (separation factors of 1.09 to 1.10).Because of the high resolution of the two sn-1 and sn-3 enantiomers, the separation between the sn-2 and sn-3 isomers was very good (separation factors of 1.22 to 1.24).

The elution order of the 12 isomers reported in the 1st column of Table I indicates that for each isomer type the retention time (2nd column) increased with decreasing chain length and increasing unsaturation of the constituent fatty acid, that is with increasing polarity of the fatty acid chain. For the two studied saturated monoacylglycerols (16:0 and 18:0), the effect of chain length increase was higher for the sn-3-isomer (5,6 %) and lower for the sn-2-isomer (4,6 %). For the 3 fatty acids of the same chain length and of increasing number of double-bonds (18:0, 18:1, 18:2), retention time increased from 5.0 to 6.6 % by adding a double-bond to the saturated fatty acid and from 5.1 to 6.2 % by adding another double-bond.

From 18:0 to 18:1, the retention time increase was maximum for the sn-3-isomers (6.6 %) and minimum for the sn-2-isomers (5.0 %) but the reverse was true from 18:1 to 18:2 (5.1 and 6.2 % increase for the sn-3 and sn-2-isomers respectively). The change in the molecule polarity between a saturated and a monounsaturated fatty acid and between a monounsaturated and a diunsaturated fatty acid did not identically affect each type of isomer.

Figure 4 shows the relationship between log retention volume (V_R) and the equivalent carbon number (ECN) in each homologous series of isomers. For the four studied fatty acids the relationship was practically linear and the 3 straight lines traced from the 2 saturated fatty acids (16:0, 18:0) were practically parallel. However the retention volumes of the unsaturated monoacylglycerols were a little higher than predicted by the relationship, especially for the monolinoleoyglycerols (18:2) and the retention volume of the sn-3-isomers seemed to increase a little more rapidly with increasing ECN than those of the sn-1- and sn-2-isomers.

Between the sn-1, sn-2 and sn-3 isomers of a given monoacylglycerol the relationships can be approximately expressed by the following equations in the analytical conditions used :

$$\log V_R \text{ (sn-3)} = \log V_R \text{ (sn-1)} + 0.131$$
$$\log V_R \text{ (sn-2)} = \log V_R \text{ (sn-1)} + 0.040$$
$$\log V_R \text{ (sn-3)} = \log V_R \text{ (sn-2)} + 0.091.$$

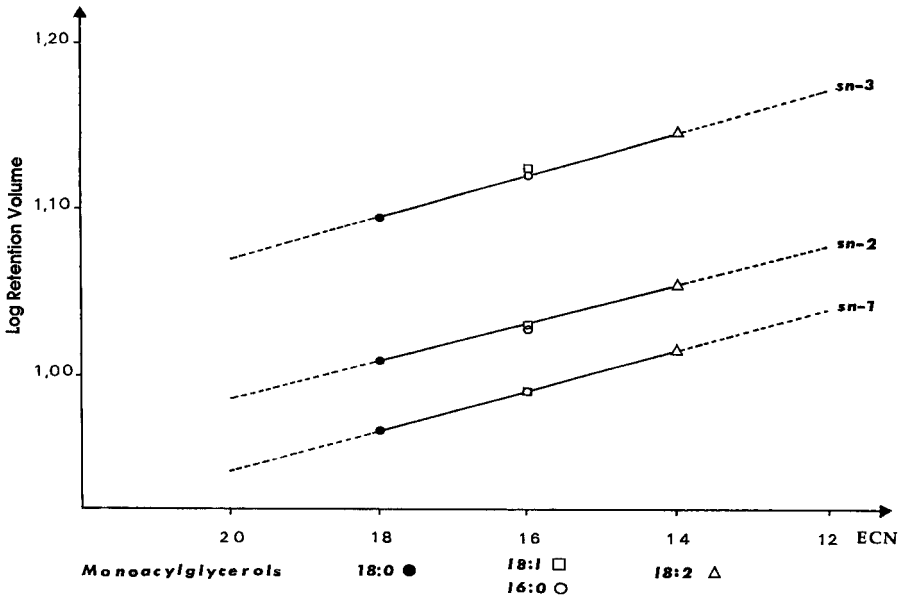

Figure 4. Plot of logarithm of retention volume versus partition number of monoacylglycerol enantiomers analyzed by chiral-phase HPLC as urethane derivatives. Retention volume (ml) = retention time (min) corrected from the column void volume x solvent flow-rate (ml min^{-1}). Equivalent Carbon Number (ECN) was calculated from the formula : ECN = CN - 2 DB, where CN is the total number of acyl carbon atoms and DB the total number of double bonds of the constituent fatty acid.

These 3 equations determine 3 parallel straight lines.

Such relationships are interesting to be established in an attempt to identify monoacylglycerol isomers by their retention volumes or conversely to predict the retention volumes of known isomers.

Data in Figure 4 and in Table I show that the retention volume of a sn-2-monoacylglycerol is practically identical to that of a sn-1 monoacylglycerol of the same chain length but with 2 double-bonds (for example sn-2-18:0 and sn-1-18:2) or of the same unsaturation but with 4 additional carbon atoms (for example sn-2-18:0 and sn-1-14:0). These two couples of isomers constitute two critical pairs whose constituents are

uneasily separable from each other. This would also be the case with the couples sn-2-16:0 or sn-2-18:1 and sn-1-12:0 or sn-1-18:3 or with the couple sn-2-20:0 and sn-1-16:0 (or sn-1-18:1).

Data in Figure 4 and Table I also indicate that the difference of retention volume between the sn-1 and sn-3-isomers of a given monoacylglycerol is so high (ca 3.5 ml) that for a wide range of monoacylglycerols, all the sn-1-isomers would always elute earlier than the sn-3-isomers. Table I shows that for 4 monoacylglycerols (16:0, 18:0, 18:1, 18:2) the retention times ranged from 9.25 to 10.36 min for the sn-1 isomers and from 12.48 to 13.98 min for the sn-3 isomers. This is also illustrated in Figure 3 for the 16:0 and 18:2 sn-1 and sn-3 isomers. Figure 4 shows that the sn-1-monoacylglycerol of ECN=10 (such as 10:0 or 18:4) would still elute earlier than the sn-3-18:0. Takagi and Ando [10] also observed this pecularity for the 18:0, 18:1 18:2 and 18:3 series.

Data reported in this work show that only sn-1 and sn-3-isomers differing from 10 units in ECN (10 carbon atoms or 5 double-bonds or any combination of the the two) would practically elute together. Except in very complex oils with a wide range of fatty acids of very different chain lengths (bovine milk fat) or of very different degrees of unsaturation (fish oils), the range of fatty acids encountered in most natural oils will be generally narrow enough to allow complete separation of the sn-1 and sn-3-isomers. However in each series of enantiomers the critical pairs, such as monopalmitoyl- and monooleoylglycerols (16:0 and 18:1) will be very incompletely separated so that prior fractionation by reversed-phase HPLC [3] is still needed.

From the overall results reported here, it follows that on the chiral phase, the separation of monoacylglycerols differing from 2 carbon atoms or 1 double-bond is low when compared to separation of enantiomers, but the retention times (or volumes) are very moderate. It is possible to highly improve separation as demonstrated by Takagi and Ando [10] by increasing the column length and decreasing the solvent flow rate, but the retention was considerably increased, since for the sn-3-18:0 the retention volume was ca 150 ml for a retention time of 5 hours.

The alternative we choose was to proceed to a prior fractionation of the monoacylglycerols by HPLC [3] in very moderate retention times, before enantiomer analysis on a chiral phase in conditions still allowing low retention times. The combined method additionally solves the problem of critical pairs resolution.

Detection

Since no commercial urethane derivatives of monoacylglycerol enantiomer were available, the quantitative aspect of chiral-phase HPLC analysis of the monoacylglycerol

TABLE II

ANALYSIS BY CHIRAL-PHASE HPLC OF RACEMIC MONOACYLGLYCEROL ENANTIOMERS AS URETHANE DERIVATIVES

Racemic monoacylglycerols	18:2		16:0		18:1		18:0	
Enantiomers	*sn*-1	*sn*-3	*sn*-1	*sn*-3	*sn*-1	*sn*-3	*sn*-1	*sn*-3
mol % (a)	49.6	50.4	50.2	49.8	49.9	50.1	50.4	49.6
	± 0.3		± 0.1		± 0.2		± 0.2	

Results are means of n = 3 analysis ± S.E.

(a) Peak area percentages.

stereoisomers was studied using commercial racemic monoacylglycerols known to contain an equal proportion of sn-1 and sn-3 isomers. After derivatization they were analyzed on the chiral column and detected by their absorption in UV light. Proportion (Percentile) of the two enantiomers in the mixtures, calculated from the registered peak areas, is reported in Table II. Data show that the values obtained were very close to those expected i.e. 50:50, since the maximum difference was lower than 1 %. They demonstrate that UV absorption was of the same magnitude for each enantiomer. They also show that no isomerization occured during the derivatization procedure. These two conditions were a prerequisite for accurate analysis of monoacylglycerol enantiomers.

These results confirm others [9] also obtained with rac. monoacylglycerols separated on another type of chiral column and detected in UV light.

CONCLUSION

We have shown in this work that monoacylglycerol enantiomers could be accurately analyzed by high performance liquid chromatography on a chiral phase both qualitatively and quantitatively, provided the mixture of monoacylglycerol isomers were previously fractionated into simple mixtures of sn-1(3)-isomers [3]. The HPLC methods of fractionation and analysis of the monoacylglycerol isomers will now be used to study the stereospecific distribution of fatty acids in natural triacylglycerols by means of the monoacylglycerols generated by enzymatical and chemical deacylation.

Results obtained will be compared to those determined by studying the diacylglycerols formed by partial chemical deacylation [15, 16] in order to check the reliability of the monoacylglycerol method which is simpler to use than the diacylglycerol method.

REFERENCES

1. Y. Kondoh, S. Takano, J. Chromatogr., 393: 427-432 (1987).
2. S. Takano, Y. Kondoh, J. Am. Oil Chem. Soc., 64: 1001-1003(1987).
3. B.G. Semporé, J.A. Bézard, J. Chromatogr., 596: 185-195 (1992).
4. N.Oi, M. Nagase, T. Doi, J. Chromatogr., 257: 111-117 (1983).
5. N. Oi, M. Nagase, Y. Inda, T. Doi, J. Chromatogr., 265: 111-116 (1983).
6. N. Oi, H. Kitahara, J. Liquid Chromatogr., 9: 511-517 (1986).

7. T. Takagi, Y. Itabashi, Yukagaku, 34: 962-963 (1985).
8. T. Takagi, Y. Itabashi J. Chromatogr., 366: 451-455 (1986).
9. Y. Itabashi, T. Takagi, Lipids, 21: 413-416 (1986).
10. T. Takagi, Y. Ando, Lipids, 25: 398- 400 (1990).
11. M. Yurkowski, H. Brockerhoff, Biochim. Biophys. Acta, 125: 55-59 (1966).
12. W.W. Christie, J.H. Moore, Biochim. Biophys. Acta, 176: 445-452 (1969).
13. F.E. Luddy, R.A. Barford, S.F. Herb, P. Magidman, R.W. Riemenschneider, J. Am. Oil Chem. Soc., 41: 693-696 (1964).
14. J.A. Bézard, M.A. Ouédraogo, J. Chromatogr., 196: 279-293 (1980).
15. B.G. Semporé, J.A. Bézard, J. Chromatogr., 547: 89-103 (1991).
16. B.G. Semporé J.A. Bézard, J. Chromatogr., 557: 227-240 (1991).
17. J. Bézard, M.A. Ouédraogo, G. Semporé, Rev. Franç. Corps Gras, 37: 171-175 (1990).
18. A.E. Thomas III, J.E. Scharoun, H. Ralston, J. Am. Oil Chem. Soc., 42: 789-792 (1965).
19. N. Oi, H. Kitahara, J. Chromatogr., 265: 117-120 (1983).
20. L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, John Wiley and sons, Inc., New-York, 2nd ed. 1979.
21. C. Litchfield, Analysis of Triglycerides, Academic Press, New-York, London, 1972.
22. A. Kuksis, in Handbook of Chromatography H.K. Mangold (editor) CRC, vol. 1, CRC Press, Boca Raton, F.L., 1984, p. 381.

Received: October 7, 1993
Accepted: November 11, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1695-1707 (1994)

USE OF A MACROCYCLIC ANTIBIOTIC AS THE CHIRAL SELECTOR FOR ENANTIOMERIC SEPARATIONS BY TLC

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ABSTRACT

The macrocyclic antibiotic, vancomycin, was used as a chiral mobile phase additive for the thin layer chromatographic (TLC) resolution of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatized amino acids, racemic drugs and dansyl-amino acids. Excellent separations were achieved for most of these compounds in the reversed phase mode. Both the nature of the stationary phase and the composition of the mobile phase strongly influenced enantiomeric resolution. The best results were obtained using diphenyl stationary phases. Acetonitrile was the organic modifier that produced the most effective separations with the shortest development times. It is highly likely that macrocyclic antibiotics will play a major role in future enantiomeric separations.

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INTRODUCTION

Enantiomeric separations in thin layer chromatography (TLC) are accomplished using either chiral stationary phases (1-8) or chiral mobile phase additives, CMAs (9-15). In contrast to HPLC, in which many chiral stationary phases are available, most TLC enantiomeric separations utilize CMAs. This is because only ligand-exchange-type stationary phases are available commercially for TLC. The most widely utilized CMAs are cyclodextrins and their derivatives (10-13,15). However, a few other classes of additives have been used successfully as well such as chiral ion interaction agents (14) and ligand exchange types (9). In this work we report on a member of a new class of chiral selectors (i.e., macrocyclic antibiotics). Vancomycin is an antibacterial compound that inhibits mucopeptide biosynthesis. Previously we found that it was useful in the HPLC separation of enantiomers when immobilized on 5 μ silica gel (16). Recently we have used other macrocycles in HPLC and capillary electrophoresis (16,17). To our knowledge this is the first report on their use as chiral mobile phase additives in chromatography.

EXPERIMENTAL SECTION

Materials. All racemic analytes were obtained from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO). Chiral macrocyclic antibiotics such as vancomycin were obtained from Advanced Separation Technologies, Inc. (Whippany, NJ). All dansyl amino acids were purchased from Sigma. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatizing kit was from Waters (Bedford, MA). Chemically bonded Diphenyl-F reversed phase plates (5 x 20 cm, 250 μ layer thickness) were from Whatman Chemical Separation Division, Inc. (Clifton, NJ).

Methods. 0.6 M sodium chloride was added to all mobile phases. Sodium chloride was used to stabilize the plate binder. Acetonitrile was used as the organic modifier. Occasionally 1% triethylammonium acetate buffer (pH = 4.1) was also used as indicated in the Tables and Figures. Chiral selectors were first dissolved in the sodium chloride solution and placed in an ultrasonic bath for about 15 minutes, then the organic modifier was added to complete the mobile phase mixture.

The plates were developed at room temperature (22°C) in 6 (i.d.) x 23 cm cylindrical glass chambers. It took approximately 1-3 hrs to completely develop a 5 x 20 cm TLC plate. All compounds were fluorescent. Spot visualization was done by using a fixed-dual wavelength (254 nm and 365 nm) ultraviolet hand lamp.

The AQC derivatives were obtained according to reference 18. Approximately 100 pmol of each compound was dissolved in 60 μL of sodium borate buffer (0.2 M. pH 8.8) in a vial; vortexed for several seconds and then 20 μL of AQC solution was added (3 mg per 1 lL of acetonitrile). The vial was heated in an oven for 10 minutes at 50°C. The resulting solutions were used in TLC without further purification.

RESULTS AND DISCUSSION

Recently we proposed the use of macrocyclic antibiotics as a new class of chiral selectors for enantiomeric separations (16,17). In this work it is shown that vancomycin is an effective chiral mobile phase additive for the TLC resolution of several enantiomeric compounds.

Vancomycin consists of three fused macrocycles (Figure 1). This amphoteric molecule contains five aromatic rings as well as peptide and carbohydrate moieties. It has a molecular weight of 1,449 and an

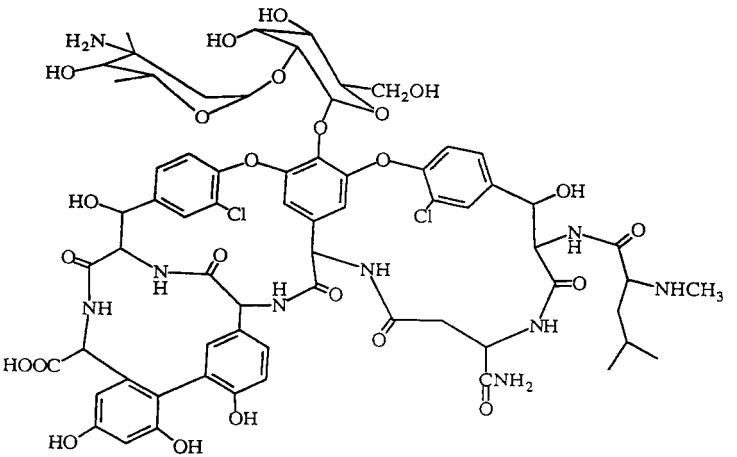
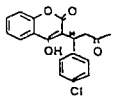
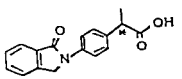
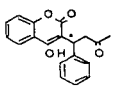
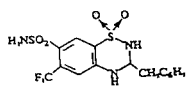
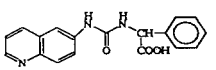
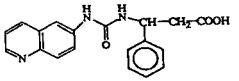
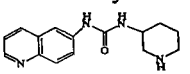


Figure 1. Molecular structure of vancomycin.

isoelectric point of ~ 5 (16). Vancomycin is produced by the bacteria (*Streptomyces orientalis*). It is soluble in water, partially soluble in methanol and hydro-organic solvent mixtures, and insoluble in higher alcohols and nonpolar organic solvents (16).

A variety of different reversed phase TLC stationary phases were used in an attempt to achieve good enantiomeric separation and resolution. Diphenyl-type stationary phases gave the best results in terms of low streaking and good spot integrity during development (see Experimental). The separation results are collected in Table I. The racemates resolved are of three types: underivatized pharmaceuticals, AQC-amino acids and dansyl amino acids. When enantiomerically pure standards were available, the retention order was determined (Table I). The mobile phase generally consisted of between 17 and 40 volume percent acetonitrile plus 0.6 M $NaCl_{(aq)}$ (Table I, footnote a). The salt in the water helped to stabilize the

Table I Reversed-Phase TLC Enantiomeric Separation Data Using a Vancomycin Chiral Selector on Diphenyl-F Plate

Compound	R_{f1}	R_{f2}	α	R_s	Conc.(M)	Mobile Phase[a]
1. Coumachlor 	0.14	0.20	1.4	2.5	0.05	4/6/0
2. Indoprofen 	0.58	0.63	1.1	1.6	0.05	4/6/0
3. Warfarin 	0.04	0.06	1.5	1.2	0.04	2/10/0
4. Bendroflume-thiazide 	0.02	0.06	3.0	1.8	0.05	0.5/8.5/1
5. AQC[b]-α-amino phenylacetic acid 	0.13(L)	0.16(D)	1.2	1.9	0.025	2/10/0
6. AQC[b]-3-amino-3-phenylpropinic acid 	0.12 0.11	0.18 0.19	1.5 1.7	2.5 2.2	0.04 0.025	2/10/0 2/10/0
7. AQC[b]-3-aminopiperidine dihydrochloride 	0.24	0.28	1.2	1.7	0.025	2/10/0

(continued)

Table I (continued)

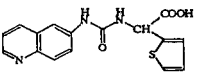
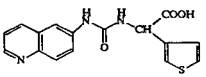
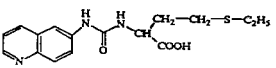
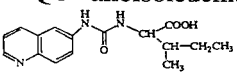
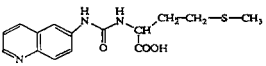
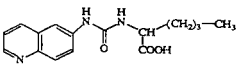
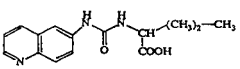
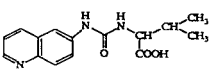
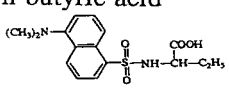
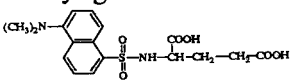
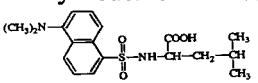
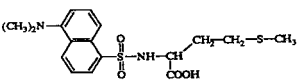
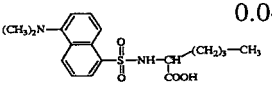
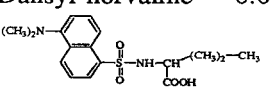
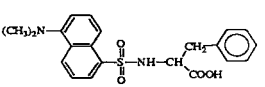
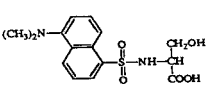
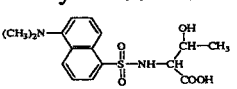
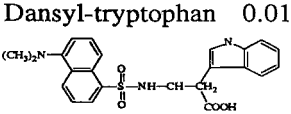
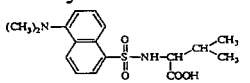
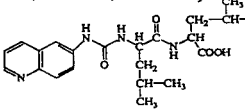
8. AQC[b]-α-amino-2-thiopheneacetic acid 	0.16 0.16	0.18 0.19	1.1 1.2	1.2 1.6	0.025 0.04	2/10/0 2/10/0
9. AQC[b]-α-amino-3-thiopheneacetic acid 	0.17	0.22	1.3	2.6	0.025	2/10/0
10. AQC[b]-ethionine 	0.14	0.17	1.2	1.4	0.025	2/10/0
11. AQC[b]-alloisoleucine 	0.14(L)	0.21(D)	1.5	3.1	0.025	2/10/0
12. AQC[b]-methionine 	0.19(L)	0.23(D)	1.2	1.5	0.025	2/10/0
13. AQC[b]-norleucine 	0.13(L)	0.16(D)	1.2	1.6	0.025	2/10/0
14. AQC[b]-norvaline 	0.21(L)	0.25(D)	1.2	2.3	0.025	2/10/0
15. AQC[b]-valine 	0.23(L)	0.27(D)	1.2	2.0	0.025	2/10/0
16. Dansyl-α-amino-n-butyric acid 	0.09(L) 0.09(L)	0.15(D) 0.21(D)	1.7 2.3	2.7 4.1	0.04 0.08	2/10/0 2/10/0

Table I (continued)

17. Dansyl-glutamic acid	0.21(L)	0.23(D)	1.1	1.4	0.04	2/10/0
						
18. Dansyl-leucine	0.03(L)	0.09(D)	3.0	3.0	0.04	2/10/0
						
19. Dansyl-methionine	0.05(L)	0.12(D)	2.4	3.4	0.04	2/10/0
						
20. Dansyl-norleucine	0.03(L)	0.07(D)	2.3	1.6	0.04	2/10/0
	0.04(L)	0.16(D)	4.0	4.9	0.08	2/10/0
						
21. Dansyl-norvaline	0.05(L)	0.12(D)	2.4	3.3	0.04	2/10/0
						
22. Dansyl-phenylalanine	0.03(L)	0.05(D)	1.7	1.5	0.04	2/10/0
						
23. Dansyl-serine	0.15(L)	0.20(D)	1.3	1.8	0.04	2/10/0
	0.16(L)	0.24(D)	1.5	2.8	0.08	2/10/0
						
24. Dansyl-threonine	0.13(L)	0.17(D)	1.3	2.5	0.05	2/10/0
						
25. Dansyl-tryptophan	0.01(L)	0.03(D)	3.0	1.3	0.04	2/10/0
						

(continued)

Table I (continued)

26. Dansyl-valine 	0.06(L)	0.10(D)	1.7	1.5	0.04	2/10/0
27. AQC[b]-leu-leu (0.04M, 2/10/0)[a] 	R_{f1}(D-L) 0.03	R_{f3}(L-D) 0.10		α_1(DLLD) 3.3	R_{S1}(DLLD) 5.0	
	R_{f2}(L-L) 0.04	R_{f4}(D-D) 0.24		α_2(LDLD) 6.0	R_{S2}(LDLD) 11.6	
(0.02M, 1.5/4.5/0)[a]	R_{f1}(D-L) 0.17	R_{f3}(L-D) 0.22		α_1(DLLD) 1.3	R_{S1}(DLLD) 4.5	
	R_{f2}(L-L) 0.18	R_{f4}(D-D) 0.42		α_2(LDLD) 2.3	R_{S2}(LDLD) 18.5	

[a] mobile phase compositions listed indicate the volume ratios of acetonitrile/0.6 M NaCl/1% triethylammonium acetate buffer (pH = 4.1).
[b] AQC stands for 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, a fluorescent-tagging-agent (see Experimental section).

binder on the TLC plate. Most of the racemates were better than baseline resolved. Interestingly, the retention order of all AQC-amino acids and dansyl amino acids (for which standards were available) was the same. The D-enantiomer always had a greater R_f value than the L-enantiomer (Table I).

Figure 2 shows the TLC separation of indoprofen and coumachlor using vancomycin as the chiral mobile phase additive. Figure 3 shows analogous separations of five racemic AQC-amino acids. Figure 4 shows the complete resolution of all four stereoisomers (two pairs of enantiomers) of the dipeptide leucyl-leucine. In this case the relative retention of each isomer is known.

It appears that two factors contribute to the effectiveness of this technique in resolving enantiomeric compounds. One is the obvious

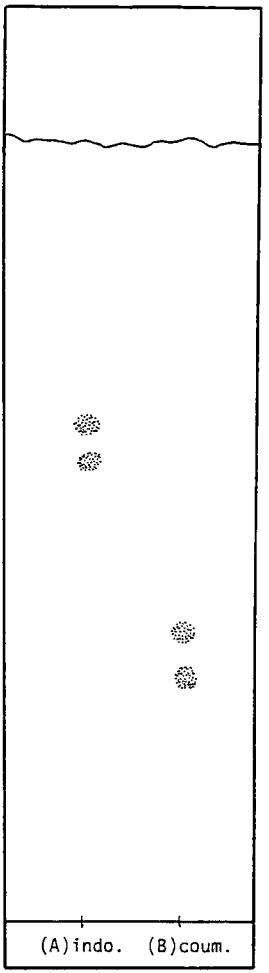


Figure 2. TLC chromatogram showing the separation of (A) indoprofen, and (B) coumachlor. The mobile phase consisted of 0.05 M vancomycin in 4:6 (by volume) acetonitrile: 0.6 M $NaCl_{(aq)}$. Diphenyl-F TLC plates (5 x 20 cm) were used. Spots were detected using a 365 nm UV hand lamp (see Experimental).

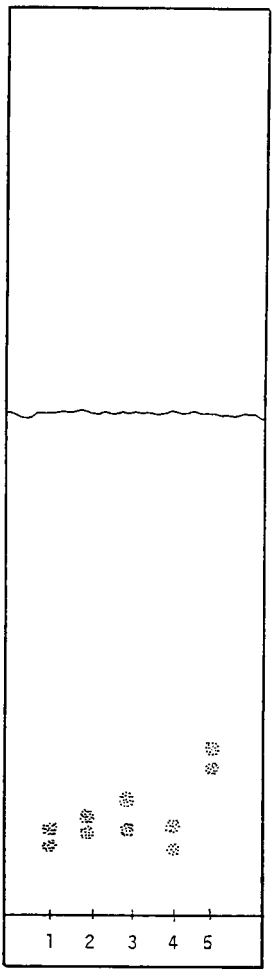


Figure 3. TLC chromatogram showing the separation of racemic: (1) AQC-ethionine, (2) AQC-α-amino-2-thiopheneacetic acid, (3) AQC-α-amino-3-thiopheneacetic acid, (4) AQC-α-amino phenylacetic acid, and (5) AQC-3-aminopiperidine dihydrochloride. The mobile phase consisted of 0.025 M vancomycin in 1:5 (by volume) acetonitrile: 0.6 M $NaCl_{(aq)}$. Other experimental conditions were the same as indicated in Figure 2.

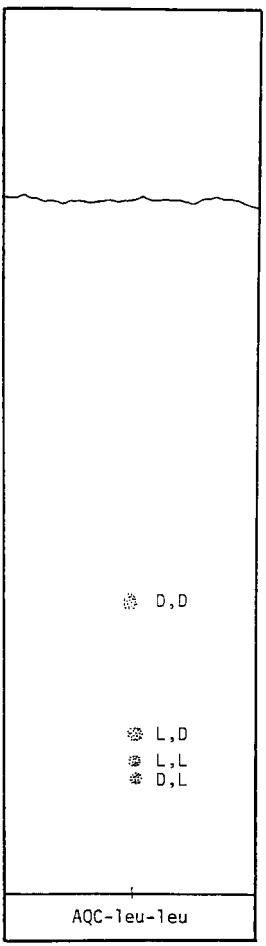


Figure 4. TLC chromatogram showing the separation of all four isomers (2 pairs of enantiomers) of AQC-leucyl-leucine. The stereochemistry of the compound represented by each spot is indicated. This was determined by developing pure standards in a separate experiment. The mobile phase consisted of 0.02 M vancomycin in 1:3 (by volume) acetonitrile: 0.6 M $NaCl_{(aq)}$. Other experimental conditions were the same as indicated in Figure 2.

enantioselectivity of the vancomycin CMA. However, the ability to maintain a relatively small spot size during development also is an important factor. In some cases TLC separations are inhibited by poor efficiency. That does not seem to be a limiting factor in these particular experiments, however.

Currently several other macrocyclic compounds are being evaluated as chiral selectors. Given the great structural variety of this class of compounds, it is likely not only that other analogous and effective chiral selectors will be found, but that they will have different enantioselectivities as well.

ACKNOWLEDGMENT

Support of this work by the Department of Energy, Office of Basic Sciences (grant DE FG02 88ER13819) is gratefully acknowledged.

REFERENCES

1. Yuasa, S.; Shimada, K.; Kameyama, M.; Yasui, M. and Adzuma, K., *J. Chromatogr. Sci.*, **18**, 311 (1980).
2. Wainer, I.W.; Brunner, C.A.; Doyle, T.D. *J. Chromatogr.*, **264**, 54 (1983).
3. Weinstein, S. *Tetrahedron Lett.*, **25**, 985 (1984).
4. Guenther, K.; Martens, J.; Schickedanz, M., *Angew. Chem. Int. Ed. Engl.*, **23**, 506 (1984).
5. Brinkman, U.A.T.; Kamminga, D., *J. Chromatogr.*, **330**, 375 (1985).
6. Alak, A.; Armstrong, D.W., *Anal. Chem.*, **58**, 582 (1986).
7. Gont, L.K.; Neuendorf, J., *J. Chromatogr.*, **391**, 343 (1987).
8. Martens, J.; Guenther, K. Schickedanz, M., *Arch. Pharm.*, **319**, 572 (1986).

9. Marchelli, R.; Virili, R.; Armani, E.; Dossena, A., *J. Chromatogr.*, **355**, 354 (1986).
10. Armstrong, D. W.; He, F.; Han, S., *J. Chromatogr.*, **448**, 345 (1988).
11. Armstrong, D. W.; Faulkner, Jr., J.R.; Han, S.M., *J. Chromatogr.*, **452**, 323 (1988).
12. Han, S.M.; Armstrong, D.W. in: "Planar Chromatography in the Life Sciences" Ed., Touchstone, J. C., John Wiley & Sons, N.Y. (1990) Ch. 7, pp 81-99.
13. Duncan, J.D.; Armstrong, D. W., *J. Planar Chromatogr.*, **3**, 65 (1990).
14. Duncan, J.D.; Armstrong, D.W.; Stalcup, A.M., *J. Liq. Chromatogr.*, **13**, 1091 (1990).
15. Duncan, J.D.; Armstrong, D.W., *J. Planar Chromatogr.*, **4**, 204 (1991).
16. Armstrong, D.W.; Tang, Y.; Chen, S.; Zhou, Y.; Bagwill, C.; Chen, J.-R., *Anal. Chem.* **66**, in press (1994).
17. Armstrong, D. W.; Rundlett, K.; Reid, G.L. III, *Anal. Chem.* submitted (1993).
18. Pawlowska, M.; Chen, S.; Armstrong, D.W., *J. Chromatogr.*, **641**, 257 (1993).

Received: January 6, 1994
Accepted: January 14, 1994

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1709-1719 (1994)

OPTIMIZATION OF THE RESOLUTION OF THE ENANTIOMERS OF β-DIMETHYLAMINO-BUTYROPHENONE BY HPLC ON A β-CYCLODEXTRIN COLUMN

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ABSTRACT

The enantiomers of β-dimethylaminobutyrophenone have been separated by HPLC on a native β-cyclodextrin bonded column using a polar organic eluent consisting of acetonitrile/methanol/acetic acid/triethylamine. To achieve this separation, the mobile phase composition has been optimized with respect to peak resolution and analysis time using a two-levels full factorial design. The important factors controlling the separation have been identified : the resolution is mainly affected by the composition and the quantity of buffer and the retention by the amount of methanol. The polynomial equations provided by the factorial design have been modified in order to enhance their reliability in an experimental domain larger than the factorial design domain.

INTRODUCTION

Among various phenylpropylamides exhibiting analgesic activity, one of the most important is the dextropropoxyphene (α-

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d-4-dimethylamino-3-methyl-1,2-diphenyl-2-butanol propionate) and its derivatives. The α-dl- and α-d-diastereoisomers possess marked analgesic activity, in contrast to the β-diastereoisomers which are substantially inactive.

The preparation of dextropropoxyphene involves 3 steps (1) : preparation of β-dimethylaminobutyrophenone by a Mannich reaction, formation of the aminocarbinol (4-dimethylamino-1,2-diphenyl-3-methyl-2-butanol) by a Grignard reaction, followed by an esterification with propionic anhydride. The α-form was obtained by fractional crystallization of the pair of diastereoisomeric salts formed by reaction of the aminocarbinol with d-camphorsulfonic acid. In 1963, Pohland et al. (2) have shown that the (-) enantiomer of the phenone is optically stable and can be used as a raw material for the stereoselective synthesis of dextropropoxyphene. The phenone racemate was resolved by fractional crystallization of its dibenzoyltartrate salt.

