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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(13), 2759-2775 (1994)

HPLC DETERMINATION OF ENANTIOMERIC PURITY OF PROTECTED AMINO ACID DERIVATIVES USED IN PEPTIDE SYNTHESIS

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ABSTRACT

An improved RP-HPLC method on ODS-Hypersil column with pre-column derivatization with Marfey's reagent were used to monitor racemization in N-, C- and/or side-chain protected amino acid derivatives by separation of a series of new diastereoisomeric Marfey's compounds. Chromatographic samples were obtained by partial deprotection of different starting materials. In a simple two-step procedure (deprotection and derivatization) the compounds of amino acids formed stable diastereomeric derivatives having facile resolutions.

INTRODUCTION

Synthetic, biologically active peptides should be very pure stereochemically. The therapeutic and biological applications require

very high chiral (optical) purity from peptides to be investigated. Their synthesis also requires optically pure amino acid derivatives as starting material. Unfortunately, the racemization is one of the more common side reaction that occur during a peptide synthetic work. There is an increased demand for a method to detect and quantitate minor amounts of enantiomeric impurity in the N or C protected amino acids used to prepare these peptides. The up-to-date solid phase synthesis methods apply BOC or FMOC-chemistry (29, 30), so it became important to check the enantiomeric purity in intermediate amino acid derivatives as BOC and FMOC protected starting compounds. HPLC is the method most frequently used for this purpose. For enantiomer resolution of chiral amino acids or their derivatives, direct techniques can be applied, in which either chiral stationary phase (19-21) or chiral additives to the mobile phase are used (22-24). Derivatization procedures with chiral reagents have also been developed to produce diastereomeric derivatives, which can be separated by ordinary stationary phases and eluents. Using enantiomeric derivatization more favourable resolutions could be achieved (usually with higher α and R_S values), than applying very specific and not always available chiral columns.

After hydrolysis the chiral amino acid analysis also gives the possibility to check the mentioned intermediates. The chiral reagents used in amino acid analysis (for derivatization via amino or carboxyl group) include

2,3,4,6-tetraacetyl-D-glycopyranosyl isothyocyanate (6)

(-)-α-methoxy-α-methyl-1-naphthalene-acetic acid (7a)

(+)-1-aminoethyl-4-dimethylaminonaphthalene (7b)

(+)-1-(9-fluorenyl)-ethyl-chloroformate (8)

and some amino acid derivatives, such as the N-protected cysteine and o-phthalaldehyde (9, 13) or napthalenedicarbaldehyde (11) adduct, tert.-butoxycarbonyl-L-amino acid N-hydroxysuccinimide esters, N-carboxy-L-leucine anhydride (10, 18), D or L-O-(4-nitrobenzyl)tyrosine methylester (12) and Marfey's reagent (1) and its analogs: structurally related chiral variants of Sanger's reagent (14-15) and monohalo-s-triazine-L-alanine amide (16).

In the present paper we describe some applications of the separation method for new Marfey's derivatives in improved analyses of optical purity and racemization of amino acid derivatives.

MATERIALS

The amino acid derivatives studied (Table 1) were synthesized by the Research Group for Peptide Chemistry of the Hungarian Academy of Sciences, the Department of Organic Chemistry, Eötvös University, Budapest, the Chemical Works, G. Richter, Budapest, Fine Chemicals Co. Reanal, Budapest and the Institute of Medical Chemistry, Szent-Györgyi Albert Medical University of Szeged. The abbreviations used follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (4).

Amino Acid Derivatives Studied

BOC-Asn-OH
H-Asn-OH
BOC-Ser(Bzl-)OH
H-Ser(Bzl)-OH
Z-Asn-OMe
BOC-His(DNP)NH_2
BOC-Cys(Bzl)NH_2
BOC-Leu-NH_2

TABLE I

Chromatographic and Racemization Data of Marfey's Compounds of Amino Acid Derivatives

Amino acid (AA)	Derivatives	α-NH_2 compounds	k' L	k' D	α	D-amino acid %	RP-C18-HPLC Eluent (v/v)	
Asn	BOC-Asn	Asn	1.5	5.1	3.4	0.38	MeOH-CH_3CN-0.02 M	
		$Asp(OH)_2$	1.4	3.7	2.6	0.37	NaOAc (pH 4)	20:5:75
Glp	BOC-Glp-OH	$Glu(OH)_2$	2.2	5.9	2.7	19.95		20:5:75
	BOC-Glp-OPFP	$Glu(OH)_2$	2.2	5.9	2.7	0.39		20:5:75
Ser	BOC-Ser(Bzl)OH	Ser(Bzl)OH	3.0	7.4	2.47*	0.42	MeOH-0.02M NaOAc (pH 4)	
		Ser(Bzl)OH	3.0	7.4	2.47	0.08		50:50
Thr	Thr(Bzl)OH	Thr(Bzl)OH	3.25	6.6	2.3	0.07		50:50
Arg	BOC-Arg(Tos)OH	Arg(Tos)OH	3.5	14.5	4.1	0.37		49.5:50.5
	FMOC-Arg(NO_2)OH	Arg(NO_2)OH	3.17	7.17	2.2	49.50		20:5:75
His	BOC-His(DNP)NH_2	His(DNP)NH_2	2.0	7.5	3.75	0.75		50:50
		His(DNP)	1.8	6.2	3.4	0.73		50:50
Cys	BOC-Cys(Bzl)NH_2	Cys(Bzl)NH_2	7.0	16.8	2.4	1.51		50:50
		Cys(Bzl)	5.8	14.1	2.4	1.54		50:50
	Cys(Acm)	Cys(Acm)	2.71	5.80	2.07	0.83		20:5:75
		Tin-OH	5.57	10.14	1.8	0.80		20:5:75
Leu	BOC-Leu-NH_2	LeuNH_2	7.1	22.5	3.2	44.0		25:10:60
		LeuOH	21.1	45.4	2.1	43.94		20:10:70
Asn	Z-Asn-OMe	$Asp(OH)_2$	1.4	3.7	2.6	0.74		20:5:75
Thr	BOC-Thr(Bzl)OH	Thr(Bzl)OH	3.25	6.6	2.3	0.18		50:50
HomoArg	BOC-HomoArg(OH)	HomoArg(OH)	11.3	4.10	3.6	41.91		20:7:73
Tin	BOC-Tin-OH	Tin-OH	5.57	10.14	1.8	0.40		20:5:75
Kpc	Kpc-OBu^t	α-AA-OH	3.46	9.76	2.5	0.71		20:5:75
Gla	Z-Gla(OBu^t)$_2$OH	$Glu(OH)_2$	2.2	5.9	2.7	0.85		20:5:75
		Gla(OBu^t)$_2$	5.75	8.75	1.52	0.9		60:40

*Using a hydroxypropyl derivatized β-cyclodextrin bonded phase column, α = 1.13 (20).

BOC-Thr(Bzl)-OH	Z-Gla(OBu^t)$_2$-OH
H-Thr-(Bzl)-OH	BOC-Homo-Arg-OH
BOC-Arg(Tos)-OH	Boc-Cys(Acm)-OH
FMOC-Arg(NO_2)-OH	
KpC-OH (6-keto)-pipecoline-2-carboxylic acid)	Ac-Glp-OH.DCHA
KpC-NH_2	
H-Cys(AcM)-OH	Glp-OPcp
KpC-OBut,	BOC-Glp-OH.DCHA
4-thiazolidine-carboxylic acid	Glp-OPfp
BOC-Tin-OH	Z-Glp-OH.DCHA
KpCOPfp	BOC-Glp-OPfp

Marfey's reagent was prepared by J. Horváth (Dept. Org. Chem., Eötvös University).

Abbreviations:

BOC - tert.-butoxycarbonyl; Tin - thiazolidine-carboxylic acid; Gla - γ-carboxy-glutamic-acid; FMOC - fluorenyl methoxycarbonyl; Kpc - ketopipecolic acid, Z - benzyloxycarbonyl; HomoArg - homoarginine; Bzl - benzyl; DNP - 2,4-dinitrophenyl; OBu^t - tert-butyl; AcM - acetamidomethyl.

METHODS

High-Performance Liquid Chromatography

Separations were performed on a laboratory-assembled instrument consisting of a reciprocating piston pump (Model 1515, Orlita, Giessen, F.R.G.), a variable-wavelength UV monitor fitted to an 8 µl flow-cell (Model 212, Cecil, Cambridge, U.K.) and a sample injector; Rheodyne, Berkeley, CA, U.S.A.), or on a Knauer HPLC system con-

sisting of two pumps Model 64 with analytical pumphead, a gradient programmer Model 50B, an injection valve with 20 µl sample loop and a spectral photometer with analytical flow cell (Knauer GmbH, Bad Homburg, FRG).

Column effluents were monitored at 340 nm (for Marfey's derivatives). The packing materials were ODS-Hypersil-ODS-6, MOS-Hypersil-6 (Shandon Southern Products, Runcorn, U.K.) and Nucleosil C-18-5 (Macherey Nagel, Düren, FRG). Peaks were recorded on a Model OH-314/1 chart recorder (Radelkis, Hungary) and the areas under them were calculated using programmed Simpson's rule. The chromatographs were operated isocratically with flow rates between 0.8 and 2.0 cm^3/min.

Deprotection of amino acid derivatives

a) Hydrolysis

Amino acid derivatives were subjected to acidic hydrolysis. The samples were treated with propionic-acid-6M HCl mixtures (1:1, v/v), or TFA-6M HCl mixtures (1:1, v/v) for 1-24 hours in sealed tubes at 105°C or some minutes (1-4) in a microwave staff. Volatile acids were removed in vacuo, samples were neutralized by $NaHCO_3$ and the acid-free hydrolysates were derivatized with Marfey's reagent.

b) BOC-derivatives (30)

1 mg of BOC-amino acid derivative was dissolved in 0.5 ml TFA. After 1 hr standing at room temperature the reaction was checked by TLC, the acid was removed in vacuo, and the acid-free hydrolysate was derivatized further.

c) FMOC-derivatives (29)

1 mg of FMOC-amino acid derivative was dissolved in 1 ml of 25% piperidine/DMF. After 1 hr standing at room temperature, the reaction was checked by TLC, the solution was evaporated and lyophilized with water.

d) Z-derivatives

1 mg of Z-amino acid derivative was deprotected by catalytic transfer hydrogenation (31-36). 10% Pd/C and 100 µl saturated NH_4 formate in MeOH were added to its solution in 200 µl MeOH. After 10 minutes the deprotection was checked by TLC (silica gel, n-BuOH-pyridine-acetic acid-water 60:20:6:24 v/v). In order to eliminate NH_4 formate, the evaporated substance was twice lyophilized from water.

Derivatization (1-5)

Derivatization was carried out according to Marfey (2) with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Pierce, Rockford, IL. U.S.A.). The hydrolysate or deprotected compound prepared from 2-5 µmol of starting material was dissolved in 100 µl of 0.5 M $NaHCO_3$ solution (if it's necessary: 50 µl MeOH, DMF or DMSO are used at first and diisopropyl-ethylamine serves as base) and 200 µl of a 1% solution of Marfey's reagent in acetone was added. The solution was incubated at 40°C for 90 min, cooled and 25 µl of 2 M HCl was added. After to 20-fold dilution in methanol or eluent, 10-20 µl aliquots were used for HPLC injection.

RESULTS AND DISCUSSION

We have previously shown (2) that the rates of racemization in peptides can be measured by conventional HPLC using pre-column derivatization with enantiomerically very pure 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) for amino acids after hydrolysis of starting peptides (1). Marfey's reagent reacts with the optical isomers of amino acids without kinetic fractionation and racemization to form stable diastereomeric N-aryl derivatives which can be separated by RP-HPLC. The derivatives have an absorption maximum at 340 nm with an extinction coefficient of ~3.10^4, and thus can be detected with high sensitivity by UV spectroscopy, the diasteromers give identical detector response (2). Recently the HPLC separation of these derivatives has been described for all common amino acids (1-4, 37).

For rapid, accurate determination of the optical purity of the synthesized amino acid derivatives the rates of racemization were planned to be mesaured by conventional HPLC using pre-column derivatization with Marfey's reagent. In order to apply the original method elaborated for all common acids (1-4, 37), the amino acid derivatives should be hydrolyzed.

According to the acidic hydrolysis free amino acids were formed from all derivatives: X-AA-OR $\xrightarrow[H_2O]{H^{\oplus}}$ H-AA-OH

eg.: BOC-Asn-OH → Asp

BOC-Ser(OBzl)OH → Ser

Z-Gla(OBu^t)OH → Glu

BOC-Glp-OH → Glu

Kpc-OH → α-aminoadipic acid

BOC-Cys(Acm) → 4-thiazolidine-carboxylic acid

So, their determination were reduced to free amino acid measurements.

However, the hydrolysis step is also a potential source of racemization so that this step must be carefully monitored. All amino acid residues undergo some racemization during acid hydrolysis (25, 26, 3). Aspartic acid appears to be the most sensitive amino acid during acidic treatment (26, 3).

Thus, a "background" racemization must be taken into account in the exact determination of racemization rates in amino acid derivatives to be hydrolyzed.

Conventional amino acid analysis utilizes azeotropic hydrochloric acid (6 M) at 110°C for 24 hours for hydrolysis (38). We, instead, made use of microwave radiation for sample hydrolysis (27, 28). Rapid hydrolysis was achieved by using a commercial microwave oven as heating source. It was not only possible to hydrolyze amino acid derivatives quantitatively within 1 min, but also with substantially less racemization (Table II). The values are in good correlation with Chen's data (39).

Because of high "background" values we have improved the method with racemization-free, very simple deprotection steps (well-known in peptide chemistry) liberating α-amino group of the derivatives.

In the case of N-protected amino acid derivatives the N-protecting groups were quantitatively removed at first:

$$\text{X-AA-OR} \xrightarrow{-X} \text{H-AA-OR} \xrightarrow{MR} \text{diastereomeric derivatives}$$

X = FMOC, BOC, Z-

R = H, Me, Et, Bzl, (pNO_2)Bzl, Bu^t, NH_2, NH-R.