In this work, we have investigated an alternative method of separation of the enantiomers of the β-dimethylaminobutyrophenone by HPLC on a chiral stationnary phase (CSP) in view of a future preparative application. The α_1-acid glycoprotein (AGP) CSP appears to have a wide applicability to molecules of pharmacological interest and is able to resolve the propoxyphene enantiomers (3). However, the AGP column has a limited stability and a low capacity. This makes preparative separations difficult or impossible. An efficient alternative method was found by Armstrong et al. (4) by using an unusual mobile phase consisting of a mixture of polar organic solvents in conjunction with the original native cyclodextrin bonded phase, to resolve racemic β-adrenergic blocking agents such as propranolol and analogous compounds. These CSP's have exceptional stability when used with these mobile phases. In addition, the separations are easily scaled to preparative proportions.

In this work, a cyclodextrin CSP with a polar organic mobile phase was used. The objectives were (i) to achieve a complete

separation of enantiomers, (ii) to understand the importance of the chromatographic conditions and (iii) to get a statistical model of the effect of the experimental variables on the separation.

First attempt of resolution using the best chromatographic conditions of Armstrong et al. (4) produced only a partial separation. An optimization procedure, varying several factors at a time was investigated. Among these, the Simplex method, that is mathematically simple and easy to implement has been widely used in analytical chemistry and sometimes in the optimization of chiral separations (see for example Ley et al., (5)). However, this method does not give informations concerning the relative importance of the experimental factors. Therefore, a two-levels full factorial design was applied to determine the magnitude of the effect of the chromatographic variables and their interactions (6). In addition, the factorial design provides a model that was modified to enhance its reliability and checked on a new set of experimental data. The advantage of the factorial design is to bring a simplified representation of a phenomenon from very few experiments. The idea was that, with some modifications of the form of the model, it could be possible to approximate more closely the behavior of the chromatography. Some of these modifications could be suggested by models existing in the chromatographic literature.

EXPERIMENTAL

Chromatographic experiments were carried out at room temperature using an HPLC pump equipped with a 20-µL injection loop and an U.V. detector. A Cyclobond-I column (250x4.6 mm i.d., Advanced Separation Technologies) packed with native β-cyclodextrin bonded to a 5 µm silica gel was used. In all cases the flow rate of the eluent was 1 ml/mn.

FACTORIAL DESIGN RESULTS

According to Armstrong et al. (4), the separation of ß-blockers can be obtained with acetonitrile as main solvent and requires small amounts of methanol (1-10% by volume) and very small amounts of glacial acetic acid and triethylamine (0.2-1.2% by volume).

After some vain attempts with other additives (water, isopropanol), three experimental variables were selected : the amount of acetic acid/triethylamine buffer, X_1, the acetic acid/triethylamine ratio, X_2 and the amount of methanol, X_3. For each natural variable, the two levels associated to the +1 and -1 levels of the corresponding coded variables are indicated in Table 1. The quality of the separation was estimated through the resolution Rs, measured on the chromatogram as:

$$Rs = 2\,(t_2 - t_1)/\,(t_{w1} + t_{w2}),$$

and the capacity factor of the first eluted peak,

$$k'_1 = t_1/t_0 - 1.$$

t_i being the retention time and t_{wi} the peak width at half-height.

The chromatographic conditions of the eight (2^3) experiments are reported in Table 2, together with the separation results. In the formalism of full fractional design, the results of the separation can be approximated by a linear combination of the coded variables :

$$Y = a_0 + a_1 X_1 + a_2 X_2 + a_3 X_3 + a_{12} X_1 X_2 + a_{13} X_1 X_3 + a_{23} X_2 X_3 + a_{123} X_1 X_2 X_3 \qquad (1)$$

It should be noted that the capacity factor was correlated in a logarithm form, for reasons discussed in the "Modelization..." section. The magnitude of the coefficients corresponding to main factors and two-factors interactions, tabulated in Table 2, indicates the relative importance of each variable. It can be seen that the resolution mainly increases with the acid/amine ratio, X_2 and slighly with the buffer percentage, X_1. The interaction term $b_{23} = -0.029$ means that the influence of the percentage of

TABLE 1
Values of the Experimental Factors in the Factorial Design.

	Factors	level -1	level +1
X_1	percentage of buffer	0.02	0.2
X_2	acid/amine ratio	6	30
X_3	percentage of methanol	1	10

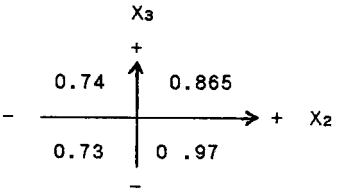
TABLE 2
Chromatographic Conditions and Responses (Resolution Rs and Capacity Factor k'_1 of the First Eluted Enantiomer) in the Factorial Design.

Run	X_1	X_2	X_3	Rs	k'_1
1	+1	+1	+1	0.93	3.19
2	-1	+1	+1	0.80	3.95
3	+1	-1	+1	0.74	1.95
4	-1	-1	+1	0.74	2.60
5	+1	+1	-1	1.04	9.25
6	-1	+1	-1	0.90	10.38
7	+1	-1	-1	0.78	4.50
8	-1	-1	-1	0.68	3.71

methanol depends on the level of X_2. This is clearly shown on the interaction diagram (scheme 1) obtained in the following way : the right top number (0.865) corresponds to the mean value of Rs for the two experiments performed with both high acid/amine ratio (X_2 = +1) and high percentage of methanol (X_3 = +1).

The amount of methanol has a negative effect on the resolution only at high acid/amine ratio. The same result is observed for the interaction X_1- X_3.

On the contrary, the capacity factor mainly depends on the amount of methanol, and whatever the level of the others factors, an increase of methanol will reduce the capacity factor.



Scheme 1

SUPPLEMENTARY EXPERIMENTS

In the factorial design, the best resolution (1.04) has been obtained at low methanol amount, and high acetic acid amount in the eluent (high acid/amine ratio and high percentage of buffer). But the separation is not complete and further experiments have been performed in the direction indicated by the factorial design, first to improve this result and second to investigate the effect of the individual parameters in order to test the models. These experiments are referred in the following as the "new set of experiments". It consists of 43 experiments, in which the percentage of buffer was varied between 0.02 and 0.4 %, the acetic acid/triethylamine ratio r between 5 and 100 and the percentage of methanol between 0 and 20 %.

A maximum resolution of 1.17, corresponding to a baseline separation was obtained with 2 % methanol, 0.15 % buffer, and r = 50. In these conditions, the capacity factor is quite high (12.0).

Figures 1 and 2 show that when only the percentage of methanol, or of buffer, is varied the resolution reaches a maximum value, around 3 % of methanol, or 0.15 % of buffer. In both cases, the retention factor decreases with an increasing percentage of additives. When the acid/amine ratio r is increased, both k' and Rs increase and reach a plateau at r = 50 (Fig. 3).

It can be noted that Armstrong et al. (4) observed the same behavior in the resolution of propranolol. They interpret the

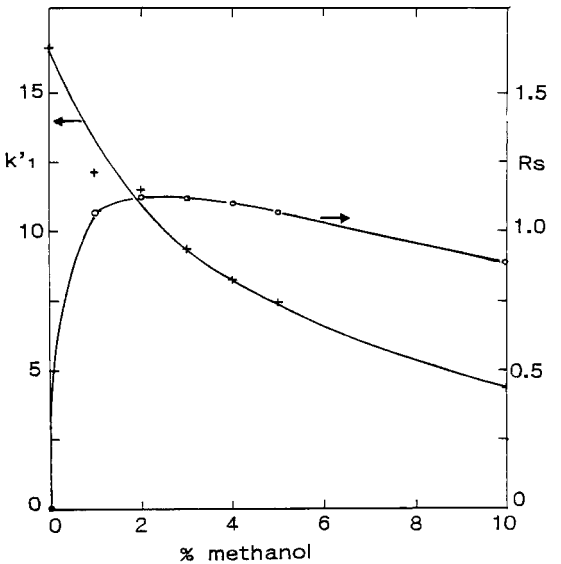


FIGURE 1. Influence of the amount of methanol in the mobile phase on the capacity factor k'1 (+) and the resolution Rs (o). Conditions: Column, Cyclobond-I (250x4.6 mm, 5 µm); mobile phase : 0.2 % (by volume) of the acetic acid/triethylamine buffer at an acid/amine ratio, 50 in acetonitrile; flowrate, 1 mL/mn; U.V. detection at 254 nm.

retention results by a better affinity for the stationary phase of the protonated amine relatively to the free enantiomer, leading to a slower elution. Actually, because of the high acid/amine ratio, there is an excess in acid that will be able to protonate the amine group of the enantiomer. But, as on the one hand, the acid excess is produced by an increase in either the buffer amount or the r ratio, and on the other hand, the buffer amount decreases the retention while the r ratio increases it, an additional mechanism, involving direct competition between buffer and enantiomers for the inclusion in the ß-cyclodextrin cavity, can be

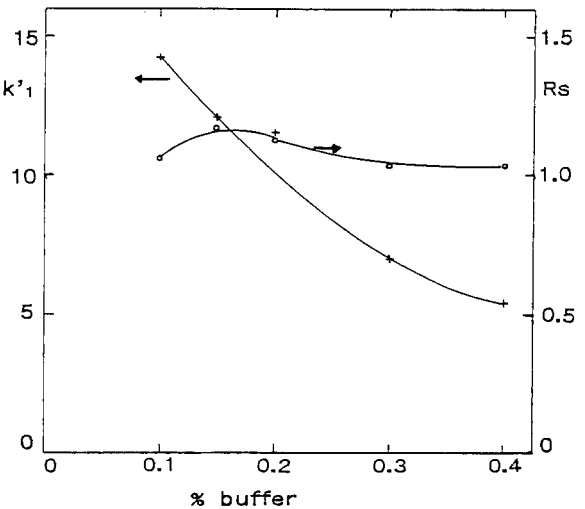


FIGURE 2. Influence of the amount of acetic acid/triethylamine buffer in the mobile phase on the capacity factor k'_1 (+) and the resolution Rs (o). Conditions : Column, Cyclobond-I (250x4.6 mm, 5 µm); mobile phase : 2 % (by volume) of methanol in acetonitrile, acid/amine ratio, 50; flowrate, 1 mL/mn; U.V. detection at 254 nm.

invoked. Inclusion of methanol in the cavity is also probably the cause of the observed reduction in retention time.

MODELIZATION OF RETENTION AND RESOLUTION

The polynomial model provided by the factorial design (Eq. 1) is expressed in terms of coded variables (level +1 or -1). To check the reliability of the model over the new set of experimental data, a transformation of natural variables to coded variables is needed. The simplest one is a linear transformation :

$$X_{coded} = -1 + 2\,(X_{natural} - X_{-1})/(X_{+1} - X_{-1})$$

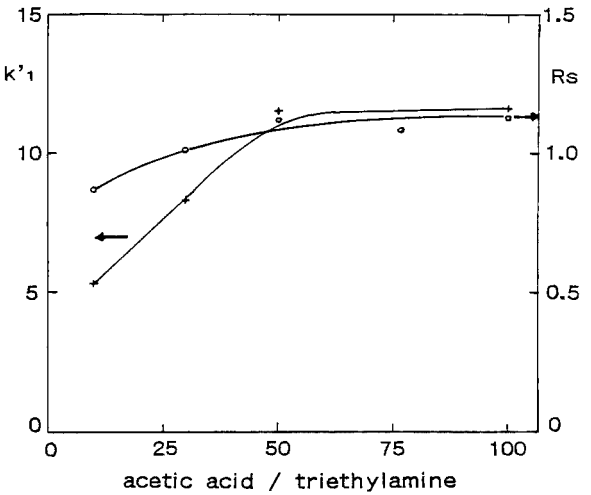


FIGURE 3. Influence of the acetic acid/triethylamine ratio in the buffer on the capacity factor k'_1 (+) and the resolution Rs (o). Conditions : Column, Cyclobond-I (250x4.6 mm, 5 µm); mobile phase : 2 % (by volume) of methanol and 0.2 % (by volume) of the buffer in acetonitrile; flowrate, 1 mL/mn; U.V. detection at 254 nm.

x_{+1} and x_{-1} being the values of the natural variables taken in the factorial design (Table 1).

Such models, with coefficients given in Table 3, represent the experimental data with a mean error of 15 % on Rs and 23 % on Log k'_1. This is a bad representation since the dispersion of the experimental points, expressed in standard deviation, is 16 % on Rs and 27% on Log k'_1. It must be noted that the capacity factor was correlated in a logarithm form, after the physicochemical model of Snyder et al. (7) giving the mobile phase effect on retention in non-chiral chromatography, in form of Log k'_1 in function of the additives molar fractions. In this work, it has been verified that the Log k'_1 model gives more accurate results than the k'_1 model.

TABLE 3
Coefficients of the Effects and Interactions of the Variables Considered in the Factorial Design, Calculated for the Resolution and for the Capacity Factor of the First Eluted Peak.

Factors		Rs	Log k'_1
average	b_0	0.826	1.442
X_1, % buffer	b_1	0.046	-0.053
X_2, acid/amine ratio	b_2	0.091	0.332
X_3, % methanol	b_3	-0.024	-0.403
interaction X_1-X_2	b_{12}	0.021	-0.029
interaction X_1-X_3	b_{13}	-0.014	-0.072
interaction X_2-X_3	b_{23}	-0.029	-0.105

The main discrepancy between model and experiments comes from the linear representation of the response when one parameter at-a-time is varied, whereas figures 2-4 show a very different behavior. Since the model is established using only two values of each variable, a certain curvature can be introduced by a non-linear transformation of natural variables to coded variables, and that without changing the coefficients of the models. In this way, the mean errors have been reduced by a factor 2-3, by modifying the acid/amine ratio r in Log(r-1) : a mean error of 5 % on the resolution, and of 12 % on the capacity factor are obtained. It is noteworthy that this transformation has a physical meaning : Log(r-1) is closely connected to the pH of the buffer solution, i.e. to the concentration of proton.

It may be added that other models have been built using a more theoretical approach, particularly by decomposing the resolution in selectivity, column efficiency and retention terms, but the final mean error has the same magnitude as in the statistical models presented here.

REFERENCES

(1) Pohland, A. and Sullivan, H.R., Preparation of α-d- and α-l-4-Dimethylamino-1,2-diphenyl-3-methyl-2-propionyloxybutane, J. Am. Chem. Soc., 77, 3400, 1955.

(2) Pohland, A., Peter, L.R. and Sullivan, H.R., Analgesics. Stereoselective Syntheses of α-(+)- and α-(-)-4-Dimethylamino-1,2-diphenyl-3-methyl-2-propionyloxybutane, J. Org. Chem., 28, 2483, 1963.

(3) Schill, G., Wainer, I.W. and Barkan, S.A., Chiral Separation of Cationic Drugs on an α_1-Acid Glycoprotein Bonded Stationary Phase, J. Liq. Chromatogr., 9, 641, 1986.

(4) Armstrong, D.W., Chen, S., Chang, C. and Chang, S., A New Approach for the Direct Resolution od Racemic beta-Adrenergic Blocking agent by HPLC, J. Liq. Chromatogr., 15, 545, 1992.

(5) Ley, G.W., Fell, A.F. and Kayes, B., Recent Advances in Chiral Separations, Plenum Press, New York, 1991, p. 97.

(6) Box, G.E.P. and Draper, N.R., Empirical Model-Building and Response Surfaces, Wiley, New York, 1987, p. 105.

(7) Snyder, L.R., Glajch, J.L. and Kirkland, J.J., Practical HPLC Method Development, Wiley, New York, 1988, p.202.

Received: November 4, 1993
Accepted: November 12, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1721-1735 (1994)

REMOVAL OF ORGANIC COMPOUNDS FROM WATER VIA ADSORPTION ONTO POLYMETHYL-HYDROSILOXANE PENTENYL-β-CYCLODEXTRIN

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ABSTRACT

A co-polymer of polymethylhydrosiloxane and pentenyl-β-cyclodextrin (PMSP-β-CD) is synthesized and used to extract various aromatic materials from water by adsorption onto the co-polymer matrix. The method of synthesis, separation scheme and parameters associated with adsorption are discussed. Salt and pH effects are also examined. The problems associated with other methods of removal are compared with those encountered by extraction using PMSP-β-CD. Most compounds studied can be quantitatively extracted from water if the extraction conditions are optimized. Removal is not greatly influenced by the addition of salt or change in pH. PMSP-β-CD is easily recycled at 80-100°C and both the organic analytes and PMSP-β-CD are recovered with a high efficiency. The results also indicate that extractions using PMSP-β–CD are not only possible but also advantageous in some cases.

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INTRODUCTION

Removal or preconcentration of organic compounds from water has been accomplished using various chemical techniques, such as polymeric membranes (1, 2), cloud point phenomena of surfactants (3, 4), cloud point phenomena of certain derivatized cyclodextrins (5) and, to a greater extent, liquid-liquid extractions (6, 7) and activated carbon (8, 9). Every method has certain strengths and limitations. A few of the more common problems associated with the aforementioned techniques include difficulties and expense in the regeneration of activated carbon, thermal degradation of analytes at the high temperatures required for surfactant cloud point extraction, as well as narrow surfactant concentration ranges in which separation by cloud point is feasible, small ranges of salt concentration or pH in which the extraction can be performed, cost of producing the temperatures needed for separation or regeneration and difficulties in monitoring various processes in the uv-vis spectral region.

Two of the more successful methods for the extraction of organic materials from water that have been used for many years are liquid-liquid extraction and adsorption onto activated carbon. The major disadvantage of liquid-liquid extraction is that large quantities of potentially hazardous organic solvents must be used to extract the required percentage of organic compounds from water. The purchasing, recycling and disposal of these solvents can also become expensive over time. Another widely successful extraction technique involves running wastewater containing organic compounds over a bed of activated carbon, where the compounds adsorb onto the surface of the carbon. The greatest drawback of this process is the limited capacity and the relatively high cost of producing temperatures required to reactivate the carbon after use. Producing these high temperatures usually represent up to 75% of the cost of the total recycling process (10). Consequently, any novel method that can perform nearly as well as activated carbon or liquid-liquid extraction, but does not require

the use of large amounts of hazardous and expensive solvents and can circumvent the high cost of regeneration would be of general interest.

In this paper polymethylhydrosiloxane pentenyl-β-cyclodextrin (PMSP-β-CD) is synthesized and used to remove and recover aromatic organic compounds from water. The method of removal is adsorption onto the co-polymer and separation by sedimentation. Regeneration of PMSP-β-CD is accomplished by heating the polymer which has organic material adsorbed onto it, at 80-100° C in water. The solution is simply decanted or the the co-polymer is filtered while the solution is hot. The organics are released from the water insoluble PMSP-β-CD, forming a concentrated aqueous solution of organic compounds. This process operates with a high recovery of the organic materials in aqueous solution, as well as for the adsorbing media (PMSP-β-CD). This technique can be used for concentration of organic compounds as well. The results of this study indicate that removal of aromatic organic material from water using PMSP-β-CD is a viable alternative to other methods of extraction. This technique is particularly attractive because of the ease of recovery of organics, nonhazardous media, low temperatures required for regeneration of PMSP-β-CD, ease of recovery for PMSP-β-CD and the ability to successfully remove numerous compounds under a variety of conditions.

EXPERIMENTAL

Materials

Chemicals were obtained from various companies as follows: Aldrich Chemical Company: acetanilide, aniline, o,p-chloroaniline, dimethyl sulfoxide - 99% anhydrous, o-nitroacetanilide, o,m,p-nitroaniline, nitrobenzene, o,m,p-nitrophenol, p-nitrotoluene, 4-phenylazophenol, 2-phenylbenzimidazole, 3-phenylphenol and phosphorous pentoxide; Fisher Chemical Company: bromopentene, sodium hydride, sodium propionate, chlorisplatinic acid,

tetrahydrofuran, methanol, polymethylhydrosiloxane (PS 122), glacial acetic acid and anhydrous sodium sulfate; Sigma Chemical Company: 2,2' dihydroxybiphenyl, sodium chloride and 2-naphthol; Matheson, Coleman and Bell: 1-naphthol ; Mallinkrodt Chemical Company: benzoic acid; ASTEC: β-cyclodextrin.

Synthesis of Cross Linked Polymethylhydrosiloxane Pentenyl-β-Cyclodextrin

In a nitrogen atmosphere, 6.79 g of β-cyclodextrin which had been previously dried in a heated vacuum drying chamber overnight with phosphorous pentoxide as the drying agent, was added to 150 ml of dimethyl sulfoxide in a three neck round bottom flask. With continuous stirring, 0.72 g of sodium hydride was added and allowed to react for 90 minutes at 50° C. Bromopentene (3.55 ml) was added to 25 ml DMSO. The solution was dropped into the flask over a one hour period and stirred for 25 hours at 50° C. The flask was then removed from the nitrogen atmosphere, the DMSO layer removed by vacuum distillation at 60° C and 100 ml of methanol was added. The methanol was removed by rotorevaporation until only 5 ml remained. This product (pentenyl-β-CD in methanol) was added to 100 ml of ice water, filtered and rinsed with 3 x 50 ml of ice water. The pentenyl-β-CD was dried overnight in a drying chamber. In a nitrogen atmosphere, 5.0 g of previously prepared pentenyl-β-CD was dissolved in 100 ml tetrahydrofuran at 53° C. To this, 3.5 g of polymethylhydrosiloxane (PS 122) and 10 mg of sodium propionate were added with stirring. Chlorocisplatinic acid (8.8 mg) was dissolved in 1 ml THF and added to the system. The solution was stirred for 4 hours at 53° C and then removed from the nitrogen atmosphere. The product, PMSP-β-CD, was filtered and dried overnight in a drying chamber.

Apparatus and Procedure

A Shimadzu LC-6A liquid chromatographic system with a SPD-2AM variable wavelength spectrophotometric detector and Linear 1200 strip chart recorder was used to quantitate each sample. The detector was operated at a

different wavelength for each compound analyzed. The flow rate and sample loop were 1.0 ml/min and 20 μm respectively. A C_8 or C_{18} column (5μm x 25cm) from Advanced Separation Technologies Incorporated was used to separate each compound.

All samples were prepared using the following method. Distilled water was added to each compound to make the desired concentration. Two ml of the aqueous solution, to which the specified amount of cross linked PMSP-β-CD had been added, was mechanically shaken for twenty hours. The cyclodextrin was allowed to settle to the bottom and the sample was decanted. This sample was then injected into the HPLC system without further preparation. The pure compound was also analyzed to obtain percent removal data. Sodium hydroxide and acetic acid were used for pH adjustment in the pH study. Sodium chloride was used in varying concentrations for the ionic strength study. The mobile phases were mixtures of methanol and water which were filtered through a membrane filter of 0.45 μm pore size and degassed by vacuum-ultrasonication method prior to use. Peak areas of both the sample containing the PMSP-β-CD and the pure compound were calculated by triangulation and used to determine percent of compound removed.

Regeneration of Polymethylhydrosiloxane Pentenyl-β-Cyclodextrin

PMSP-β-CD that had been used to remove organic material from water was added to 500 ml of water. After one hour of heating above 80° C, the mixture was vacuum filtered using Whatman filter paper. The polymer was allowed to air dry for one day. The extracting power of this recycled polymer was measured using a test compound and compared with that of freshly synthesized PMSP-β-CD.

RESULTS AND DISCUSSION

PMSP-β-CD appears to be a hard, brittle solid with negligible solubility in most solvents, including methanol, acetonitrile, ether, water, methylene chloride,

hexane, isopropanol, chloroform, DMSO and DMF. Since this material has both a siloxane and cyclodextrin portion, it may be possible to have both nonspecific, hydrophobic adsorption as well as complexation into the cyclodextrin cavity as means for removing organic compounds from water. Using an optical microscope with 22x and 57x magnification, a photograph of PMSP-β-CD was taken (Figure 1). This image suggests that PMSP-β-CD has a nonporous, glass-like structure. The average partial size was 0.9 mm and the range was from about 0.5 - 1.6 mm.

In the course of studying data from different removal trials, it became obvious that the time allowed for adsorption was important. Consequently, a time study was performed. This data for the unstirred, "stagnant" system is shown in Table I. Three compounds were studied at two different PMSP-β-CD concentrations. After 48 hours, equilibrium is reached in all cases. In a stationary system, the dissolved components must diffuse through the solution until an available adsorption site is found. Shaking or stirring produces rapid mixing, which brings analytes into contact with the surface more efficiently. As expected, equilibrium times were greatly reduced. Consequently all further experiments were done with mechanically shaken mixtures. Table II is a typical comparison of equilibration times in mixed versus unmixed extractions. The time in which maximum removal is achieved is reduced to three hours with mechanical shaking.

Table III shows the relative removal of various aromatic compounds from water using PMSP-β-CD. At the high PMSP-β-CD concentration of 500 g/mmol, all but three compounds were removed at levels greater than 90%. The three that are removed at less than 90% are acetanilide, o-nitroacetanilide and p-nitroacetanilide. This suggests that acetanilide and substituted acetanilides are more poorly adsorbed onto PMSP-β-CD than other compounds. The addition of slightly more PMSP-β-CD will raise removal of the acetanilides to nearly 100%. Clearly the capacity of the PMSP-β-CD in its present state (*i.e.*, relatively large average particle size) is not high. Further grinding of the adsorbent to increase

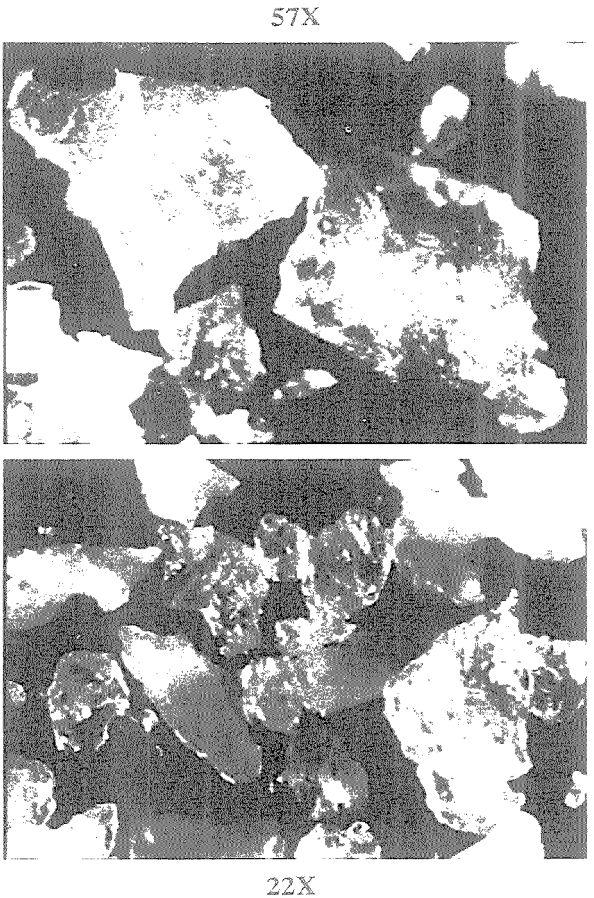

Figure 1: Optical Microscope Photograph of PMSP-β-CD at 22X and 57X magnification.

TABLE I. EFFECT OF TIME ON PERCENT REMOVAL[a]

COMPOUND	TIME (hours)	% REMOVED (0.100 mmol/g)[b]	% REMOVED (0.025 mmol/g)[b]
m-nitrophenol	1	2	13
	6	8	30
	23	27	47
	48	24	62
	68	20	63
	140	27	62
o-nitroaniline	1	8	23
	6	19	37
	23	18	56
	48	32	62
	68	34	68
	140	31	69
p-nitrophenol	1	5	17
	6	12	28
	23	20	44
	48	22	53
	68	24	50
	140	28	58

[a]Two ml of aqueous sample is used. All compound concentrations are $1x10^{-3}$ M.
[b]mmol/g refers to mmol of analyte per gram of PMSP-β-CD.

TABLE II. PERCENT REMOVAL OF M-NITROPHENOL: TIME SHAKEN VERSUS TIME STATIONARY[a]

TIME (hours)	STATIONARY % REMOVED	SHAKEN % REMOVED
0.5	--	40
1.0	13	--
1.5	--	60
2.0	--	78
3.0	--	77
4.0	--	80
6.0	30	--
23.0	47	--
48.0	62	--
68.0	63	--

[a]Two ml of aqueous sample is used. Concentration of m-nitrophenol is 1×10^{-3} M.
Analyte per PMSP-β-CD concentration is 0.025 mmol/g for stationary sample and 0.020 mmol/g for shaken sample.

TABLE III. PERCENTAGE OF AROMATIC MATERIAL REMOVED FROM WATER USING PMS PENTENE-β-CYCLODEXTRIN[a]

ANALYTE	% REMOVED (0.002 mmol/g)[g]	% REMOVED (0.025 mmol/g)[g]	ANALYTE CONC (M x 10^{-5})
acetanilide	81	34	1.0
aniline	98[b]	--	2.5
benzoic acid	90[c]	--	5.0
2,2' dihydroxybiphenyl	93	--	1.0
o-chloroaniline	99[d]	--	1.5
p-chloroaniline	99	--	1.0
N-methylaniline	99[e]	--	1.9
1-naphthol	99	--	1.0
2-naphthol	98	57[f]	1.0
o-nitroacetanilide	70	20	1.0
p-nitroacetanilide	89	--	1.0
o-nitroaniline	95	59	1.0
m-nitroaniline	90	--	1.0
p-nitroaniline	96	--	1.0
nitrobenzene	99	91[f]	1.0
o-nitrophenol	99	--	1.0
m-nitrophenol	96	63	1.0
p-nitrophenol	91	58	1.0
p-nitrotoluene	97	--	1.0
4-phenazophenol	99	--	1.0
3-phenylphenol	90	--	1.0
2-phenylbenzimidazole	98	--	1.0

[a]A two ml aqueous sample is used in all cases. All samples were shaken mechanically for 20 hours.

[b]PMSP-β-CD concentration is 0.005 mmol/g.

[c]PMSP-β-CD concentration is 0.01 mmol/g.

[d]PMSP-β-CD concentration is 0.003 mmol/g.

[e]PMSP-β-CD concentration is 0.004 mmol/g.

[f]PMSP-β-CD concentration is 0.025 mmol/g.

[g]mmol/g refers to mmol of analyte per gram of PMSP-β-CD.

the surface to volume ratio can increase the capacity by nearly two orders of magnitude. However, in this initial study the coarse material was preferred due to the ease of isolation via decantation or fast filtering. Also shown in Table III, PMSP-β-CD is able to extract a significant portion of extremely dilute organic compounds (1×10^{-5} M). With EPA guidelines on allowable organic levels in wastewater becoming more stringent, novel techniques may be needed to remove such trace organics from water. Also, it should be noted that o-chloroaniline and p-chloroaniline are effectively removed with PMSP-β-CD. This is significant because many halogenated organics cannot be removed by derivatized cyclodextrins in cloud point extractions (5). This is because these particular compounds interfere with the phase separation process.

Figure 2 illustrates the effect of PMSP-β-CD concentration on percent removal of m-nitrophenol and 2-naphthol. As expected, until maximum removal is reached, a higher percentage of both compounds is removed as more PMSP-β-CD is introduced into the system. For m-nitrophenol, the maximum point of removal occurs after the addition of 0.005 mmol/g. For 2-naphthol, maximum removal occurs after only 0.016 mmol/g. This suggests that each compound has a different adsorption affinity for PMSP-β-CD. Any given compound's affinity for PMSP-β-CD is probably dependent on many properties such as size of the molecule and functional groups present in the compound. Adsorption isotherms are easily measured for most compounds. Figure 3 shows the isotherm for m-nitrophenol. Adsorption on PMSP-β-CD seems to be a classic Langmurian process. It seems that there are primary adsorption sites but little, if any, secondary adsorption under the conditions of this experiment. Hence, characterization of this adsorbent may be more straight forward than would be indicated from its relatively complex chemical make-up.

Table IV compares extraction values of four compounds at three pHs. 2-Naphthol and m-nitrophenol are weak acids which become ionized under basic conditions. o-Nitroaniline is a weak base which is ionized in acidic solutions. Nitrobenzene does not ionize to an appreciable extent under basic or acidic

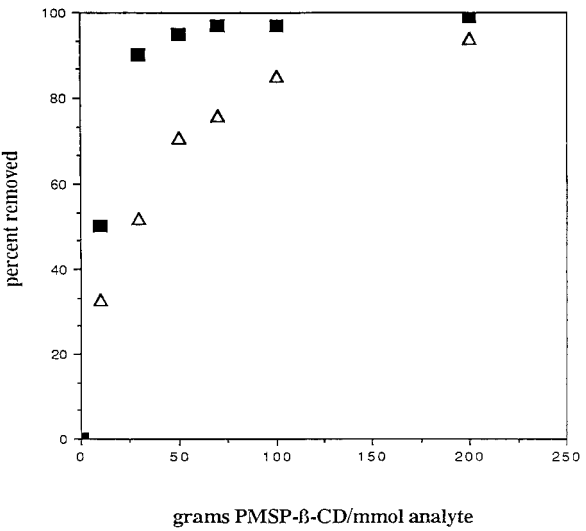

Figure 2: Effect of PMSP-β-CD concentration on percent removal of 2-naphthol (■) and m-nitrophenol (▲). Two ml aqueous samples of varying concentrations were analyzed in all cases. All samples were mechanically shaken for 20 hours.

conditions. While removal is constant for 2-naphthol and nitrobenzene, a slight change is recorded for o-nitroaniline and m-nitrophenol. The small difference in removal for o-nitroaniline and m-nitrophenol is likely due to the ionized species (pH 10.0 for m-nitrophenol and pH 1.5 for o-nitroaniline) having a different affinity of the adsorbent than the corresponding neutral species. Apparently 2-naphthol is sufficiently hydrophobic that it is strongly absorbed whether or not it is ionized. As expected, the percent removal remains constant for nitrobenzene, which was chosen as the reference compound since it should not ionize within the pH range studied. It should be noted that the pH dependence is only a factor when small amounts of PMSP-β-CD are used. If large amounts of PMSP-β-CD are added to the aqueous system, the more weakly absorbing species still can be quantitatively removed.

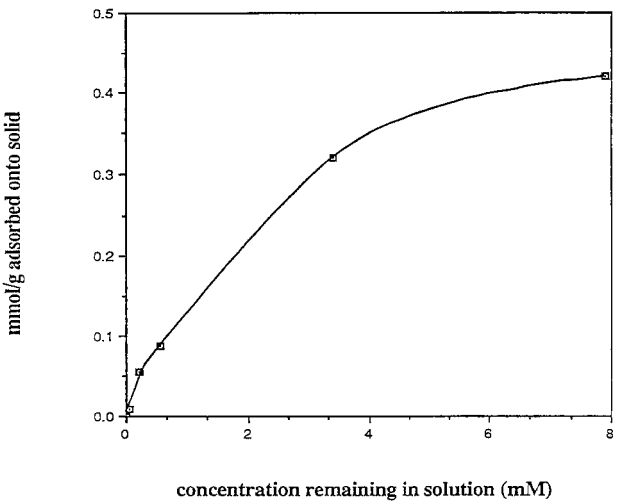

Figure 3: Adsorption isotherm of m-nitrophenol on PMSP-β-CD. Two ml aqueous samples of varying concentrations were analyzed in all cases. All samples were mechanically shaken for 20 hours. All analyzes were performed at room temperature (see Experimental). PMSP-β-CD concentration was 50 mg/ml for each sample.

The effect of salt on percent removal is presented in Table V. Surprisingly extraction of many organic compounds from aqueous solution are not effected by a salt concentrations as high as 0.5 M NaCl. There seems to be no evidence for the 'salting out' effect of organic compounds that is seen in many other circumstances. Another important aspect of removal in salt water is that the separation mechanism (sedimentation) is not effected by the addition of salt. Most cloud point extractions are influenced by even a small salt concentrations (3). This could be an important consideration in applications that require a constant separation mechanism in conditions of varying ionic strength.

Perhaps the most interesting aspect of this method for the separation and removal of organic compounds from water is that it is possible to easily recover both the PMSP-β-CD and organic compounds at high efficiencies. To recycle the

TABLE IV. pH EFFECT ON PERCENT REMOVAL[a]

COMPOUND	pH	%REMOVED (0.017 mmol/g)[b]	% REMOVED (0.002 mmol/g)[b]
o-nitroaniline	1.5	45	99
	7.0	59	99
	10.0	55	99
2-naphthol	3.0	58	92
	7.0	-	98
	10.0	56	94
nitrobenzene	3.0	91	99
	7.0	91	99
	10.0	89	99
m-nitrophenol	3.0	63	92
	7.0	58	90
	10.0	48	95

[a]Two ml of 5 x 10^{-5} M aqueous sample is analyzed in all cases. All samples are mechanically shaken for 20 hours.

[b]mmol/g refers to mmol of analyte per gram of PMSP-β-CD.

PMSP-β-CD, all that is needed is a small amount of water and a source of energy sufficient to heat the water to between 60 and 80° C. Different compounds are released from the adsorbent at different temperatures. However all compounds investigated were completely removed at temperatures between 80-100° C. The PMSP-β-CD is simply added to the water and heated at 80-100° C for a few minutes or until all adsorbed organic material is released. The PMSP-β-CD used in this study was recycled approximately thirty times over a six month period. There was no significant difference found between new and recycled adsorbent. The fact it is relatively easy to induce the release of organic compounds by PMSP-β-CD means that this method can be used in analytical procedures as a preconcentration technique. The low temperature recycling combined with the hardiness of this material makes PMSP-β-CD an interesting alternative for either the concentration or removal of organic materials from water.

TABLE V. SALT EFFECT ON PERCENT REMOVAL[a]

COMPOUND	SALT CONC (M)	% REMOVED (0.017 mmol/g)[b]	% REMOVED (0.002 mmol/g)[b]
o-nitroaniline	0.00	59	98
	0.01	69	98
	0.05	59	98
	0.10	62	96
	0.50	60	99
2-naphthol	0.00	57	99
	0.01	52	97
	0.05	68	98
	0.10	64	97
	0.50	57	97
nitrobenzene	0.00	91	99
	0.01	94	99
	0.05	96	99
	0.10	95	99
	0.50	97	99

[a]Two ml of 5 x 10^{-5} M aqueous sample is analyzed in all cases. All samples are mechanically shaken for 20 hours.

[b]mmol/g refers to mmol of analyte per gram of PMSP-β-CD.

CONCLUSIONS

PMSP-β-CD is a very hardy material that can be used to remove a variety of organic compounds from water. The cost of recycling or reusing this material is inexpensive because low temperatures are used (<100°C). Recovery of PMSP-β-CD is accomplished with simple decantation or filtration. The organic compound that is removed from water, also can be concentrated and quantitatively recovered making it a potentially useful analytical methodology. Both recovery of the PMSP-β-CD and the organic material are quantitatively performed.

ACKNOWLEDGEMENT

Support of this work by the Department of Energy, Office of Basic Sciences (DE FG02 88ER13819) is gratefully acknowledged.

REFERENCES

1. I. Cabasso, E. Klein and J.K. Smith, *Am. Chem. Soc., 35*, 498-502 (1975).

2. J.M. Dickson, M. Babai-Pirouz and D.R. Lloyd, *Ind. Eng. Chem. Proc. Des. Dev.,22*, 625-632 (1983).

3. T. Saitoh and W.L. Hinze, *Anal. Chem., 63*, 2520-2525 (1991).

4. H. Sekikawa, R. Hori, T. Arita, K. Ito and M. Nakano, *Chem. Pharm. Bull., 26*, 2489-2496 (1978).

5. D. Warner-Schmid, S. Hoshi and D.W. Armstrong, *Sep. Science and Tech., 28*, 1009-1018 (1993).

6. C. Bordier, *J. Biol. Chem., 256*, 1604 (1981).

7. B.R. Ganong and J.P. Delmore, *Anal. Biochem., 193*, 35 (1991).

8. R.J. McKinnon and J.E. Dyksen, *J. Amer. Wat. Works Assoc., 76*, 42-47 (1984).

9. S.J. Randtke and V.L. Snoeyink, *J. Amer. Wat. Works Assoc., 75*, 406-409 (1983).

10. H.M. Neukrieg, M.G. Smith, S.W. Maloney and H.S. Irwin, *J. Amer. Wat. Works Assoc., 76*, 158-167 (1984).

11. M.J. Semmens and A.B. Staples, *J. Amer. Wat. Works Assoc., 78*, 76-81 (1986).

Received: September 12, 1993
Accepted: December 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1737-1742 (1994)

SELECTION OF COLUMNS FOR ANALYSIS OF BLOOD UREA

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ABSTRACT

For the analysis of blood urea, the author used several kinds of columns such as conventional ion exchange chromatographic column, ion chromatographic column and reverse phase columns. The result of comparison for separation efficiency will be discussed and conventional ion exchange chromatographic column indicated a best separation efficiency. Prior to application to the columns, blood urea was treated with solid phase extraction (SPE) with a strong cation exchange column with SO_3H functional group.

INTRODUCTION

In order to avoid overlapping of blood admixtures to determine the compound of interest in blood, pretreatment using solid phase extraction (SPE), liquid-liquid extraction and chromatographic separation will be required. In advance before the determination of the compound, the reader must clarify whether the eluted peak was not overlapped and confirm totally pure using several kind of detection method such as photo diode array or mass spectrometry and so on. These detectors are expensive. The simple way to avoid overlapping in HPLC is to purify the compound of interest prior to HPLC application. Using solid phase extraction (SPE) prior to HPLC application, sufficient separation of compound of interest from blood admixtures will be attainable. Concerning SPE of blood

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urea, the paper is now under evaluation[1]. In this paper the author will present the comparison of separation efficiency of conventional ion exchange chromatographic column, ion chromatographic column and reverse phase columns for the separation and determination of blood urea.

MATERIALS AND METHOD

Blood was supplied from Wako and other reagents were special grade commercially available.

For the analysis of blood urea, ion exchange chromatographic column of Mitsubushi Kasei MCI^R CK 08S strong cation exchange column (SO_3H type, 4.6X 150 mm), ion chromatographic column of Wescan strong cation exchange column 269-004 (SO_3H type, 4.6X 250 mm), reverse phase column of Capcell Pak C-18 SG120 (4.6X 250 mm, 5 μm) from Shiseido and reverse phase column column of Capcell Pak C-18 AG120 (4.6X 250 mm, 5 μm) were compared with separation efficiency. The column capacity and particle size of MCI^R CK 08S and Wescan strong cation exchange column 269-004 was as follows: more than 1.9 meq/ml and 11-14 μm and 0.03 meq/ml and 10 μm, respectively. The material of the former is SDB polymer base (degree of crosslinking of DVB is 8%) and that of the latter is silica base, therefore residual silanol effect must be considered for the latter. The eluent of ion exchange columns is 1.5 mM HCl aqueous solution and urea is detected by ultraviolet (UV) at the wavelength of 210 nm. Flow rate is 2 ml/min.

The material of the reverse phase columns was silica with a trace amount of heavy metals and silicone was coated to prevent the residual heavy metal effect as well as residual silanol effect which may cause tailing phenomena. The eluent of reverse phase columns is a mixture of an aqueous solution of water and acetonitrile at a ratio of 90/10 adjusted to pH 9 with phosphate buffer and detection is by UV at the wavelength of 210 nm. Flow rate is 1 ml/min. HPLC apparatus of ion exchange chromatography and reverse phase chromatography was SP-8750 from Spectra-Physics.

SPE of blood urea using strong cation exchange column is as follows: blood is acidified at pH 3 with HCl or used as is. These were applied for ultrafiltration by centrifugation at 4,000 rpm using a Centricon^R (cut-off molecular weight 10,000 daltons) supplied by Amicon and the centrifugated solution was acidified to pH3 with HCl. These were applied to the strong cation exchange column (SO_3H type) of Bond Elut^R SCX (500 mg of resin weight and 0.6 ml of void volume) supplied by Analytichem (Harbor

City, CA, USA). The column was conditioned with 2 ml of methanol followed by 2ml of 0.1M HCl aqueous solution[1]. One ml of blood was applied to the conditioned SCX column at an application flow rate of 0.3 ml/min and washed with 2 ml of water. The retained urea on SCX column was eluted with 2 ml of 1M HCl aqueous solution at the flow rate of 0.3 ml/min. Conditioning and elution in SPE were carried out using a Model AP-115 AN vacuum pump supplied by Iwaki (Tokyo, Japan)[1]. Thus the treated blood urea was applied to HPLC with a column of MCI^{R} CK 08S.

When using ion chromatographic column and reverse phase columns for blood urea analysis, the recommended SPE procedure is as follows: the series of C-18 column and the strong cation exchange column were connected in this order. C-18 column is for removing hydrophobic compounds in blood. The treatment procedure of the strong cation exchange column used for SPE is the same as mentioned above. The conditioning method of C-18 column used for SPE is as follows: C-18 column has 500 mg resin weight and 0.6 ml void volume, thus the conditioning procedure is 2 ml acetonitrile followed by 2 ml water and the conditioned column was set prior to the strong cation exchange column. The conditioning procedure was identical to that presented in the paper[2]. The above treated blood was applied to ion chromatographic column and reverse phase columns.

RESULTS AND DISCUSSION

I Comparison of separation efficiency between ion exchange chromatographic column and ion chromatographic column

The major difference between them is a column capacity. Chromatograms obtained by ion exchange chromatography and ion chromatography, respectively, was presented in Figures I and II. As shown in Figure I, after SPE treatment blood urea was completely separated from blood admixtures. On the contrary, in Figure II by ion chromatography after SPE treatment the chromatogram was found to be successfully separated from blood admixtures, however many admixtures was still present. If the column deteriorates in the process of time and theoretical plate number is decreased, the separation will become worse, thus the former is more superior to the latter.

However there are several ways to overcome these weak points. One is the use of lower flow rate. The other is the use of C-18 column in SPE prior to the strong cation exchange column to remove hydrophobic components by C-18. By the latter procedure, the peaks after elution of urea was diminished. In Figure II, the chromatogram treated

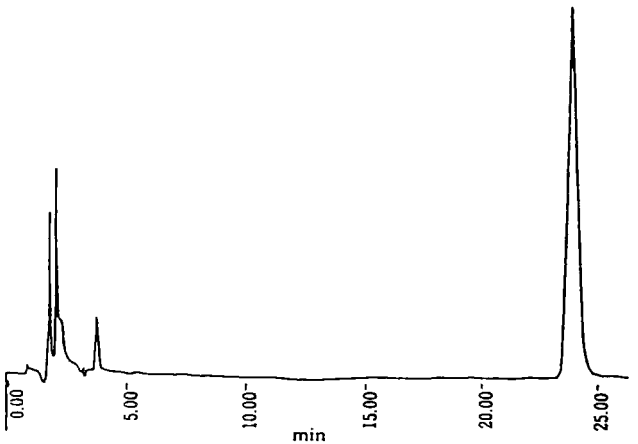

FIGURE I HPLC chromatogram of blood urea after SPE treatment by conventional ion exchange chromatographic column of MCI^{R} CK 08S

The peak eluted at around 24 min is blood urea.

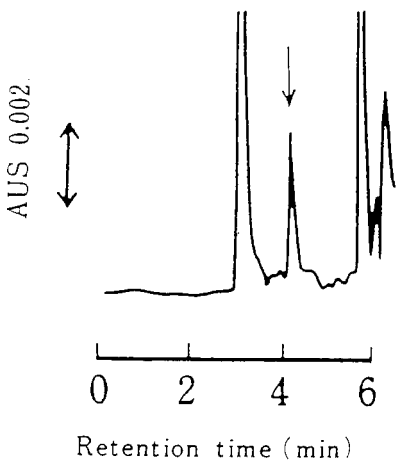

FIGURE II HPLC chromatogram of blood urea after SPE treatment by ion chromatographic column of Wescan 269-004

The peak eluted at around 4 min is blood urea.

without the use of C-18 column for SPE was presented. When using C-18 in addition to the strong cation exchange column for SPE, the peaks eluted after urea in Figure II was diminished.Therefore, both ion exchange columns are found to be appropriate to the analysis of blood urea combined with SPE if the series of C-18 and the strong cation exchange columns were used when using ion chromatographic column of Wescan 269-004. The conventional strong cation exchange column of MCI^{R} CK 08S is superior to ion chromatographic column due to needlessness of C-18 column for SPE.

These results indicated that several factors must be simultaneously considered for the selection of columns.

II Blood urea analysis using SPE combined with reverse phase columns[3-6)]

Urea is a weak alkalinized compound, thus the author suspect when the alkalinized eluent was used, ionization of urea was suppressed and neutralized urea will retain in the reverse phase columns. Therefore, eluent was alkalinized at pH 9 in order to suppress ionization of urea.

Blood urea was treated with SPE procedure as mentioned in the experimental section and applied to HPLC with reverse phase columns. The blood urea was not successfully retained in C-18 column even if ionization of urea was successfully suppressed. This is due to degree of hydrophobicity of suppressed urea. The hydrophobicity of suppressed urea is insufficient as to retain in C-18 column. The k' from void volume to urea elution is around one and around there many blood admixtures eluted, which were mostly hydrophilic compounds unsuccessfully removed by C-18 column for SPE treatment, thus lead to unsuccessful separation. This phenomena is same in AG120 as well as in SG120.

After SPE treatment, urea was acidified and existed as ureonium, however this acidity will be neglected compared with the amount of alkalinized eluent, therefore urea in eluent was sufficiently alkalinized and suppressed ionization.

The alternative method is the use of C-8, phenyl or cyclhexyl in place of C-18 column. However, the author speculates significant improvement of separation will not be attained by those columns. Therefore, ion exchange chromatography will be more appropriate to the analysis of blood urea combined with SPE.

CONCLUSION

Urea was analyzed by conventional ion exchange chromatographic column, ion chromatographic column, reverse phase column after SPE treatment of blood.

Conventional ion exchange chromatographic column was found to be superior to the ion chromatographic column and reverse phase columns. This was found to be due to significant retention in the column with a greater capacity.

REFERENCES

1) H. Shintani, J. Liq. Chromatogr., in submission (1993)
2) H. Shintani, J. Liq. Chromatogr., 15: 1315-1335 (1992)
3) H.Shintani, A. Wojcik,"Enzyme Reaction Detection in Liquid Chromatography", S.K. Lam, eds, Blackie and Son, Inc., New York, 1993, in press.
4) H. Shintani, H. Suzuki, "Bioinstrumentation and Biosensors", D.L.Wise, eds, Marcel Dekker, Inc., New York, NY, 1990, pp.181-245.
5) H. Shintani, J. Chromatogr., 344: 145-155 (1985)
6) H. Shintani, J. Chromatogr., 378: 95-102 (1986)

Received: October 20, 1993
Accepted: November 24, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1743-1754 (1994)

GLYCERYLALKYLSILYLATED SILICA GELS FOR DIRECT INJECTION ANALYSIS OF DRUGS IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A new packing material for direct injection analysis of drug in serum by high-performance liquid chromatography is described. It was found out that proteins are not adsorbed but low-molecular-weight compounds are analyzed by reversed-phase high-performance liquid chromatography using glycerylalkylsilylated silica gels with alkyl moiety ranging from C3 to C6. On chromatography using glycerylundecylsilylated silica gel, on the other hand, retention of low-molecular-weight compounds increased but recovery of proteins decreased from it. Reversed-phase separation of drugs on the new packing materials by direct injection of serum samples is demonstrated.

INTRODUCTION

Drugs in serum are analyzed commonly by reversed-phase high-performance liquid chromatography, in advance of which the samples need time-consuming pretreatment for removing protein

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ingredients. Direct injection of serum samples has long been unfavorable because of resulting in quick deterioration of HPLC columns caused by the denaturation and accumulation of proteins in serum.

Recently developed were new packing materials, in which serum samples can be injected directly without any pretreatment[1-7]. They are called restricted access packing materials[8,9]. The stationary phases consist of two parts. One part, to which proteins can access, are hydrophilic against adsorption of proteins. The other part, shielded from access of proteins, is hydrophobic for partition of low-molecular-weight compounds. Owing to this structure, these packing materials selectively retain low-molecular-weight compounds, while proteins are eluted in the void volume with high recovery. In general, preparation of these packing materials is not so simple and consists of at least two reaction steps; all surface of silica gel is covalently covered with hydrophobic compounds, then hydrophilic feature is introduced only the external surface.

It is known, on the other hand, that glycerylpropylsilylated silica gel can be used for size exclusion chromatography of proteins without denaturation nor adsorption[10]. We have found out that glycerylalkylsilylated silica gels have the function of restricted access packing materials[11]. Because the glycerylalkylsilyl group consists of hydrophilic and hydrophobic moieties, it is expected to possess both functions as a surface barrier against access of proteins and as a partition phase for low-molecular-weight compounds. The present results suggest that the glycerylalkylsilyl stationary phases form bilayer structure (Fig. 1). Further, these packing materials have an advantage over ISRP from the preparative point of view, because they are prepared with one-step silylation. Here we report the details of their preparation and characteristics in HPLC.

EXPERIMENTAL

Reagents and Materials

3-Buten-1-ol, 3-methyl-3-buten-1-ol, 5-hexen-1-ol, 10-undecene-1-ol, epichlorohydrin and dicyclohexyl-18-crow-6 were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). $H_2PtCl_6 \cdot 6H_2O$, K_2PtCl_4, Bovine serum albumin, phenobarbital, phenytoin and carbamazepine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Trimethylsilane and 3-glycidoxypropylsilane were form Shinetsu Chemical (Tokyo, Japan). Silica gel was Develosile (particle size 5 μm, pour size 60 Å and surface area 500 m^2/g) from Norura Chemicals (Seto, Japan).

Preparation of 4-Glycidoxybutyltrimethoxysilane

4-(2-Hydroxy-3-chlorobutoxy)-1-butene: To a mixture of 72.1 g (1 mol) of 3-buten-1-ol and 9.3 g (0.1 mol) of epichlorohydrin were added 12 drops of conc.H_2SO_4 at room temperature. The mixture was refluxed for 4 h and cooled to room temperature, and 1.2 g of $BaCO_3$ was added to it. The mixture was stirred for 30 min and 1 mol of chloroform was added to it. The mixture was filtered, evaporated and distilled under vacuum to give 17.2 g (0.1 mol, 100 %) of 4-(2-hydroxy-3-chlorobutoxy)-1-butene[12].

4-Glycidoxy-1-butene: To 500 ml of diethyl ether containing 22 g (0.55 mol) of powder NaOH was added gradually 55 g (0.33 mol) of 4-(2-hydroxy-3-chlorobutoxy)-1-buten and the suspenson was stirred for 15 h at room temperature. To the stirred suspension was added gradually 150 ml of water. The organic layer was washed by 300 ml of water, dried on $MgSO_4$ and evaporated. Vacuum distillation gave 28.6 g (0.22 mol, 67 %) of 4-glycidoxy-1-butene[13].

4-Glycidoxybutyltrimethoxysilane: To a solution of 15 g (0.12 mol) of 4-glycidoxy-1-butene in 10 ml of toluene was added 0.6 ml

of 1 % $H_2PtCl_6 \cdot 6H_2O$ in t-butanol at room temperature. A solution of 14.4 g (0.12 mol) of trimethoxysilane in 10 ml of toluene was added to the suspension. The suspension was refluxed for 1 h, evaporated and distilled under vacuum to give 11.9 g (0.05 mol, 42 %) of 4-glycidoxybutyltrimethoxysilane[14]. ^1H NMR (60 MHz) δ 0.67 (m, $SiCH_2$, 2H), 1.52 (m, CH_2, 4H), 2.65 (m, OCH_2, 2H), 3.05 (m, OCH, 1H), 3.47 (m, OCH_2, OCH_3, 13H).

Preparation of 4-Glycidoxy-2-methylbutyltrimethoxysilane

4-Glycidoxy-2-methylbutyltrimethoxysilane (13 %) was prepared form 3-methyl-3-buten-1-ol by the method similar to the preparation of 4-glycidoxybutyltrimethoxysilane. ^1H NMR (60 MHz) δ 0.66 (m, $SiCH_2$, 2H), 0.95 (d, CH3, 3H), 1.58 (m, CH, CH_2, 3H), 2.53 (m, OCH_2, 2H), 2.965 (m, OCH, 1H), 3.35 (m, OCH_2, OCH_3 13H).

Preparation of 6-Glycidoxyhexyltrimethoxysilane

6-glycidoxy-1-hexene: 6-Glycidoxy-1-hexene (37 %) was prepared from 5-hexen-1-ol by the method similar to the preparation of 4-glycidoxy-1-butene

6-Glycidoxy-1-hexyltrimethoxysilane: To a mixture of 40 mg of K_2PtCl_4 and 200 mg of dicyclohexyl-18-crown-6 was added 20 ml of benzene. The mixture was refluxed for 2 h and allowed to stand for 16 h to give the catalyst. The catalyst was added to a mixture of 5 g (32 mmol) of 6-glycidoxy-1-hexene and 3.9 g (32 mmol) of trimethoxysilane at room temperature. The mixture was stirred at 100°C for 2 h, evaporated and distilled under vacuum to give 3.6 g (13 mmol, 41%) of 6-glycidoxyhexyltrimethoxysilane[15]. ^1H NMR (60 MHz) δ 0.65 (m, $SiCH_2$, 2H), 1.50 (m, CH_2, 8H), 2.67 (m, OCH_2, 2H), 3.08 (m, OCH, 1H), 3.50 (m, OCH_2, OCH_3, 13H).

Preparation of 11-Glycidoxyundecyltrimethoxysilane

11-Glycidoxy-1-undecyltrimethoxysilane (9.5 %) was prepared form 10-undecen-1-ol by the method similar to the preparation of 6-glycidoxyhexyltrimethoxysilane. ^{1}H NMR (60 MHz) δ 0.67 (m, $SiCH_2$, 2H), 1.40 (m, CH_2, 18H), 2.65 (m, OCH_2, 2H), 3.05 (m, OCH, 1H), 3.48 (m, OCH_2, OCH_3, 13H).

Preparation of Glycerylalkylsilylated Silica Gels

Silica gel (10 g) in a vial was dried under vacuum at 120°C for 2 h. To the silica gel was added 0.6 ml of water, the vial was sealed and allowed to stand for 24 h. To the silica gel was added 34 ml of toluene. To the stirred suspension was added 0.1 ml of 10 % p-toluenesulfonic acid in acetonitrile and 22 mmol of glycidoxyalkyltrimethoxysilane. The suspension was stirred at 120°C for 16 h, cooled, filtered and washed sequentially with 200 ml of toluene and 200 ml of acetone. To the silica gel was added 50 ml of 0.01 N H_2SO_4 in water. The suspension was refluxed for 1 h, cooled and washed with water. To the silica gel was added 50 ml of 10 mM phosphate buffer (pH 8.0). The suspension was refluxed for 1 h, cooled and washed sequentially with water, methanol and diethyl ether. The suspension was filtered and dried under vacuum at 60°C for 4 h to give glycerylalkylsilylated silica gel. In the case of glyceryhexcylslylation and glycerylundecylsilylation, the suspensions were refluxed for 3 h in each operation.

Apparatus

The prepared glycerylalkylsilylated silica gels were packed into stainless steel tubes (150 mm x 4.6 mm I.D.) by conventional high-pressure slurry-packing procedures.

The HPLC system consisted of a pump (LC-6A, Shimadzu, Kyoto, Japan), a UV detector (SPD-6A, Shimadzu), a data processor (CR-4A, Shimadzu) and a injector (model 7125, Rheodyne, U.S.A).

RESULTS AND DISCUSSION

Surface Coverage of Glycerylalkylsilylated Silica Gel

Table 1 shows the surface coverage of the packing materials estimated from the value of carbon contents. The values of surface coverage fall between 2.03 and 3.00 $\mu mol/m^2$. These values suggest that the stationary phases on these silica gels are not polymeric[16] but are monomeric layers of glycerylalkylsilyl groups composed of hydrophilic and hydrophobic moieties.

Chromatography of Low-molecular-weight Compounds

Tables 2 and 3 show the capacity factors of aromatics (benzene and naphthalene) and drugs (phenobarbital and carbamazepine), respectively, on the glycerylalkylsilylated silica gels. The packing materials with a longer alkyl moiety gave larger capacity factor, and more hydrophobic compounds gave larger capacity factor. This result indicates that the low-molecular-weight compounds can be analyzed by reversed-phase chromatography.

Recovery of BSA

Kimatas' method[6] was referred to for testing recovery of proteins from the packing materials. A dilute solution of BSA was used to detect slight differences among the packing materials. As the recovery of BSA from the columns increased with repeat number of injection, a newly prepared column was used in each run. Table 4 shows the results. The recovery was high from glycerylalkylsilylated silica gels with an alkyl moiety of C3 to C6. On the other hand, the recovery was very low from the packing material with an alkyl moiety of C11, indicating that there was a strong interaction between the hydrophobic undecyl moiety and BSA. When in contact with a highly aqueous mobile phase, the stationary phase with an alkyl moiety of C3 to C6 is

TABLE 1

Surface Coverage of Glycerylalkylsilylated Silica Gels

Stationary phase	Carbon contents (%)	Surface coverage ($\mu mol/m^2$)
Glycerylpropyl	8.35	3.00
Glycerylbutyl	8.82	2.69
Glyceryl-2-methylbutyl	8.86	2.32
Glycerylhexyl	11.1	2.72
Glycerylundecyl	13.0	2.03

TABLE 2

Capacity Factor of Benzene and Naphthalene on Glycerylalkylsilylated Silica Gels

Stationary phase	Capacity factor k'	
	Benzene	Naphthalene
Glycerylpropyl[a]	0.702	3.96
Glycerylbutyl[a]	0.994	6.51
Glyceryl-2-methylbutyl[a]	1.03	6.57
Glycerylhexyl[a]	4.40	-
Glycerylhexyl[b]	1.82	6.21
Glycerylundecyl[b]	4.83	-

[a]Mobile phase: acetonitrile-water (1:9)
[b]Mobile phase: acetonitrile-water (3:7)

TABLE 3

Capacity Factor of Drugs on Glycerylalkylsilylated Silica Gels

Stationary phase	Capacity factor k'	
	Phenobalbital	Carbamazepine
Glycerylpropyl	0.162	0.675
Glycerylbutyl	0.310	1.22
Glyceryl-2-methylbutyl	0.418	1.43
Glycerylhexyl	1.55	4.53

Mobile phase: acetonitrile-100mM phosphate buffer (pH 6.9) (2:8)

TABLE 4

Recovery of BSA from the Glycerylalkylsilylated Silica Gels

Stationary phase	Recovery of BSA (%)
Glycerylpropyl	102
Glycerylbutyl	98.0
Glyceryl-2-methylbutyl	97.0
Glycerylhexyl	94.9
Glycerylundecyl	5.7

HPLC conditions: mobile phase, acetonitrile-100mM phosphate buffer (pH 6.9) (2:8); Flow rate, 1ml/min; UV detection, 295 nm; sample, 10mg/ml; injection volum, 20 µl

in a "bristle" state, whereas with a C11 moiety it is in a "folded" state[17-19]. Hence, it is suggested that the stationary phases with an alkyl moiety of C3 to C6 form bilayer structure (Fig. 1) but that with an alkyl moiety of C11 does not in the present condition.

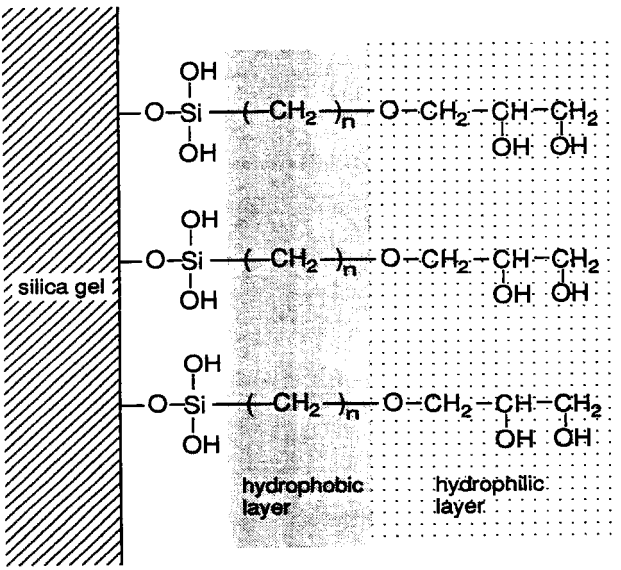

FIGURE 1. Structure of glycerylalkylsilylated silica gel.

Analysis of Drugs in Derum by Direct Injection

Fig. 2 shows separation of drugs in serum by direct injection on the glycerylalkylsilylated silica gels. The serum proteins are eluted in the void volume, and the drugs can be analyzed quantitatively.

In the case of direct injection analysis of serum, in general, concentration of organic solvent in mobile phase is limited to low in order to avoid denaturation of proteins[20]. This is a strong restriction when only one kind of packing materials can be available, because it is quite probable that hydrophobic drugs hardly elute whereas hydrophilic ones elute too easily. This difficulty is to be solved by changing the nature of the stationary phase. The present packing material is one of

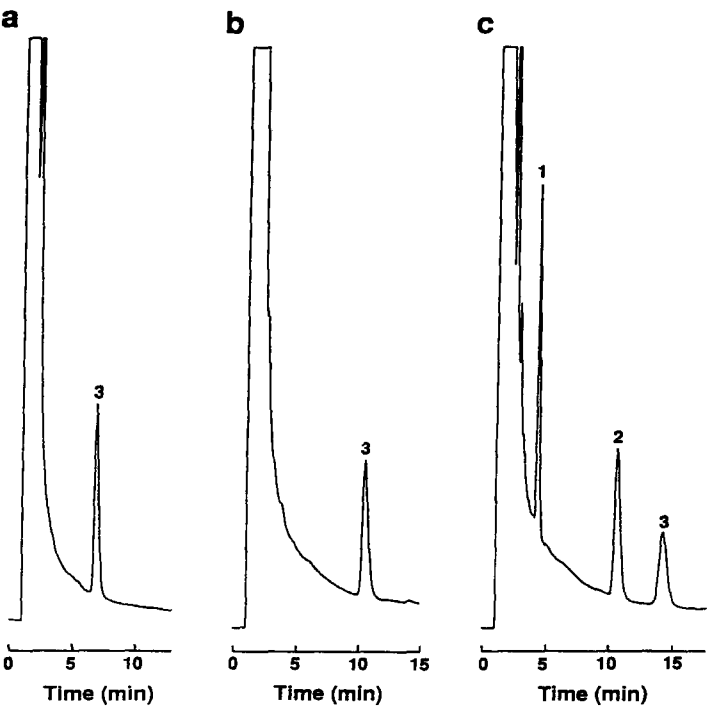

FIGURE 2. Chromatograms of human serum spiked with drugs by direct injection on (a) glycerylpropylsilylated silica gel, (b) glycerylhexylsilylated silica gel and (c) glyceryl-2-methylbutylsilylated silica gel. Solutes: 1, phenobarbital (40 μg/ml); 2, phenytoin (20 μg/ml); 3, carbamazepine (10 μg/ml). HPLC conditions: mobile phase, (a) acetonitrile-100mM phosphate buffer (pH 6.9) (1:9), (b) acetonitrile-100 mM phosphate buffer (pH 6.9) (2:8), (c) THF-100mM phosphate buffer (pH 6.9) (8:92); flow rate, 1 ml/min; UV detection, 220nm; injection volume, 20 μl.

such examples; if the glycerylalkylsilylated silica gel with an alkyl moiety of suitable length to the drugs of consideration is selected, the drugs in serum with various hydrophobicity or hydrophilicity can be analyzed quantitatively.