TABLE II

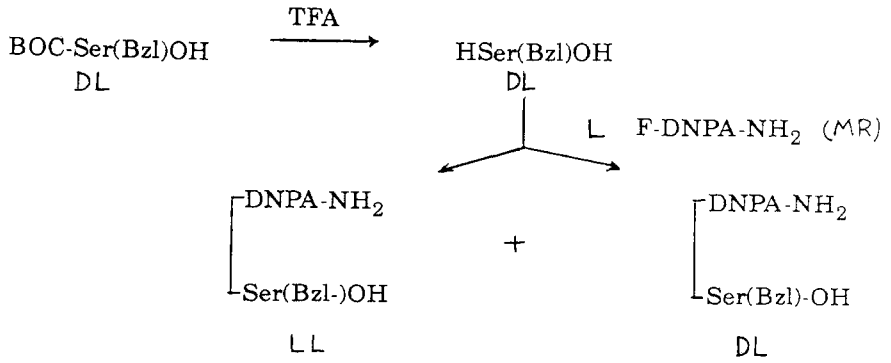
Optical Behaviour of L-Amino Acids During Acid Treatment

AA	D-enantiomer content % and time of treatment				
	105°C		microwave radiation		
	1 h	24 h	10 sec	20 sec	60 sec
Asp	1.68	11.8	0.55	0.94	1.40
Glu	0.80	2.65	0.24	0.32	0.77

The C-protected amino acid derivatives (chiefly esters) react directly via arylation with Marfey's reagent without preparation problems. Quantitative reactions were achieved after optimization (solvent pH, reaction time), using a molar reagent excess of approximately 1,4-1,6 or greater.

Our modification of the original procedure requires the separation of a series of new Marfey's derivatives, in which the amino acid residues are modified with other protecting groups at the C-terminal and in the side chains. These groups modify the chromatographic behaviour of the Marfey's derivatives according to their hydrophobic or hydrophilic character. So, new eluent systems should be elaborated (see Table I).

The BOC-amino acid derivatives were deprotected quantitatively and selectively at amino group by trifluoroacetic acid treatment (checked by RP-HPLC and NP-TLC). Derivatization was carried out according to Marfey:



The FMOC α-amino protecting groups were removed quantitatively by piperidinolysis. The Z groups were splitted quantitatively from α-amino groups by catalytic transfer hydrogenation. The chromatographic data of new Marfey's derivatives are summarized in Table I. It was concluded, that the all new diastereomeric compounds could be separated well on RP-columns. The chromatographic conditions were optimized to achieve large separation coefficients (α) (see Table I), and baseline separations with methanol-Na-acetate buffer (pH 4) or methanol-acetonitrile-Na-acetate buffer eluent systems, isocratically. It seems that the L-diastereomers are usually eluted before the D-isomers, and the protecting groups of ester and amide type increase the retention times because of their high hydrophobic characters (Bzl, Et, Me, OBu^t, NH_2, NH-R, etc.). So the eluent systems contain more organic modifiers (CH_3OH or CH_3CN) as usually (see e.g. $Gla(OBu^t)_2$).

The protecting groups (NO_2, Bu^t, Bzl, DNP, Tos) of side chain protected amino acid derivatives have a similar effect on the retention.

Fig.1. demonstrates the separation of D and L-Ser-OBzl-Marfey's derivatives.

Racemization of amino acid derivatives could be determined by measuring quantitatively the D-amino acid (or derivative) content of the sample, or directly the D/L compound ratio. Thus, the optical purity of amino acids used in peptide syntheses were checked very precisely: since the detection limit was about 5-8 pmol of an amino acid enantiomer, 0.02-0.05% of the racemization could be determined accurately.

Racemization rate depends on the preparation methods, of course.

It seems from Table I, that BOC-Leu-NH_2, FMOC-Arg(NO_2)OH and BOC-Homo-Arg(OH) were strongly racemized (>80%) during their synthesis because of strong basic reaction conditions. The other compounds contain only minimum quantities of D-amino acids, so their enantiomeric purity is suitable enough for peptide synthesis.

On the other hand especially important is the question of the enantiomeric purity of N-protected amino acids after activation of the C-terminal carboxyl group with peptide coupling reagents. Coupling of the carboxyl activated N-protected amino acid with an amino components can lead to epimeric products because of racemization (40).

Our improved method was applied to check the racemization level of an efficient, new amidation method of protected amino acids, when N-tert-butoxycarbonyl derivatives of L-leucine, S-benzyl-cysteine and N-(2,4-dinitrophenyl)-L-histidine were amidated with crystalline ammonium salts of 3-hydroxy-1.2.3-benztriazin-4(3H)-one and N-hydroxysuccinimide to give after activation the corresponding amino acid amides in good yields (5). Table I shows (Cys and His amides),

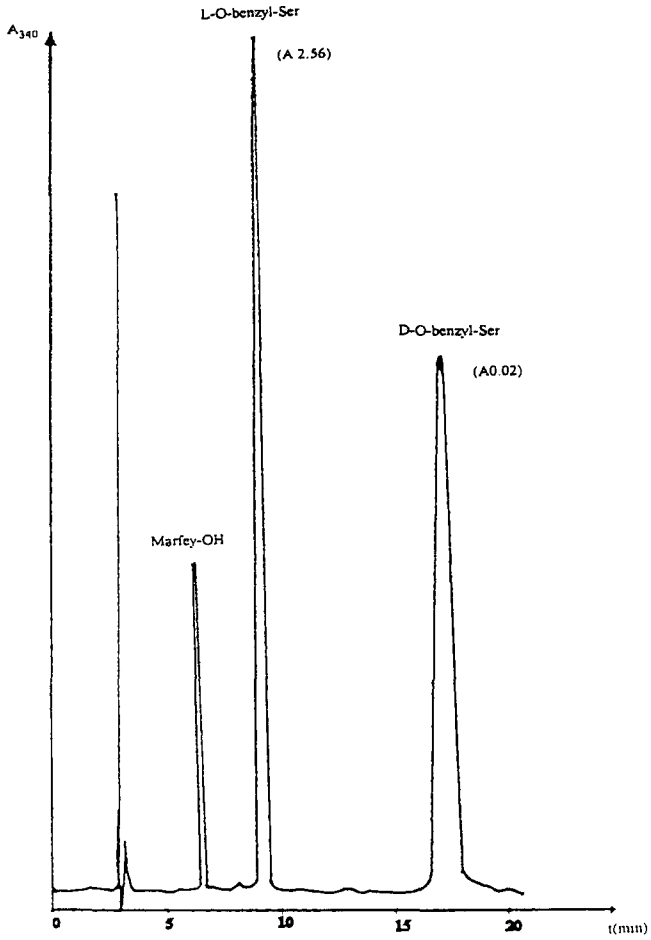


FIGURE 1. Chromatographic pattern of racemization of O-benzyl-L-serine (Fine Chemicals, REANAL, Hungary)
Column: Nucleosil C18-5 μm. Eluent: methanol - 0.02 M sodium acetate buffer (pH 4) 50:50 (v/v). Flow rate: 1.0 mL/min. Detection: 340 nm. Derivatization: 5 mmol. D-isomer content: 0.34%.

that only minimal quantities of D-amino acids could be detected using the novel amidation reagents. The endproducts have high enantiomeric purity.

The method can be used for determination of L-impurities in D-compounds, of course. Our data were supported by optical rotation measurements, too. If we took into account the enantiomer-contents as corrections, the optical rotation data of the samples became identical with the literature ones (e.g. in the case of Z-Gla(OBu^t)$_2$).

In order to validate this technique, it was found, that the values of D isomer content obtained after hydrolysis and deprotection were practically identical in the case of BOC-Asn, BOC-Leu-NH_2, BOC-Cys(Bzl)NH_2 and BOC-His(DNP)NH_2 (see Table I).

It seems from the recent papers, that the popularity of Marfey's reagent is increasing further in the laboratories of peptide chemists (41, 42).

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REFERENCES

1. P. Marfey, Carlsberg Res. Commun. 49: 591-596 (1984)

2. Gy. Szókán, G. Mező, F. Hudecz, J. Chromatogr. 444: 115-122 (1988)

3. Gy. Szókán, G. Mező, F. Hudecz, Zs. Majer, I. Schön, O. Nyéki, T. Szirtes, R. Dölling, J. Liquid. Chrom. 12: 2855-2875 (1989)

4. Gy. Szókán, G. Mező, F. Hudecz, in Peptides, B. Penke, A. Török, eds., Walter de Gruyter, Berlin, 1988, pp. 83-86.

5. Cs. Somlai, Gy. Szókán, L. Baláspiri, Synthesis, 285-287 (1992)

6. N. Nimura, H. Ogura, T. Kinoshita, J. Chromatogr. 202: 375 (1980)

7a. J. Goto, M. Hasegawa, S. Nakamura, K. Shimada, T. Nambara, J. Chromatogr. 152: 413-419 (1978)

7b. T. Nambara, S. Ikegawa, M. Hasegawa, J. Goto, Anal. Chim. Acta, 101: 111-118 (1978)

8. S. Einarsson, B. Josefsson, P. Möller, D. Sanchez, Anal. Chem. 59: 1191-1198 (1987)

9. R.H. Buck, K. Krummen, J. Chromatogr. 315: 279-285 (1984)

10. A.R. Mitchell, S.B.H. Kent, I.C. Chu, R.B. Merrifield, Anal. Chem. 50: 637-640 (1978)

11. T. Takaya, Y. Kishida, S. Sakakibara, J. Chromatogr. 215: 249-256 (1981)

12. S. Görög, B. Herényi, M. Löw, J. Chromatogr. 353: 417-424 (1986)

13. H. Brückner, R. Wittner, H. Godel, Chromatographia, 32: 383-388 (1991)

14. H. Brückner, C. Keller-Hoehl, Chromatographia 30: 621-629 (1990)

15. H. Brückner, C. Gah, J. Chromatogr. 555: 81-89 (1991)

16. H. Brückner, B. Strecker, J. Chromatogr. 627: 97-105 (1992)

17. IUPAC-IUB Commission on Biochemical Nomenclature, J. Biol. Chem. 247: 977-983 (1972)

18. W.R. Cahill, Jr., E.P. Kroeff, D.J. Pietrzyk, J. Liquid Chromatogr. 3: 1319-1334 (1980)

19. W.H. Pirkle, J.E. McCune, J. Chromatogr. 479: 419-423 (1986)

20. S.C. Chang, L.R. Wang, D.W. Armstrong, J. Liquid Chromatogr. 15: 1411-1429 (1992)

21. G. Gübitz, F. Juffmann, W. Jellenz, Chromatographia, 16: 103-106 (1982)

22. S. Weinstein, Trends in Anal. Chem. 3: 16-20 (1984)

23. N. Nimura, A. Toyama, Y. Kasahara, T. Kinoshita, J. Chromatogr. 239: 671-675 (1982)

24. F. Federici, A.M. Girelli, A. Messina, I. Nicoletti, M. Sinibaldi, Anal. Letters, 23: 1565-1549 (1990)

25. A. Neuberger, Adv. Protein Chem. 4: 339-385 (1948)

26. P.J.M. van der Oetaelaar, J.R.C.M. van Beckhoven, H.J. Hoenders, J. Chromatogr. 388: 441-448 (1987)

27. L.B. Fischer, Anal. Chem. 58: 261-268 (1986)

28. S.T. Chen, S.H. Chiou, Y.H. Chu, K.T. Wang, Int. J. Peptide Protein Res. 30: 572-576 (1987)

29. E. Atherton, R.C. Sheppard, Solid Phase Peptide Synthesis, IRL Press at Oxford Univ. Press, 1989.

30. J.M. Stewart, J.D. Young, Solid Phase Peptide Synthesis, Pierce Chem. Co., Rockford, Illinois, USA, 1984.

31. S.A. Khan, K.M. Sivanandaiah, Synthesis, 750-751 (1978).

32. K.M. Sivanandaiah, S. Gurusiddappa, J. Chem. Research (S) 108-109 (1979)

33. A.M. Felix, E.P. Heimer, T.J. Lambros, C. Tzougraki, J. Meienhofer, J. Org. Chem. 43: 4194-4196 (1978)

34. M.K. Anwer, S.A. Khan, K.M. Sivanandaia, Synthesis, 751-752 (1978)

35. M.K. Anwer, A.F. Spatola, C.D. Bossinger, E. Flanigan, R.C. Lin, D.B. Olsen, D. Stephenson, J. Org. Chem. 48: 3503-3509 (1983)

36. M.K. Anwer, A.F. Spatola, Synthesis, 929-932 (1980)

37. S. Kochbar, P. Christen, Anal. Biochem. 178: 17-21 (1989)

38. D.H. Spackman, W.H. Stein, S. Moore, Anal. Chem. 30: 1190-1215 (1958)

39. S.-T. Chen, S.-H. Wu, K.-T. Wang, Int. J. Peptide Protein Res. 33: 73-75 (1989)

40. D.S. Kemp in The Peptides, E. Gross, J. Meienhofer, eds. Vol. I. Acad. Press, New York, 1979 pp. 317-383.

41. A. Scaloni, M. Simmaco, F. Bossa, Anal. Biochem. 197: 305-310 (1991)

42. J.G. Asamson, T. Hoang, A. Crivici, G.A. Lajoie, Anal. Biochem. 202: 210-214 (1992)

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TRYPTIC MAP VARIATION OF ERYTHROPOIETIN RESULTING FROM CARBOXYPEPTIDASE B-LIKE ACTIVITY

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ABSTRACT

The recombinant human erythropoietin (r-HuEPO) is monitored by tryptic mapping for its purity and identity. Occasionally, these maps have produced an additional peak not evident in the reference standard. This peak was isolated, sequenced and characterized by mass spectrum. It is derived from a known r-HuEPO tryptic peptide 144-150 with the C-terminal arginine removed. Its formation can be reduced or totally eliminated by the addition of inhibitors of carboxypeptidase B activity. These results indicate the presence of residual carboxypeptidase B-like activity in some r-HuEPO lots. Similar enzymatic activity has been shown in varying amounts in the concentrated diafiltered media before purification. The peptide 144-150 is one of the first to be affected by the carboxypeptidase B-like activity. This peptide seems to locate in the most trypsin-sensitive part of r-HuEPO.