REFERENCES

1 H. Yoshida, I. Morita, G. Tamai, T. Masujima, T. Tsuru, N. Takai and H. Imai, Chromatographia, 19: 466-472 (1984)

2 H. Hagestam and T. C. Pinkerton, Anal. Chem., 57: 1757-1763 (1985)

3 Y. Sudo, R. Miyagawa and Y. Takahata, Chromatography, 9: 179-180 (1988)

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

5 J. Haginaka, N. Yasuda, J. Wakai, H. Matsunaga, H. Yasuda and Y. Kimura, Anal. Chem., 61: 2445-2448 (1989)

6 K. Kimata, R. Tsuboi, K. Hosoya, N. Tanaka and T. Araki, J. Chromatogr., 515: 73-84 (1990)

7 J. Haginaka and J. Wakai, Chromatographia, 29: 223-227 (1990)

8 J. Haginaka, Trends Anal. Chem., 10: 17-22 (1991)

9 K. K. Unger, Chromatographia, 31: 507-511 (1991)

10 F. E. Regnier and R. Noel, J. Chromatogr. Sci., 14: 316-320 (1976)

11 Y. Sudo, M. Akiba, T. Sakaki and Y. Takahata, Jpn. Kokai Tokkyo Koho, 90 45758

12 D. Swern, G. N. Billen and H. B. Knight, J. Am. Chem. Soc., 71: 1152-1156 (1949)

13 H. Flores-Gallard and C. B. Pollard, J. Org. Chem., 12: 831-833 (1947)

14 E. P. Plueddemann and G. Fanger, J. Am. Chem. Soc., 81: 2632-2635 (1959)

15 T. Satou and H. Okinoshima, Jpn. Kokai Tokkyo Koho, 77 93718

16 L. C. Sander and S. A. Wise, Anal. Chem., 56: 504-510 (1984)

17 R. K. Gilpin and J. A. Squires, J. Chromatogr. Sci., 19: 195-199 (1981)

18 R. K. Gilpin, M. E. Gangoda and A. E. Krishen, J. Chromatogr. Sci., 20: 345-348 (1982)

19 L. C. Sander, J. B. Callis and L. R. Field, Anal. Chem., 55: 1068-1075 (1983)

20 T. C. Pinkerton, T. D. Miller, S. E. Cook, J. A. Perry, J. D. Rateike and T. J. Szczerba, BioChromatography, 1: 96-105 (1986)

Received: November 11, 1993
Accepted: January 14, 1994

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1755-1772 (1994)

THE ELECTROCHEMICAL DETECTION OF PENICILLINS IN MILK

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ABSTRACT

Penicillins can be detected in milk samples using pulsed amperometric detection following reversed-phase high performance liquid chromatography. A detection limit of $2*10^{-6}$ M was achieved for penicillin G, with similar values obtained for the other penicillins tested. To improve these detection limit values, an on-column dual-solvent concentration scheme was adapted. A concentrating solvent forced the deposition of penicillins onto the C-18 column. Afterward, the normal chromatographic eluent carried the penicillins off the stationary phase and allowed a separation. Work with standard solutions proved the viability of the scheme and also demonstrated the linearity of detector response at low concentrations. Using the concentration procedure, a detection limit of $2*10^{-7}$ M was found for penicillin G in milk solution, and in general the concentration scheme allowed detection limits for all of the penicillins to be improved by approximately a factor of 10.

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INTRODUCTION

The penicillins are a group of antibiotics commonly prescribed for a variety of bacterial infections. The natural products penicillin G and 6-aminopenicillanic acid are produced by fermentation of *Penicillium chrysogenum*; further members of this class are produced by synthetic modification of these natural products (1). The impact penicillins have made on human medicine is well understood; not as well known is the degree to which penicillins have been adapted for use in veterinary medicine for treatment of infections and as a prophylactic during surgical procedures (2-6).

One group of animals that may be treated with penicillins are dairy cattle (7,8). These drugs are commonly applied by dairy farmers to fight lung and udder infections in their herd. It is legal to use penicillins to treat these infections as long as the bovine system is clear of the antibiotic during milking. Nevertheless, there is the potential for the transfer of penicillins into milk. This may have serious consequences to the milk consumer, as the incidence of allergic response to penicillins may be as high as 10% (9). While this response may be just the appearance of a mild skin rash, in acute cases the response may be an anaphylactic reaction, which can be fatal. The Food and Drug Administration has recently announced plans for a nationwide milk screening program for penicillins, as well as other drugs, in response to criticism from consumer groups (10).

Analytical methodology for penicillin screening in milk must meet stringent standards. Milk is a fairly complex substance, providing a challenging sample matrix. As well, the screening tests must feature detectability to trace concentration levels. Several different analytical approaches have been used for these screening tests. Microbiological assays have shown the requisite sensitivity, but they are time-consuming, lack specificity, and do not lend themselves well to

quantitative work (11). Colorimetric assays lack both sensitivity and specificity. Recent work has focused on high performance liquid chromatography (HPLC) usage to provide selectivity in conjunction with a sensitive detector. Several groups have detected penicillins by direct UV absorbance following HPLC (12-15) with detection limits reported as low as 0.1 ppm. The low wavelengths chosen (typically 200-230 nm) led to poor detector selectivity, which was problematic when applied to milk samples. Despite selectivity problems, detection limits to .03 ppm for milk samples were reported (11) for a method incorporating a chemical concentration step into the sample preparation procedure before injection into the HPLC. Moats (16, 17) was able to detect less than 10 ppb of penicillin, but only after extensive sample preparation that involved a concentration step and "heart cutting" (18), where a fraction from a preparative HPLC trial is collected and rechromatographed on the analytical HPLC system.

Other detector options are available for coupling with HPLC. Several indirect methods and derivatization schemes have allowed more selective photometric detection at higher wavelengths (19-22) but have lacked sufficient sensitivity. Fluorescent penicillin adducts can be produced using o-phthaldialdehyde (23,24), but this is limited to species having a primary amine group on their side chain. Electrochemical detection has been reported using amperometric oxidation after photolytic derivatization with a UV flashlamp (25). Recent work in this group (26,27) has shown that pulsed amperometric detection (PAD) at a gold electrode can provide a simple approach to detecting all penicillins without the necessity of derivatization. PAD allows sensitive response to the penicillins either by direct oxidation of the compound or indirectly by the suppression of residual current from background processes, depending on the waveform chosen. Detector sensitivity is comparable to direct UV detection, but

the selectivity of PAD is superior considering the UV wavelengths necessary. This work sought to apply this detection methodology following reversed-phase HPLC to milk samples containing penicillins.

EXPERIMENTAL

Materials and Reagents

Penicillins were obtained from Sigma Chemical Co. (St. Louis, MO, USA). A listing of the penicillins used and their abbreviations are given in Table 1. Reagent grade acetic acid from Fisher (Pittsburgh, PA, USA) and sodium acetate from Baker (Philipsburg, NJ, USA) were used to produce acetate buffer solutions. For milk extractions, GC/pesticide grade hexane and HPLC grade methylene chloride from Alltech (Deerfield, IL, USA) were used. HPLC grade acetonitrile and methanol from Fisher were used as mobile phase organic modifiers. Water for aqueous solutions was distilled and deionized before use. Chromatographic mobile phases were vacuum filtered through an Alltech 0.2-micrometer nylon

TABLE 1
A Listing of the Penicillins Used

PENICILLIN	ABBREVIATION
Amoxicillin	Amox
Ampicillin	Amp
Cloxacillin	Cloxa
Dicloxacillin	Dicloxa
Methicillin	Meth
Nafcillin	Naf
Oxacillin	Oxa
Penicillin G	Pen G
Penicillin V	Pen V

filter and sonicated before use. A nylon 0.45-micrometer syringe-tip filter was used to screen samples before their injection into the HPLC.

Chromatographic Apparatus

A Waters (Milford, MA, USA) 625 Gradient LC System was used as the primary pump for all HPLC work. A flow rate of 2.0 ml/minute was standard for most applications. The pump was run in "silk mode", a Waters feature designed to reduce pump noise. The injection loop had a volume of 50 microliters. When the dual pump concentration scheme was adapted, a Spectra Physics (San Jose, CA, USA) Isochrom LC pump was used as the concentrating pump. To apply the concentrating system, the main valve in the Waters 625 was replumbed as can be seen in Figure 1. In the concentrating mode, the Spectra Physics pump concentrated milk samples onto the column and then pumped to waste. Normally, the flow rate for the Spectra Physics pump was 3 ml/minute and the concentrating solvent was .02 M acetate buffer. In this mode, the Waters 625 LC system pumped the normal mobile phase, the eluting solvent, through the detector and then out to waste. When the valve was switched to the analytical mode, the concentrating pump was diverted directly to waste. The analytical pump now delivered the mobile phase to the column eluting the concentrated milk sample. Finally, the concentrated sample entered the detector for analysis. The standard gradient program used for penicillin separation, adapted from reference 27, is given in Table 2.

A Waters 8 x 10 Radial-Pak Compression Module housed a 10-micrometer μBondaPak C-18 Radial-Pak cartridge stationary phase. The cartridge had an internal diameter of 8 mm and a length of 100 mm. All separations were done at ambient laboratory temperature (ca. 20 +/- 2 degrees C.) Sample preconcentration was always done on the analytical column.

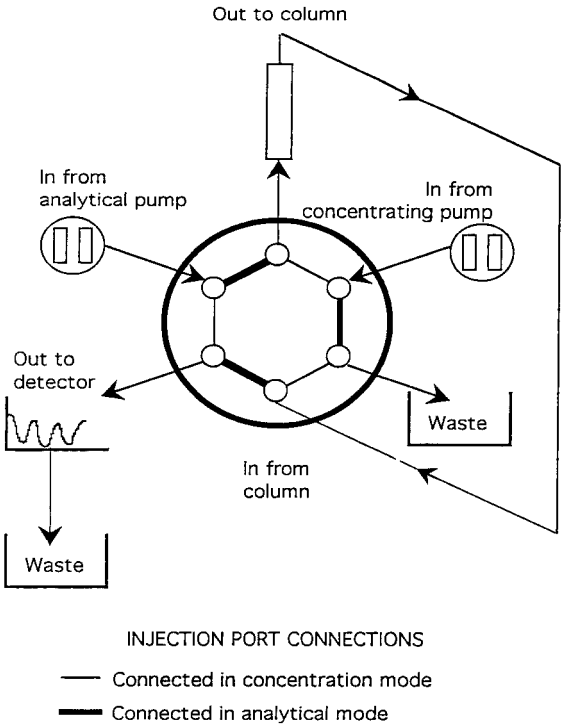

Figure 1. Valve setup in concentration mode.

TABLE 2
Gradient Program For Penicillin Separations
(Flow Rate 2 ml/min)

TIME (MIN)	COMPOSITION
Initial	15% Acetonitrile 10% Methanol 75% .02 M Acetate Buffer
0-15	Linear Ramp
15	30% Acetonitrile 0% Methanol 70% .02 M Acetate Buffer
15 +	Isocratic Hold

Electrochemical detection used the Waters 464 Pulsed Electrochemical Detector. A thin-layer cell was utilized that had an electrode block containing dual gold electrodes in the series configuration. The upstream element was used as the working electrode, while the downstream electrode served as the counter electrode for this work. To minimize detector noise, the detector was configured in the floating ground mode throughout this project, which served to ground the detector at a virtual point rather than to the chassis of the instrument. A Ag/AgCl reference electrode was used in the thin-layer cell. An indirect PAD waveform, given below, was used for all milk applications.

E1 = 1300 mV for .166 seconds

E2 = 1500 mV for .166 seconds

E3 = -200 mV for .333 seconds

Chromatographic data was collected with a Gateway (N. Sioux City, SD, USA) 386SX computer using a Keithley MetraByte (Taunton, MA, USA) Chrom-1AT interface board. Recorded PAD data for quantitative studies was treated with a fourier transform smoothing algorithm to minimize high frequency noise.

Milk Sample Preparation

Skim milk of various brands was purchased at local grocery stores. Milk samples were prepared by spiking measured volumes of the skim milk with known amounts of penicillins. Proteins were then precipitated with two volumes of acetonitrile. The solution was left standing for five minutes during which time the precipitated proteins settled to the bottom. The aqueous portion was decanted and filtered by suction. Fats were then extracted with an equal volume of 1:1 methylene chloride:hexane. The aqueous phase was centrifuged at 3000 rpm for 10 minutes. Finally, the remaining solution was diluted 3:1 with 0.02 M acetate buffer and passed through a 0.2 micrometer nylon filter. For detection without on-

column preconcentration of the milk samples, there was no dilution with acetate buffer.

RESULTS AND DISCUSSION

Direct injection of milk samples was not advisable to insure a reasonable HPLC column lifetime, yet an important concern was to make the sample preparation as simple and quick as possible. The complexity of the milk sample matrix forced past workers to introduce various lengthy sample preparation schemes, as noted in the Introduction. It was hoped that the selectivity of PAD would permit the investment of less sample preparation time. A detailed description of the basic sample preparation procedures can be found in the Experimental section. This procedure removed largely the proteins and fats, leaving behind lower molecular weight water-soluble species. However, it was known that this portion was still relatively complex in composition (28).

Injections of the milk extract spiked with penicillins illustrated that most of the response to the milk sample matrix was confined to a large void response eluting within 4 minutes of injection. The major effect on the penicillin separation was that any species eluting before ampicillin would coelute with this void response. Although ampicillin suffers from a slight overlap with this region, it can be clearly discerned as well as all following peaks. (see Figure 2). A set of experiments were devised to find the percentage of penicillins recovered from the milk samples, and to examine the reproducibility of response. Five injections of a standard solution (typically $1*10^{-4}$ M) in a water matrix were made into the C-18 column. Three separate milk extractions were run, and a series of 5 injections made from each of the extracts. The resulting statistics from these experiments, based on peak height measurements, are summarized in Table 3. The recovery

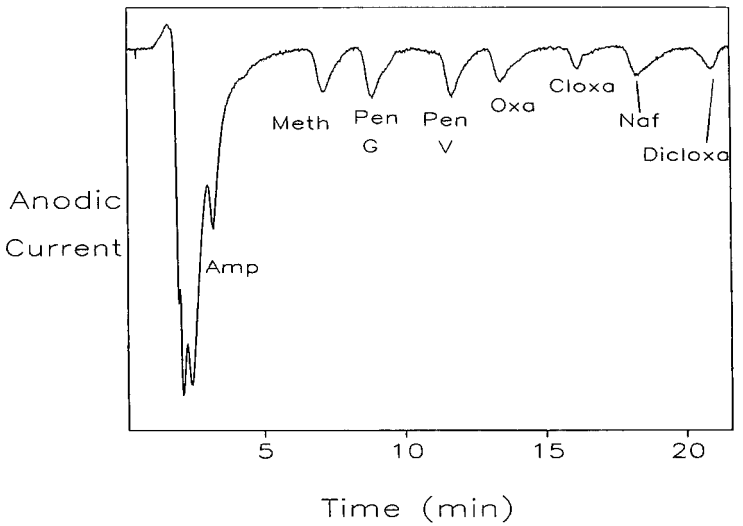

Figure 2. Direct detection of 8 pencillins within a milk sample. 50 µl injections on a µBondapak C-18 column. All penicillins are at $1*10^{-4}$ M. Waveform and gradient given in the Experimental section.

TABLE 3
Statistics from HPLC separations in Milk.

	Amp	Meth	Pen G	Pen V	Oxa	Cloxa	Naf	Dicloxa
Gradient k' value	0.82	3.57	4.61	6.42	7.55	9.59	10.83	12.74
Recovery % from milk samples	56.4	91.3	87.7	84.9	78.1	66.3	63.1	71.0
Standard solution RSD (%)	4.19	2.24	2.38	2.13	3.32	2.84	8.57	2.74
Intra-extract variability (RSD, %)	4.93	5.12	4.55	4.96	8.57	9.73	8.51	6.73
Inter-extract variability (RSD, %)	3.50	2.68	8.90	3.35	6.55	9.50	10.4	4.14

percentages are good considering the difficulty of recovering the penicillins quantitatively during the extraction step. It is interesting to note the inverse correlation between k' and recovery percentage. This is likely due to the more non-polar penicillins not being extracted efficiently into the aqueous phase during sample preparation. Ampicillin is an exception to this trend, which may be due in part to its overlap with the void response. For the most part, the variabilities are below 10% relative standard deviation. The later eluting peaks tended to have larger variabilities, but this was largely due to the fact that these are expressed in terms of relative standard deviations (RSD). The later peaks are also the smallest ones, inflating the RSD's relative to the earlier, larger peaks. If the variabilities are expressed in terms of absolute standard deviation, these later eluting compounds are only slightly less reproducible than their earlier counterparts.

Detection limits for direct injection of the milk extracts containing penicillin spikes are given in the first row of Table 4. These values are from 2-5 times higher than what can be obtained with a cleaner sample matrix. As the recovery percentages in Table 3 attest, this is mainly due to an increased noise level when

TABLE 4
Milk Detection Limits

	Amp	Meth	Pen G	Pen V	Oxa	Cloxa	Naf	Dicloxa
Detection limit for direct 50 µl injections of milk samples (µM)	2	2	2	2	3	4	4	7
Detection limit for on-column concentration of milk samples (µM)	0.3	0.3	0.2	0.2	0.2	0.3	0.2	0.3

using the milk samples rather than a loss in sensitivity. The detectability of the later-eluting peaks suffered somewhat due to the expected chromatographic peak broadening, and some slight degree of improvement could be seen by employing alternative HPLC allowing them to elute earlier (but causing coelution of the initial peaks). Otherwise the PAD response was close to the maximum that could be obtained with our detector.

Other routes to improve detectability by employing a concentration step were examined. The first attempt at concentration was directed toward simply evaporating the sample under reduced pressure to lower the volume and concentrate the penicillins. The problem with this approach, though, was that the concentrated solutions were simply too viscous for HPLC analysis. Those samples that were injected caused clogging problems. Further steps to thin the sample were examined, but there appeared to be no way to avoid a time-consuming and labor-intensive process. Thus, an alternative method for concentration was sought.

Another method tested to improve detectability was an on-column concentration scheme. The basic plumbing for the system is shown in Figure 1 and described in the Experimental section. Early chromatographic work during this project tested aqueous mobile phases containing only buffer and/or electrolyte but no organic modifier. Under these conditions injected penicillins were infinitely retained on the C-18 stationary phase and not recovered from the column. Upon decreasing the polarity of the mobile phase by the addition of ca. 30% by volume methanol and/or acetonitrile, reasonable retention times were achieved. Therefore, an aqueous solvent was used to concentrate the penicillins on the C-18 column, followed by a switch to the regular gradient program containing organic modifier to elute the concentrated sample. By doing this, penicillin solutions that would be

undetectable using the direct method could be concentrated to the point of detectability. One drawback to this type of procedure was that a much larger sample volume would be required. This was much less a concern for bovine milk samples than for samples from human body fluids. One major advantage to this type of concentration was that it could easily be automated to run as an on-line process. A similar method could probably be developed using an off-column concentration step with a solid phase extraction cartridge.

Before any milk samples were analyzed, extensive testing of the method on standard penicillin solutions was undertaken. Figure 3 shows the detection of 10 ppb penicillin V dissolved in .02 M acetate buffer. The sample solution was concentrated for 20 minutes using a flow rate of 3 ml/minute before switching to an elution solvent of 30% acetonitrile/70% .02 M acetate buffer. This solvent was used for samples containing only a single penicillin instead of the standard gradient program in order to save time during repetitive trials. The large baseline shift at around 2.5 minutes is due to the transition from the concentrating solvent to the eluting solvent at the detector. The concentration scheme kept the elution solvent flowing through the thin-layer detector cell even during the concentration period to minimize detector noise. However, upon switching from concentration to elution a fairly large volume of the aqueous concentrating solution would be trapped in the system and had to pass through the detector cell, causing the perturbation at short times following valve switching.

As long as the final eluted concentration of the penicillin was less than $3.5*10^{-4}$ M, linear detector response could be expected for the concentration method (27). At greater concentrations the current was less than predicted by extrapolation of the linear region, as current was proportional to adsorbed analyte surface coverage rather than concentration, giving isotherm-like response (26). A

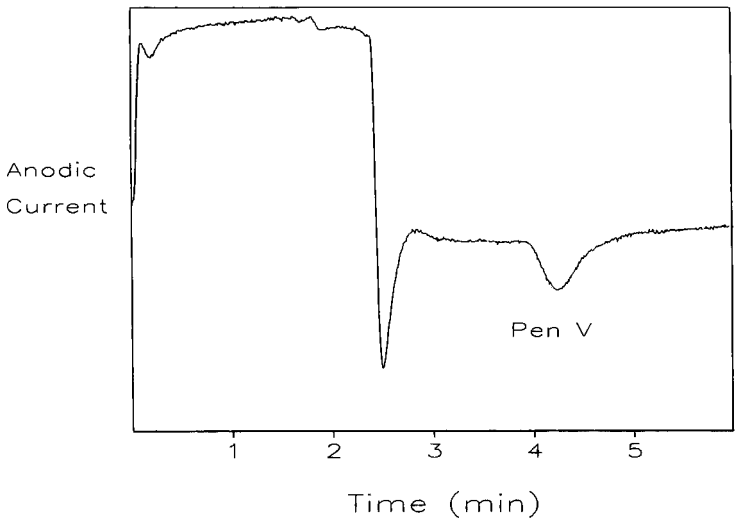

Figure 3. 10 ppb penicillin V detected after concentration. 20 minutes of concentration at 3 ml/min. Analytical solvent was 30% acetonitrile/ 70% .02 M acetate buffer, 2 ml/min. Waveform given in the Experimental section.

plot of peak area vs. concentration was made for a series of penicillin V solutions, each concentrated for 6 minutes at 3 ml/min. The linearity was good; the statistics below describe the plot for solutions ranging from 15 to 35 ppb in concentration.

$$y = -28.360 + 5.9880x \quad R^2 = 1.000$$

Alternatively, response was also linear with concentration time. A 20 ppb penicillin V solution was concentrated for various times ranging from 4 to 10 minutes at a fixed flow rate. A plot of peak area vs. concentration time gave a linear plot as described by the following statistics.

$$y = -17.450 + 15.450x \quad R^2 = 0.998$$

One major limitation of other methods designed to achieve trace penicillin detection is that they are limited to measuring a single penicillin specie. This

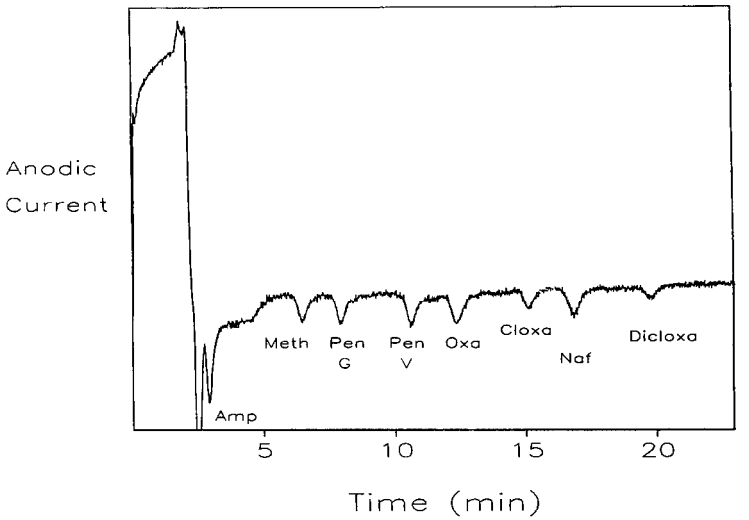

Figure 4. Separation of 8 penicillins at $2.6*10^{-8}$ M after concentration. 30 minutes of concentration at 3 ml/min. μBondapak C-18 column. Waveform and gradient given in the Experimental section.

concentration method can be used for samples containing multiple penicillins. Figure 4 shows a sample containing $2.6*10^{-8}$ M each of 8 different penicillins after on-line concentration.

When applied to milk samples, usage of the on-line concentration method allowed detection limits to be lowered versus determination by direct injection. As noted in the Experimental section, the milk extract had to be diluted before use with the concentration system. Without this thinning step, the viscous solution could not be pumped through the system without column clogging and HPLC pump pressure overloads. Figure 5 shows a separation of 8 penicillins in milk, all at $1*10^{-5}$ M. The milk was concentrated on-column for 8 minutes at a flow rate of 3 ml/min before the separation took place. Although longer times were useful

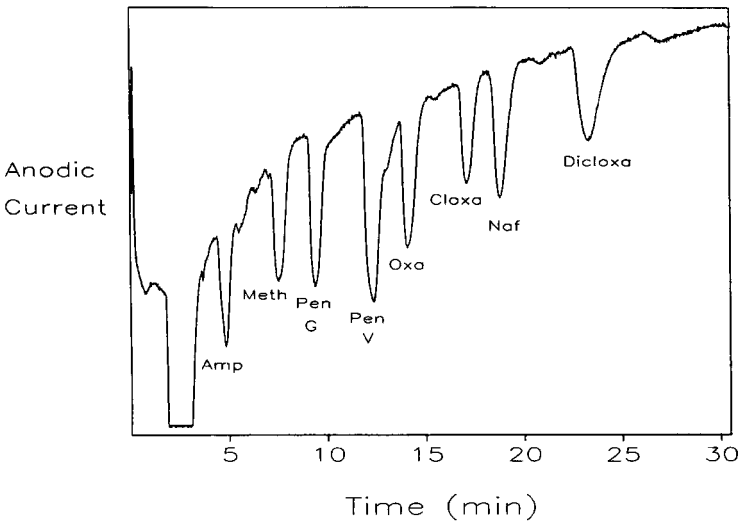

Figure 5. The separation of 8 penicillins in a milk sample following concentration. 8 minutes of concentration at 3 ml/min. All penicillins were present at $1*10^{-5}$ M in the milk. μBondapak C-18 column. Waveform and gradient given in the Experimental section.

when concentrating standard solutions, better output was obtained for the milk samples with concentration times of less than 10 minutes (at 3 ml/min), as the concentration step for the penicillins served to accentuate a few species from the milk sample matrix that were not previously found to be problematic during direct injections of the milk extract. The interference from these species ultimately limited detectability; a detection limit of $2*10^{-7}$ M was achieved for penicillin G, and in general detection limits were improved by ca. a factor of 10 for all the penicillins, as shown in Table 4. All of these values were obtained with 8 minutes of concentration time, requiring 24 ml of milk sample.

CONCLUSION

Detection of penicillins within milk samples can be done directly to low micromolar concentrations using PAD following HPLC. Application of the on-column concentration scheme allowed 8 penicillins to be separated and quantitated to less than micromolar concentrations. Although this scheme requires the use of 2 HPLC pumps, the concentration scheme is relatively quick, can be completely automated if necessary, and would not need any further labor than that already required for preparation of milk samples. In contrast to some current procedures, this method is adaptable for sensitive detection of any one of 8 penicillins, either as solitary analytes or mixed together.

ACKNOWLEDGEMENTS

This research was supported by an award from Research Corporation. Financial support for RLE was the result of a grant to the college from the U.S. Department of Education's Ronald E. McNair Post-Baccalaureate Achievement Program. The generous donation of equipment from the Waters Chromatography Division of the Millipore Corporation is acknowledged with gratitude. Graphics assistance has been provided by Ara Kooser and Dan Mossman. Special thanks to Dr. Lee Patel.

REFERENCES

1. G. J. Tortora, B. R. Funke and C. L. Case, Microbiology: An Introduction,3rd Ed., Benjamin/Cummings, Redwood City, CA, 1989.

2. P. A. Okewole, E. M. Uche, I. L. Oyetunde, P. S. Odeyemi and P. B. Dawal, Lab. Anim., 23, 275, (1989).

3. E. P. Tulleners, J. Am. Vet. Med. Assoc., 198, 1765, (1991).

4. T. L. Seahorn and J. Schumacher, J. Am. Vet. Med. Assoc., 199, 368, (1991).

5. S. Y. Gardner, V. B. Reef and P. A. Spencer, J. Am. Vet. Med. Assoc., 199, 370 (1991).

6. K. A. Johnson and S. C. Roe, J. Am. Vet. Med. Assoc., 192, 1573, (1988).

7. J. M. Reimer, R. W. Sweeney and J. Saik, J. Am. Vet. Med. Assoc., 192, 1297, (1988).

8. R. R. Leder, V. M. Lane and D. P. Barrett, J. Am. Vet. Med. Assoc., 192, 1299 (1988).

9. A. G. Gilman, L. S. Goodman and A. Gilman, The Pharmacological Basis of Therapeutics, 6th Ed., Macmillan, New York, 1980.

10. K. Schneider, Chicago Tribune, Dec. 28, 1990, Section 1, Page 5.

11. H. Terada and Y. Sakabe, J. Chromatogr., 348, 379, (1985).

12. F. Jehl, P. Birckel and H. Monteil, J. Chromatogr., 413, 109, (1987).

13. W. A. Moats, J. Chromatogr., 317, 311, (1984).

14. A. M. Lipczynski, Analyst, 112, 411, (1987).

15. A. Marzo, N. Monti, M. Ripamonti, E. A. Martelli and M. Picari, J. Chromatogr., 507, 235, (1990).

16. W. A. Moats, J. Chromatogr., 507, 177, (1990).

17. W. A. Moats, J. Assoc. Off. Anal. Chem., 75, 257, (1992).

18. J. Carlqvist and D. Westerlund, J. Chromatogr., 344, 285, (1965).

19. A. Besada and N. Tadros, Mikrochimica Acta, 2, 225, (1987).

20. E. Mendez-Alvarez, R. Soto-Otero, G. Sierra-Paredes, E. Aguilar-Veiga, J. Galan-Valiente and G. Sierra-Varcuno, Biomed. Chromatogr., 5, 78, (1991).

21. W. T. Kok, J. J. Havax, W. H. Voogt, U. A. T. Brinkman and R. W. Frei, Anal. Chem., 57, 2580, (1985).

22. B. Morelli and M. Mariani, Anal. Letters, 20, 1429, (1987).

23. M. E. Rogers, M. W. Adlard, G. Saunders and G. Holt, J. Chromatogr., 297, 385, (1984).

24. M. E. Rogers, M. W. Adlard, G. Saunders and G. Holt, J. Chromatogr., 257, 91, (1983).

25. C. M. Selavka, I. S. Krull and K. Bratin, J. Pharm. Biomed. Anal., 4, 83, (1986).

26. L. Koprowski, E. Kirchmann and L. E. Welch, Electroanalysis, 5, 473, (1993).

27. E. Kirchmann and L. E. Welch, J. Chromatogr., 633, 111, (1993).

28. J. M. Orten and O. W. Neuhaus, Human Biochemistry, 10th ed., C. V. Mosby Co., St. Louis, MO, 1982.

Received: November 4, 1993
Accepted: November 11, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1773-1783 (1994)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF TOREMIFENE AND METABOLITES

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ABSTRACT

The separation of toremifene and its metabolites 4-hydroxytoremifene, N-desmethyltoremifene, N-desdimethyltoremifene and deaminohydroxytoremifene by reversed-phase high performance liquid chromatography is described. The effects of pH, buffer concentration and type and proportion of organic modifier on the retention and resolution of the compounds have been studied. This allows optimum conditions for a particular biological application to be developed by simple modification of these paramaters. For the separation of toremifene and metabolites in microsomal metabolism and in plasma, the optimum conditions were 65% (v/v) acetonitrile in 0.25M ammonium acetate-acetic acid buffer, pH 5.0-5.2.

INTRODUCTION

Toremifene (Figure 1), the chloroethyl analogue of tamoxifen, is a non-steroidal antiestrogen drug currently under clinical trial for the treatment of breast cancer (1).

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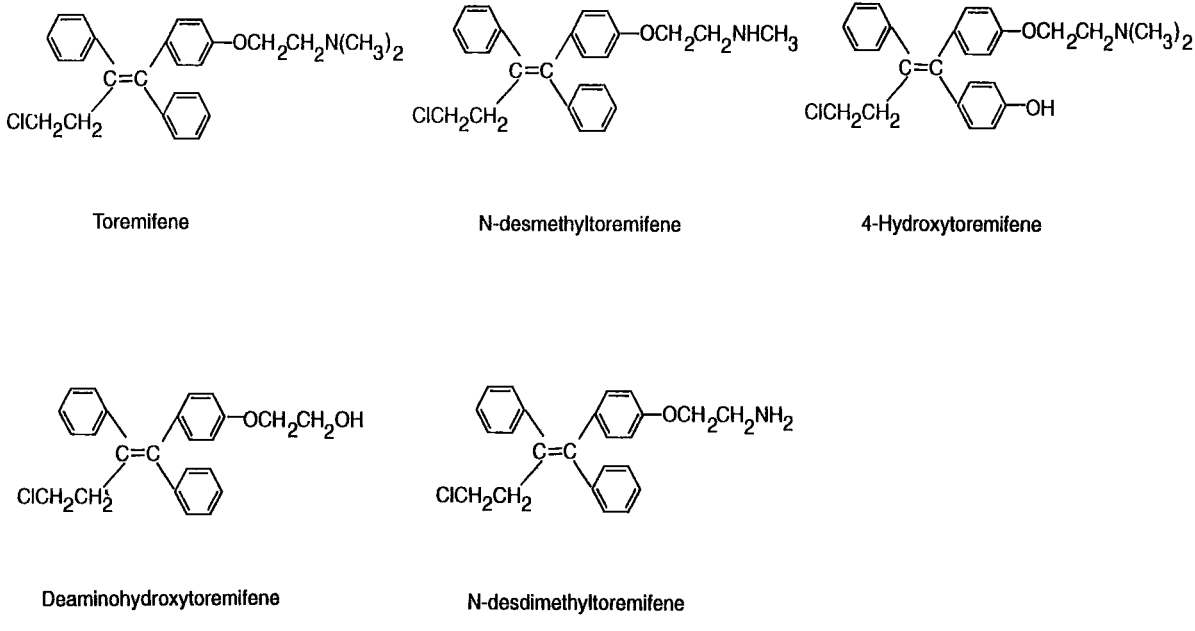

Figure 1 Structures of toremifene and its major metabolites.

It has similar estrogenic / antiestrogenic properties to tamoxifen but, in contrast to tamoxifen , has been shown not to cause hepatocellular carcinoma in rats in long term high dose feeding experiments (1, 2). We are interested in studying the metabolism and pharmacokinetics of toremifene in order to find out whether the difference in carcinogenesis between the two compounds can be explained in terms of metabolism and pharmacokinetics. This requires a simple and efficient analytical method. There are only two high performance liquid chromatographic (HPLC) methods reported for the separation of toremifene and metabolites (3, 4). We describe here a novel reversed-phase system for the separation of toremifene and metabolites on a Hypersil-ODS column. The retention behaviours of these compounds in the acetonitrile-ammonium acetate buffer system have been studied in detail to allow simple optimization of the system for a particular biological application. The applications of the method were demonstrated by the separation of toremifene and metabolites in rat liver microsomes following incubation in the presence of NADPH and in spiked human plasma.

EXPERIMENTAL

Materials and Reagents

Toremifene and metabolites were gifts from Farmos Gronp Ltd. (Oulu, Finland). Ammonium acetate, glaical acetic acid, $MgCl_2$, NaOH, Hepes and dimethyl sulphoxide (DMSO) were AnalaR grade from BDH (Poole, Dorset, U.K.). NADPH was from Sigma Chem. Co. (poole, Dorset, U.K.). Acetonitrile and methanol were HPLC grade from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland).

Toremifene Metabolism in Rat Liver Microsomes

Rat liver microsomes (1mg protein) were incubated with toremifene (50μM) in 0.05M Hepes-NaOH buffer (pH 7.4) in the presence of NADPH (0.5mM) and

$MgCl_2$ (5mM) at 37° for 15min. The reaction was stopped by vortex-mixed with 2 volumes of methanol / DMSO (4:1 v/v). The supernatant after centrifugation was analysed by HPLC.

Extraction of Toremifene and Metabolites in Plasma

Plasma (200µl) was vortex-mixed with 400µl of methanol / DMSO (4:1 v/v) for 30 sec. The mixture was centrifuged at 5000×g for 10min and the supernatant was analysed by HPLC.

High Performance Liquid Chromatography

A Varian Ltd. (Walton-on-Thames, Surrey, U.K.) model 9010 liquid chromatograph was used with a Varian 9050 UV-Vis detector set at 280nm. A Rheodyne 7125 injector (Cotati, CA, U.S.A.) fitted with a 200µl loop was used for sample injection. The column was Hypersil-ODS (5µM particle size, 250×4.6mm I.D.) and the mobile phase was acetonitrile in ammonium acetate buffer at various pH and molarity. The flow-rate was 1ml / min.

RESULTS AND DISCUSSION

The Effect of Buffer Concentration on the Retention and Resolution of Toremifene and Metabolites

The retention and resolution of toremifene and its metabolites are significantly affected by the molar concentration of ammonium acetate buffer used in the mobile phase. The variation of the capacity factor (k') with the buffer concentration is shown in Figure 2. The optimum buffer concentration for the rapid and complete separation of toremifene, 4-hydroxytoremifene, N-desmethyltoremifene and deaminohydroxytoremifene was between 0.25 and 0.38M. At concentrations below

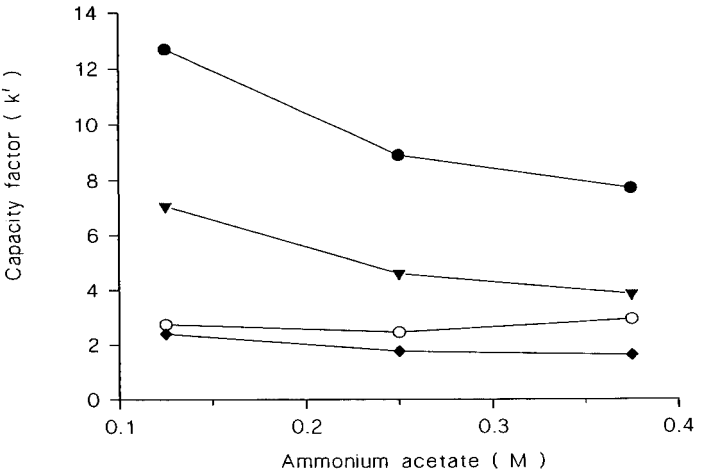

Figure 2 The effect of ammonium acetate buffer molar concentration on the capacity factors (k') of toremifene and metabolites. The mobile phasd was maintained at pH 5.16 and contained 65% acetonitrile. ● = Toremifene, ▲ = N-desmethyltoremifene, ❍ = deaminohydroxytoremifene and ◆ = 4-hydroxytoremifene.

0.2M excessive retention of N-desmethyltoremifene and particularly of toremifene was observed with the consequence of peak broadening. Ammonium acetate is an excellent general purpose mobile phase additive for improving the efficiency of reversed-phase columns (5). The observed trend of decreasing k' values with the increased in ammonium acetate concentrations is in common with other compounds studied (5-8).

The Effect of pH on the Retention and Resolution of Toremifene and Metabolites

The pH of the mobile phase can greatly influence the retention of compounds, especially those which tend to ionize in solution, in reversed-phase HPLC. The k'

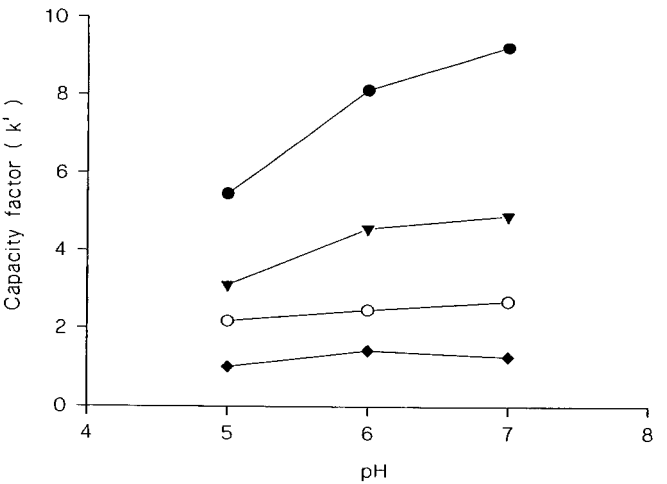

Figure 3 The effect of mobile phase pH on the capacity factors of toremifene and metabolites. The eluent was 65% acetonitrile in 0.25M ammonium acetate buffer at the various pH studied. ● = Toremifene, ▲ = N-desmethyltoremifene, ○ = deaminohydroxytoremifene and ◆ = 4-hydroxytoremifene.

values of toremifene and N-desmethyltoremifene increased significantly with increasing mobile phase pH (Figure 3). This is because increasing the pH suppressed the protonation of the amino nitrogen of these compounds. This increased their hydrophobicity and therefore their retention. The effect of pH on the retention of deaminohydroxytoremifene was negligible. This is to be expected since with the lose of the amino group this compound is essentially neutral. For 4-hydroxytoremifene, there was an initial increased of retention with increasing pH up to a value of about 6. This was followed by a decreased in k' as the pH increased. These results are consistent with the fact that 4-hydroxytoremifene has two ionizable groups, *i.e.* dimethylamino and phenol groups. The former ionizes at low while the latter at high

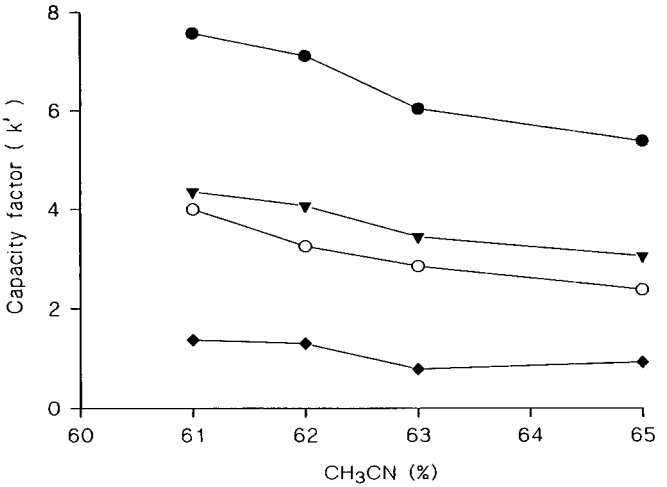

Figure 4 The effect of mobile phase acetonitrile content on the capacity factors (k') of toremifene and metabolites. The eluent was 0.25M ammonium acetate buffer, pH 5.16 containing the various concentration of acetonitrile. ● = Toremifene, ▲ = N-desmethyltoremifene, ○ = deaminohydroxytoremifene and ◆ = 4-hydroxytoremifene.

pH. The compound is therefore less hydropholic at either low or high pH and is most hydropholic at pH around 6 when it was retained the longest.

Figure 3 clearly shows that the optimum pH for the rapid separation of toremifene and its major metabolites is between 5.0 and 5.2.

The Effect of organic Modifier Concentration on the Retention and Resolution of Toremifene and Metabolites

Two organic modifiers, acetonitrile and methanol, were investigated for the separation of toremifene and metabolites. Acetonitrile was found to be better than methanol in terms of speed of separation and column efficiency. The effect of

acetonitrile concentration on the k' values of toremifene and metabolites (Figure 4) is that expected for reversed-phase chromatography. The k' values decreased with increasing acetonitrile content in the mobile phase. The optimum concentration of acetonitrile was between 64-65%.

The Optimum Solvent System for the Separation of Toremifene and Metabolites

From the results obtained above it becomes obvious that for the fast and efficient separation of toremifene and its major metabolites on the Hypersil-ODS column a mobile phase of 65% (v/v) in 0.25M ammonium a cetate, pH 5.1-5.2 is required. The separation of a standard mixture consisted of 4-hydroxytoremifene, deaminohydroxytoremifene, N-desdimethyltoremifene, N-desmethyltoremifene and toremifene is shown in Figure 5, with the elution order as in the order of the compounds listed. The elution order is that expected for reversed-phase HPLC.

Applications of the Separation

Two examples of application of the method are given here. The separation of toremifene and metabolites in rat liver microsomes following incubation at 37°C in the presence of NADPH is shown in Figure 6. The metabolites detected were 4-hydroxytoremifene, deaminohydroxytroemifene, N-desdimethyltoremifene and N-desmethyltoremifene, cllearly indicated that the major pathway of toremifene metabolism in this species is the demethylation reactions.

Figure 7 shows the separation of deaminohydroxytoremifene (0.25μg /mL), N-desdimethyltoremifene (0.5 μg / mL), N-desmethyltoremifene (1 μg / mL) and toremifene (2.5 μg / mL) in a human plasma sample spiked with these compounds. No interfering peaks were detected when control plasma samples were analysed.

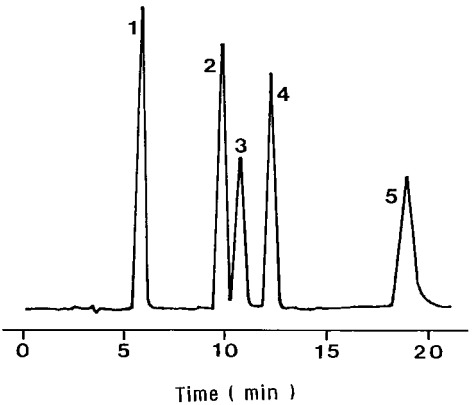

Figure 5 Separation of a standard mixture of toremifene and metabolites. Column, Hypersil-ODS; mobile phase, 65% (v/v) acetonitrile, pH 5.16; flow-rate, 1mL / min; detector, UV 280nm. Peaks: 1 = 4-hydroxytoremifene, 2 = deaminohydroxytoremifene, 3 = N-desdimethyltoremifene, 4 = N-desmethyltoremifene, and 5 = toremifene.

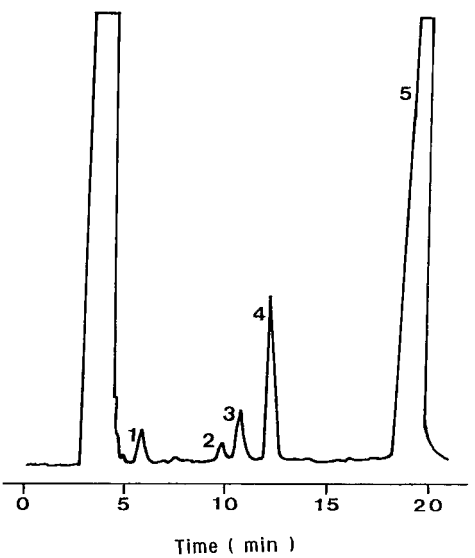

Figure 6 Separation of toremifene and metabolites in rat liver microsome metabolism. HPLC conditions and peak identification as in Figure 5.

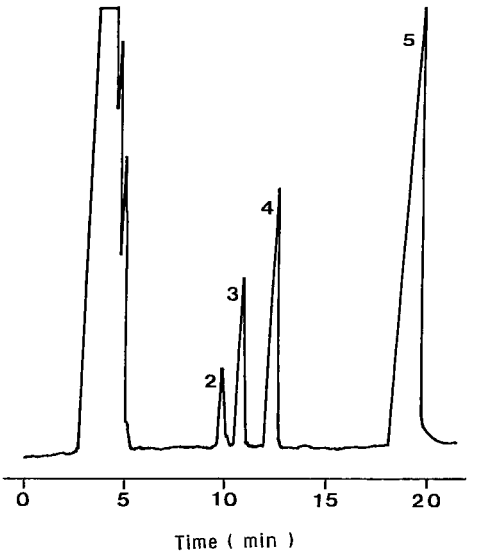

Figure 7 Separation of toremifene and metabolites in plasma spiked with deaminohydroxytoremifene (0.25μg / mL), N-desdimethyltoremifene (0.5 μg / mL), N-desmethyltoremifene (1 μg / mL)and toremifene (2.5 μg / mL . HPLC and peak identification as in Figure 5.

Since toremifene is given to patients at relatively high dose (200mg / m^2 daily) the present method is sensitive enough for the detection of toremifene and metabolites in plasma.

We expect the flexibility of the method will allow other toremifene metabolites in tissues, urine or faecal samples to be analysed by simple modification of the mobile phase system, *e.g.* by adjusting the buffer concentration, pH and / or organic modifier content in the eluent.

The method is currently being used in our laboratory for the study of toremifene metabolism and pharmacokinetics in rat liver. The results will than be compared to

those obtained with tamoxifen. It is hoped that such a study will lead to a better understanding of the mechanism of carcinogenesis caused by tamoxifen and the lack of it by toremifene.

REFERENCES

1. Abstracts of the UICC symposium, Aug 21-27, 1986 Budapest. Abstract numbers 2120, 2122, 2988, 2991, 2993, 2994 and 2995.

2. Kangas, L., In: Progress in Cancer Research and Therapy, Vol.15 : Hormones and Cancer 3 (Eds. F. Bresciani, R.J.B. King, M.E. Lippman and J.P. Raynaud). Raven Press, New York, 1988. pp. 374-377.

3. De Gregorio, M.W., Ford, J.M., Benz, C.C. and Wiebe, V.J., J. Clin. Oncol., 7, 1399, 1989.

4. Webster, L.K., Crinis, N.A., Stokes, K.H. and Bishop, J.F., J. Chromatogr., 565, 482, 1991.

5. Lim, C.K. and Peters, T.J., J. Chromatogr., 316, 397, 1984.

6. Rideout, J.M., Wright, D.J. and Lim, C.K., J. Liq. Chromatogr., 6, 383, 1983.

7. Wright, D.J., Rideout, J.M. and Lim, C.K., Biochem. J., 209, 553, 1983.

8. Lim, C.K. and Peters, T.J., Methods Enzymol., 123, 389, 1986.

Received: November 5, 1993
Accepted: November 11, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1785-1794 (1994)

OXOLINIC ACID AND FLUMEQUINE IN FISH TISSUES: VALIDATION OF AN HPLC METHOD; ANALYSIS OF MEDICATED FISH AND COMMERCIAL FISH SAMPLES

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ABSTRACT

This paper describes a simple method for residue analysis of oxolinic acid and flumequine in fish tissues by HPLC and fluorometric detection. The quinolones were extracted with ethyl acetate, purified with hexane and then analyzed using a RP-8 Lichrosorb reversed phase column. The method was validated with satisfying results. The high sensitivity of the fluorometric detection allowed to reach a quantification limit of 2 µg oxolinic acid and 5 µg flumequine/kg fish tissue and an absolute detection limit of 20 pg and 50 pg respectively. Medicated fish as well as commercial cultured fish samples were analyzed with the described method. Oxolinic acid and flumequine were detected in both of them.

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INTRODUCTION

During the last decades commercial aquaculture has been developed and widely extended. For the prevention and the treatment of infectious diseases of cultured fish chemotherapeutic agents are used. Oxolinic acid and flumequine are synthetic antibacterial drugs, structurally related to each other and belonging to the group of quinolones. They are very active against gram-negative microorganisms even at low concentrations and frequently used by fish farmers. The antimicrobial activity of quinolones, their efficacy and toxicity (1) as well as pharmacokinetics and bioavailability of flumequine and oxolinic acid in Atlantic Salmon were studied (2).
Recently I. Steffenak and al.(3) showed that "oxolinic acid and flumequine seem to be especially entrapped in bone..... This reservoir seems to act as a depot from which the drug is slowly released into other tissues." Both drugs are extensively used in Europe. Neither EEC reglementation nor legislation do exist. The proposed waiting time between the end of a treatment and the moment when the fishes are killed may not be less than 6 days if oxolinic acid is used and 3 days if flumequine is used as drug (4). To protect the consumers health, we were interested to know, whether or not, residues of oxolinic acid and flumequine may be found in commercial fish tissues. Several methods using HPLC were published concerning the determination of oxolinic acid and flumequine alone or simultaneously with other antibacterials (5-10). Simple analytical methods with high sensitivity are necessary to detect residues. Lyse Larocque and al. (11) determined oxolinic acid in salmon muscle tissue using a very simple and rapid extraction procedure. We extended this method for the extraction of flumequine as well and worked out new chromatographic conditions suitable for both quinolones.

EXPERIMENTAL

Apparatus

A Stomacher Lab-Blender 80 from L.E.D. Techno (Belgium) was used for the extraction procedure. Centrifugations were achieved with a centrifuge GLC-2B from Sorvall (Dupont, USA). Ethyl acetate was evaporated using a Reacti-Therm heating module from Pierce (Illinois, USA) and nitrogen. A Vortex super mixer from Lab-Line Instruments (Illinois, USA) was used for the purification procedure. HPLC analyses were performed with a 5000 liquid chromatograph from Varian (USA). Oxolinic acid and flumequine were detected with an LS-4 fluorescence spectrometer from Perkin-Elmer (USA). The chromatograms were registered with a recorder A 41 from Ankersmit (The Netherlands) (paper speed: 2 mm/min).

Solvents and reagents

Ethyl acetate, acetonitrile and hexane were delivered by Labscan (Ireland), sodium sulphate anhydrous by UCB (Belgium) and oxalic acid G.R. by Merck (Germany). For the extraction of oxolinic acid and flumequine an oxalic acid solution 0.01 M, pH 3 was prepared. The eluent for the HPLC analyses consisted of acetonitrile and a solution of oxalic acid 0.025 M, pH 3.2 (32 + 68; v/v), both were filtered and degassed by helium before use.

Standards and standard solutions

Oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-g]quinoline-7-carboxylic acid) (figure 1) and flumequine (9-fluoro-6,7-dihydro-5-methyl-1-

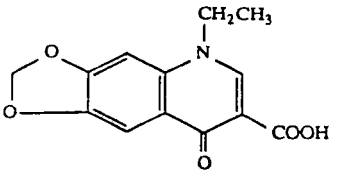

FIGURE 1. Oxolinic acid

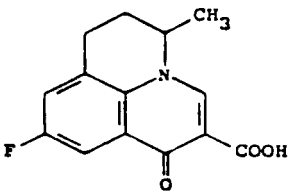

FIGURE 2. Flumequine

oxo-1H,5H-benzo[ij]quinolizine-2-carboxylic acid) (figure 2) were supplied by Sigma (USA). The standard stock solutions contained 1 mg/ml NaOH 0.1 M. The working standard solution contained both quinolones, each at a concentration of 1 µg/ml water.

Sample preparation

Oxolinic acid and flumequine were extracted from 2 g minced fish tissue, dried by 2 g sodium sulphate anhydrous, with 24 ml ethyl acetate in a plastic bag by means of a Stomacher Lab-Blender 80 during 1 minute. The mixture was poured into a 100 ml glass tube and centrifuged at 2000 rpm during 5 minutes. The solvent was transferred into a small glass tube

and evaporated under a stream of nitrogen at 45 °C. The residue was rinced with 5 ml ethyl acetate, centrifuged and the solvent was added to the first portion of solvent to be evaporated. An oily residue of approximately 0.5 ml was left to which were added successively: 2 ml 0.01 M oxalic acid, pH 3 and 2 ml hexane. After each addition the tube was shaken vigourously on a Vortex during 1 minute. The mixture was then centrifuged at 2000 rpm during 5 minutes and the upper organic layer discarded. The lower aqueous layer was the final extract from which a 100 µl portion was injected into the liquid chromatograph.

Chromatography

The liquid chromatograph was fitted with a 5 µm RP-8 Lichrosorb reversed phase column (125 mm x 4 mm, cat. Nr. 50432) from Merck (Germany). The flow rate was 1 ml/minute, the analyses were performed at room temperature. Oxolinic acid and flumequine were detected with a fluorescence detector (excitation wavelength: 327 nm; emission wavelength: 369 nm; slits: 15 and 20; fixed scale: 2 or 5).

RESULTS AND DISCUSSION

Validation of the HPLC - method

The described method was validated using cultured rainbow trout tissues. For the extraction procedure we used a Stomacher Lab-Blender 80 which homogenizes very well sample, solvent and sodium sulphate, a required condition to extract efficaciously residues. For the HPLC analyses we have chosen a 5 µm RP-8 Lichrosorb reversed phase column and a mixture of acetonitrile and oxalic acid 0.025 M, pH 3.2 (32 + 68; v/v) as

eluent. A very good elution and separation of both quinolones could be achieved. The analyses of oxolinic acid and flumequine standards resulted in a linear response within a range of 1 to 100 ng. The correlation coefficients were 0.999 for both quinolones. Table 1 summarizes the recoveries, standard deviations and coefficients of variation of oxolinic acid and flumequine. The recoveries were checked on spiked rainbow-trout tissues (50, 25, 12.5 and 6.25 µg of each quinolone/kg tissue). Each result is the mean of 5 extractions. Even 6.25 µg/kg gave satisfying results with maximum recoveries of 89 % for oxolinic acid and 67 % for flumequine. This allowed us to fix the quantification limit at 2 µg oxolinic acid and 5 µg flumequine/kg fish tissue. The absolute detection limit was 20 pg for oxolinic acid and 50 pg for flumequine (fixed scale: 20), which is shown in figure 3 A. A typical chromatogram of a standard solution is shown in figure 3 B, a

TABLE 1

Recovery Data of Oxolinic Acid and Flumequine in Fish by HPLC and Fluorometric Detection

Added drug	Added quantity (µg/kg)	Mean recovery (n=5) (µg/kg)	Standard deviation	Coefficient of variation (%)	Recovery (%)
Oxol. acid	50.00	41.33	2.21	5.35	82.67
Flumequine	50.00	34.88	2.43	6.97	69.77
Oxol. acid	25.00	19.04	1.08	5.70	76.17
Flumequine	25.00	13.32	0.93	7.02	53.27
Oxol. acid	12.50	8.74	0.83	9.50	69.95
Flumequine	12.50	6.36	0.83	13.11	50.85
Oxol. acid	6.25	5.81	0.17	2.84	89.43
Flumequine	6.25	4.41	0.07	1.51	67.86

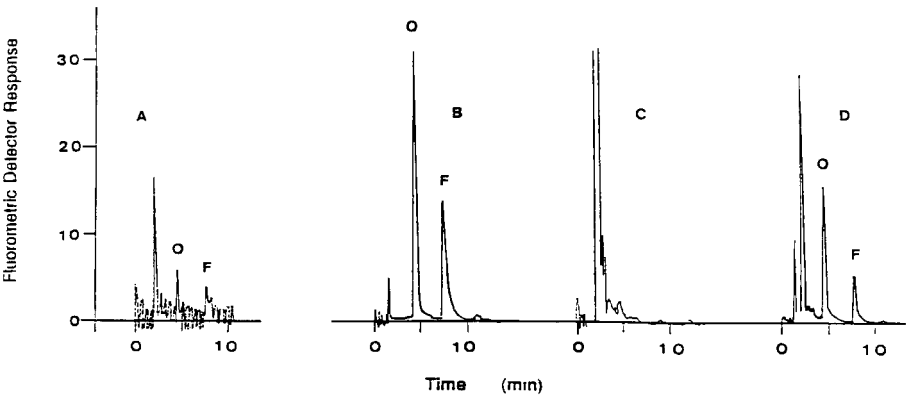

FIGURE 3. Chromatograms of Oxolinic Acid (O) and Flumequine (F). -A. Absolute Detection Limit (20 pg Oxolinic Acid. 50 pg Flumequine); -B. Standard Solution (12.5 ng Oxolinic Acid and 12.5 ng Flumequine); -C. Blank Fish Tissue Sample; -D. Spiked Fish Tissue Sample (12.5 µg Oxolinic Acid and 12.5 µg Flumequine/kg Fish Tissue); Injection Volume: 100 µl; Fixed Scale of the Fluorescence Spectrometer: 20 (A), 5 (B,C,D).

blank fish tissue sample in figure 3 C and a spiked fish tissue sample in figure 3 D. The retention time for oxolinic acid was 4.5 minutes and for flumequine 7.5 minutes under the described LC conditions.

Other quinolones are used as well for the prevention and the treatment of infectious diseases of cultured fish, such as enrofloxacin, sarafloxacin and difloxacin. We did not include their analysis in this method, since their detection wavelengths are different. Their extraction procedures have to be adapted as well, except for difloxacin, for which we obtained very good results, but at an excitation wavelength of 278 nm and an emission wavelength of 440 nm.

Analysis of medicated fish and commercial fish samples

13 rainbow trouts were treated with flumequine (12 mg/kg weight/day) (4), 3 other-ones were fed without any additive and were used as blank

samples. The trouts (> 50 g) were hold in a pool with constant temperature (10 - 11 °C) and a flow of 6 litres water/minute approximately. Flumequine was mixed to the feed and distributed automatically during 12 hours a day. The quantity of feed was 1 % of fish weight. The trouts were treated during 6 days and then killed successively, 4 trouts 1 day after the end of the treatment, 2 trouts 4 days after it, 4 trouts 6 days after it and 3 trouts 7 days after it. The trout tissues were analyzed using the experimental conditions described. The results are given in table 2. Each result is the mean of (n) analyzed trouts. These results are similar to those obtained by I. Steffenak and al.(3). Flumequine is very slowly eliminated and it seems to be clear that the proposed waiting time of 3 days after the end of the treatment with flumequine is far to short to get commercial fish free of residues.

The method has been applied to the analysis of commercial samples of different fish tissues coming from different fish farms. 84 samples have been analyzed until now. 2 samples contained flumequine at a concentration of 46 and 75 µg/kg fish tissue. 4 samples contained oxolinic acid ranging from 17 µg/kg to 1.1 mg/kg fish tissue.

TABLE 2

Recovery Data of Flumequine in medicated Rainbow Trouts

Days after end of medication	Concentration of flumequine (µg/kg fish tissue)
1	20 (n=4)
4	8.75 (n=2)
6	2.81 (n=4)
7	1.41 (n=3)

CONCLUSION

The developed method has proved to be suitable as a routine analysis method for the detection of residues of oxolinic acid and flumequine in fish tissues. More than 20 samples can be analyzed a day. Fishes were medicated with flumequine. Considering the fact that these fishes contained still flumequine 7 days after the end of the medication as well as commercial cultured fishes contained residues of flumequine and oxolinic acid, it seems to be necessary to study more precisely the waiting time after the end of a medication and the moment when the fishes are free of residues. This study has to cover flumequine and oxolinic acid. Modern analytical methods, such as HPLC and fluorometric detection, are very sensitive and able to detect low residue levels of drugs. Maximum residue levels (MRL) may be adapted to it.

ACKNOWLEDGEMENT

We wish to thank Mrs. Khari Tsilikas for her technical assistance and Dr. F. Lieffrig from CER (Marloie) who treated the rainbow trouts.

REFERENCES

1. P.M. Vancutsem, J.G. Babish, W.S. Schwark, Cornell Vet., 80: 173-186 (1990)

2. A. Rogstad, O.F. Ellingsen, C. Syvertsen, Aquaculture, 110(3-4): 207-220 (1993)

3. I.Steffenak, V. Hormazabal, M. Yndestad, Food Addit. Contam., 8: 777-780 (1991)

4. P. De Kinkelin, La Pisciculture Française, 92: 17-29 (1988)

5. Yoshitomo Ikai, Hisao Oka, Norihisa Kawamura, Masuo Yamada, J. Chromatogr., 477: 397-406 (1989)

6. Masakazu Horie, Kouichi Saito, Youji Hoshino, Norihide Nose, J. Chromatogr., 402: 301-308 (1987)

7. O. B. Samuelsen, J. Chromatogr., 530: 452-457 (1990)

8. A. Rogstad, V. Hormazabal, M. Yndestad, J. Liq. Chromatogr., 12: 3073-3086 (1989)

9. M. Horie, K. Saito, N. Nose, M. Tera, H. Nakazawa, J. Liq. Chromatogr., 16: 1463-1472 (1993)

10. V. Hormazabal, A. Rogstad, I. Steffenak, M. Yndestad, J. Liq. Chromatogr., 14: 1605-1614 (1991)

11. L. Larocque, M. Schnurr, S. Sved, A. Weninger J. Assoc. Off. Anal. Chem., 74: 608-611 (1991)

Received: October 1, 1993
Accepted: November 4, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1795-1809 (1994)

DETERMINATION OF 4-METHYL UMBELLIFERONE AND METABOLITES IN WILLIAMS E MEDIA AND DOG PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A gradient high performance liquid chromatographic method has been developed for the determination of 4-methylumbelliferone (4MU), 4-methylumbelliferyl sulfate (4MUS), and 4-methylumbelliferyl β-D-glucuronide (4MUG) in dog plasma and William's E media. Samples containing the internal standard, umbelliferone were prepared for analysis by precipitation with acetonitrile prior to injection onto a 150 x 2.1mm C18 Hypersil reversed-phase column. The compounds were eluted in less than 7 minutes with a fast linear gradient from 9.5% acetonitrile to 32% acetonitrile in pH 4.5 acetate buffer with a flow rate of 0.35 mL/min. All compounds were detected at 314 nm. Total sample cycle time was 10 minutes. Intra-run (n=5) and inter-run (n=18) precision was less than 15% relative standard deviation across the entire calibration range. The lower limit of quantitiation with a 50 µL sample size was 2.5 µM.

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INTRODUCTION

4-Methylumbelliferone (7-hydroxy-, 4-methyl-coumarin, Hymecromone, 4MU) is a cholerectic agent that is available in Europe [1]. The compound has also been used to treat spasms of the sphincter of Oddi and the biliary ducts [2]. 4MU is almost entirely excreted in the urine or bile of animals as a glucuronide or sulfate conjugate at the 7-hydroxy position [3]. As the compound is highly fluorescent and has a simple pattern of metabolism, it has been used as an attractive model compound to study phase II (conjugative) metabolic pathways for both *in vitro* an *in vivo* drug metabolism experiments.

The glucuronidation of 4MU is catalyzed by UDP-glucuronosyltransferases (UGTs) located in the endoplasmic reticulum of the liver, lung, intestine, and kidney. 4MU is a high activity substrate in microsomal preparations, but is not a specific substrate for any single isozyme. For example, 4MU glucuronidation is catalyzed by at least three isozymes of the human *UGT1* gene family [4]. The *UGT2* gene family specifically catalyzes the glucuronidation of steroids or bile acids, and these isozymes appear to either have low or no activity for 4MU [5,6]. The only isozymes in the *UGT2* family that are capable of catalyzing 4MU glucuronidation are those encoded by *UGT2B8* and *UGT2B11*, however the specific activity appears to be much lower than with *UGT1* isoenzymes [6]. Sulfotransferases are located in the cytosol of various tissues. 4MU is a substrate for the phenol sulfotransferases [3]. 4MU may not be a substrate for isozymes that catalyze sulfation of steroids such as dehydroepiandrostenedione, since other simple phenols such as p-nitrophenol or naphthol are not substrates for steroidal sulfotranserases

[7]. *In vivo*, sulfation appears to be saturable as the excretion of the sulfate conjugate is proportionally lower at higher doses [8].

Quantitative analyses of 4MU, 4MU glucuronide, and 4MU-sulfate have been accomplished by both direct and indirect methods. Indirect methods to quantitatively determine 4MU and its conjugates involve extraction of the unconjugated substrate, 4MU, which can then be measured fluorimetrically (excitation 360-390 nm, emission 450 nm). The conjugates in the aqueous fraction can then be hydrolyzed by treatment with b-glucuronidase/sulfatase, followed by fluorometric determination of the released 4MU. This method has been applied to samples from *in vitro* incubations [9], liver perfusate [10], and whole rat blood [11]. Another approach is to separate 4MU from its sulfate and glucuronide conjugates by ion exchange chromatography on Dowex AG50 resin, followed by hydrolysis of the sulfate conjugate at 80°C for 30 min [12] or by treatment with β-glucuronidase/sulfatase to release 4MU [13]. With the above methods, the sulfate and the glucuronide cannot be independently determined, unless one uses a preparation of β-glucuronidase that is free of sulfatase activity. For example, Anaundi et al. incubated liver perfusate samples with an *E. coli* β-glucuronidase preparation (sulfatase free) to determine the free 4 MU from hydrolyzed 4MUG [10]. To determine the amount of 4MUS, β-glucuronidase activity was inhibited with 10 mM saccharo-1,4-lactone in enzyme preparations from *Helix pomatia* containing both β-glucuronidase and sulfatase, followed by fluorometric determination of the released 4MU [10].