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone that induces mammalian red blood cell differentiation (1-7). Recombinant human EPO

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(r-HuEPO, also called EPO in this text) is marketed worldwide for the treatment of the anemia associated with chronic renal failure. Also, in the U.S, r-HuEPO is marketed for the anemia associated with human immunodeficiency virus (HIV)-infected patients under zidovudine (azidothymidine, AZT)- therapy and for anemia related to chemotherapy for cancer. The EPO molecule has been extensively characterized (8-12) and consists of 165 amino acids with two disulfide bonds and about 40% of carbohydrate by weight. The EPO used in the formulations is rigorously tested for its identity and purity by several analytical techniques. One of these tests is tryptic mapping which is a well established technique for protein identity and purity determination. As a routine specification test for EPO reference standard and bulk lots, it requires the chromatogram produced for the sample tryptic map to correlate with that of the reference standard.

Occasionally, an extra peak has appeared in some EPO lots, not evident in the reference standard. Interestingly enough, this anomaly was not observed by any other analytical tests and did not affect the activity. Regardless, the presence of the extra peak was persistent enough in some lots to prompt the study of its identity and the source of the unexpected fragmentation. This report describes the successful determination of the unknown peak as an anomalous EPO peptide fragment. Various experiments are described in the investigation to determine the cause of this unusual fragmentation pattern.

MATERIALS AND METHODS

Materials

Trifluoroacetic acid (TFA) was protein sequencer grade (Applied Biosystems, Foster City , CA) and acetonitrile was high performance liquid

chromatographic (HPLC) grade (Baxter, Burdick & Jackson, Muskegon, MI). All the other chemicals were of reagent grade (Sigma Chemical Co., St. Louis, MO). The TPCK-treated trypsin solutions were prepared in distilled water fresh daily. Carboxypeptidase B was diluted with distilled water as required in the experiments. The solutions of ethylenediamine tetraacetic acid, disodium salt (EDTA) , L-arginine, L-histidine and 2,2'-dipyridyl were also prepared in distilled water in the concentrations specified in the Results section.

Chromatography

The EPO peptide mapping method consisted of a reversed phase (RP) C4 column (250 x 4.6 mm, 5 micron, 300 A, Vydac 214TP54) and a gradient mobile phase of 0.06% TFA in water (A) and in acetonitrile (B). Gradient 1, which was used in the routine tryptic map method, had Step 1: 0% to 55% B in 125 min and Step 2: 55% to 75% B in 10 min. This gradient was modified to Gradient 2 , used in this study, which consisted of Step 1: 0% to 38% B in 80 min and Step 2: 38% to 100% B in 10 min. The column temperature was ambient and the detection was by ultra-violet (UV) at 215 and 280 nm.

Sample Preparation

In the trypsin digests, 200 µL of EPO sample in 20 mM citrate buffer was mixed with 10 µL of trypsin, both at concentration of 1.0 mg/mL, to reach the substrate to enzyme ratio of 20:1. For preparative studies, the original sample concentration (usually 1.5-2.0 mg/mL) was used and the added trypsin adjusted accordingly to maintain the 20:1 ratio. The length of incubation time at 37°C was usually 18 hours. All the samples were frozen immediately after the digestion and stored in the freezer (-20°C) until ready for analysis. Sample aliquots used for the chromatography

varied from 20 μL to 200 μL depending on the original sample concentration.

Unknown Peak Isolation

Using a 200-μL column load, the extra peak was collected under the same chromatographic conditions as in the analytical studies. The collected peak was concentrated on SpeedVac to a final volume of about 50 μL, and the concentrate was used for further analysis.

Unknown Peak Identification

The sequencing was done by an ABI 477A Pulsed Liquid Sequencer using the conditions recommended by the manufacturer. The Fast Atom Bombardment (FAB) mass spectral data were acquired using a Finnigan-MAT (San Jose, CA) TSQ70 triple stage mass spectrometer equipped with an Ion Tech saddle field source (ion gun) and high voltage conversion dynode (15kV) detection. The samples were prepared in a thioglycerol matrix and bombarded with 8 keV xenon atoms. The instrument was scanned initially from 500 to 1500 u (mass units) in a total time of 2 sec. Follow-up scans were acquired over the mass range of 1500 to 3000 u (scan time 3 sec).

RESULTS

Isolation and Identification of Peak 14

The typical tryptic maps of EPO reference standard (A) and the variant EPO lot (B), recorded at 215 nm, highlight the presence of the extra peak

14 (Figure 1, B). The peptide 18, that eluted at around 100 minutes, was not critical to the study results and was omitted to shorten the total runtimes. The sequences of all the tryptic peptide peaks 1-18 have been determined as shown in Figure 1. The complete 165-amino acid sequence of EPO structure has been previously published (10). The tryptic map chromatograms, recorded at 280 nm, on the other hand, showed only five major peaks, 8, 13, 16, 17 and 18 for (A) and also the new peak 14 for (B). These were the tryptic peptides that contained either tyrosine and/or tryptophan amino acids. The ultraviolet (UV) spectra of each peak were recorded by a photo-diode array detector during the chromatographic run. The scans for peaks 8, 13 and 14 indicated the presence of tyrosine and were identical for 13 and 14. Peak 16 showed the typical UV-pattern of a tryptophan containing peptide and 17 and 18 showed the mixture of tyrosine and tryptophan. In addition to the identical UV scans, the two peaks 13 and 14 were interrelated by the peak 13 decreasing as the peak 14 was forming. The peak 13 is a known EPO tryptic fragment, Val-Tyr-Ser-Asn-Phe-Leu-Arg (VYSNFLR), at a position of 144-150. This indicated that the unknown fragment 14 was some kind of derivative of the peptide 13, but less polar, due to its later elution in reversed phase HPLC.

The peak 14 in the variant EPO chromatogram was isolated by reversed phase HPLC and analyzed by sequencer and FAB-mass spectrometry. The amino-terminal sequence data revealed the peptide 14 to be a hexapeptide, Val-Tyr-Ser-Asn-Phe-Leu (VYSNFL), which was the same sequence as for the heptapeptide 13, except the terminal arginine removed. A closely eluting heptapeptide peak 15, Ser-Leu-Thr-Leu-Leu-Arg (SLTTLLR), with arginine in sequence position 7, was interfering in the identification until very pure 14 was obtained. The FAB mass spectrum of the purified 14 yielded an intense signal with m/z 742, consistent with the sequence of VYSNFL. This mass ion differed only by

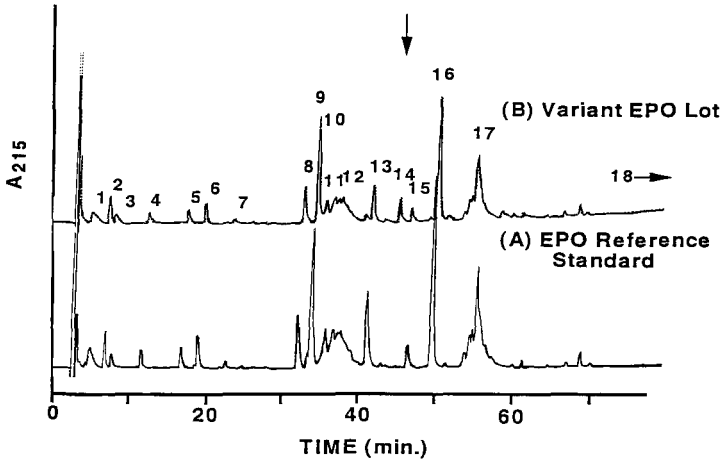

Figure 1. Typical tryptic-map chromatograms of a variant EPO lot (B) and EPO reference standard (A). Chromatographic conditions: Column, Vydac 214TP54, 5u, 300 A, 250 x 4.6 mm. Mobile Phase, A: 0.06 % TFA in water, B: 0.06 % TFA in aceto-nitrile. Gradient: Step 1, 0 to 38 % B in 80 min., Step 2, 38 to 100 % B in 10 min.
Detection was at 215 nm. Identified peak sequences: **1:** 1-4, **2:** not identified **3:** 111-116, **4:** 11-14, **5:** 98-103, **6:** 141-143, **7:** 140-143, **8:** 15-20, **9:** 132-139, **10:** 5-10 and 155-162, connected with disulfide bond CYS7-CYS161, **11:** 21-45*, **12:** 117-131*, **13:** 144-150, **14:** 144-149, **15:** 104-110, **16:** 46-52, **17:** 77-97*, **18:** 54-78.
* Indicates presence of carbohydrates.

one arginine (156 u) residue, from the molecular weight of 898 u for peak 13 (VYSNFLR). The loss of arginine from 13 was also consistent with the new peptide 14 being less polar in the reversed phase chromatogram. The synthetic peptide VYSNFL was also shown to coelute with the peak 14 in the tryptic map chromatogram (data not shown).

Effect of Heat and Inhibitors

The presence of a metallo-protease in the variant EPO lots became a suspect when either a 3-minute preboiling (Figure 2) or addition of 8.5

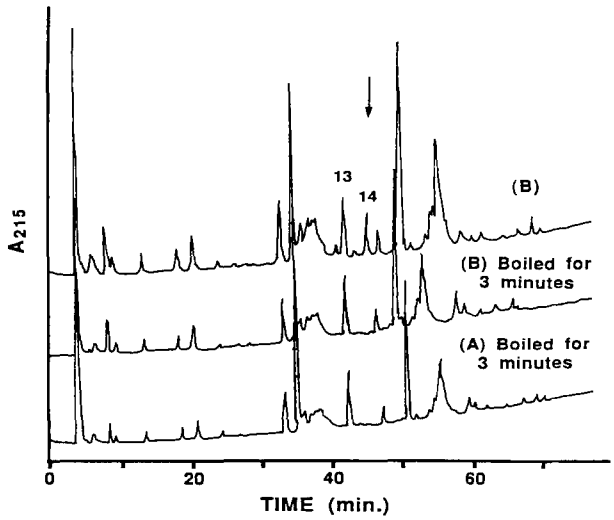

Figure 2. Effect of 3 minute boiling on the tryptic maps of a variant EPO lot (B) and EPO reference standard (A). Chromatographic conditions as in Fig. 1.

mM EDTA (Figure 3) prior to trypsin digestion, caused the extra peak to disappear. No effect was observed on the reference standard EPO under same conditions. Also, addition of 50 mM of specific Carboxypeptidase B inhibitors such as dipyridyl (Figure 4) or arginine hydrochloride (data not shown) to the samples before digestion caused the peak 14 to disappear. The inhibitor, L-lysine, was not equally effective in hindering the peak 14 formation, but 200 mM addition caused the peak to decrease (data not shown).

Effect of Concentrated Diafiltered Media (CDM)

Addition of CDM (without EPO) in the samples before digestion had an opposite effect from the additives above. The undiluted CDM caused extensive changes in the EPO tryptic map. However, when the CDM

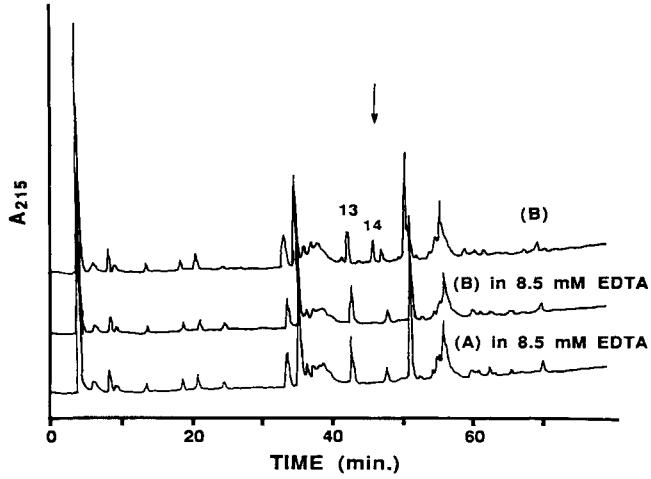

Figure 3. Effect of 8.5 mM of EDTA on the tryptic maps of a variant EPO lot (B) and EPO reference standard (A). Chromatographic conditions as in Fig. 1.

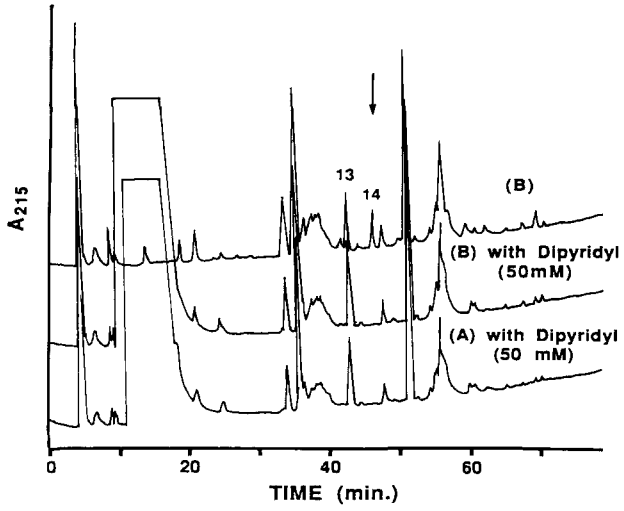

Figure 4. Effect of 50 mM of dipyridyl on the tryptic maps of a variant EPO lot (B) and EPO reference standard (A). Chromatographic conditions as in Fig. 1.

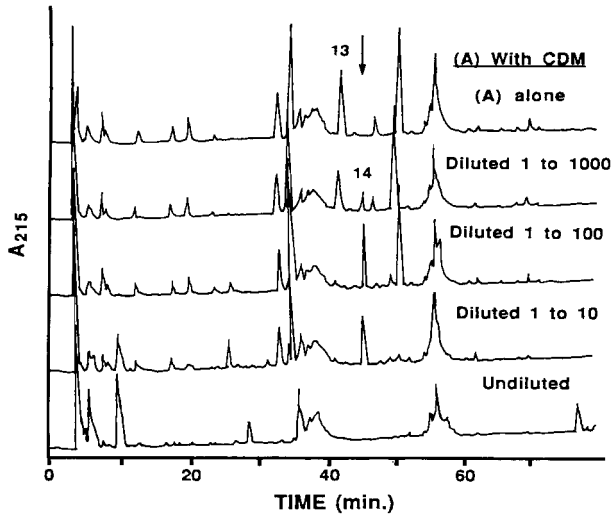

Figure 5. Effect of concentrated diafiltered media (CDM) on the tryptic map of EPO reference standard (A). Chromatographic conditions as in Fig. 1.

concentrate was diluted 1 to 10, 1 to 100 and 1 to 1000 with distilled water and then added to EPO reference standard, the extra peak 14 formation was observed. At the 1 to 1000 dilution level, the peak 14 size was close to those seen in the variant EPO lots (Figure 5, trace 2). The more concentrated dilution levels of CDM caused some additional degradations to occur (Figure 5, traces 3 and 4).

Effect of Carboxypeptidase B (CPB)

Addition of CPB in the samples before digestion had the same effect as CDM. Concentrated CPB caused very extensive changes in the tryptic maps of EPO reference standard. However, when the enzyme level was reduced to around 1-20 mU/mL, the tryptic map became similar to that of

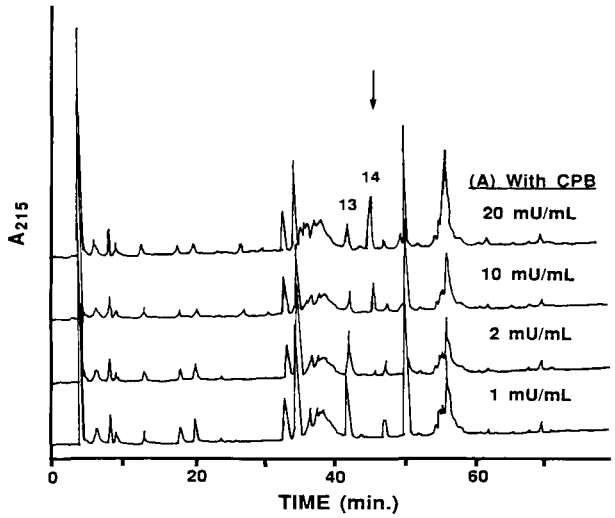

Figure 6. Effect of Carboxypeptidase B (CPB) on the tryptic map of EPO reference standard (A). Chromatographic conditions as in Fig. 1.

the variant EPO lot (Figure 6). The minimum amount of CPB in EPO reference standard required to produce the peak 14, as seen in the variant EPO lots , was in the range of 2-10 mU/mL (Figure 6, traces 2 and 3). On the other hand, the addition 10 mU/mL of CPB into the variant EPO lot caused a total conversion of peak 13 to the new peak 14.

DISCUSSION

Trypsin cleaves proteins at the carboxyl side of basic amino acids, arginine and lysine. On occasion, the trypsin digestion may be incomplete or variant, especially when adjacent proline, arginine or lysine residues are present, or when the bond is buried within the 3-

dimensional structure of a native protein. In general, variations in the peptide mapping can be caused by both structural changes in the protein and by differences in digestion and chromatography. The last two variables are usually minimized by running a reference standard side-by-side with the sample, as was done in our studies. Therefore, what was observed in this study was very unusual in that only one normal tryptic peptide lost the C-terminal arginine in the variant EPO lot digestion.

EPO carbohydrate heterogeneity did not seem to be the source of the anomaly. The extra peak 14 was sharp which was indicative of a non-glycosylated fragment. Also, based on the elution order on RP/HPLC, the peak 14 was less polar than the peptide peak 13, the potential parent peptide. Attached carbohydrate would have made the peptide more polar. In addition, the sequencing data gave a normal serine which was the most likely attachment site for the carbohydrate moiety on the peptide 14.

The total loss of the extra peak in the boiling and EDTA experiments, indicated that we were dealing with some type of metallo-protease. The boiling destroyed the enzyme activity, without altering the rest of the tryptic map, and EDTA formed a chelating complex with the divalent cation of the enzyme. Among the known metallo-proteases, there are a few specific ones that are known to cleave exclusively basic C-terminal amino acids, arginine and lysine. Carboxypeptidase B (CPB) is one such exopeptidase with a Zn-cofactor and a molecular weight at around 34,000, very close to that of EPO. It can be specifically inhibited by another complexing agent, dipyridyl or by L-arginine and L-lysine.

L-lysine is known to be a much less effective inhibitor (inhibitor constant, $Ki=13\times10^{-3}M$, pH 8) than L-arginine ($Ki=0.5\times10^{-3}M$, pH 8), which was also shown to be the case experimentally. Even 200 mM of L-lysine did not cause as much suppression of the extra peak formation as did 50 mM of L-arginine. One might suspect that the trypsin used in the study contained the metallo-protease as a trace contaminant. This, however, was not likely for two reasons: 1. CPB is known to be inactivated by

lyophilization, and the trypsin solution was made from lyophilized powder, and 2. The EPO reference standard, run side-by-side, did not produce the extra peak.

While the peak 14 formation was reduced or eliminated in the variant EPO tryptic map by various techniques, like adding 8.5 mM EDTA, it could also be produced in EPO reference standard mixed with 10 mU/mL of CPB or various levels of CDM. Since there was an indication of a high level of CPB-like activity in CDM, no C-terminal arginine could be assumed to be unaffected in native EPO. According to DNA sequence data, EPO should have an arginine in the C-terminal position of 166. However, no arginyl residue at 166 has been observed by C-terminal analysis by us and others (11). The 166-arginine clipping seems to be part of the post-translational modification of the EPO molecule.

It was also intriguing to note that mainly one specific peptide 13 (144-150) was the primary target of the CPB-like enzymatic activity. In addition, there never was a complete conversion of 13 into 14 in all the variant lots studied. It, therefore, seemed that this reaction consumed all the available enzyme in the sample. If another 10 mU/mL of CPB were added into a variant EPO lot, a total conversion of 13 to 14 was observed. Only when a large excess of CPB was added, did the other peptides become targets of attack, and very altered tryptic maps were observed. Why the tryptic peptide, 144-150, was the primary target for the residual CPB-like activity, is still under investigation. The reason may be that this peptide locates in the most trypsin-sensitive part of the molecule. The results, however, demonstrate the level of sensitivity that can be achieved by peptide mapping in detecting such minute enzymatic activity.

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REFERENCES

1. Goldwasser E. and Kung CKH. Ann. N.Y. Acad. Sci. 149, 49-53 (1968).

2. Miyake T. Kung, CKH. and Goldwasser E. J. Biol. Chem 252, 5558-5564 (1977).

3. Goldwasser E. Blood Cells 10, 147-162 (1984).

4. Jacobs K., Shoemaker C., Rudersdorf R., Neil SD., Kaufman RJ, Mufson A., Seehra, Jones SS., Hewick R., Fritch EF., Kawakita M., Shimizu T. and Miyake T. Nature (London) 313, 806-810 (1985).

5. Lin FK., Suggs S., Lin CH.,Browne JK., Smalling R., Egrie JC., Chen KK., Fox GM., Martin F., Stabinsky Z., Badrawi SM., Lai PH. and Goldwasser E. Proc. Natl. Acad. Sci. USA 82, 7580-7584 (1985).

6. Powell JS., Berkner KL., Lebo RV. and Adamson JW. Proc. Natl. Acad. Sci. USA 83, 6465-6469 (1986).

7. Golde DW. and Gasson JC. Scientific American, July, 62-71 (1988).

8. Goldwasser E. Control of Cellular Division and Development, Part A., Cunningham D. and Goldwasser H. (eds), A. R. Liss, New York, NY., 487 (1981).

9. Browne JK., Cohen AM., Egrie JC., Lai PH., Lin FK., Strickland T., Watson E. and Stebbing N. Cold Spring Harbor Symp. Quant.Biol., 51, 693-702 (1986).

10. Lai PH., Everett R., Wang FF., Arakawa T. and Goldwasser E. J. Biol.Chem. 261, No. 7, 3116-3121 (1986).

11. Recny MA., Scoble HA. and Kim Y. J. Biol. Chem. 262, 17156-17163 (1987).

12. Davis JM., Arakawa T., Strickland TW. and Yphantis DA. Biochem. 26, 2633-2638 (1987).

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SEPARATION OF POLYBUTYLENE GLYCOLS ON C_{18} AND C_4 STATIONARY PHASES

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ABSTRACT

Polybutylene glycol (PBG) samples largely differing in molecular weight M_r are separated by gradient reversed-phase high performance liquid chromatgraphy on C_{18} and C_4 stationary phases by use of either acetonitrile or methanol as organic modifiers and signal monitoring by means of evaporative light scattering detection. Neither acetonitrile nor methanol are sufficient for the quantitative elution of all samples from the extremely hydrophobic C_{18} matrix. However, the elution potency of methanol is markedly better compared with acetonitrile presumably attributable to a solubility increase of sample molecules via hydrogen bonding between its hydroxy group and the ether oxygens of the solutes. Marked lower retention of high M_r oligomers is observed on the more polar C_4 matrix and the elution power of acetonitrile is now sufficient for the quantitative release of all investigated polyether samples. A substantial increase of signals eluting at higher retention times and thus representing oligomers with higher M_r becomes evident in the range PBG 650 < PBG 1000 < PBG 2000 < PBG 3000. Although the PBGs exhibit a broad "within-sample" oligomeric M_r distribution and thus substantial peak overlapping by superposition of the individual chromatograms a clear assignment to individual samples can be done on the basis of the different chromatographic patterns.

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INTRODUCTION

Besides the polypropylene glycols the polytetrahydrofurans, i.e. the polybutylene glycols (PBGs) play a more and more increasing role for the use as flexibiliser and toughener in formulated epoxy-based systems. Further, they are used as long-chain α,ω- dialcohol components in the synthesis of either polyurethane fibres, polyester and polyurethane plastics or as starting materials in the synthesis of cross-linked polyurethane casting elastomers. In many applications PBGs are first reacted with diisocyanates to form isocyanate prepolymers, which are subsequently converted to polyurethanes. For quality control as well as for discussion of structure-property relationships there is a need to know the oligomeric composition of PBGs. Structural assignment of the PBGs can be easily accomplished by gradient reversed-phase high performance liquid chromatography (RP-HPLC) of the cleavage products obtained after acid or base catalysed hydrolysis of the samples. Signal monitoring by evaporative light scattering detection (ELSD) offers the advantage to use the native hydrolysis products without prior derivatisation with a chromophoric agent. Recently we reported on our chromatographic investigations of PBG 1000 by use of different stationary phases and different organic modifiers (1,2). In this paper an improved and very efficient separation system for the analysis of samples largely differing in M_r is described, which further permits a selective attribution to the type of PBG on the basis of the individual chromatographic patterns. For comparative purpose we have applied a C_{18} as well as a C_4 stationary phase and both acetonitrile and methanol as the organic modifiers.

EXPERIMENTAL

- *Separation media*

The polybutylene glycol samples PBG 650, PBG 1000, PBG 2000 and PBG 3000 ("technical quality") were obtained from BASF (Ludwigshafen, Germany). Nucleosil $5C_{18}$ and $5C_4$ (each column 125 x 4.6 mm I.D., 5 µm particle size, 100 Å pore diameter) from Macherey-Nagel (Oensingen, Switzerland) were used as the stationary phases for RP-HPLC. For gel permeation chromatography (GPC) a series

of four PLgel columns (each 300 x 7.5 mm I.D., 5 μm particle size) with pore diameters in the range of 10^5 Å, 10^3 Å, 500 Å and 100 Å and a PLgel precolumn (50 x 7 mm I.D., 5 μm particle size, 100 Å) for column protection were purchased from Polymer Labs. (Church Stretton, Shropshire, UK).

- *Chemicals and solvents*

Narrow-range polystyrene molecular weight calibration standards for determination of M_n and M_w values were obtained from Polymer Labs. (Church Stretton, Shropshire, UK). Acetonitrile and methanol (both HPLC grade) were from Fluka (Buchs, Switzerland). Tetrahydrofuran ("pro analysi") stabilised with 0.025 % of 2,6-di-tert. butyl-4-methyl phenol (Fluka) was used for GPC. Water for the use in HPLC was purified with a Milli-Q reagent water system from Millipore-Waters (Milford, MA, USA).

- *Analytical equipment*

The HPLC apparatus consisted of a SP 8800 ternary HPLC pump, an SP 8880 autosampler equipped with a 10 μl sample loop, a PC 1000 data acquisition unit, all obtained from Spectra Physics (San Jose, CA, USA). For ELSD a type Sedex 45 apparatus from SEDERE (Vitry sur Seine, France) equipped with a 20 W iodine lamp was applied. For GPC a SP 8810 precision isocratic pump, a SP 8875 autosampler equipped with a 100 μl sample loop, a SP 8430 refractive index detector, a SP 4270 integrator (all from Spectra Physics) and a column thermostat from Henggeler Analytic Instruments (Riehen, Switzerland) was used. A 2 micron filter (Rheodyne, Cotati, CA, USA) was inserted between pump and autosampler in order to avoid clogging of the columns by non-soluble solvent and sample impurities.

- *Chromatographic separation*

The gradient system depicted in Table 1 was used for the separation with either acetonitrile or methanol as organic modifier. Chromatography was performed at ambient temperature (ca. 22°C) and a flow-rate of 1.5 ml/min. Aliquots of 10 μl of ca. 2 % methanolic solutions (w/v) of the polyethers were injected onto the HPLC column. For detection by means of ELSD the nebulisation chamber was heated to 40°C and the nitrogen flow was adjusted to 4.5 l/min corresponding to an inlet pressure of 200 kPa. GPC was done at a flow-rate of 1 ml/min and the column

Table 1: Gradient Programme for the Separation of PBGs

Time (min.)	Organic Solvent (%)	Water (%)
0	20	80
40	100	0
75	100	0
76	20	80
90	20	80

temperature was adjusted to 29°C. Aliquots of 100 µl of PBG samples (0.5 %, w/v) were injected and signals were monitored at a range of 0.02 x 10^{-3} refractive index units full scale (RIUFS) measured against tetrahydrofuran in the reference cell.

- Calculation of M_n and M_w values:

This was performed on the basis of "low-molecular weight" calibration by use of 17 narrow range polystyrene calibration standards covering the M_r range from 104 D (styrene monomer) to 120'000 D (approx. n ≅ 1150).