Direct separations of 4MU, 4MUG, and 4MUS have been achieved by thin-layer chromatography and by HPLC. Morita et al. separated the

three compounds on silica gel plates and visualized the spots under UV light at 365 nm [14]. The spots were then scraped, extracted with pH 10.5 Sorensen buffer and 4MUS and 4MUG were hydrolyzed by addition of an equal volume of 2N HCl followed by boiling for 30 min and 2 hr, respectively; 4MU was then measured fluorometrically. Determination of 4MU and it conjugates has also been achieved by HPLC [15-18]. Sandman developed a method for the determination of lysosomal enzyme activity in urine, by determination of the enzymatically produced 4MU from a series of 4MU conjugates including the glucuronide and sulfate [15]. The conjugates were separated from the 4MU on a styrene-divinyl benzene polymer column with a mobile phase consisting of methanol/pH 10.3 glycine buffer. The 4MU was measured on a fluorescence detector. This procedure did not directly measure the conjugates but could be modified for this purpose. Femfert et al. developed a method for the simultaneous determination of 4MU and its conjugates in serum or plasma from humans [16]. This chromatographic method employed tetrabutylammonium bromide as an ion-pairing reagent. Samples (100 µL) were treated with an equal volume of perchloric acid/perchlorate to precipitate proteins and a 10 µL aliquot of the supernatant was injected directly. Compounds were detected at 254 or 280 nm. This method was rapid (less than 9 min), showed good reproducibility, and had a limit of sensitivity of 0.2 µg/mL with a coefficient of variation of 7.5%. More recently, Zimmerman et al. developed a direct method for the determination of 4MU and its conjugates in rat liver perfusate plasma [17]. Sample preparation consisted of addition of umbelliferone as an internal standard to 100 µL plasma samples followed by precipation of perfusate

plasma protein with 4 volumes of methanol. After centrifugation, an aliquot of the supernatant was removed, reconsituted in 200 µL 25% methanol/H20 and injected onto the HPLC column. 4MU and its conjugates were separated on C18 reversed-phase column by gradient elution from 25% to 40% methanol in a phosphate buffer and detected at 313 nm with a total cycle time of 40 min per injection. The sensitivity of this method was 0.5 µg/mL. A separate, more sensitive assay for 4MU in perfusate containing red blood cells that utilized an ethyl acetate extraction procedure was also reported by these authors (limit of quantitation = 0.05 µg/mL). To determine the pharmacokinetics of 4MU and its conjugates in human volunteers, Garrett et al. developed an HPLC method for plasma and urine samples [18]. The compounds were separated on a ODS Hypersil column with an ion-pairing mobile phase similar to that used by Femfert et al. [16]. 4MU was detected by fluorescence (excitation 330 nm, emission 450 nm), 4MUS by UV absorbance at 315 nm, and 4MUG by fluorescence (excitation 330 nm, emission 380 nm). Sample preparation consisted of addition of the internal standard, umbelliferone, followed by an acetonitrile precipitation step. The limit of quantiation for this assay with 20 µL of plasma was 5ng/mL for 4MU, 63 ng/mL for 4MUG, and 409 ng/mL for 4MUS. Sensitivity for 4MU could be increased via an extraction step with larger volumes of sample.

Due to the well documented metabolic profile associated with 4MU, this compound was selected as an *in vivo* and *in vitro* model for phase II drug metabolism in a series of experiments associated with the development of a bioartificial liver at the University of Minnesota [19].

The large number of samples associated with these experiments required the development of an assay with a short analysis cycle time while maintaining accuracy and precision. A sensitive assay was required for the quantitation of 4MU and its conjugates in dog and rabbit plasma and from a modified tisssue culture media that is used to sustain the hepatocytes in the bioartificial liver. Experiments designed to optimize hepatocyte function were conducted with 4MU conjugation as one of the indicators of the performance of the bioartificial liver. As several thousand samples were anticipated, the assay was developed on a small bore column (2.1 mm i.d.) in order to save on solvent costs. A short gradient elution method was selected in order to eliminate late eluting peaks that were encountered with an isocratic, ion-pairing mobile phase.

EXPERIMENTAL

Chemicals

All chemicals were of analytical grade. 4MU, 4MUS, 4MUG, umbelliferone, lidocaine, glacial acetic acid, sodium acetate, and sodium hydroxide were purchased from Sigma (St. Louis, MO). Umbelliferone was recrystallized two times from ethyl acetate to remove a minor impurity. Acetonitrile (Fisher, Fairlawn, N.J.) was HPLC grade. William's E media (Gibco, Grand Island, NY) was supplemented with 200 U/L insulin, 40,000 U/L penicillin G, 400 mg/L streptomycin sulfate, and 0.292 g of L-glutamine per liter. The media also contained 5 or 12 μg/mL of lidocaine and 60 μM 4MU as biotransformation markers.

Instrumentation and Chromatography

Chromatography was performed on a Hewlett-Packard 1090L liquid chromatograph (Palo Alto, CA) outfitted with a column oven, autoinjector, and a diode array detector set at 314 nm with a 10 nm bandwidth for analyte monitoring and 510 nm for reference. Mobile phase was delivered at 0.35 mL/min through a Keystone Scientific (Bellefonte, PA) 5μ C18 Hypersil column, 150 x 2.1 mm i.d., maintained at 40°C in a column oven. The A mobile phase consisted of 0.5% Na acetate buffer, pH 4.5 (prepared by mixing solutions of 0.5% Na acetate and 0.5% acetic acid to pH 4.5) and acetonitrile (19:1 v/v). Mobile phase B consited of 0.5% acetate buffer, pH 4.5 and acetonitrile (1:1 v/v). The initial conditions consisted of 10% B and increased to 60% B in a linear gradient over 6 min which was then maintained at 60% B for an additional 1 min. This was followed by a three min equilibration period at the initial conditions. A personal computer with Chromperfect Direct software (Justice Innovations, Palo Alto, CA) was used to acquire data.

Sample and Standard Curve Preparation

A 50μL aliquot of blank plasma or media or samples of plasma or media containing 4MU and its metabolites was pipetted into a 12 x 75 mm disposable test tube to which 20 μL of a 100 μM solution of umbelliferone (internal standard) in methanol was added. Protein precipitation was accomplished by addition of 2.0 mL of acetonitrile, mixing on a vortex mixer for 10 sec, and centrifugation at 1500 g for 10 min. The acetonitrile was transferred to a clean 10 x 75 mm disposable test tube and evaporated to dryness under N_2 in a 50°C water bath. The residue was

reconstituted in 75 µL of Mobile phase A, transferred to autoinjector vials, and 5 µL was injected onto the column.

RESULTS

4MU, 4MUG, and 4MUS were detected at 314 nm with a diode-array detector. This high wavelength is the UV maximum for the three compounds and is relatively free of interference from potential contaminants in plasma or tissue culture media. Chromatograms of a blank dog plasma extract and a plasma sample from a dog administered 4MU are shown in Figure 1. Figure 2 shows the results obtained from extracts of 50µL samples of a modified William's E media. No interfering peaks were observed under the peaks of interest. The total cycle time for the assay was 10 minutes and the retention times for the peaks of interest were as follows: 4MUG = 2.2 min, 4MUS = 4.0 min, umbelliferone (internal standard, I.S.) = 5.0 min, and 4MU = 6.3 min.

Recovery, Precision, and Accuracy.

Peak heights were used for quantitation. Linear regression of the peak height ratios versus the drug concentration were performed to determine the slope, intercept, and correlation coefficient of the standard curves. Within-run calibration curves consisted of 5 replicates at each level. Between-run calibration standards and quality control standards were assayed in triplicate on 6 different working days.

Recovery was determined by comparing the peak heights of treated plasma and WEM samples with the peak heights of standard injections of the same concentration. Recoveries (mean $\pm$ S.D., n = 5) at 10 nMol/mL

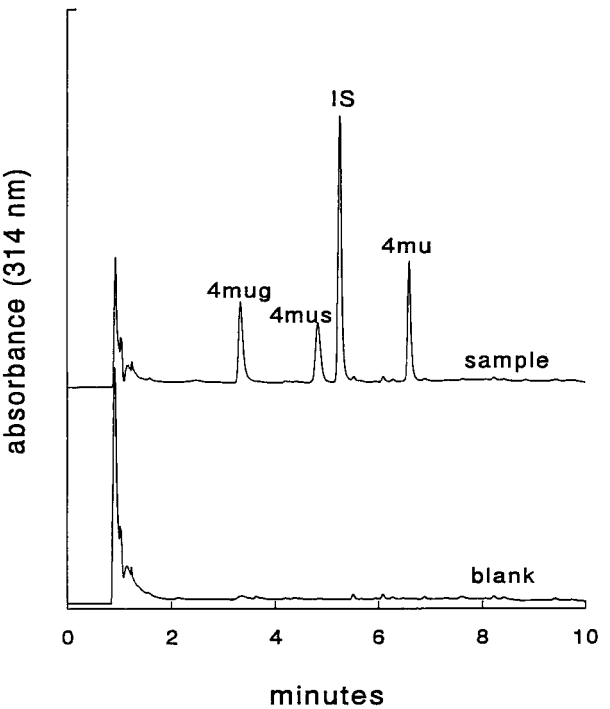

FIGURE 1.

Blank dog plasma and dog plasma sample. Separation occurred on a Keystone ODS (150 x 2.1mm i.d., 5 µm Hypersil) reversed-phase column. The compounds were eluted at a flow rate of 0.35 mL/min with a linear gradient from 9.5% acetonitrile to 32% acetonitrile in pH 4.5 acetate buffer over 6 minutes then maintained at 32% acetonitrile for 1 minute. This was followed by a three min equilibration period at the initial conditions. All compounds were detected at 314 nm.

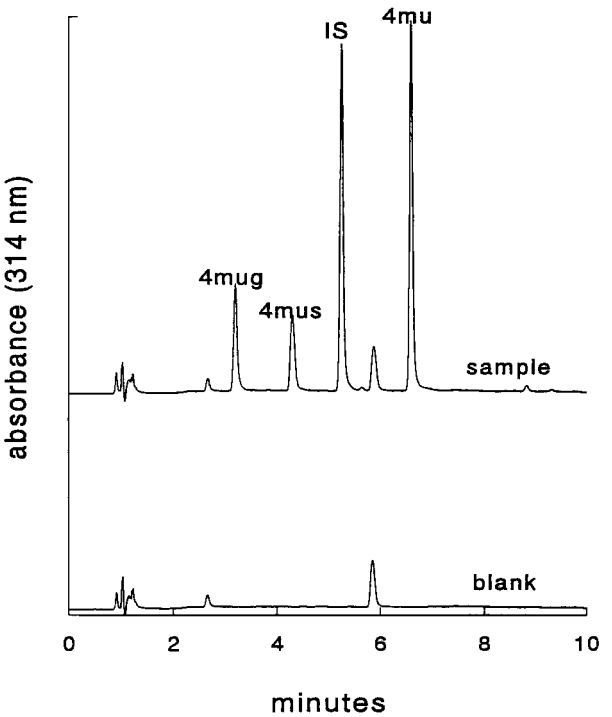

FIGURE 2.

Blank WEM and WEM sample. Conditions as in Figure 1.

in plasma and WEM were respectively 89.4 ± 1.2% and 91.4 ± 1.6% for 4MU, 92.1 ± 7.3% and 91.1 ± 1.1% for 4MUG, 89.4 ± 1.2% and 92.4 ± 2.2% for 4MUS, 88.3 ± 2.3% and 91.6 ± 2.1% for U. Accuracy and precision for the assay in dog plasma and modified William's E media are shown in Tables 1 and 2. Table 1 contains the data for within-run precision (n=5) and Table 2 shows the between-run accuracy and

TABLE 1.

WITHIN RUN PRECISION FOR 4-METHYLUMBELLIFERONE AND METABOLITES IN DOG PLASMA AND WILLIAMS E MEDIA (n=5).

plasma	4mu	rsd	4mug	rsd	4mus	rsd
µMol	avg±sd	(%)	avg±sd	(%)	avg±sd	(%)
2.5	2.50 ± 0.32	12.63	2.45 ± 0.13	5.35	2.53 ± 0.26	10.25
10.0	9.94 ± 0.27	2.73	10.53 ± 0.28	2.68	9.65 ± 0.59	6.16
20.0	20.58 ± 0.61	2.98	21.22 ± 0.68	3.22	19.10 ± 1.14	5.95
50.0	49.96 ± 1.29	2.59	51.10 ± 2.33	4.56	51.57 ± 2.45	4.75
125.0	123.17 ± 4.36	3.54	117.72 ± 3.36	2.86	130.86 ± 6.10	4.66
250.0	248.53 ± 7.71	3.10	235.63 ± 8.48	3.60	247.39 ± 9.56	3.86

media	4mu	rsd	4mug	rsd	4mus	rsd
µMol	avg±sd	(%)	avg±sd	(%)	avg±sd	(%)
2.5	2.46 ± 0.31	12.62	2.49 ± 0.34	13.80	2.46 ± 0.35	14.34
10.0	10.65 ± 0.58	5.49	10.18 ± 0.82	8.06	10.52 ± 0.88	8.38
20.0	20.29 ± 0.78	3.84	19.99 ± 1.68	8.39	20.52 ± 1.75	8.54
50.0	46.59 ± 0.97	2.08	48.73 ± 0.63	1.29	50.22 ± 0.68	1.36
125.0	122.44 ± 5.49	4.48	123.31 ± 4.29	3.48	120.04 ± 4.91	4.09
250.0	255.93 ± 9.44	3.69	256.22 ± 8.10	3.16	243.22 ± 4.97	2.04

TABLE 2.

BETWEEN RUN PRECISION FOR 4-METHYLUMBELLIFERONE AND METABOLITES IN DOG PLASMA AND WILLIAMS E MEDIA (n=18).

plasma	4mu	rsd	4mug	rsd	4mus	rsd
µMol	avg±sd	(%)	avg±sd	(%)	avg±sd	(%)
2.5	2.47 ± 0.21	8.44	2.48 ± 0.25	9.90	2.50 ± 0.18	7.27
10.0	10.19 ± 0.39	3.84	9.76 ± 1.17	11.94	10.43 ± 0.93	8.93
20.0	20.97 ± 0.86	4.11	20.45 ± 1.60	7.83	21.14 ± 1.91	9.05
50.0	51.53 ± 1.86	3.62	53.00 ± 5.91	11.14	52.20 ± 2.31	4.42
125.0	123.20 ± 7.67	6.22	127.34 ± 3.99	10.99	124.10 ± 13.09	10.55
250.0	244.29 ± 10.33	4.23	243.20 ± 25.99	10.69	230.65 ± 5.16	10.91

media	4mu	rsd	4mug	rsd	4mus	rsd
µMol	avg±sd	(%)	avg±sd	(%)	avg±sd	(%)
2.5	2.48 ± 0.18	7.31	2.50 ± 0.38	15.42	2.44 ± 0.23	9.34
10.0	10.21 ± 0.58	5.68	10.05 ± 0.63	6.25	10.64 ± 0.63	5.93
20.0	20.35 ± 0.74	3.65	20.14 ± 1.23	6.11	21.54 ± 1.38	6.39
50.0	49.13 ± 2.14	4.36	49.84 ± 1.47	2.96	51.95 ± 1.45	2.79
125.0	124.24 ± 5.41	4.36	125.68 ± 5.91	4.71	120.41 ± 5.03	4.18
250.0	247.85 ± 10.26	4.14	246.87 ± 15.67	6.35	220.60 ± 8.49	8.38

precision data for the three compounds of interest. The lower limit of quantitation for the three compounds was 2.5 μM. The within-run precision in media at the lower limit of quantitation (2.5 μM) ranged from 10-15% relative standard deviation. At all other concentrations, the precision of the assay was less than 10%.

No degradation of QC samples was noticed over a two week period with samples stored at -70°C. All samples were processed within two weeks of collection and working stocks were made fresh each day.

DISCUSSION

This new assay procedure for 4MU and its metabolites has several advantages over the three previously reported HPLC methods [16-18]. The analysis time is much shorter than the gradient method reported by Zimmerman et al. [17]. For our purposes, detection at 314 nm had adequate sensitivity. The lowest limit of quantitation (2.5 μM = 0.44 μg/mL of 4MU) was comparable to the previously reported methods [15,16] that also used UV detection despite the use of a smaller sample size. Sensitivity for 4MU and 4MUG can be improved with fluorescence detection, as described by Garrett et al. [18], but the emission maxima are different for the two compounds requiring either separate analyses or the availability of a time programmable fluorescence detector. 4MUG and 4MUS display significalntly less native fluorescence than 4MU [15], and consequently, the sensitivity for these compounds with fluorescence detection is not dramatically improved compared to UV detection. Pre

4MU, 4MUG, and 4MUS were detected at 314 nm with a diode-array detector. This high wavelength is the UV maximum for the three

compounds and is relatively free of interference for potential contaminants in plasma or tissue culture media. Chromatograms of a blank dog plasma extract, and extract of dog plasma with the addition of standards, and a plasma sample from a dog administered 4MU are shown in Figure 1. Figure 2 shows the results obtained from extracts of 50μL samples of a modified William's E media. No interfering peaks were observed under the peaks of interest. The total cycle time for the assay was 10 minutes and the retention times for the peaks of interest were as follows: 4MUG = 2.2 min, 4MUS = 4.0 min, umbelliferone (internal standard, I.S.) = 5.0 min, and 4MU = 6.3 min. Accuracy and precision for the assay in dog plasma and modified William's E media are shown in Tables I and II. Table I contains the data for within-day precision (n=5) and Table II shows the between-day accuracy and precision data for the three compounds of interest. The lower limit of quantitation for the three compounds was 2.5 μM. The within-run precision in media at the lower limit of quantitation (2.5 μM) ranged from 10-15% relative standard deviation. At all other concentrations, the precision of the assay was generally less than 10% .

ACKNOWLEGEMENT

This work was performed at the University of Minnesota with project grants from NIH, Proctor and Gamble, and Endotronics.

REFERENCES

[1] L. Fontaine, M. Grand, D. Molho, J. Chabert, and E. Boschetti, *Therapie*, **23**: 51-62 (1968).

[2] L. Fontaine, M. Grand, D. Molho, E. Boschetti, *Therapie*, **23**: 63-74 (1968).

[3] G.J. Mulder, S. Brouwer, J.G. Weitering, E. Scholtens, K.S. Pang, *Biochem. Pharmacol.* **34**:1325-1329 (1985)

[4] L. Sutherland, S. bin Sinafi, T. Ebner, D.J. Clarke, and B. Burchell, FEBS Lett. **308:**161-164 (1992).

[5] P.I. Mackenzie, *J. Biol. Chem.* **262**:9744-9749 (1987).

[6] C.S. Jin, J.O. Miners, and P.I. Mackenzie, *Biochem. Biophys. Res. Comm.* **194**:496-503 (1993).

[7] R.D. Sekura and W.B. Jakoby, *Arch. Biochem. Biophys.* **211**:352-359 (1981).

[8] G.J. Mulder, J.G. Weitering, S. Brouwer, and E. Scholtens, In *Advances in Glucuronide Conjugation. Falk Symposium 40.* S. Matern, K.W. Bock, and W. Gerok (eds.), MTP Press Ltd, Lancaster, UK, 1985, pp. 21-30.

[9] S. Miyauchi, Y. Sugiyama, T. Iga, and M. Hanano, *J. Pharm. Sci.* **77:**688-692 (1988).

[10] I.M. Anundi, F.C. Kaufmann, M. El-Mouelhi, and R.G. Thurman, *Mol. Pharmacol.* **29:**599-605 (1986).

[11] G.J. Mulder, S. Brouwer, and E. Scholtens, *Biochem. Pharmacol.* **33:**2341-2344 (1984).

[12] G.M.J. van Kempen and G.S.I.M. Jansen, *Anal. Biochem.* **46:**438-442 (1972).

[13] E.-M. Suolinna and E. Mantyla, *Biochem. Pharmacol.* **29:**2963-2968 (1980).

[14] K. Morita, Y. Sugiyama, and M. Hanano, *J. Pharmacobio-Dyn.* **9:**117-124 (1986).

[15] R. Sandman, *J. Chromatogr.* **272:**67-73 (1983).

[16] U. Femfert, H.D. Kuntz, B. May, *J. Chromatogr.* **278:**452-457 (1983).

[17] C.L. Zimmerman, S. Ratna, E. LeBoeuf, and K.S. Pang, *J. Chromatogr.* **563:**83-94 (1991).

[18] E.R. Garrett, J. Venitz, K. Eberst, and J.J. Cerda, *Biopharm. Drug Disp.* **14:**13-39 (1993).

[19] S.L. Nyberg, R.A. Shatford, M.V. Peshwa, J.G. White, F.B. Cerra, and W-S. Hu, *Biotech. Bioeng.***41:**194-203 (1993).

Received: October 27, 1993
Accepted: November 14, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1811-1819 (1994)

SEPARATION OF ACROLEIN AND ITS POSSIBLE METABOLITES USING DIFFERENT MODES OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Ion-exclusion, reverse phase, anion exchange and partition chromatography were used to separate acrolein and related compounds including 3-hydroxypropanal, 3-hydroxypropionic acid, 1,3-propanediol, allyl alcohol, acrylic acid, HCO_3^-, glycerol, glycidol, oxalic acid, malonic acid, propionic acid, ethanol, and propanol. The ion-exclusion chromatography employed an Interaction ORH-801 organic acid analysis column and was capable of separating these small and polar molecules. The reverse phase chromatography was used to resolve co-eluting compounds on the ion-exclusion column. The anion exchange and partition chromatography were used to separate organic acids and carbohydrates, respectively.

INTRODUCTION

Acrolein is the active ingredient in an aquatic herbicide and biocide that has been widely used to clear weeds and aquatic plants from irrigation ditches and canals and microorganisms from oil field equipments. Acrolein is also widely used as an important precursor in industrial synthesis. Acrolein was known to hydrolyze rapidly in water with first order kinetic half-lives ranging from 14 to 92 hours (1), and 3-Hydroxypropanal was identified as the major hydrolytic degradation product. Acrolein was also known to

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undergo rapid biotransformation under both aerobic and anaerobic conditions to form a group of structurally related compounds. Major hydrolytic and biotransformation products included 3-hydroxypropanal, 3-hydroxypropionic acid, allyl alcohol, acrylic acid, propionic acid, HCO_3^-, glyceric acid, 1,3-propanediol, oxalic acid, propanol, and butyric acid. The metabolism of acrolein in fish (studies conducted at Springborn Laboratories, Inc., 1993) and rat (2,3) suggested the formation of various metabolic products including acrylic acid, glycerol, glycidol, and sugar conjugates. The chemistry of acrolein posed an unique challenge to analytical chemists because of its high reactivity and volatility. To understand the degradation mechanism of acrolein in the environment, an adequate analytical separation technique is essential to identify and quantify metabolites of acrolein (especially those of transient nature). The separation techniques described in this paper provided a fast and relatively simple method to monitor acrolein and its metabolites.

MATERIALS AND METHODS

Chemicals

All reference standards were purchased from commercial sources and were analytical grade. All solvents were HPLC grade. ^{14}C-Acrolein was obtained from Sigma Chemical Company, St. Louis, MO. ^{14}C-$NaHCO_3$ was obtained from New England Nuclear, Boston, MA. 3-Hydroxypropanal (^{14}C-) was synthesized by adding ^{14}C-acrolein to water and allowing the mixture to react at room temperature for approximately seven days. The 3-hydroxypropanal was the major product (approximately 95%) of this reaction. The identification of this major product was achieved by derivatization with pentafluorophenylhydrazine (Aldrich Chemical Company) and subsequent analysis of the derivative by particle beam LC/MS using electron impact ionization. HPLC standards of each compound were prepared in reagent grade water.

High Performance Liquid Chromatography (HPLC)

HPLC chromatograms were collected using following instrumentations: a Waters model 510 solvent pump, a Hewlett Packard model 1050 autosampler, a Radiomatic model A-280 radiometric detector with data acquisition systems, a FlAtron CH-30 column heater, a Hewlett Packard model 1047A refractive index detector, a Hewlett Packard model 1040A photo diode-array detector and a Hewlett Packard model 3396A integrator. The Radiomatic A-280 radiometric detector equipped with a 500-μL liquid scintillation cell was used to monitor radiolabeled compounds (^{14}C-acrolein, ^{14}C-3-hydroxypropanal, and ^{14}C-HCO_3^-), while refractive index and photo diode-array detectors were used to monitor all non-radiolabeled compounds.

Five HPLC chromatographic systems were developed to separate acrolein and related compounds. They were one ion-exclusion system, two reverse phase systems, one anion exchange system, and one partition system. The ion-exclusion chromatographic system used an Interaction ORH-801 organic acid column (300 X 6.5 mm) at 35°C and 1 mM H_2SO_4 mobile phase at 0.8 mL/minute. The first reverse phase chromatographic system used a MetaChem Inertsil ODS-2 column (5 μm, 250 X 4.6 mm) maintained at 35°C,

and using a 0.05% H_3PO_4 mobile phase at 1 mL/minute. The second reverse phase chromatographic system used a Phenomenex Ultremex C18 (3 μm, 250 X 4.6 mm) at ambient temperature and using a 0.1 M KH_2PO_4 (pH 2.5) mobile phase at 0.8 mL/minute. The anion-exchange chromatographic system used a Phenomenex Spherex 10 SAX column (150 X 4.6 mm) at ambient temperature and using a 5/95 acetonitrile/potassium hydrogen phthalate (2 mM, pH 6.5) mobile phase at 1.5 mL/minute. The partition chromatographic system employed a Waters Carbohydrate Analysis column (10 μm, 300 X 3.9 mm) at ambient temperature and using a 80/20 acetonitrile/water mobile phase at 2 mL/minute.

RESULTS AND DISCUSSION

Acrolein is a small (molecular weight of 56), very polar, and highly water soluble compound. Most of its degradation products also bear these physical properties. The structural similarities of these small molecules made it very difficult, if not impossible, for signal-run separations using a single mode of chromatography. Integrations of various modes of chromatography were used in this work to separate these polar molecules.

The ORH-801 column is a polymer based ion-exclusion column designed specifically for separating weak organic acids, carbohydrates, alcohols, and inorganic anions. The separation using this column was satisfactory for most of the compounds tested. A list of the compounds investigated and their retention times are presented in Table I. An HPLC chromatogram showing the separation of a standard mixture is shown in Figure 1. There were three retention zones that were critical to acrolein studies. The first one (zone #1) was the early eluting region (retention time ~ 4.3 - 5.3 minutes) contained mostly strong organic acids and carbohydrates. Zone #2 (retention time ~ 8.3 minutes) contained lactic acid, glycerol, 3-hydroxypropionic acid, and glycidol. Zone #3 (retention time ~ 11 minutes) contained propionic acid, acrylic acid, and HCO_3^-.

The two reverse phase systems were developed primarily to resolve co-eluting compounds in zones #2 and #3. A list of retention times of selected compounds using these two reverse phase systems was also presented in Table 1. An HPLC chromatogram showing the separation of selected compounds using the Inertsil ODS-2 system is shown in Figure 2. The Inertsil ODS-2 system separated glycerol and glycidol, while glycidol and 3-hydroxypropionic acid still co-eluted. This system could also separate acrylic acid, propionic acid, and HCO_3^-. The Ultremex C18 system was used to separate glycidol and 3-hydroxypropionic acid. Acrolein chromatographed poorly (excessive retention and severe peak tailing) on both reverse phase columns due to the 100% aqueous mobile phases. These two systems were therefore, only used to monitor acrolein metabolites.

To retain strong organic acids (zone #1), a strong anion exchange chromatographic system (4) was used.

TABLE 1

Retention Times of Selected Compounds Using Ion-Exclusion and Reverse Phase Chromatographic Systems.

Compounds	Ion-Exclusion System retention time (minutes)	Inertsil ODS-2 System retention time (minutes)	Ultremex C18 System retention time (minutes)
Oxalic acid	4.32	3.52	4.12
Maltose	4.99	4.00	
Lactose	5.11	3.83	
Lactulose	5.12	4.22	
Citric acid	5.24	10.23	
Glucuronic acid	5.30	4.04	4.11
Propiolic acid	5.30	6.60	
Malonic acid	6.11	6.90	6.20
Glyceric acid	6.83	3.66	4.81
Glyceraldehyde	7.10		4.19
Lactic acid	7.95	6.22	7.02
Glycerol	8.32	3.95	4.35
3-Hydroxypropionic acid	8.40	5.80	6.87
Glycidol	8.41	5.83	7.71
Formic acid	8.70		
3-Hydroxypropanal	9.00	4.90	
Acetic Acid	9.30	6.67	
Ethylene glycol	9.68		
1,2-Propanediol	10.10	5.80	6.98
1,3-Propanediol	10.35	5.00	6.19
Propionic acid	10.98	16.31	19.2
$NaHCO_3$	11.36	9.90	
Acrylic acid	11.41	13.32	16.2
Adipic acid	11.81		
Ethanol	12.11	7.07	8.55
Allyl alcohol	12.63	10.07	12.7
Butyric acid	13.40		
Propanol	15.13	15.80	21.6
Acrolein	15.62		

Organic acids could be easily distinguished from alcohols, since alcohols (neutral) had no retention on the anion exchange column. This anion exchange system was used primarily to confirm the formation of oxalic acid in acrolein metabolism studies.

The other class of early eluting compounds on the ORH-801 column was carbohydrate. A partition chromatography utilizing a Carbohydrate Analysis column was used to selectively retain carbohydrates. Alcohols and carboxylic acids either had no retention or excess retention on this column. A list of selected compounds and their retention times using the anion exchange and carbohydrate systems are presented in Table 2.

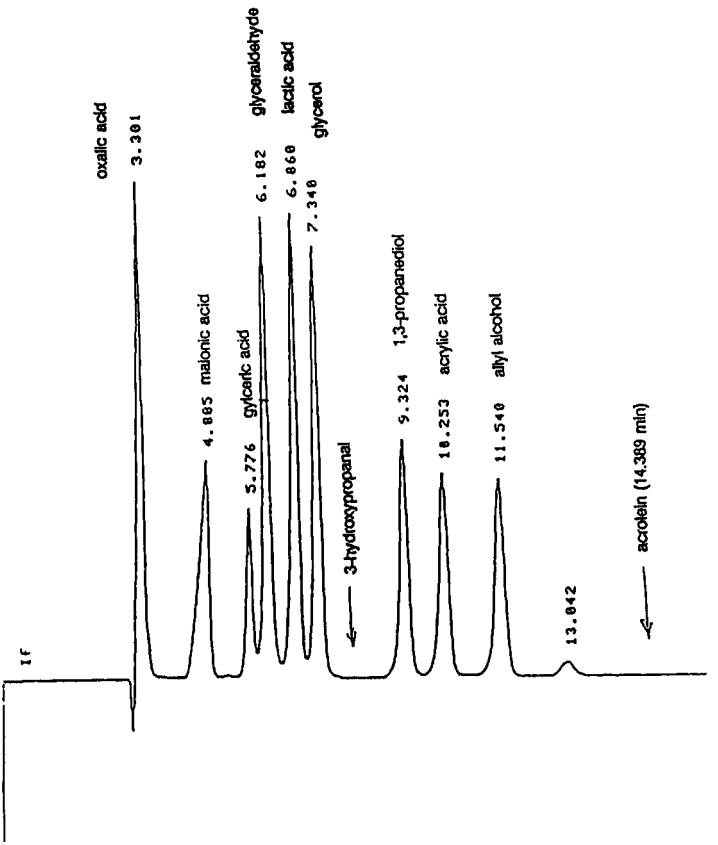

Figure 1. Separation of a standard mixture using ion-exclusion chromatography. HPLC conditions: ORH-801 column at 35°C, 1 mM H_2SO_4 at 0.8 mL/minute, RI detection. The retention times of acrolein and 3-hydroxypropanal were determined on separate runs. 3-Hydroxypropanal was detected using a radiometric detector. (Note: Retention times in this chromatogram were different from those listed in Table I due to the differences in HPLC dead volumes.)

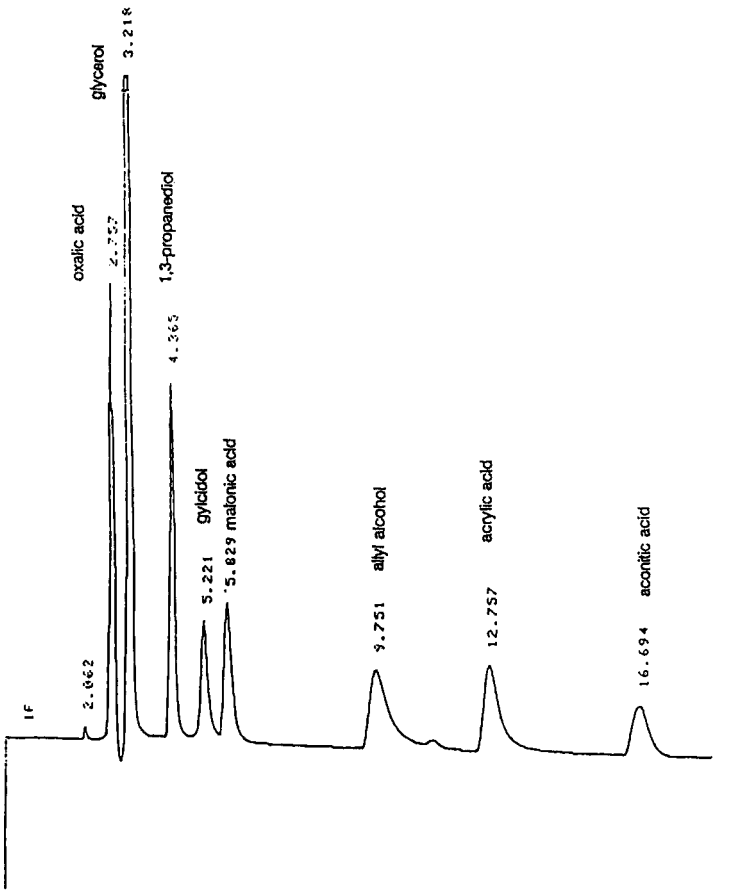

Figure 2. **Separation of a standard mixture using reverse phase chromatography. HPLC conditions: Inertsil ODS-2 column at 35°C, 0.05% H_3PO_4 at 1 mL/minute, RI detection. (Note: Retention times in this chromatogram were different from those listed in Table I due to the differences in HPLC dead volumes.)**

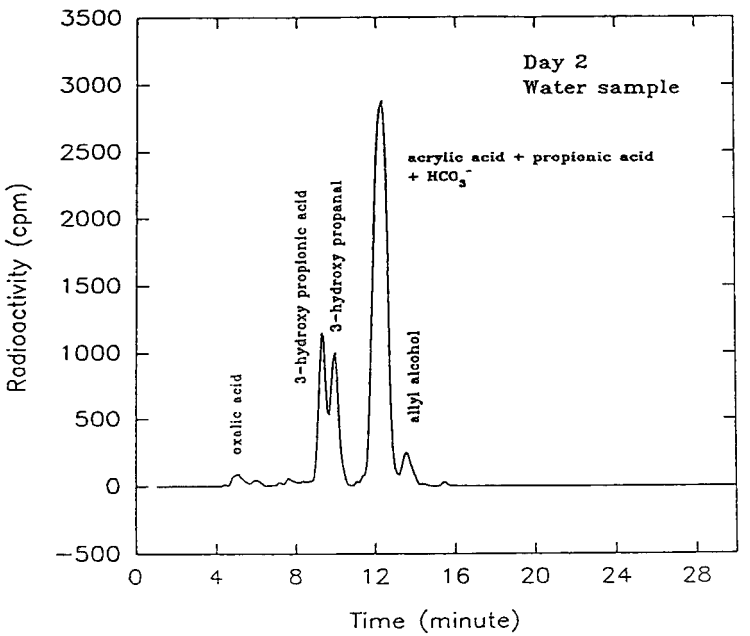

Figure 3. **HPLC radiochromatogram of a water sample collected during an aquatic metabolism study with acrolein. The separation of acrolein metabolites was achieved using the ion exclusion chromatographic system.**

TABLE 2

Retention Times of Selected Compounds Using Anion Exchange and Partition Chromatographic Systems.

Compounds	Spherex SAX System retention time (minutes)	Carbohydrate Analysis System retention time (minutes)
Oxalic acid	6.40	
Malonic acid	6.63	
Propiolic acid	3.27	
3-Hydroxypropionic acid	2.80	
Glyceric acid	2.72	
Acetic acid	3.43	
Glucose		6.17
Lactulose		12.45
Maltose		12.36
Lactose		14.47

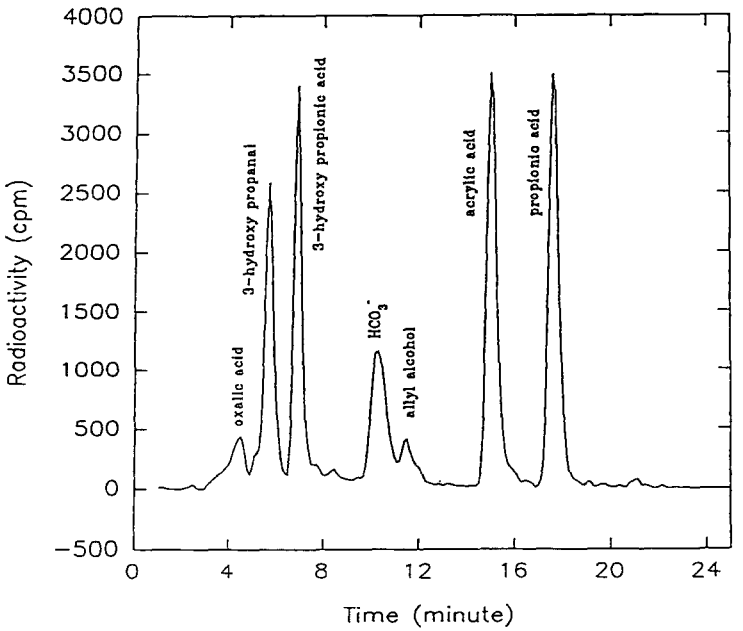

Figure 4. **HPLC radiochromatogram of the same sample as in Figure 3 using the Inertsil ODS-2 chromatographic system.**

The separation techniques developed in this work could serve as a good method reference for separating small and polar organic compounds. These techniques were applied to aquatic (aerobic and anaerobic) and fish (freshwater fish and shellfish) metabolism studies with acrolein. These chromatographic systems were proved to be adequate to separate a large number of acrolein metabolites. Aqueous samples and tissue extracts could be chromatographed directly without further manipulations (eg. derivatization, extraction). The relatively simple sample preparation and rapid analysis time permitted the identification and quantification of transitory metabolites. Two representative chromatograms collected during an aquatic metabolism study with acrolein are presented in Figures 3 and 4 showing the formation of several microbial biotransformation products. Three major metabolites, acrylic acid, propionic acid, and HCO_3^-, co-eluted on the ion-exclusion column (Figure 3), while they were separated on the reverse phase column (Figure 4).

ACKNOWLEDGMENT

We thank Baker Performance Chemical, Inc. for the financial support to this work. We also thank Jeffrey Bob and Ann Smith of Springborn Laboratories, Inc. for generating aquatic metabolism samples.

REFERENCES

1. K. Bowmer, M. L. Higgins, Arch. Environ. Contam. Toxicol., 5: 87-96 (1976)

2. W. Draminski, E. Eder, D. Henschler, Arch Toxicol., 52: 243-247 (1983)

3. J. M. Panel, J. C. Wood, K. C. Leibman, Drug Metab. Dispos., 8, 305-308 (1980)

4. T. A. Walker, N. Akbarl, T. V. Ho, J. Liq. Chromatogr., 14 (4): 619-641 (1991)

Received: October 16, 1993
Accepted: December 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1821-1835 (1994)

CHARACTERISTICS OF CENTRIFUGAL PARTITION CHROMATOGRAPHY FOR LANTHANOID SEPARATION IN HDEHP EXTRACTION SYSTEM

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ABSTRACT

Separation of light lanthanoids with centrifugal partition chromatography (CPC) was investigated in extraction system of 30% HDEHP/CCl_4/n-paraffin and HNO_3. Since separation factors between lanthanoids, Ce, Nd, Pr, Sm, Eu and Gd, were independent of volume ratio of CCl_4 and n-paraffin diluents, difference in the density between the mobile phase and the stationary phase was controlled by varying the volume ratio of two diluents in order to operate at reasonable pump-pressure. The effects of volume of the stationary phase and rotational speed on the separation characteristics of CPC were examined to find the optimum operation conditions. The effect of chain length of n-paraffin (number of carbon atoms: 6 - 15) as diluents was also examined. The best separation efficiency was obtained with the longest, n-pentadecane. On the basis of the above results, mutual separation of lanthanoids was successfully performed in 30% HDEHP/15% CCl_4/55% n-pentadecane and 0.5 mol/dm^3 HNO_3 extraction system at a low pump pressure of 4.5 kgf/cm^2.

INTRODUCTION

Centrifugal partition chromatography (CPC) is a new liquid-liquid separation method, in which the centrifugal force enables retention of a stationary phase

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in the partition cells, dispersion of a mobile phase in the stationary phase and rapid phase separation (1). The method was first developed for separation of organic substances. Recently it has been also applied to separation of inorganic substances (2)(3)(4). The authors reported separation of light lanthanoids with CPC in 30% TBP extraction system using lithium nitrate as a salting-out reagent (5)(6). Difference in density between the aqueous and organic phases could be controlled by using carbon tetrachloride (CCl_4: 1.6 g/cm^3 density) as a heavy diluent and n-dodecane (0.75 g/cm^3) as a light diluent, and then the CPC apparatus was operated at low pump pressure.

In order to achieve effective separation with CPC apparatus, it is important to get both large separation factor and high theoretical plate number. The separation factor depends on the extraction conditions such as acidity of aqueous phase and concentration of extractant. The theoretical plate number is affected by the operation conditions of CPC apparatus such as rotational speed and volume of the stationary phase, and by the physical properties of the two phases such as interfacial tension and viscosity.

Di-2-ethylhexyl phosphoric acid (HDEHP) has been widely used for mutual separation of lanthanoids and actinoids. Separation of lanthanoids in extraction system of HDEHP and hydrochloric acid by using CPC apparatus was also reported (7)(8). This paper deals with the separation characteristics of light lanthanoids with CPC apparatus in extraction system of HDEHP/CCl_4/n-paraffin and nitric acid (HNO_3). To obtain the optimum extraction conditions, distribution coefficients were determined by batch experiments. Then, to find the operation conditions of CPC apparatus giving high theoretical plate number, the effects of volume of the stationary phase, rotational speed and the physical properties were examined. For the effect of the physical properties of the two phases, n-paraffin with different number of carbon atoms (C: 6 – 15) was used as a diluent because their chemical properties seem similar to each other. After that, the separation of lanthanoids with CPC apparatus was demonstrated.

EXPERIMENTAL

Reagents

An extractant of HDEHP (98.2%) and diluents of CCl_4 (97.8%), n-hexane (95 – 97%) and other n-paraffin (99.0%) were used without further purification.

As solutes, Li and Ce nitrates and Pr, Nd, Sm, Eu and Gd oxides of reagent grade were used by dissolving them in HNO_3 solution. Lithium was an unextractable spike to evaluate a dead volume of CPC chromatogram.

Apparatus

Two types of CPC apparatus, Model-LLN and Model-LLB, were supplied by Sanki Engineering Co. Ltd. The former apparatus was used in order to study the effect of the stationary phase volume on the separation efficiency of CPC. The apparatus was equipped with different number of partition-cell cartridges, Model 250W, which consisted of 400 microcells. The volume of each cartridge was 20.8 cm^3. The latter apparatus, Model-LLB, was used for the other experiments because the latter is superior to the former in the pressure-stability. The apparatus was equipped with 6 rotors, in which 720 micro-cells per rotor were molded. The total volume was 135 cm^3.

An inductively coupled plasma (ICP) spectrometer was used for determining the concentration of lanthanoids.

Procedures

In batch experiments, the initial concentration of each solute in the aqueous phase was 500 mg/dm^3. Both aqueous and organic phases of 10 cm^3 were shaken at 25 °C for 5 h. Since the solutes could not be back-extracted quantitatively, the concentration of the solutes in the organic phase was determined by subtracting concentration of the remained solutes in the aqueous phase from that in the initial aqueous phase.

In CPC experiments, at first, the stationary and mobile phases were balanced in the partition cells with the same method previously described (6). Then, sample solution of 1.1 cm^3 was injected through a sample loop to the partition cells by a pump. Each solute was eluted at the flow rate of 1.5 cm^3/min and 25 °C.

Analysis of the chromatogram

Distribution coefficient (K_d), separation factor (α) and resolution between two peaks (R_s) were calculated from chromatograms with the same equations (6). For estimation of the separation efficiency of CPC, relative peak width (W_p) was used instead of the theoretical plate number because of too large dead volume;

$$W_p = V_{wh}/V_R'$$

where V_{wh} in cm^3 is peak width at half height and V_R' in cm^3 is corrected retention volume which was obtained by subtracting retention volume of Li.

RESULTS AND DISCUSSION

Determination of the K_d and α values by batch method

Effect of HNO_3 and CCl_4: TABLE 1 shows the K_d values of lanthanoids obtained by the batch experiments. It was confirmed that Li was not extracted in all experimental conditions. The effect of the concentration of HNO_3 on the K_d values in 30% HDEHP/ 70% n-dodecane is shown in FIGURE 1. The K_d values decreased with increasing concentration of HNO_3. This decrease with the slope of -3 indicates that extraction of lanthanoids with HDEHP is due to cation exchange reaction as follows (9);

$$M^{3+}_{aq} + 3(HY)_{2\ org} \longrightarrow M(HY_2)_{3\ org} + 3H^+_{aq}$$

where M^{3+} is a lanthanoid ion (III) and HY is a HDEHP. On the other hand, the α values between lanthanoids were almost independent of the concentration of HNO_3.

FIGURE 2 shows the effect of concentration of CCl_4 on the K_d values in 30% HDEHP/n-dodecane and 0.5 mol/dm^3 (M) HNO_3. The values decreased with increasing concentration of CCl_4 in analogy with the TBP extraction system. This seems a result of the decrease of the activity of HDEHP in the organic phase with increasing concentration of CCl_4 because CCl_4 has a larger acceptor number than n-dodecane (10). On the other hand, the α values were almost independent of the concentration of CCl_4.

From these results, it was found that the K_d value for adequate retention volume and the difference in density for reasonable pump pressure in CPC operation could be set without decrease of the α value.

Effect of number of carbon atoms of n-paraffin: FIGURE 3 shows effect of number of carbon atoms of n-paraffin on the K_d values in 30% HDEHP/70% n-paraffin and 0.7M HNO_3. The K_d values increased with increasing the number and the α values were almost independent of the number.

Since HDEHP is polar, the association among themselves takes place through the dissolving water in the organic phase. Solubility of water in n-paraf-

TABLE 1
The K_d Values of Lanthanoids Obtained by Batch Experiments (30% HDEHP)

CCl_4: 0%, n-dodecane: 70%

$[HNO_3]$ M	Ce	Pr	Nd	Sm	Eu	Gd
0.25	2.8	3.9	4.9	28	67	123
0.50	0.56	0.72	0.77	4.2	9.1	16
0.75	0.22	0.21	0.24	1.2	2.4	4.3
1.00	0.15	0.11	0.13	0.56	1.0	1.7

CCl_4: 10%, n-dodecane: 60%

$[HNO_3]$ M	Ce	Pr	Nd	Sm	Eu	Gd
0.25	1.9	2.6	3.4	20	45	82
0.50	0.38	0.46	0.53	3.0	6.2	11
0.75	0.15	0.16	0.17	0.88	1.7	3.0
1.00	0.12	0.093	0.12	0.38	0.74	1.2

CCl_4: 20%, n-dodecane: 50%

$[HNO_3]$ M	Ce	Pr	Nd	Sm	Eu	Gd
0.25	1.3	1.8	2.4	15	34	61
0.50	0.26	0.34	0.31	2.1	4.2	7.7
0.75	0.10	0.10	0.12	0.61	1.2	2.2
1.00	0.085	0.056	0.083	0.25	0.50	0.82

CCl_4: 30%, n-dodecane: 40%

$[HNO_3]$ M	Ce	Pr	Nd	Sm	Eu	Gd
0.25	0.87	1.2	1.7	10	25	45
0.50	0.18	0.23	0.22	1.5	3.0	5.5
0.75	0.084	0.066	0.062	0.40	0.84	1.4
1.00	0.077	0.033	0.054	0.17	0.36	0.58

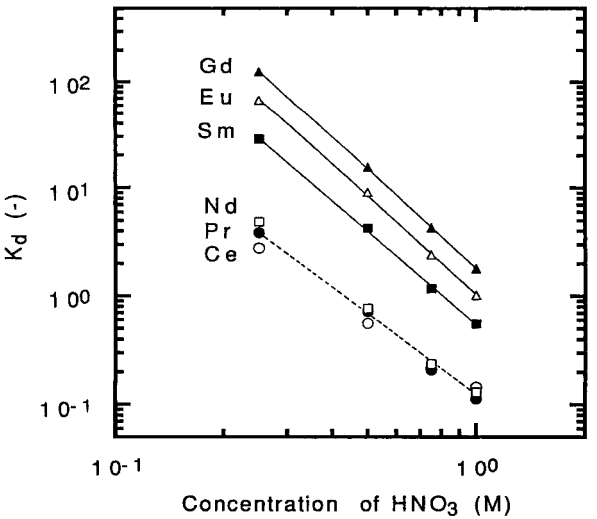

FIGURE 1. Effect of HNO_3 on the K_d values of lanthanoids. Organic phase: 30% HDEHP/70% n-dodecane.

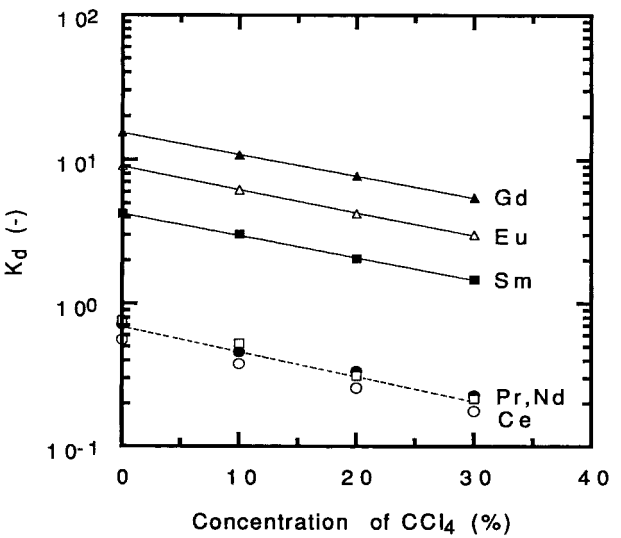

FIGURE 2. Effect of CCl_4 on the K_d values of lanthanoids. Aqueous phase: 0.5 M HNO_3, Organic phase: 30% HDEHP/CCl_4/n-dodecane

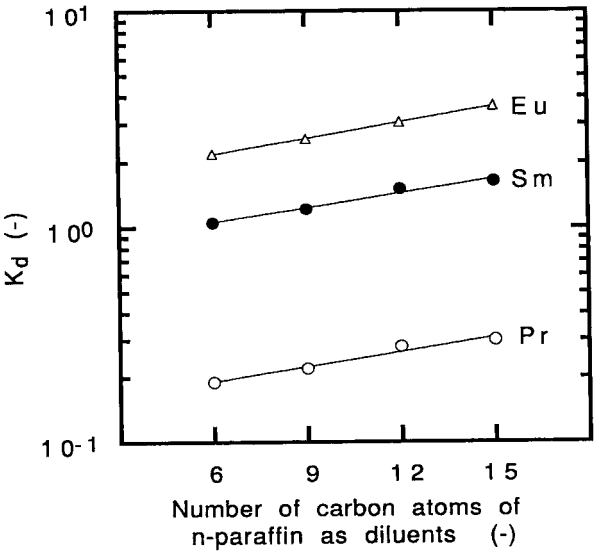

FIGURE 3. Effect of number of carbon atoms of n–paraffin as diluents on the K_d values of lanthanoids. Aqueous phase: 0.7 M HNO_3, Organic phase: 30% HDEHP/ 70%n–paraffin.

fin decreases with increasing the number (11). Therefore, the authors interpret the increase of the K_d values as the increase of the HDEHP activity in the organic phase.

Measurement of the separation efficiency of CPC

Effect of the stationary phase volume: FIGURE 4 shows the effect of volume of the stationary phase on the separation efficiency of CPC, which was examined by changing number of the partition cell cartridges. The relative peak width of Pr and Eu decreased and the resolution between them increased with increasing volume of the stationary phase.

It was observed that the maximum volume of the stationary phase being retained in the partition cells varied with the experimental conditions of CPC. Therefore, the effects of rotational speed and difference in the density on the

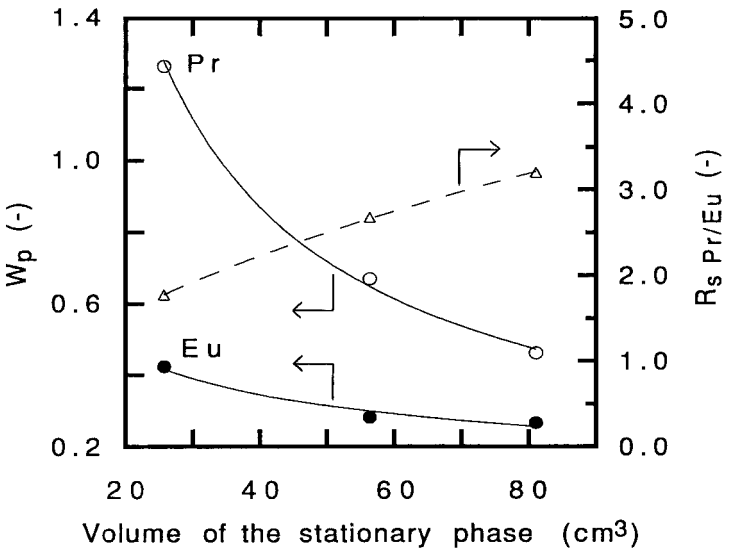

FIGURE 4. Effect of volume of the stationary phase on the separation efficiency of CPC. Mobile phase: 0.5 M HNO_3, Stationary phase: 30% HDEHP/15% CCl_4/ 55% n-dodecane, Rotational speed: 800 rpm.

maximum volume were examined. The results are shown in FIGURE 5. The maximum volume increased with increasing the rotational speed and the difference in density. Since the volume of the stationary phase affects the separation efficiency of CPC as described above, the volume of the stationary phase was attempted to keep constant in the CPC experiments below.

Effect of the rotational speed: FIGURE 6 shows the effect of the rotational speed of the partition cells in the region of 800 – 1400 rpm on the separation efficiency of CPC. The operation conditions and the analytical results of chromatograms are shown in TABLE 2 (Serial No.1). The pump pressure increased with increasing the rotational speed. The best separation efficiency was observed at 800 rpm. It was reported that the separation efficiency was improved with increasing the rotational speed because the dropsize of the mobile phase decreased with increasing gravity resulting from rotation (2). In this experimental condi-

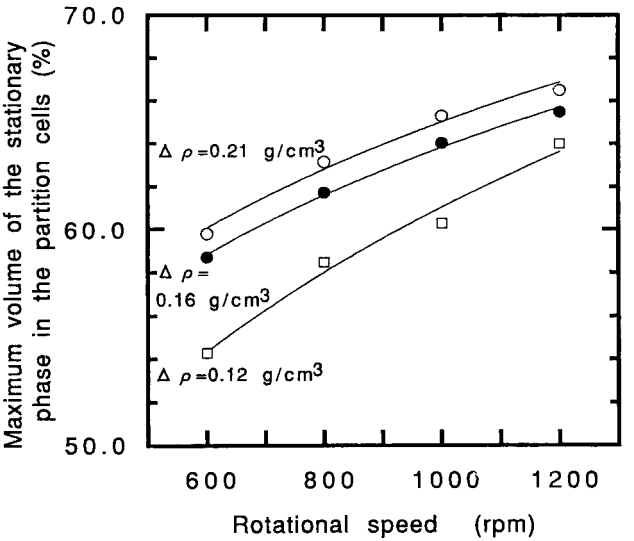

FIGURE 5. Effects of rotational speed and difference in density ($\Delta\rho$) on the maximum volume of the stationary phase in the partition cells. Mobile phase: H_2O, Stationary phase: CCl_4/n-dodecane.

tions, however, this tendency was not observed. It might be necessary to examine the effect of the rotational speed under other experimental conditions such as another flow rate.

Effect of number of carbon atoms of n-paraffin: FIGURE 7 shows the effect of chain length of n-paraffin (number of carbon atoms: 6 - 15) as diluents on the separation efficiency of CPC. The operation conditions and the analytical results of chromatograms are shown in TABLE 2 (Serial No.2). The relative peak width of Sm and Eu decreased and the resolution increased with increasing the number. Since the α values were not affected by the difference of the number, the net improvement of the resolution between them is ascribed to the decrease in the peak width.

In CPC, the dropsize of the dispersed mobile phase is presumed one of the important parameters affecting the separation efficiency. It is predicted that the

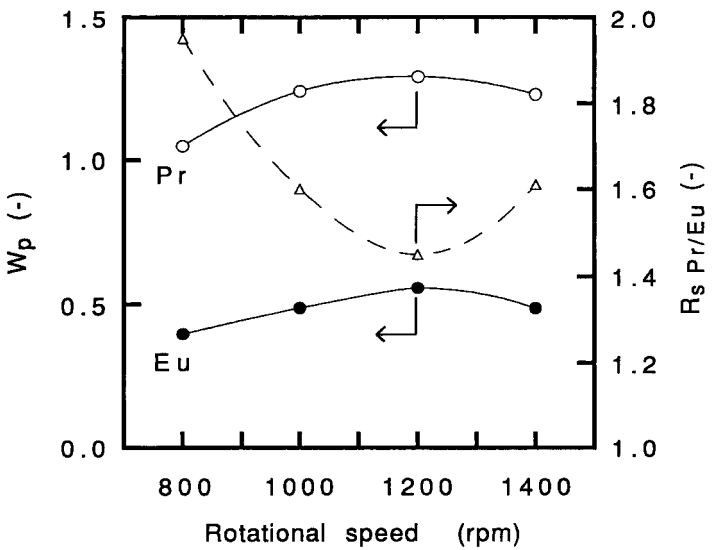

FIGURE 6. Effect of the rotational speed on the separation efficiency of CPC. Mobile phase: 0.5 M HNO_3, Stationary phase: 30% HDEHP/15% CCl_4/55% n-dodecane.

dropsize is reduced with decreasing interfacial tension and viscosity of the stationary phase (12).

The measured values of some physical properties of 30% HDEHP/70% n-paraffin and 0.7 M HNO_3 are shown in TABLE 3. The interfacial tension and the difference in density decreased and the viscosity increased with increasing the number. Since the peak width decreased with increasing the number, the interfacial tension seems to affect strongly the dropsize. However, it is difficult to discuss the effect of the difference in density on the dropsize. The quantitative evaluation of the effect of other physical properties on the dropsize must be necessary for investigation of the separation efficiency of CPC.

Mutual separation of lanthanoids with CPC apparatus

On the basis of the results of determination of the K_d values and measurement of the separation efficiency of CPC, mutual separation of light lanthanoids

TABLE 2
Operation Conditions and Analytical Results of Chromatograms in CPC experiments

Serial number		1				2				3
Mobile phase [HNO_3] M		0.5				0.7				0.5
Stationary phase	[HDEHP] %	30				30	30	30	30	30
	[CCl_4] %	15				—	—	—	—	15
	[n-paraffin] %	55(C12)				70 (C6)	70 (C9)	70(C12)	70(C15)	55(C15)
Stationary phase volume	cm^3	53.6	54.1	53.1	55.1	64.6	65.1	65.6	66.0	55.6
Rotational speed	rpm	800	1000	1200	1400	900				800
K_d	Pr	0.35	0.36	0.34	0.37	—	—	—	—	0.45
	Sm	—	—	—	—	0.88	1.1_3	1.2_7	1.6_6	2.7_9
	Eu	5.0_6	5.1_8	5.1_2	5.3_4	1.9_1	2.5_3	2.8_7	3.5_9	5.8_3
	Gd	—	—	—	—	—	—	—	—	8.8_0
α	Pr/Sm	—	—	—	—	—	—	—	—	6.2_4
	Pr/Eu	$14._4$	$14._4$	$15._0$	$14._5$	—	—	—	—	$13._1$
	Sm/Eu	—	—	—	—	2.1_7	2.2_4	2.2_6	2.1_6	2.0_9
	Eu/Gd	—	—	—	—	—	—	—	—	1.5_1
W_p	Pr	1.0_5	1.2_4	1.2_9	1.2_3	—	—	—	—	0.73
	Sm	—	—	—	—	0.91	0.81	0.77	0.62	0.40
	Eu	0.40	0.49	0.56	0.49	0.77	0.69	0.64	0.55	0.31
	Gd	—	—	—	—	—	—	—	—	0.30
R_s	Pr/Sm	—	—	—	—	—	—	—	—	1.6_3
	Pr/Eu	1.9_6	1.6_0	1.4_5	1.6_1	—	—	—	—	2.5_5
	Sm/Eu	—	—	—	—	0.46	0.52	0.57	0.63	1.0_5
	Eu/Gd	—	—	—	—	—	—	—	—	0.68
Pump pressure	kgf/cm^2	7	11	15	20	32	29	27	25	4.5

1: Effect of rotational speed, 2: Effect of number of carbon atoms as diluents, 3: Demonstration of mutual lanthanoids separation.

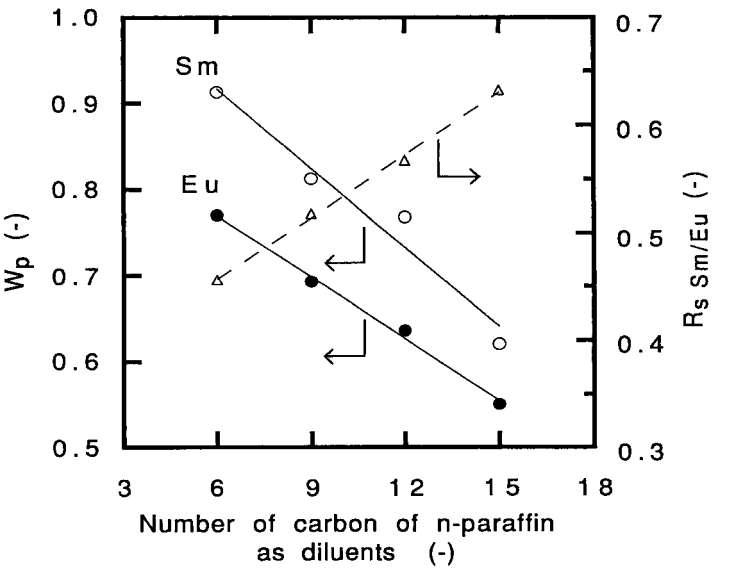

FIGURE 7. Effect of number of carbon atoms of n-paraffin as diluents on the separation efficiency of CPC. Mobile phase: 0.7 M HNO_3, Stationary phase: 30% HDEHP/ 70% n-paraffin, Rotational speed: 900 rpm.

TABLE 3
Some Physical Properties of 30% HDEHP/70% n-Paraffin at 25 °C
(Aqueous phase: 0.7 M HNO_3)

n-Paraffin	Interface tension (10^{-2}N/m)	Viscosity (mPa·s)	Difference in the density (g/cm^3)
n-hexane	5.31	0.56$_4$	0.41$_0$
n-nonane	4.94	1.39	0.23$_1$
n-dodecane	4.70	2.60	0.19$_9$
n-pentadecane	4.52	4.32	0.18$_7$

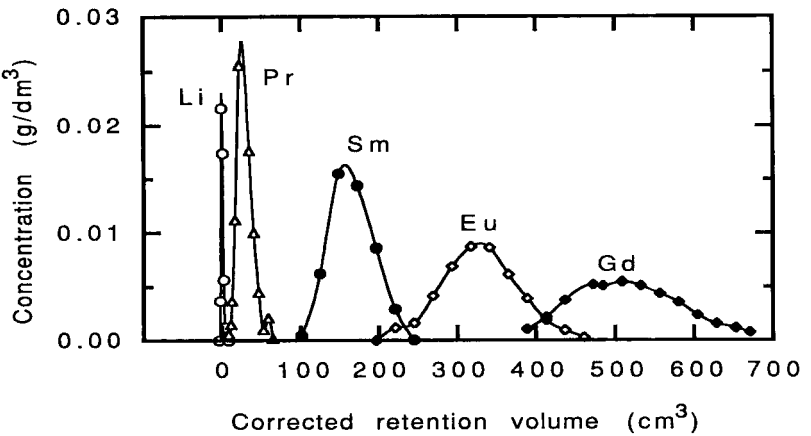

FIGURE 8. Chromatogram of Li, Pr, Sm, Eu and Gd. Mobile phase: 0.5 M HNO_3, Stationary phase: 30% HDEHP/15% CCl_4/55% n-pentadecane, Rotational speed: 800 rpm.

was demonstrated. Mobile phase of 0.5 M HNO_3 was selected for the adequate retention volume. In order to operate the CPC apparatus under a stable condition, the difference in density between the two phases was adjusted to be 0.054 by use of 15% CCl_4 as a heavy diluent. Since the separation efficiency was improved with increasing number of carbon atoms, n-pentadecane was used as a light diluent. The rotational speed was fixed at 800 rpm.

FIGURE 8 shows a chromatogram of Li, Pr, Sm, Eu and Gd. The analytical results of the chromatogram are also summarized in TABLE 2 (Serial No.3). Since the relative peak width of Pr and Eu was reduced as compared with the n-dodecane diluent system (Serial No.1), the resolution between Pr and Eu increased from 1.9_5 to 2.5_5. The resolution between Eu and Gd (R_s=0.68) was not so good because of small α value (α=1.5), but the others were good ($R_s \geq 1$). Furthermore, the CPC apparatus could be stably operated under very low pressure (4.5 kgf/cm^2).

CONCLUSIONS

The characteristics of CPC for lanthanoid separation were investigated in the extraction system of 30% HDEHP/CCl_4/n-paraffin and HNO_3. The K_d values showed a dependency on concentration of HNO_3 and the diluents but the α values did not. Therefore, the adequate K_d values and difference in the density were obtained without decrease of α values for the reasonable retention volume and pump pressure, respectively.

The separation efficiency of CPC was improved with increasing the number of carbon atoms of the n-paraffin used as a light diluent. It is of interest that the separation efficiency of CPC could be improved not only by the optimization of the operation conditions but also by the selection of diluent. In future, the authors intend to examine the effect of the physical properties of the diluent on the separation efficiency more quantitatively.