Results

From the chromatographic patterns it is obvious that the different samples show a broad M_r distribution. This view is also corroborated by calculation of the polydispersity index M_w/M_n of the different PBGs from the GPC measurements using polystyrene calibration, which yields values markedly differing from unity and thus attributable to substantial sample heterogeneity (Table 2). Although the choice of polystyrene calibration standards may not be an optimum means of M_r determination and therefore the true may differ markedly from the measured values (see Discussion below), it is nevertheless evident that the individual M_n and M_w values within the range of investigated PBGs differ at a factor of about 8 (Table 2). When measured by HPLC an optimum peak resolution R_s[1)] of the low M_r oligomers of the PBG 650 and

[1)] $R_s = 2 \times (t_2-t_1)/w_1+w_2)$, *where* t_1 and t_2 are the retention times of two adjacent peaks and w_1 and w_2 their base widths.

Table 2: *M_n, M_w and M_w/M_n Values of Polybutylene Glycols*

	PBG 650	PBG 1000	PBG 2000	PBG 3000
M_n	1070	1552	3938	6095
M_w	2089	3452	12073	15796
M_w/M_n	1.95	2.28	3.07	2.61

PBG 1000 samples was achieved on a C_{18} stationary phase with a linear solvent strength (LSS) system consisting of acetonitrile and water but the medium-to-high M_r sample constituents were not eluted. This view becomes evident from the vast decrease of the signal responses for the oligomers in the range PBG 650 > PBG 1000 > PBG 2000 > PBG 3000 (Figures 1a-d), though equal amounts had been injected and thus only the low M_r "impurities" from PBG 2000 and PBG 3000 were eluted with this system. It is further remarkable that the t_R values of all elutable oligomers of PBG 650, PBG 1000, PBG 2000 and PBG 3000 coincide completely and the last peak leaving the column appears at ca. 40 min. The only difference between the chromatographic patterns consists in a substantial relative increase of peak areas attributable to oligomers with higher M_r in the range PBG 650 < PBG 1000 < PBG 2000 < PBG 3000 (Figures 1a-d). As already shown previously (1), methanol as organic modifier strongly enhances elution of medium-to-high M_r PBG oligomers and nearly complete elution was at least observed for PBG 650 and PBG 1000 (Figures 2a,b). In contrast, marked amounts of PBG 2000 and in particular PBG 3000, which preponderably consist of high molecular weight oligomers, still remain on the stationary phase (Figures 2c,d). Nevertheless after replacement of the strongly hydrophobic C_{18} by the more polar C_4 matrix elution of the whole quantity of oligomers of PBG 650, PBG 1000, PBG 2000 and PBG 3000 was already effected with acetonitrile affording excellent peak resolution R_s (Figures 3a-d). Base-line separation of 38 PBG 650 and 40 PBG 1000 oligomers, as well as of 47 and 51, respectively, sufficiently resolved PBG 2000 and PBG 3000 oligomers (the most of them exhibiting base-line separation) are observed on the C_4 matrix with acetonitrile as modifier. In contrast resolution of signals attributable to high M_r oligomers is much

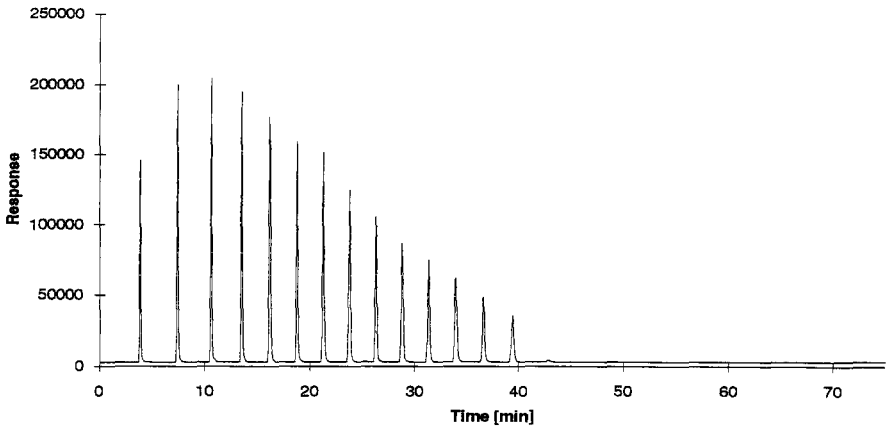

Figure 1a

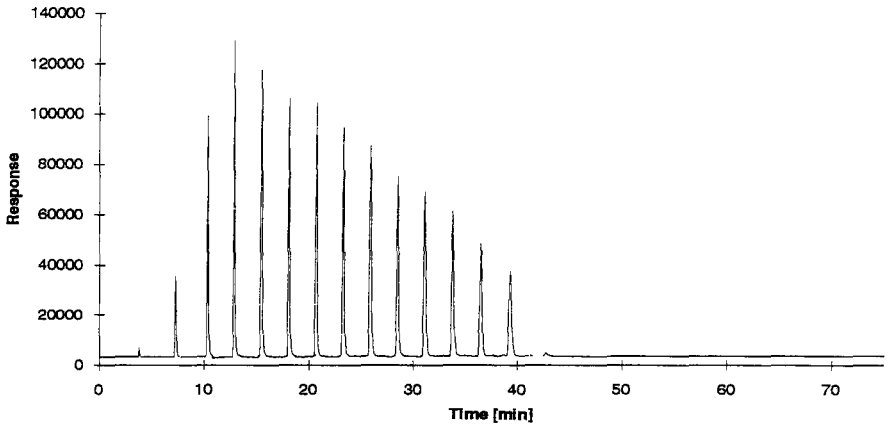

Figure 1b

Figures 1a-d: Chromatogrammes of a) PBG 650, b) PBG 1000, c) PBG 2000 d) PBG 3000 on a C_{18} column and acetonitrile as organic modifier

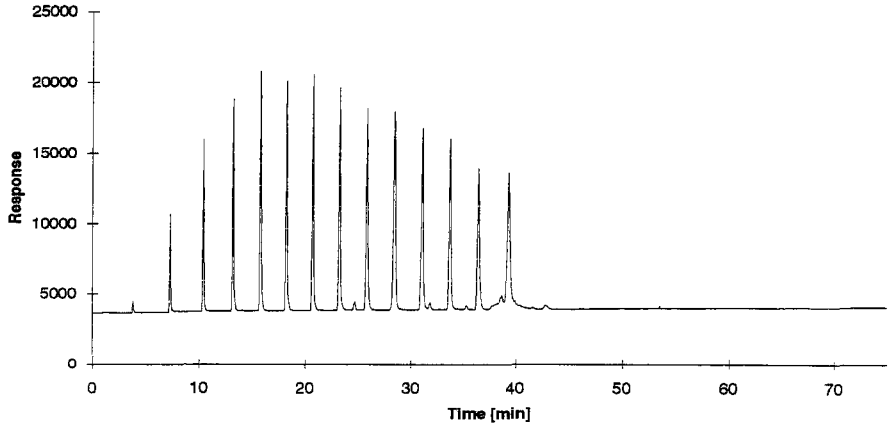

Figure 1c

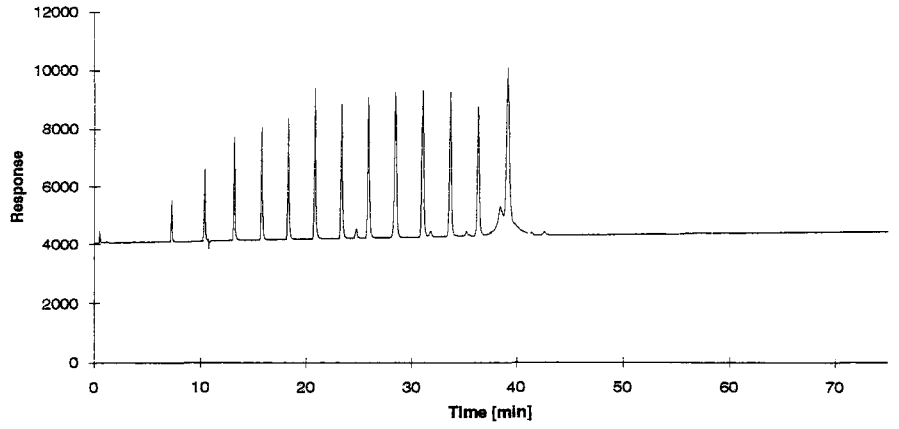

Figure 1d

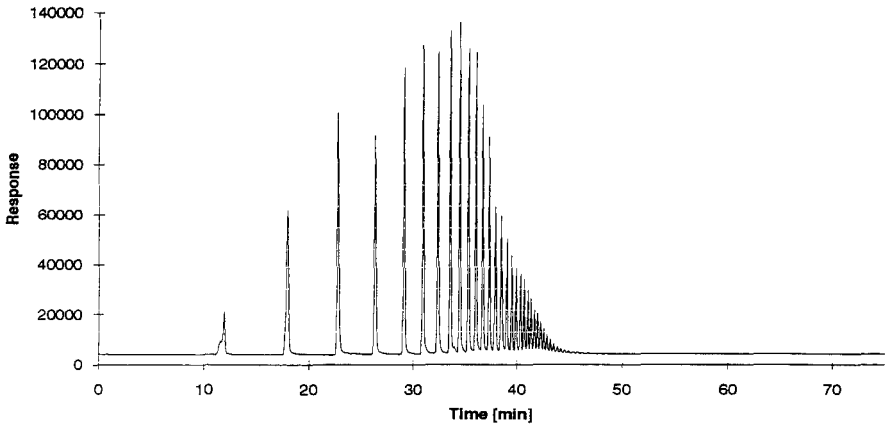

Figure 2a

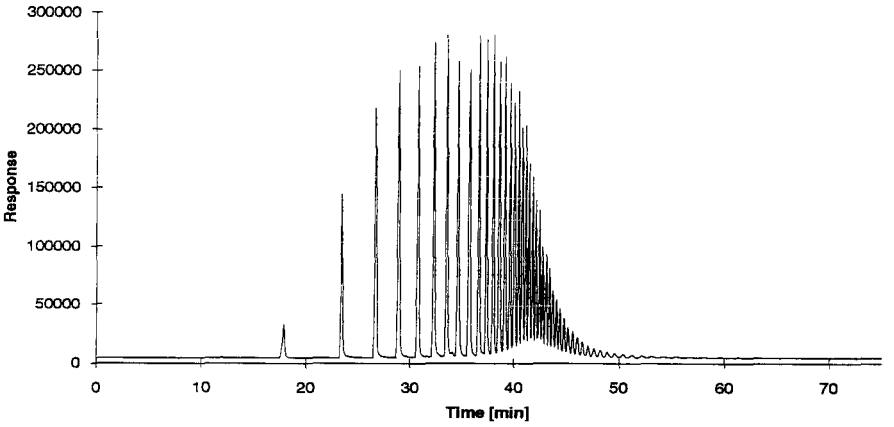

Figure 2b

Figures 2a-d: Chromatogrammes of a) PBG 650, b) PBG 1000, c) PBG 2000 d) PBG 3000 on a C_{18} column and methanol as organic modifier

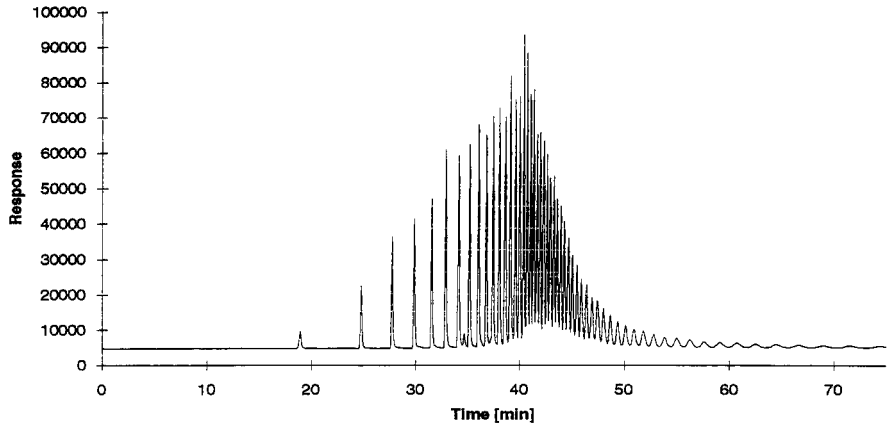

Figure 2c

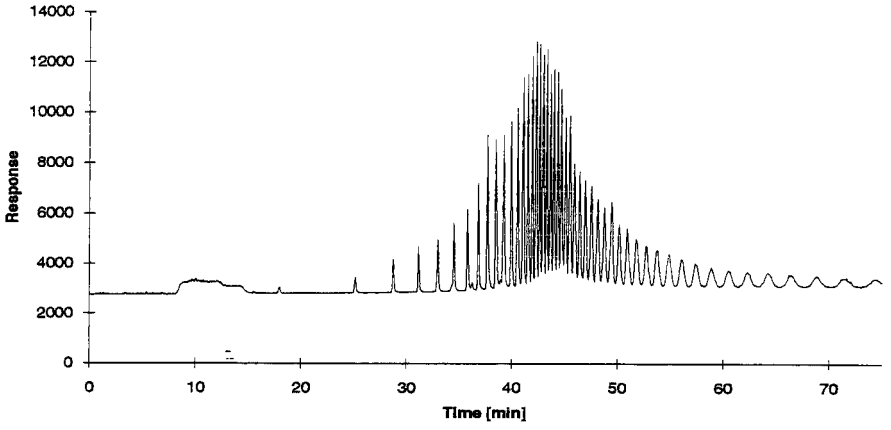

Figure 2d

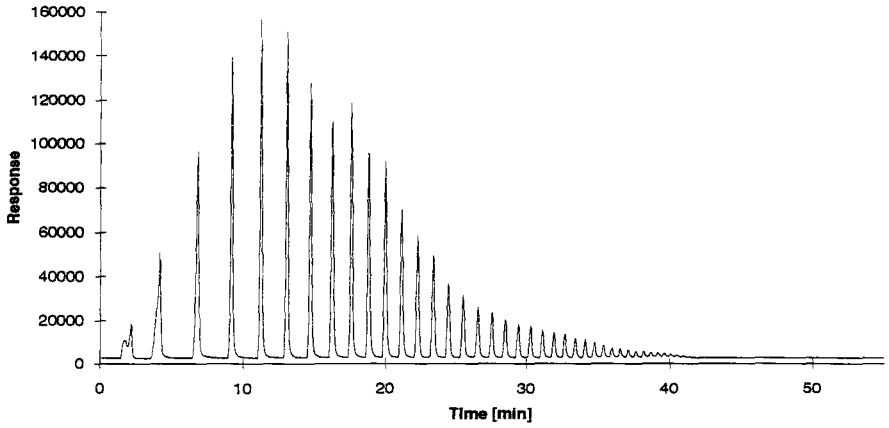

Figure 3a

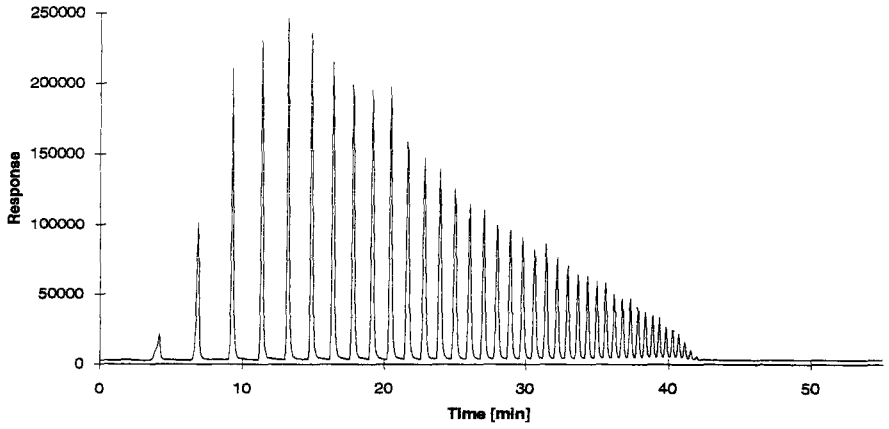

Figure 3b

Figures 3a-d: Chromatogrammes of a) PBG 650, b) PBG 1000, c) PBG 2000 d) PBG 3000 on a C_4 column and acetonitrile as organic modifier

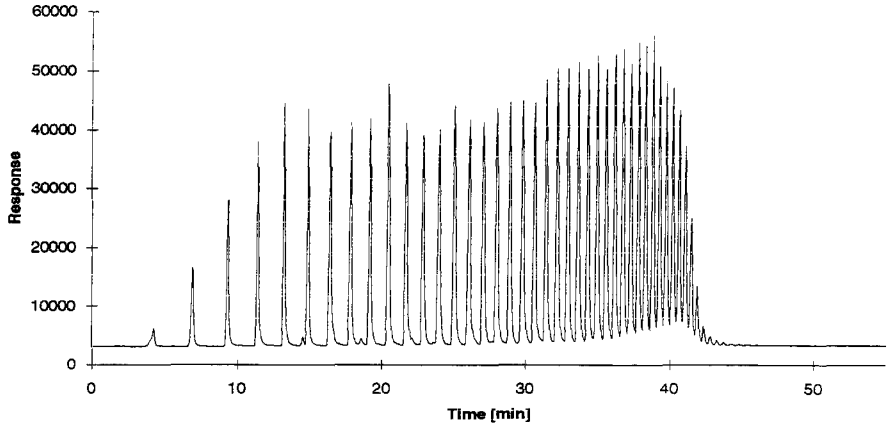

Figure 3c

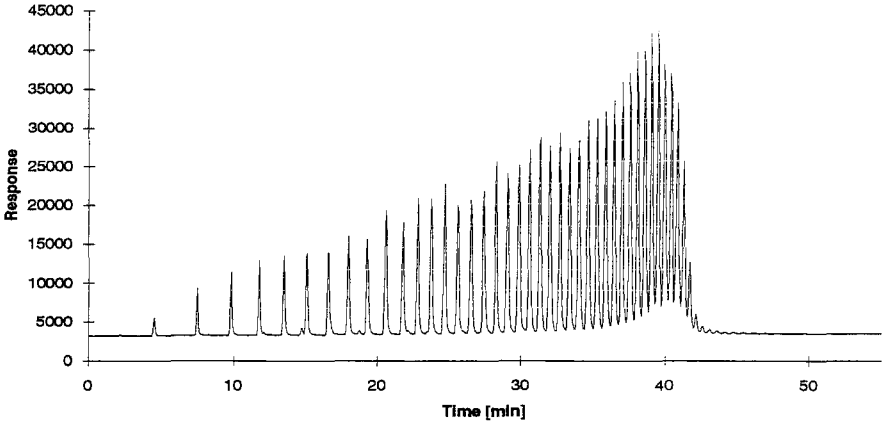

Figure 3d

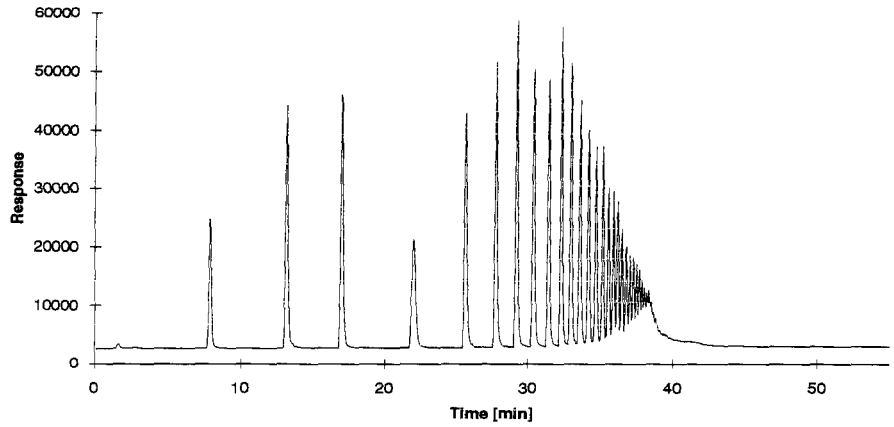

Figure 4a

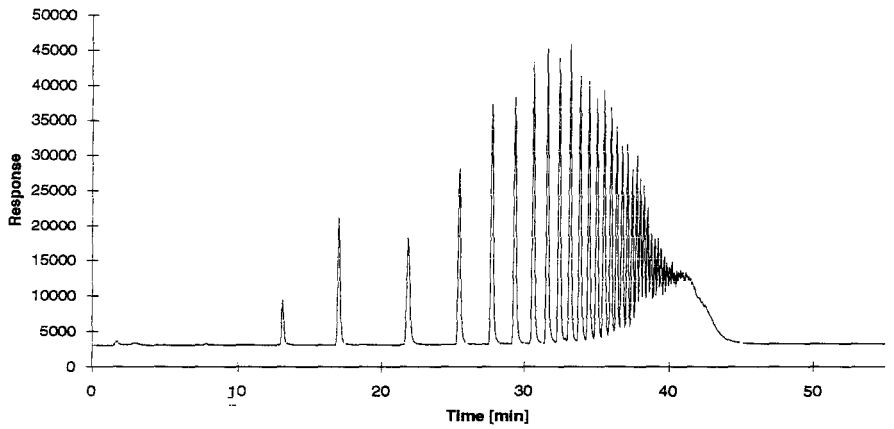

Figure 4b

Figures 4a-d: Chromatogrammes of a) PBG 650, b) PBG 1000, c) PBG 2000 d) PBG 3000 on a C_4 column and methanol as organic modifier

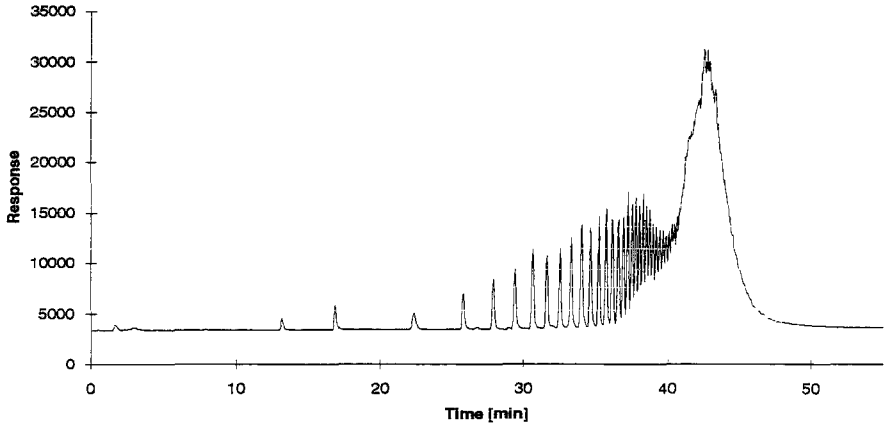

Figure 4c

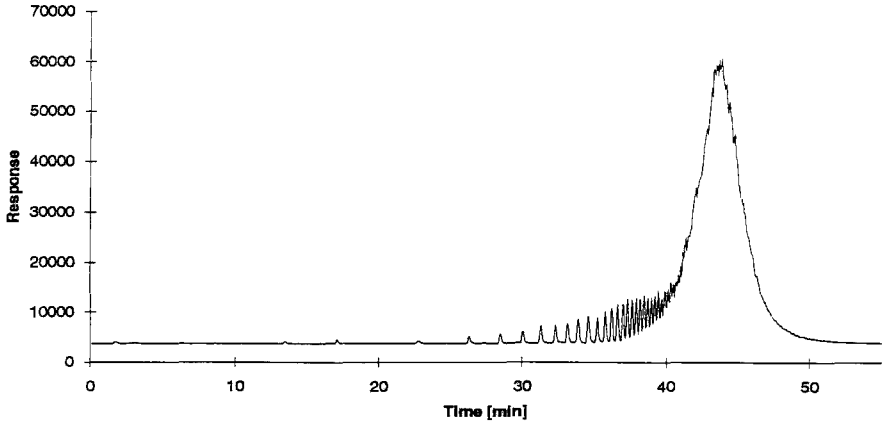

Figure 4d

lower with methanol as modifier resulting in a marked "signal compression" in the range PBG 650 < PBG 1000 < PBG 2000 < PBG 3000 (Figures 4a-d).

DISCUSSION

The native polyethers are not detectable by UV in the usual wavelength range between 210 and 280 nm due to the lack of a chromophor. When wavelength's below 200 nm are used severe baseline deterioration has to be taken into account in particular in gradient chromatography. Nevertheless several authors have described addition of trace amounts (5 ppm) of nitric acid (3) or sodium azide (4) to the aqueous phase to compensate for the baseline drift invoked by the gradual increase of the concentration of organic modifier. On the other hand tagging of the hydroxyl endgroups with a chromophoric agent (1,5,6) makes the polyethers amenable to detection in the usual wavelength range. Signal monitoring on the basis of refractive index (RI) measurement cannot be applied when a solvent gradient is used. In order to avoid an additional derivatisation step we have chosen ELSD primarily based on its wide application range for nonvolatile components, which easily form solid particles after loss of the surrounding solvent shell by nebulisation and subsequent heating of the resulting droplets in the drift tube (7-18). In contrast to low-wavelength UV detection a stable baseline is now obtained, which is either independent on the type of organic modifier or gradient shape (8-10,17). Optimisation of the conditions for signal monitoring of polyethers were described recently (1,2) and used throughout this study.

In most cases of oligomer separation gradient HPLC is required due to the broad M_r distribution of oligomers. Isocratic elution, on the one hand, will yet effect sufficient R_s of low-to-medium M_r oligomers but already a fraction of medium M_r sample constituents will show more and more increased peak broadening and thus neither unsatisfactorily detected nor quantitatively eluted from the stationary phase. On the other hand a mobile phase with stronger elution potency often provides sufficient resolution of high M_r oligomers but signals attributable to low-to-medium M_r oligomers are often unsufficiently separated or even coincide owing to their small retention.

In previous papers (1,2) we have shown that acetonitrile yet provides excellent peak resolution of PBG 1000 oligomers on a C_{18} stationary phase but unfortunately the higher molecular weight sample constituents are not eluted from the matrix. In contrast, methanol as organic modifier effected an almost quantitative "release" of PBG oligomers. We assume that the substantially hydrophobic polyether samples undergo strong interactions with the highly hydrophobic C_{18} chains of the stationary phase. It is known that in n-alkyl bonded silicas the high mobility of the n-alkyl chain results in a liquid-like behaviour of the hydrophobic ligands as was concluded from cross-polarisation magic angle spinning nuclear magnetic resonance experiments (CP-MAS NMR) (19). As a logical consequence the more hydrophobic this "liquid layer", i.e. the longer the n-alkyl chain covalently bound to silanol groups on the surface of the solid silica support, the better its solvation potency towards the relatively hydrophobic PBG samples. Starting from this point of view it will now be easily comprehensible that the power of acetonitrile as a polar and aprotic solvent will be too small to effect elution of medium to high M_r PBG homologues. In contrast, the marked increase of the elution potency of methanol may be ascribed to an "affinity-shift" of solute molecules away from the hydrophobic layer on the silica surface towards the protic solvent methanol due to its ability to undergo hydrogen bonding with PBG ether oxygens (1). This effect may thus provide a substantial solubility increase of the solutes in the mobile phase, which cannot be achieved with the aprotic modifier due to its lack of "exchangeable" protons and, as a consequence, the inability to undergo hydrogen bonding. These conclusions can be drawn from the elution profiles depicted in Figures 1a-d and 2a-d, respectively.

It is clearly shown that methanol effects a marked decrease in retention on a C_{18} matrix compared with acetonitrile but unfortunately a part of the medium and the whole amount of high M_r PBG-2000 and PBG-3000 oligomers still remain on the stationary phase. As reported recently (2) ethanol and isopropanol yet further improve elution of PBG 1000 from a C_{18} phase but the high M_r oligomers coincide and elute as a broad and poorly resolved signal. Hence it might be possible to effect either sufficient R_s or complete elution by combining the separation efficiency of acetonitrile and the excellent eluotropic properties of the protic modifiers ethanol and isopropanol by use of a ternary solvent gradient programme. This would include the start of chromatography with a binary gradient of acetonitrile and water for several

minutes to separate the low M_r oligomers, subsequent admixture of the protic solvent in the gradient mode and a concomitant decrease of the concentration of the aprotic modifier at higher t_R values to achieve quantitative elution of the sample. It should, however, be emphasised that ternary solvent (gradient) systems are only easily applicable when the percentage of one mobile phase component is kept constant, i.e. when small amounts (between 0 and 10 %) e. g. of "modulating" solvent like tetrahydrofuran or dioxan are used (20). In contrast, optimisation of elution conditions would be tremendously complicated when all three components of the solvent mixture are changed simultaneously.