ACKNOWLEDGMENTS

The authors are grateful to Drs. Z. YOSHIDA and H. NAGANAWA of JAERI for their helpful comments and valuable discussions and also to Mr. H. TAKEISHI of JAERI for his help in ICP operation.

REFERENCES

1. W.Murayama, T.Kobayashi, Y.Kosuge, H.Yano, Y.Nunogaki, K.Nunogaki, J. Chromatogr., 239: 643-649 (1982).

2. K.Akiba, S.Sawai, S.Nakamura, W.Murayama, J.Liq.Chromatogr., 11: 2517-2536 (1988).

3. S.Muralidharan, R.Cai, H.Freiser, J.Liq.Chromatogr., 13: 3651-3672 (1990).

4. Y.Surakitbanharn, S.Muralidharn, H.Freiser, Solv.Extr.Ion.Exch., 9: 45-49 (1991).

5. S.Usuda, H.Abe, S.Tachimori, H.Takeishi, W.Murayama, "Application of Centrifugal Partition Chromatography to Separation of Actinides and

Lanthanides in TBP Extraction System", in Solvent Extraction 1990, Part A, T.Sekine, ed., Elsevier Sci. Pub., Amsterdam, 1992, pp. 717–722.

6. H.Abe, S.Usuda, H.Takeishi, S.Tachimori, J.Liq.Chromatogr., 16: 2661–2672 (1993).
7. T.Araki, T.Okazawa, Y.Kubo, H.Ando, H.Asai, J.Liq.Chromatogr., 11: 267–281 (1988).
8. T.Araki, H.Asai, H.Ando, N.Tanaka, K.Kimata, K.Hosoya, H.Narita, J.Liq. Chromatogr., 13: 3673–3687 (1990).
9. D.F.Peppard, G.W.Mason, G.Giffin, J.Inorg.Nucl.Chem., 27: 1683–1691 (1965).
10. U.Mayer, V.Gytmann, W.Gerger, Monatshefte Für Chemie, 106: 1235–1257 (1975).
11. P.Schatzberg, J.Phys.Chem., 67: 776–779 (1963).
12. C.B.Hayworth, R.E.Treybal, Ind.Eng.Chem., 42: 1174–1181 (1950).

Received: October 28, 1993
Accepted: November 14, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1837-1848 (1994)

A SENSITIVE ISOCRATIC LIQUID CHROMATOGRAPHY ASSAY FOR THE DETERMINATION OF DIPYRIDAMOLE IN PLASMA WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

For many years, dipyridamole (DP) has been used in the treatment of hypertension as a vasodilatator, but recently it has been recognized as an anti-platelet aggregation agent and to potentiate cytotoxic. A rapid and very sensitive (1 nM) procedure for the determination of free and protein-bound DP in plasma, using reversed-phase high-performance liquid chromatography on a μBondapark C18 (10μm) column (300 x 3.9 mm I.D.) with coulometric detection (+ 0.65 V), is reported. Free and bound DP were separated using ultrafiltration. The liquid-liquid extraction from plasma included solvent extraction using diethyl-ether and a preparative column to separate DP from constituents normally found in plasma. The particular columns used contained a specially modified form of diatomaceous earth which requires no-preconditioning washes. The overall recovery from plasma was 52 ± 12 % at the concentration of 0.5 ng/ml (1 nM). Concentrations of DP between 1 nM and 1 μM were measured in plasma with relative standard deviations under 7.6 % (n = 6). The subsequent determination of DP levels in patients orally administered from 107 to 500 mg/m^2/day showed that DP binding to plasma protein was higher than 90 % with a mean at 97.2 % (95.5 % to 92.3 %).

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INTRODUCTION

Despite intensive efforts to discover new and effective anticancer drugs, the number of clinically useful cytotoxic agents has remained relatively stable. However, great progress has been made in understanding the mechanisms of cytotoxicity of currently available agents and in associating them with drugs which allow a biochemical modulation of cytotoxic agents like the anti-metabolite compounds. Dipyridamole (DP) is a structural analogue of the purine and pyrimidine nucleosides and is used clinically as a vasodilatator and recently, it was shown to potentiate anti-metabolite activity in a dose-dependent manner (1 - 3).

It therefore appears important to control the plasma level of this compound, which is mainly bound to plasma-protein. Several methods, e.g., spectrofluorimetry and high-performance liquid chromatography (HPLC) with spectrophotometric, fluorescence or amperometric detection were previously reported for the determination of DP in biological samples (4 - 6). In addition to being time consuming, spectrofluorimetric methods suffers from the low specificity inherent in non-chromatographic methods. The three reported HPLC methods suffered from a relative lack in sensitivity. Moreover, the HPLC assay using electrochemical detection was only applicable for the determination of DP in pharmaceuticals without developing any procedure for plasma preparation (6).

Yet, no method is available for the rapid determination of free and protein bound DP with a sensitivity lower than 5 ng/ml (10 nM).

The specificity of this method allowed the quantitation of free and total DP in plasma. It describes also a rapid, sensitive (1 nM) and selective HPLC method using a reversed-phase column and coulometric detection involving only a simple liquid-liquid extraction with a preparative column which requires no-preconditionning washes.

The assay was employed during a recent clinical pharmacology study to characterize the high protein binding rate of DP (> 90 %) after *per os* administration (7).

MATERIALS AND METHODS

Apparatus

The HPLC system was a computer-monitored GOLD PC apparatus (Model 126 pumps, Model 406 Interface), (Beckman, Gagny, France), an ESA Coulochem

5100A dual-electrode coulometric detector with an ESA guard cell (Cunow, Cergy Saint-Christophe, France), a μBondapak C18, 10 μm column (300 x 3.9 mm I.D.) and a WISP 512 autosamples (Waters, Millipore, Molsheim, France).

Reagents, Standard Solutions and Internal Standard

DP was purchased from Boehringer-Ingelheim (Reims, France) and indometacin (INDO) from Merck (Darmstadt, Germany). All other reagents were obtained from Prolabo (Paris, France) and were of the highest purity available.

A stock solution containing 5 mg/ml of dipyridamole (DP) was prepared in methanol. The internal standard stock solution indometacin (INDO) 5 mg/ml was also prepared in methanol. Plasma standard solutions of DP for the calibration curves were prepared by appropriate detection of the stock DP solutions with drug-free plasma so that concentrations from 1 to 200 nM were obtained. The plasma standards and the internal standard solution were freshly prepared each day of analysis.

HPLC Conditions

Isocratic elution conditions were adopted. The eluting solvent was methanol - 0.1 M sodium acetate buffer (pH 4.0) (55 : 45, $^{v}/_{v}$). Chem-Elut® columns (Analytichem International, Ann Harbor, Mi, USA) were used for plasma samples preparation. Throughout the study, deionized water (Milli Q water purification system, Millipore) was used.

Room temperature and a flow-rate of 1.0 ml/min were maintained throughout the analyses. A pressure of 100 bars (1 500 p.s.i.) was used. A volume of 20 μl of the samples in methanol was injected into the column.

Electrochemical Detection

The potentials corresponding to the limiting current wave of the DP and INDO were determined by generation of hydrodynamic voltammogram and the potential of the upstream electrode was set to + 0.05 V being lower than the rising portion of the voltammogram of the analyte.

This stabilized the baseline and allowed a better reproducibility of the chromatographic results. The second electrod was used to detect the analyte at its appropriate potential corresponding to the limiting current wave. Amounts of DP were quantified by comparison of peak height with that of a standard sample.

Sample Pretreatment

Blood samples were collected in EDTA tubes and immediately centrifuged at 3 000 g for 10 minutes. The separated plasma was rapidly frozen in polypropylene tubes at - 20°C until analysis. 500 µl of plasma was mixed for 10 s using a rotating mixing and then supplemented with 100 µl of INDO as internal standard.

Free and bound DP were separated using SM 13243 ultrafiltration units (Sartorius, Palaiseau, France) by centrifugation (4 000 g for 30 min).

The aqueous samples were added to the dry Chem Elut® column and left 3 to 5 minutes to absorbe and distribute as a thin film over the hydrophilic packing material. Two milliliters of diethyl-ether were used to eluate the first aliquot.

As the solvent was trickled through the column, it extracted DP from the aqueous layer. Four additioned milliliters of diethyl-ether were used to end the extraction. The extract was concentrated by evaporating the 6 ml of eluant under a gentle steam of nitrogen at ambiant temperature (10 min). The residue was reconstituted in 100 µl of methanol by placing the tube in an ultrasonic water bath for 10 min. The reconstituted residue was then vortexed and transferred to an autosampler microvial.

Plasma samples

A pharmacological clinical study was performed in patients who received oral administration of DP. Blood samples were collected at 8 a.m. and 5 p.m. every day from patients receiving three times daily (morning, midday and evening) DP doses ranging from 107 to 500 mg/m^2/day. Two daily blood samples were analysed by our HPLC method, one at 8 a.m. and the other one at 5 p.m. The blood samples collected at 8 a.m. were analysed before the morning administration of DP, therefore the results shown in table 4 took into account the total dose of the day before whereas the blood sample collected at 5 p.m. took into account only the morning and midday doses (Table 5). As the stability of freshly prepared standard solutions exposed to daylight could show considerable variations (5), all samples and standards were protected from light.

RESULTS

Electrochemical detection

The optimal potential between the two electrodes for the detection of DP and INDO was determined by making voltammograms. Figure 1 shows the

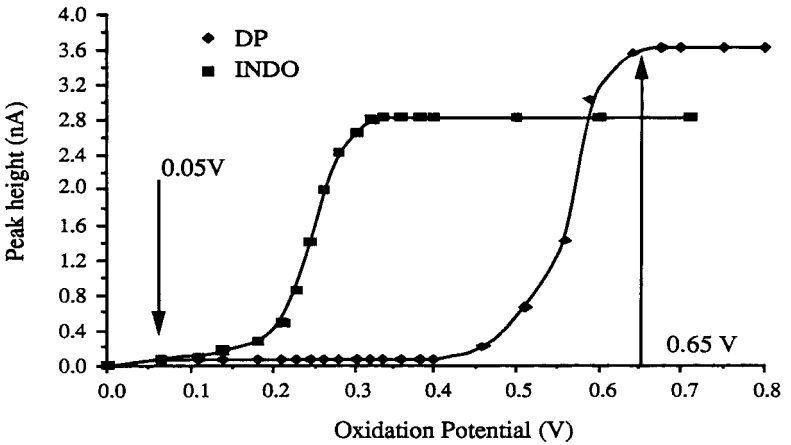

FIGURE 1
Voltammograms of dipyridamole (DP) and indometacin (INDO).

voltammograms for DP, INDO both at 200 ng/ml and the background current from mobile phase constituents. The optimal potential for the detection of DP and INDO was found to be + 0.65 V (Fig. 1).

The chromatogram from a blank plasma spiked with 100 ng of INDO (internal standard), is shown in Figure 2. At a signal-to-noise ratio of 3, the minimal detectable concentrations after the extraction procedure were 0.5 ng/ml (1nM) by injecting 20 µl of 100 µl of reconstituted plasma extract (Fig. 3).

Linearity and precision

In order to investigate the linearity of the extraction and ultrafiltration procedure, blank plasma samples were spiked with DP (1 to 200 nM). The calibration curve showed good linearity as expressed by the following equation :

$y = 0.026\ (\pm 0.010)\ x - 0.053\ (\pm 0.008)$

$r = 0.996 \pm 0.003$

The linearity of the extraction procedure without ultrafiltration. Blank total plasma samples were spiked with DP (0.2 to 10.0 µM). The calibration curve showed

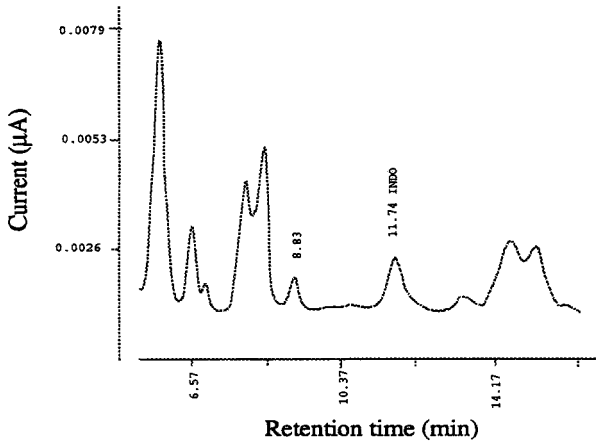

FIGURE 2

Representative chromatogram from 1 ml of plasma control extract, supplemented with 100 ng INDO as internal standard. The mobile phase was methanol - 0.1 M sodium acetate buffer (pH 4.0) (55 : 45, $^{v}/_{v}$). For chromatographic conditions, see text.

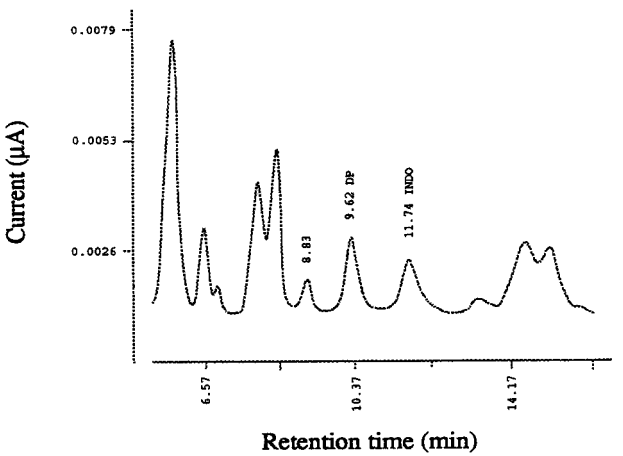

FIGURE 3

Representative chromatogram of a patient plasma sample supplemented with 100 ng INDO and containing DP at 14.6 nM. The mobile phase was as in Fig. 2. For chromatographic conditions, see text.

good linearity as can be seen from the following equation :

$y = 0.074 (\pm 0.006) x - 0.378 (\pm 0.005)$

$r = 0.998 \pm 0.001$

These calibrations curves were obtained from 3 different samples x and y are respectively the concentration of DP (μM) and the peak area, r is the correlation coefficient.

The within-day relative standard deviation (R.S.D.), based on six determinations, was less than 12.0 % for DP at concentrations ranging from 1 to 1 000 nM (Table 1).

The between-day R.S.D. was calculated by performing six analyses of plasma samples containing DP at five concentrations on different days that did not follow. A summary of these analyses is presented in table 2. The between-day R.S.D. were all under 16.0 % (4.3 to 15.3 %). The results indicate a relatively good precision of the assay.

DP stored at -20°C for up to 1 month showed no sign of decomposition and almost the same concentration values were measured (n = 6, table 2).These results suggest that in these storage conditions DP is stable for at least 1 month. As nearly the same concentration values were obtained after a storage at - 20°C for up to 6 months, we could consider that under these conditions DP is also stable for at least 6 months (data not shown).

TABLE 1

Within-day Variability of Total DP Plasma Assay (n = 6)

DP (nM)	Concentration measured (nM)	Coefficient of variation (%)	R.S.D. (%)
1	1.2	7.6	6.3
20	22.4	6.5	12.0
100	97.2	5.0	4.7
200	202.0	2.0	2.2
1 000	1 010.8	2.2	2.3

TABLE 2

Between-day Variability of Total DP Plasma Assay (n = 6)

DP (nM)	Concentration measured (nM)	Coefficient of variation (%)	R.S.D. (%)
1	0.9	9.6	12.0
20	23.0	8.5	15.3
100	96.8	7.0	6.1
200	204.4	4.3	4.7
1 000	1 008.0	4.8	4.3

TABLE 3

Mean Recovery Rates of Total DP at Five Different Concentrations (n = 3)

DP (nM)	Recoveries ± C.V. (%)
1	52 ± 12
20	74 ± 9
100	75 ± 8
200	86 ± 5
1 000	89 ± 5

Under this plasmatic extraction procedure, no major endogenous sources of interference was observed (Fig. 2 and 3) and the specificity between DP and the internal standard (INDO) was satisfactory. The retention times for DP and INDO were 9.6 ± 0.9 and 11.7 ± 1.2 minutes, respectively.

The mean recovery rates of DP from plasma over a concentration range from 1 to 1 000 nM (Table 3) were higher than 74 %, except at 1 nM (52 ± 12 %).

Absolute recovery of DP from plasma was assessed by comparing the peaks of the drug in plasma samples with those obtained by direct injection of DP standard.

Determination of DP in plasma

As DP is a base with a pKa of 6.4, accurate pH control is needed and should be ionised in the acidic sodium acetate buffer (pH : 4.0). Therefore, the mixture methanol - buffer (55 : 45, $^{v}/_{v}$) was chosen as it provides a good resolution between DP and the internal standard.

In order to evaluate the extraction procedure for the analysis of free and total DP, plasma samples of patients were investigated. Free and total DP levels in plasma were measured and the results (Tables 4 and 5) showed that DP is extensively bound to the plasma protein with a mean of free DP at 2.8 % (0.5 to 7.7 %). Statistically significant correlation coefficients were found between DP doses and plasmatic concentrations of free and total DP and also between free and total DP (7).

TABLE 4

Mean Plasma Free and Total DP Concentrations for Plasma Samples at 8 a.m.

DP dose (mg/m^2/day)	Number of patients	Total DP (μM)	Free DP (nM)	Percentage of free DP
107	1	0.146	-	
132	1	0.078	3.4	4.4
150	3	0.112	1.8	0.8
161	2	0.158	2.8	1.8
176	1	0.376	9.6	2.6
200	3	0.568	13.2	2.3
214	1	0.252	6.8	2.7
265	1	0.422	11.4	2.7
300	3	0.592	19.0	3.2
321	1	0.258	7.0	2.7
400	2	1.622	8.0	0.5
450	1	1.386	36.0	2.6
500	1	3.262	-	

- not enough plasma sample

TABLE 5

Mean Plasma Free and Total DP Concentrations for Plasma Samples at 5 p.m.

DP dose (morning + midday) (mg/m^2)	Number of patients	Total DP (μM)	Free DP (nM)	Percentage of free DP
88	1	0.590	12.8	2.2
100	3	0.728	55.4	7.7
107	2	0.794	12.0	1.5
176	1	3.990	54.4	1.4
200	3	2.858	58.4	2.0
214	1	1.298	35.2	2.7
250	2	2.332	43.2	1.8
300	2	6.222	215.4	3.5

DISCUSSION

Electrochemical detection was found to be 20 fold more sensitive than ultraviolet detection at 280 nm (4). No endogenous sources of interference from plasma was observed and the detection limit of the assay can be assigned at 1nM. Compared to other chromatographic procedures, this HPLC method has the advantage to be a very simple and rapid diethyl-ether extraction procedure. The sample preparation used is less complex than those mentioned in the literature (5) while being more sensitive. Overloading the column should be strictly avoided, since if more aqueous sample was added than the column was designed for, the sample could break through the bottom of the column into the collection tubes. The large surface area interfaced between the aqueous and organic layers which gave correct recoveries (Table 3) and eliminated emulsion problem.

These extraction columns contained a specially modified form of diatomaceous earth which requires no pre-conditioning washes however they are not reusable.

In conclusion, a very rapid and accurate method for the analysis of total and free DP has been described that can be employed for therapeutic monitoring or pharmacokinetic studies. Owing to the rapid extraction procedure by a highly

efficient liquid-liquid method and because of the sensitivity (0.5 ng/ml or 1 nM), the selectivity of the procedure and the rapidity, this HPLC assay is quite suitable for routine analysis in bioavailability studies.

As indometacin is also a drug prescribed, the ideal internal standard would be the methoxydipyridamole but this compound is not commercialized (8).

During the last decade, only few anticancer agents actually improved the therapeutic index. However, a better knowledge of the cytotoxicity mechanisms of commercialized drugs allowed to envisage the use of modulators. Such examples of modulation are found in trials associating various antineoplastic agents and DP. This vasodilating agent has recently been shown to potentiate antimetabolite activity in a dose-dependent manner (2, 3). It therefore appears very important to control the free plasma levels of this compound which is mainly bound to plasma protein (Table 4 and 5).

Acknowledgement

This study was supported by a grant from the Ligue Nationale Contre le Cancer.

REFERENCES

1. Insu, P.L. and Dixon, R.L.: Effect of Dipyridamole in the toxicity of various antineoplastic agents, Toxicol. Appl. Pharmacol. **17**, 1, 281, 1970

2. Grem, J.L. and Fisher, P.H.: Augmentation of 5-Fluorouracil cytotoxicity in a human colon cancer cells by Dipyridamole. Cancer Res. **45** : 2967-2972, 1985

3. Barberi, M., Merlin, J.L. and Weber, B.: Preclinical studies of Fluorouracil-Dipyridamole association in human cell lines and in mice. J. Cancer Res. Clin. Oncol. **116**, suppl. S 620, 1990

4. Barberi, M., Merlin, J.L. and Weber, B.: Sensitive determination of free and plasma protein-bound dipyridamole by high-performance liquid chromatography. J. Chromatogr. **565**, 511-515, 1991

5. Wolfram, K.M. and Bjornsson, T.D.: High performance liquid analysis of dipyridamole in plasma and whole blood. J. Chromatogr., **183**, 57-64, 1980

6. Deballon, C. and Guernet, M.: HPLC with electrochemical detection for the determination of dipyridamole in pharmaceuticals. J. Pharm. Biomed. Analysis 6, **8** : 1045-1048, 1988

7. Barberi-Heyob, M., Merlin, J.L. and Weber, B.: Intérêt de l'administration intrapéritonéale dans la modulation du fluorouracile (FUra) par le dipyridamole (DP). Bull. Cancer **80**, 483-485, 1993

8. Schmid, J., Beschke, K., Roth, W., Bizler, G. and Riss, F.W.: Rapid, sensitive determination of dipyridamole in human by high performance liquid chromatography. J. Chromatogr., **163**, 239-243, 1979

Received: November 11, 1993
Accepted: November 24, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1849 (1994)

corrected 3 Oct. 94/AP

ERRATUM

I. N. Acworth, et al., "Simultaneous Measurement of Monoamine, Amino Acid, and Drug Levels, using High Performance Liquid Chromatography and Coulometric Array Technology: Application to *in vivo* Microdialysis Perfusate Analysis," J. Liquid Chrom., 17(3): 685-705 (1994).

In Table II (page 695), the level of dopamine (DA) found in the hippocampus should be 6.3 pg/Collection, not 63 as shown in the published paper. The correct Table II is given below:

TABLE II
Basal Striatal and Hippocampal ECF Analyte Levels

	Basal Striatal Levels		Basal Hippocampal Levels	
Monoamines and Metabolites	pg/Collection Mean ± SEM (n = 3-6)	Typical "Ratio Accuracy"	pg/Collection Mean ± SEM (n = 3)	Typical "Ratio Accuracy"
3MT	54 ± 8.6	0.98	ND	–
5HIAA	2880 ± 585	0.99	1530 ± 63	0.99
5HT	ND	–	19.4 ± 11	0
5HTOL	ND	–	ND	–
DA	51.8 ± 6.8	0.99	6.3 ± 0.5	0.96
DOPAC	12845 ± 1485	0.70	259 ± 47	0.95
HVA	13905 ± 225	0.79	117 ± 14	0.95
NE	ND	–	ND	–
Amino Acids				
ALA	13095 ± 450	0.95	7695 ± 1215	0.98
ARG	14355 ± 1305	0.94	10170 ± 675	0.93
ASN	3420 ± 225	0	3510 ± 90	0
ASP	2835 ± 585	0	3730 ± 90	0.23
GABA	383 ± 32	0	293 ± 45	0
GLN	318195 ± 22860	0.91	21105 ± 4680	0.62
GLU	12870 ± 540	0.93	4860 ± 270	0.94
GLY	16425 ± 4590	0.93	5850 ± 765	0.75
HIS	11115 ± 495	0.91	ND	0.60
SER	21105 ± 1980	0.99	10395 ± 225	0.96
TAU	12501 ± 945	0.94	8973 ± 270	0.77
THR	21780 ± 3555	0.96	16470 ± 405	0.99
TYR	1800 ± 45	0.98	5625 ± 270	0.98

ND = Not detectable

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(8), 1851-1859 (1994)

LIQUID CHROMATOGRAPHY CALENDAR

1994

APRIL 10 - 15: 207th ACS National Meeting, San Diego, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036, USA.

APRIL 19 - 22: Rubber Division ACS, 145th Spring Technical Meeting, Palmer House Hotel, Chicago, Illinois. Contact: C. Morrison, Rubber Division, P.O. Box 499, Akron, OH 44309-0499, USA.

MAY 8 - 13: HPLC '94: Eighteenth International Symposium on High Performance Liquid Chromatography, Minneapolis Convention Center, Minneapolis, Minnesota. Contact: Mrs. Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, USA.

MAY 23 - 25: International Symposium on Polymer Analysis and Characterization (ISPAC-7), Les Diablerets, Switzerland. Contact: Howard G. Barth, ISPAC Chairman, DuPont Company, Central Research & Development, P. O. Box 80228, Wilmington, DE 19880-0228, USA or Dr. M. Rinaudo, CERMAV-CNRS, B. P. 53X, 38041 Grenoble Cedex France.

JUNE 1 - 3: Joint 26th Central Regional/27th Great Lakes Regional Meeting, ACS, Kalamazoo and Huron Valley Sections. Contact: H. Griffin, Univ of Michigan, Ann Arbor, MI 48109, USA.

JUNE 1 - 3: International Symposium on Hormone & Veterinary Drug Residue Analysis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. C. Van Peteghem, University of Ghent, L:aboratory of Food Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 5 - 7: VIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques & Applications in

Chromatography and Capillary Electrophoresis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. Dr. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Dept. of Pharmaceutical Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 6 - 9: 8th International LIMS Conference, The Westin William Penn Hotel, Pittsburgh, PA. Contact: Richard R. Mahaffey, 8th Int'l LIMS Conference, c/o Eastman Chemical Co., P. O. Box 1973/Bldg. 284, Kingsport, TN 37662, USA.

JUNE 12 - 15: 1994 Prep Symposium & Exhibit, sponsored by the Washington Chromatography Discussion Group, at the Georgetown University Conference Center by Marriott, Washington, DC. Contact: Janet E. Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 15: Fourth International Symposium on Field-Flow Fractionation (FFF'94), Lund, Sweden. Contact: Dr. Agneta Sjogren, The Swedish Chemical Society, Wallingatan 24, 2 tr, S-111 24 Stockholm, Sweden.

JUNE 17 - 19: 49th Northwest Regional ACS Meeting, Anchorage, Alaska. Contact: G. L. Trigiano, University of Alaska, 3890 University Lake Drive, Anchorage, AK 99508, USA.

JUNE 19 - 22: 68th Colloid & Surface Science Symposium, Stanford University, Stanford, California. Contact: Dept. of Chem. Engineering, Stanford, University, Stanford, CA 94305-5025, USA.

JUNE 19 - 22: 24th Northeast Regional ACS Meeting, Burlington, Vermont. Contact: W. R. Leenstra, Dept. of Chem., Iniversity of Vermont, Burlington, VT 05405, USA.

JUNE 19 - 23: 24th National Medicinal Chem. Symposium, Little America Hotel & Towers, Salt Lake City, Utah. Contact: M. A. Jensen & A. D. Broom, Dept. of Medicinal Chem, 308 Skaggs Hall, Univ of Utah, Salt Lake City, UT 84112, USA.

JUNE 19 - 24: 20th International Symposium on Chromatography, Bournemouth International Centre, Bournemouth, UK. Contact: Mrs. Jennifer A. Challis, The Chromatography Society, Suite 4, Clarendon Chambers, 32 Clarendon Street, Nottingham, NG1 5JD, UK.

JUNE 24 - 27: BOC Priestly Conference, Bucknell University, Lewisburg, PA, co-sponsored with the Royal Soc of Chem. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

JULY 31 - AUGUST 5: American Society of Pharmacognosy Annual Meeting, International Research Congress on Natural Products, joint meeting with the Assoc. Français pour l'Enseignement et la Recherche en Pharmacognosie, and the Gesellschaft fur Arzneipflanzenforschung, and the Phytochemical Society of Europe, Halifax, Nova Scotia, Canada. Contact: D. J. Slatkin, P. O. Box 13145, Pittsburgh, PA 15243, USA.

AUGUST 14 - 17: Summer National Meeting & Particle Technology Forum, AIChE, Denver, Colorado. Contact: AIChE Express Service Center, 345 East 47 Street, New York, NY 10017, USA.

AUGUST 21 - 23: Australasian Plastics & Rubber Inst. 7th Technology Convention, Melbourne, Australia. Contact: APRI, P. O. Box 241, Mont Albert 3127, Australia.

AUGUST 21 - 26: 208th ACS National Meeting, Washington, DC. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 29 - SEPTEMBER 2: Synthetic Membranes in Science & Industry, University of Tubingen, Germany. Contact: Dechema e.V., Exhibitions & Congresses, Theodor-Heuss-Allee 25, P. O. Box 150104, D-60486 Frankfurt am Main, Germany.

SEPTEMBER 4 - 9: 4th European Rheology Conference, Seville, Spain. Contact: C. Gallegos, Dept. de Ingenieria Quimica, Universidad de Seville, 41071 Seville, Spain.

SEPTEMBER 5 - 7: 25th International Symposium on Essential Oils, Grasse, France. Contact: Assoc. des Ingenieurs et Techniques de la Parfumerie (A.I.T.P.), 48 ave. Riou-Blanquet, 06130 Grasse, France.

SEPTEMBER 8 - 10: Surfaces in Biomaterials 1994, Scottsdale, Arizona. Contact: Surfaces in Biomaterials Foundation, c/o Ardel Management, P. O. Box 26111, Minneapolis, Minn, USA.

SEPTEMBER 12 - 15: 3rd International Conference on Separations in Biotechnology, University of Reading, U.K. Contact: SCI, Conference Office, 14/15 Belgrave Square, London SW1X 8PS, U.K.

SEPTEMBER 25 - 28: Future of Spectroscopy: From Astronomy to Biology, Ste.-Adele, Que., Canada. Contact: Doris Ruest, Conference Mgr., NRC Canada, Ottawa, Ont., Canada K1A 0R6.

SEPTEMBER 27 - 29: CCPC International Conference on Chemical Process Automation, Houston, Texas. Contact: AIChE Express Service Center, 345 East 47th Street, New York, NY 10017, USA.

OCTOBER 9 - 14: 186th Meeting of the Electrochemical Society. Contact: Electrochem. Soc., 10 S. Main St., Pennington, NJ 08534-2896, USA.

OCTOBER 16 - 19: 46th Southeastern Regional Meeting, ACS, Birmingham, Alabama. Contact: L. Kispert, Chem Dept, Univ of Alabama, Box 870336, Tuscaloosa, AL 35115, USA.

OCTOBER 17 - 19: 3rd Int'l Symposium on Supercritical Fluids, Strasbourg, France. Contact: Int'l Soc for the Advancement of Supercritical Fluids, 1 rue Grandville, B. P. 451, F-54001 Nancy Cedex France.

OCTOBER 18 - 22: 30th ACS Western Regional Meeting, Sacramento, California. Contact: S. Shoemaker, Calif. Inst of Food & Agricultural Res., Univ. of Calif-Davis, 258 Cruss Hall, Davis, CA 95616-8598, USA.

OCTOBER 23 - 27: Annual Meeting of the American Association of Cereal Chemists, Nashville, Tennessee. Contact: J. M. Schimml, AACC, 3340 Pilot Knob Road, St. Paul, MN 55121-2097, USA.

OCTOBER 24 - 26: 2nd Internat'l Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: SAICSC-ACS, P. O. Box 1723, Dhahrain 31311, saudi Arabia.

NOVEMBER 2 - 5: 29th Midwestern Regional Meeting, ACS, Kansas City, Kansas. Contact: M. Wickham-St. Germain, Midwest Res. Inst, 425 Volker Blvd, Kansas City, MO 64110, USA.

NOVEMBER 7 - 10: 13th Int'l Conference on the Application opf Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan, Univ. of N. Texas, Dept. of Physics, P. O. Box 5368, Denton, TX 76203, USA.

NOVEMBER 10 - 11: 17th Int'l Conference on Chemistry, Biosciences, Pharmacy & Environmental Pollution, New Delhi, India. Contact: V. M. Bhatnagar, Alena Chem of Canada, P. O. Box 1779, Cornwall, Ont., Canada K6H 5V7.

NOVEMBER 11 - 12: 3rd Conference on Current Trends in Computational Chemistry, Jackson, Mississippi. Contact: J. Leszczynski, Jackson State University, Dept. of Chem., 1400 J. R. Lynch St., Jackson, MS 39217, USA.

NOVEMBER 13 - 16: 50th Southwest Regional Meeting, ACS, Fort Worth, Texas. Contact: H. C. Kelly, Texas Christian Univ, Chem Dept, Ft. Worth, TX 76129, USA.

NOVEMBER 13 - 18: Annual Meeting of the A.I.Ch.E., San Francisco, California. Contact: AIChE Express Service Center, 345 East 47th St., New York, NY 10017, USA.

NOVEMBER 14 - 17: 33rd Eastern Analytical Symposium, Somerset, New Jersey. Contact: Sheri Gold, EAS, P. O. Box 633, Montchanin, DE 19710, USA.

NOVEMBER 16 - 18: Envirosoft'94, 5th Int'l Conference on Development and Application of Computer Techniques to Environmental Studies, San Francisco, California. Contact: Computational Mechanics, Inc., 25 Bridge St., Billerica, MA 01821, USA.

DECEMBER 4 - 6: IBEX'94, International Biotechnology Expo & Scientific Conference, Moscone Center, San Francisco, California. Contact: Cartlidge & Associates, Inc., 1070 Sixth Avenue, Suite 307, Belmont, CA 94002, USA.

1995

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 31 - JUNE 2: 27th Central regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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.

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

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.

1997

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

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

The Journal of Liquid Chromatography will publish, **at no charge**, announcements of interest to liquid chromatographers in every issue of the Journal. To be listed in Meetings & Symposia, we will need to know: Name of the meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P.O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

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