In a former study we tested solute retention with the aprotic modifier acetonitrile on less hydrophobic, i.e. more polar stationary phases like C_8, C_4, C_{phenyl} and C_1 matrices (1). As expected, solvophobic solute-matrix interactions are markedly suppressed in the range $C_{18} > C_8 > C_4 > C_{Phenyl} \cong C_1$ and PBG 1000 was quantitatively eluted from a C_4 matrix. This fact prompted us to apply a gradient-based chromatographic system for the separation of PBGs markedly differing in average M_r and further showing a broad within-sample oligomeric M_r distribution (high polydispersity index M_w/M_n) on a C_4 column by further comparing the effects of the two modifiers acetonitrile and methanol on chromatographic characteristics. On this short-chain matrix the elution power of acetonitrile is now sufficient to afford a complete "release" of the whole amount of oligomers of investigated PBG samples from the stationary phase. This effect may primarily be attributed to the marked decrease in solute-matrix interactions resulting in a relative solubility shift of the solutes towards the aprotic modifier. Fortunately these interactions are still sufficiently high to allow excellent separation of medium-to-high M_r oligomers (Figures 3a-d) without impairment of the R_s of low M_r homologues. For this reason the C_4 column offers a compromise between solute solubility in the stationary and the mobile phase, by which either complete release or separation of all PBG samples is achieved. Further, the substantial differences in the chromatographic patterns allow "recognition" of individual PBG samples within mixtures. In contrast, with methanol the "solvation equilibrium" of solutes in the stationary and mobile phase is markedly shifted towards the protic solvent, as can be concluded from Figures 4a-d. In this case it is assumed that the vast solubility increase with methanol suppresses those interactions between the individual PBG oligomers and the matrix, which are

indispensable for sufficient peak resolution. As the consequence a "levelling" effect occurs affording co-elution of medium-to-high M_r sample constituents in particular for PBG 2000 and PBG 3000 (Figures 4c,d), which now appear in the chromatogramme as broad and unresolved signals. Nevertheless the low and at least a part of the medium M_r oligomers are well-separated and a selective assignment of individual PBG samples within mixtures can be achieved in a similar manner as with acetonitrile (see above).

Further, on the basis of the impressive high resolution chromatographic patterns obtained with acetonitrile on a C_4 matrix, it seems reasonable that HPLC would provide a better means for the determination of the M_n, M_w and M_w/M_n values of polybutylene glycols than GPC as proposed recently by Trathnigg et al. (21) after their HPLC investigations of polyethylene glycols. In this context it may be worthy to note that the M_r measurements of PBGs with polystyrene calibration standards (Table 2) far exceeds the manufacturer's classification, which may be interpreted by a different solvation of calibrator and sample by the solvent tetrahydrofuran. For this reason the similar polarities of solvent and samples will favour formation of solute-solvent associates, which in turn markedly extends the hydrodynamic volume of the PBG samples yielding elevated M_n and M_w values.

CONCLUSIONS

Polybutylene glycol oligomers, which markedly differ in molecular weight as well as in polydispersity can be efficiently separated on a C_4 stationary phase with acetonitrile as organic modifier. Although methanol elicits only poor resolution of high M_r oligomers on this material it nevertheless permits a selective attribution of solutes to an individual PBG sample. On the other hand, the power of acetonitrile is unsufficient to elute the whole amount of medium-to-high M_r homologues of all investigated PBG samples from a C_{18} column and methanol only effects the "release" of the total quantity of PBG 650 and PBG 1000 oligomers from this stationary phase. This observation can be ascribed to a marked "solvation" of the solute by the highly hydrophobic matrix, by which it is tightly retained in the "liquid layer" of n-octadecyl chains covalently bound to the silica gel surface. In contrast, the

short-chain C_4 column affords complete elution of all investigated PBG samples due to lower hydrophobic solvation and a concomitant solubility shift of the solutes towards both modifiers. The "levelling" effect of methanol, which tremendously decreases the R_S of medium-to-high M_r PBG oligomers compared with acetonitrile can be satisfactorily explained by its hydrogen bonding capability, which further enhances the solubility shift towards the protic modifier. As the consequence solute-matrix interactions responsible for satisfactory oligomer separation are substantially suppressed.

References

1) *K. Rissler, H.-P. Künzi, H.-J. Grether, J. Chromatogr., 635: 89-101 (1993)*
2) *K. Rissler, U. Fuchslueger , H.-J. Grether, J. Chromatogr., 654: 309-314 (1993)*
3) *Sj. van der Val, L. R. Snyder, J. Chromatogr., 255: 463-474 (1983)*
4) *G. Barka, P. Hoffmann, J. Chromatogr., 389: 273-278 (1987)*
5) *A. Nozawa, T. Ohnuma, J. Chromatogr., 187: 261-263 (1980)*
6) *P. L. Desbène, B. Desmazières, J. J. Basselier, A. Desbène-Monvernay, J. Chromatogr., 461: 305- 313 (1989)*
7) *J. M. Charlesworth, Anal. Chem., 50: 1414- 1420 (1978)*
8) *R. Macrae, J. Dick, J. Chromatogr., 21: 138-145 (1981)*
9) *A. Stolyhwo, H. Colin, G. Guiochon, J. Chromatogr., 265: 1-18 (1983)*
10) *A. Stolyhwo, H. Colin, M. Martin, G. Guiochon, J. Chromatogr., 288: 253-273 (1984)*
11) *T. H. Mourey, L. E. Oppenheimer, Anal. Chem., 56: 2427-2434 (1984)*
12) *L. E. Oppenheimer, T. H. Mourey, J. Chromatogr., 298: 217- 224 (1984)*
13) *L. E. Oppenheimer, T. H. Mourey, J. Chromatogr., 323: 297- 304 (1985)*
14) *T. H. Mourey, J. Chromatogr., 357: 101- 106 (1986)*
15) *R. Schultz, H. Engelhardt, Chromatographia, 29: 517-522 (1990)*
16) *D. E. Schaufelberger, T. G. McCloud , J. A. Beutler, J. Chromatogr., 538: 87-90 (1991)*
17) *P. van der Meeren, J. Vanderdeelen, L. Baert, Anal. Chem., 64: 1056-1062*
A. I. Hopia, V.-M. Ollilainen, J. Liq. Chromatogr., 16: 2469-2482 (1993)
19) *M. Hanson, K. K. Unger, J. Schmid, K. Albert, E. Bayer, Anal. Chem., 65: 2249-2253 (1993)*
20) *E. Roggendorf, R. Spatz, J. Chromatogr., 204: 263-268 (1981)*
21) *B. Trathnigg, D. Thamer, X. Yan, S. Kinigasa, J. Liq. Chromatogr. 16: 2453-2467 (1993)*

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A NEW METHOD OF STUDYING TEMPERATURE DEPENDENCE AND THE EFFECT OF MOBILE PHASE COMPOSITION ON THE RETENTION MECHANISM IN REVERSED PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

In this work, a rapid procedure to examinate the effect of temperature and eluent composition on thermodynamic properties in high performance liquid chromatography is presented. The use of an experimental design is proposed to study thermodynamic solution property trends for ten Benzodiazepines. Enthalpies and entropies of transfert (mobile to stationary phase) are calculated by evaluation of Van't Hoff plots. For all cases examined enthapies of transfert are negative. These data showed that the entropic contribution to retention becomes more significant as the solvant polarity decreases. Enthalpy-enthropy compensation behavior is tested for varying mobile phase composition.

INTRODUCTION

The mechanism of retention in reversed phase liquid chromatography has been the subject of much debate. The many interactions that a solute may undergo in both the stationary and mobile phases explain the difficulty in elucidating the mechanism of retention. Explanation of solute retention in "reversed phase" separation is described qualitatively through partioning models (1,2,3,4) or by the use the hydrophobic effect (5,6,7). One of the first thermodynamic

investigations was made by Knox and Vasvari (8). Knox plotted lnk' vs $\frac{1}{T}$ for a group of compounds separated on two different columns. The resultant Van't Hoff plots gave absolute enthalpies and relative entropies of transfert for the solute. This method necessitated a great number of experiments. More recently linear Van't Hoff plots have been observed for eight retinoates (9). In this study, the effect of temperature and the nature of the organic modifier were studied. Enthalpies of transfert were studied using the slopes of the Van't Hoff plots, but $\Delta S°$ values were not provided due to an ambiguity in the calculation of the phase ratio of the commercial columns. Our aim in carrying out this research was to show the advantage of using an experimental design which can reduce the number of experiments. With only 13 experiments, thermodynamic solution property trends are examined as a function of eluent composition. The relative effect of entropy and enthalpy on k' is discussed and enthalpy-enthropy compensation behavior is tested as a function of mobile phase composition.

MATERIALS AND METHODS

CHROMATOGRAPHIC CONDITIONS

APPARATUS : The HPLC system consisted of a HPLC Waters pump 501 (Saint Quentin en Yvelines, France), an Interchim rheodyne injection valve Model 7125 (Montluçon, France) fitted with a 20µl sample loop, a Merck L 4000 variable wavelength UV spectrophotometer detector and a Merck D 2500 chromato integrator (Nogent-sur-Marne, France). A Waters 150 mm × 3.9 mm ID. RP 18 column (Nova pak, 5 µm particle size) was used with a controlled temperature in an Interchim crococil oven TM N° 701 (Montluçon, France). Overall temperature control was maintained within ± 1° C with a variation from 26° C to 50° C. The detection wavelength was 254 nm. The flow rate used varied from 0.6 to 1.6 mL/min. The mobile phase was a methanol-water mixture with varied percentages of methanol from 50 % to 80 %. Weaker percentages were not used because of the excessively high column pressure obtained with 50 % of methanol with a flow rate of 1.6 mL/min.

REAGENTS AND SAMPLES : Methanol was HPLC grade determine analytical. (1) Bromazepam (2) Nitrazepam (3) Flunitrazepam (4) Clobazam (5) Lorazepam (6) Oxazepam

(7) Tofisopam (8) Chlordiazepoxide (9) Chlorazepate dipotassic and (10) Diazepam were obtained from HOFFMANN LA ROCHE (Basel, Switzerland). These were diluted in methanol in a concentration range of 10-80 mg/mL.

METHODS

CHEMOMETRIC METHODOLOGY : The traditional approach studies each factor separately to find the influence of mobile phase composition and temperature on the retention mechanism. A chemometric approach is based on the use of matrix experiments which study the simultaneous variation of all factors. This way the number of experiments can be reduced compared with the traditional methods. A mathematical model is used which linked the observed response (Y) and the influencing factor (X). Variables were coded to have a variation from -1 to +1. The experimental quantitative factors included the mobile phase composition, its flow rate and column temperature.

THERMODYNAMIC RELATIONSHIPS : Valuable information concerning the retention mechanism in HPLC may be gained by examining the temperature dependence of retention which is given by the equation :

$$\ln k' = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \Phi$$

where k' is the capacity factor of the solute

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

t_R is the retention time of the compound and t_0 the retention time of an unretained peak such as sodium nitrate. ΔS° is the enthalpy of transfert of the solute from the mobile phase to the stationary phase, ΔH° is the entropy of transfert from the mobile phase to the stationary phase, T is the temperature and Φ the phase ratio (volume of the stationary phase divided by the volume of the mobile phase). Numerical values for the phase ratio may be estimated from the physical constant of the packing material. To estimate the phase ratio, a physical model was used which links the percent carbon loading with other physical properties of the stationary phase (10). It must be noted that ΔH° values are independent of the phase ratio. In addition any uncertainty in the phase ratio affects the ΔS° values equally and thus the ΔS° trends, as a function of the eluent compositions, are unaffected.

RESULTS AND DISCUSSION

The principle of the statistical method is based on a second order polynomial between the capacity factor k' of each compound and the factors studied [mobile phase composition-flow rate-column temperature]. In order to investigate the effect of these factors, a modified Box and Benhken experimental design was used (11). Thirteen experiments were carried out and the capacity factor of each compound was determined. The k' values were then fitted into a second order polynomial.

$$\ln k' = a_0 + a_1 \ln x_1 + a_2 \ln x_2 + a_3 \ln x_3 + a_{12}(\ln x_1)(\ln x_2) + a_{13}(\ln x_1)(\ln x_3) + a_{23}(\ln x_2)(\ln x_3) + a_{11}(\ln x_1)^2 + a_{22}(\ln x_2)^2 + a_{33}(\ln x_2)^2$$

where x_1 is the percentage of methanol in the methanol/water mixture, x_2 the flow rate, x_3 the column temperature and a_0, a_1, a_2, a_3, a_{12}, a_{13}, a_{23}, a_{11}, a_{22}, a_{33} the model coefficients (11).

Using this model, seven mobile phase compositions and seven column temperatures lnk' versus these two factors for Flunitrazepam were plotted (Fig. 1). k' decreased when x_1 and x_3 increased. Linear Van't Hoff behavior was obtained for all solutes. The Van't Hoff plot for the solute Flunitrazepam with a mobile phase composition of 50 % of methanol is given in (Fig. 2). The correlation coefficient (r) for the linear fit of this plot was 0.999. Since these data were linear, it was possible to calculate $\Delta H°$ and $\Delta S°$ for this system. $\Delta H°$ was found to be -2.36 kcalmol^{-1} and $\Delta S°$ was calculated to be -2.64 calmol^{-1} K^{-1}. Table 1 contains a complete list of the $\Delta H°$ and $\Delta S°$ values obtained for all the solutes with the different mobile phase compositions. The r values listed for each solute demonstrate the good linearity of these data over the temperature range 25 to 50° C. Three groups of compounds were distinguished according to the variation of $\Delta H°$ versus solvent polarity. In each group, most entropies transfer were negative and values increased with an increased solvent polarity (decreased methanol concentration) (Fig. 3). This phenomenon has been attributed to an ordering of water molecules adjacent to the surface of the solute molecule which is relatively large and non polar. This hydrophobic effect can be described as the tendency of a large and relatively non polar solute to reduce its surface area exposed to water either through association with other relatively large and non polar molecules or through removal from the solution by adsorption. Thereby, an increase in the mutual association of solute molecules in the mobile phase and this

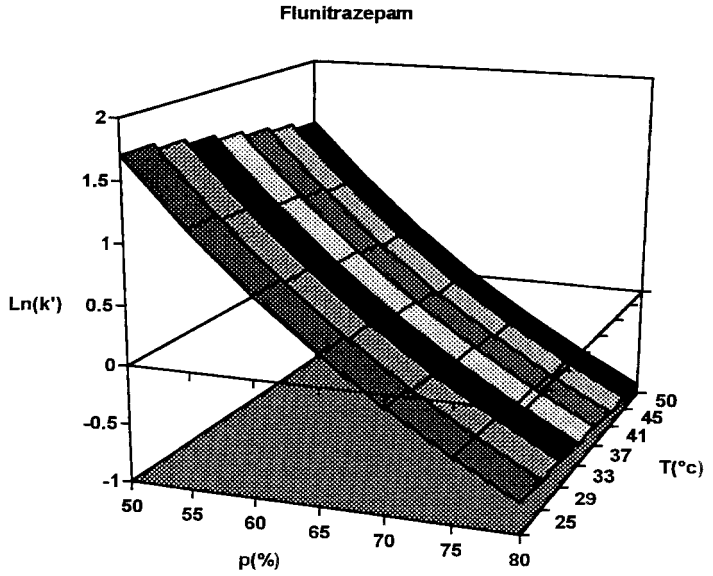

Figure 1 : plot of the logarithme of the capacity factor k' versus percentage of methanol P (%) in the mixture methanol/water and column temperature T(°C) for flunitrazepam.

process of inserting the solute molecule into water would thus explain the observed trend of Δ S° values to positively increase.

In each instance, enthalpies of transfer were negative. It is estimated that maximum variation uncertainty in the calculation ΔH° was inferior or equal to 7 %. For the first group of compounds, when the percentage of methanol in the mixture increased enthalpies of transfer values increased (Fig. 4). When the percentage of methanol increased from 50 % to 80 % ΔH° increased for Bromazepam, Nitrazepam and Flunitrazepam by respectively 28 %, 44 % and 42 % :

Bromazepam < Flunitrazepam < Nitrazepam.

This behavior can be explained since interactions between the solute molecule and a relative non polar mobile phase can be expected to be stronger than for solute-mobile phase combinations of dissimilar polarity. For the second group of solutes when the percentage of

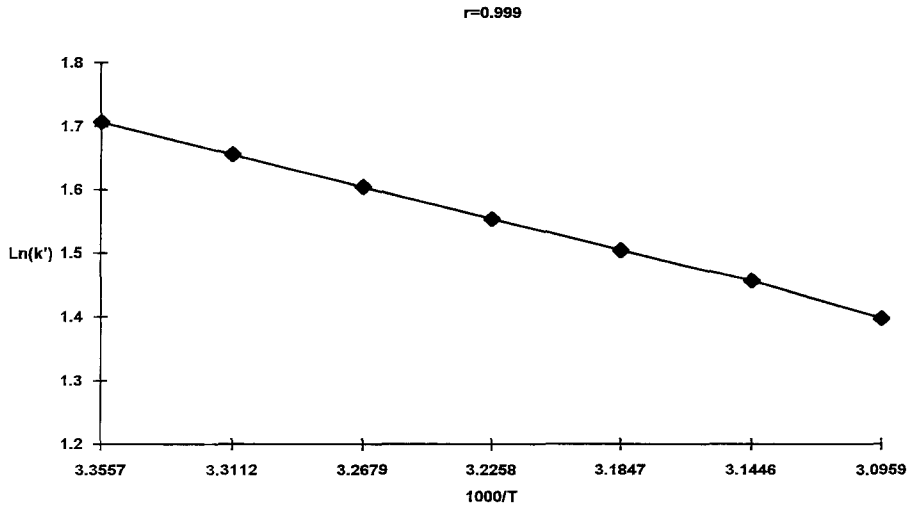

Figure 2 : Van't Hoff plot for the solute flunitrazepam with a mobile phase 50/50 methanol/water.

methanol increased $\Delta H°$ decreased . If this percentage increased from 50 % to 80 %, $\Delta H°$ decreased for Oxazepam and Diazepam by respectively 16 % and 24 % : Oxazepam < Diazepam.

It became energetically more favorable for the solute to be in the stationary phase than in the mobile phase. The small decrease of $\Delta H°$ values with a decrease of solvant polarity was attributed to decreased solute-mobile phase interaction. The third group of compounds was made up of Chlorazepate dipotassic, Chlordiazepoxide, Clobazam, Tofisopam and Lorazepam. For these solute molecules the enthalpy of transfert was constant or its decrease was not significant ($\leq$ 4 %) as the mobile phase composition of methanol was increased. Energetically it is not better for these solutes to be either in the mobile phase or in the stationary phase. For every solute when $\Delta H°$ is compared to $\Delta S°$ over the temperature range studied, the magnitude of $\Delta H°$ was always greater than that of $T\Delta S°$ (Table 2). This indicates, that enthalpy plays a greater role in the transfer of a solute from the mobile phase to the stationary phase and

Table 1 : $\Delta H°$, $\Delta S°$ values for all solutes and for seven percentages of methanol in the methanol/water mixture

	Percentage of methanol											
	50 %			55 %			60 %			65 %		
Compound [a] N°	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/mol/K)	r [b]	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/mol/K)	r	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/mol/K)	r	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/mol/K)	r
1	1.76	1.39	0.997	1.71	2.17	0.999	1.61	2.64	0.999	1.51	3.03	0.998
2	2.63	3.72	0.999	2.39	3.94	0.999	2.18	4.11	0.998	1.98	4.24	0.998
3	2.36	2.65	0.999	2.16	3.06	0.999	1.98	3.39	0.999	1.81	3.65	0.999
4	1.68	0.03	0.998	1.67	1.04	0.998	1.66	1.92	0.998	1.65	2.68	0.998
5	1.97	0.61	0.999	1.97	1.85	0.999	1.98	2.94	0.999	1.97	3.90	0.999
6	1.74	-0.19	0.999	1.82	1.15	0.999	1.88	2.33	0.999	1.94	3.38	0.999
7	1.85	-0.45	0.999	1.84	0.75	0.999	1.83	1.78	0.999	1.83	2.65	0.999
8	2.01	-0.10	0.999	2.01	0.98	0.999	2.01	1.97	0.999	2.01	2.87	0.999
9	2.15	0.11	0.999	2.16	1.30	0.999	2.15	2.37	0.999	2.15	3.32	0.999
10	1.35	-3.08	0.987	1.47	-1.50	0.990	1.56	-0.12	0.992	1.64	1.10	0.993

(continued)

Continuation of Table 1

	Percentage of methanol								
	70 %			75 %			80 %		
Compound N°	- ΔH° (kcal/mol)	- ΔS° (cal/mol/K)	r	- ΔH° (kcal/mol)	- ΔS° (cal/mol/K)	r	- ΔH° (kcal/mol)	- ΔS° (cal/mol/K)	r
1	1.42	3.36	0.998	1.34	3.63	0.998	1.26	3.86	0.997
2	1.80	4.34	0.997	1.62	4.41	0.997	1.46	4.45	0.994
3	1.65	3.86	0.999	1.51	4.03	0.999	1.37	4.16	0.997
4	1.63	3.37	0.998	1.63	3.98	0.998	1.62	4.53	0.997
5	1.97	4.76	0.999	1.97	5.52	0.999	1.97	6.21	0.999
6	1.99	4.32	0.999	2.03	5.17	0.999	2.07	5.93	0.999
7	1.81	3.42	0.999	1.80	4.07	0.999	1.79	4.63	0.999
8	2.01	3.69	0.999	2.00	4.43	0.999	2.00	5.11	0.999
9	2.15	4.18	0.999	2.15	4.95	0.999	2.16	5.65	0.999
10	1.70	2.20	0.994	1.75	3.18	0.994	1.78	4.06	0.995

a : (1) Bromazepam (2) Nitrazepam (3) Flunitrazepam (4) Clobazam (5) Lorazepam (6) Oxazepam

(7) Tofisopam (8) Chlordiazepoxide (9) Chlorazepate dipotassic (10) Diazepam

b : r value for the linear fit of the Van't Hoff plot

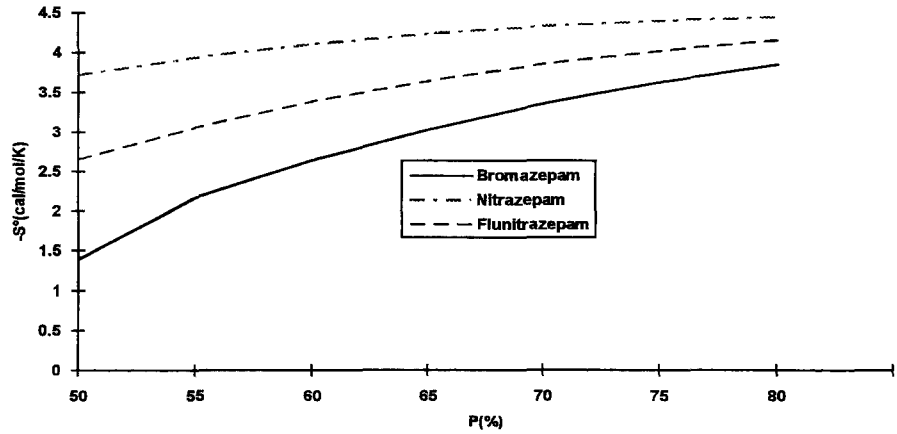

Figure 3 : dependence of the standard entropy change, - ΔS° on the percentage of methanol, P (%) in the mixture methanol/water for the first group of compounds.

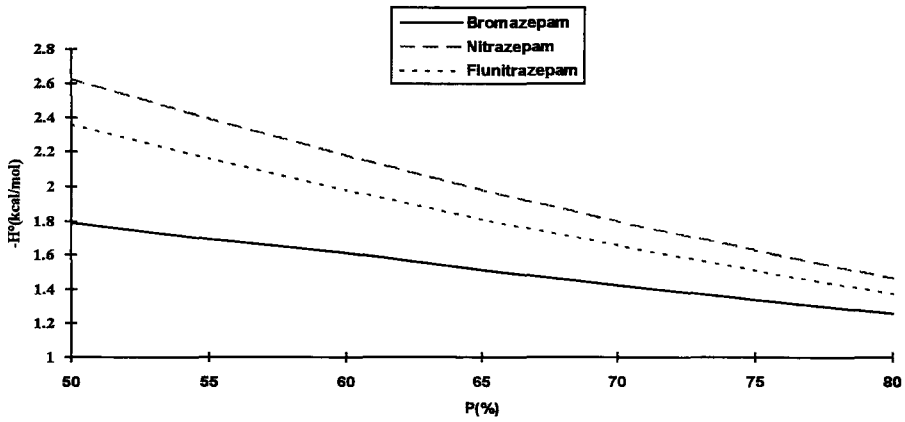

Figure 4 : dependence of the standard enthalpy change, - ΔH° on the percentage of methanol, P (%) in the mixture methanol/water for the first group of compounds.

Table 2 : $\Delta H°$, $\Delta S°$ and $T\Delta S°$ values for all solutes for two different compositions of the mobile phase :

[1] : 50 % of methanol in the methanol/water mixture

[2] : 80 % of methanol in the methanol/water mixture

Compound [a] N°	Percentage of methanol					
	[1]			[2]		
	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol/K)	$T\Delta S°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol/K)	$T\Delta S°$ [b] (kcal/mol)
1	-1.76	-1.39	-0.43	-1.26	-3.86	-1.19
2	-2.63	-3.72	-1.15	-1.46	-4.45	-1.38
3	-2.36	-2.65	-0.82	-1.37	-4.16	-1.29
4	-1.68	-0.03	-0.00	-1.62	-4.53	-1.40
5	-1.97	-0.61	-0.19	-1.97	-6.21	-1.93
6	-1.74	+0.19	+0.06	-2.07	-5.93	-1.84
7	-1.86	+0.45	+0.14	-1.79	-4.63	-1.44
8	-2.01	+0.10	+0.31	-2.00	-5.11	-1.58
9	-2.15	-0.11	-0.34	-2.16	-5.05	-1.75
10	-1.35	+3.08	+0.95	-1.78	-4.06	-1.26

[a] See Table 1

[b] For T = 310 K

therefore in the retention process than does entropy. Nevertheless, it appears that entropy plays an increasing role in retention as the percentage of methanol in the eluent increased (Table 3). It is of interest to study the similarity in the retention mechanism for this family of compounds. Enthaly-entropy compensation (12) is a term used to describe a compensation temperature which is a system that is independant for a class of experimental systems. Enthalpy-entropy

Table 3 : Correlation coefficients of regressions between $\ln k'_T$ and $\Delta H°$ for each compound of the first two groups

Group N°	Compounds	Correlation coefficient r
I	Bromazepam	0.991
	Nitrazepam	0.999
	Flunitrazepam	0.999
II	Oxazepam	0.999
	Diazepam	0.993
III	Tofisopam	For these solutes $\Delta H°$ is constant or its variation with percentage of methanol is not significant
	Clobazam	
	Chlordiazepoxide	
	Chorazepate dipotassic	
	Lorazepam	

compensation has been applied to chromatographic systems to evaluate the retention mechanism (13, 14, 15, 16, 17). The following equation relates the compensation temperature (β) to the capacity factor at temperature T (k'_T)

$$\ln k'_T = \ln k'_0 - \frac{\Delta H°}{R}\left(\frac{1}{T} - \frac{1}{\beta}\right)$$

A plot of lnk' versus (- $\Delta H°/R$) should yield a slope line of $\frac{1}{T} - \frac{1}{\beta}$. The compensation temperature (β) is the temperature at which the studied compound had the same value of k' whatever the mobile phase composition. Compensation "enthalpy-entropy" was obviously tested for the first two groups of solutes when the percentage of methanol varied from 50 % to 80 % at a temperature of 310 K. Table 3 contains a complete list of correlation coefficients r. The high degree of correlation indicates that the retention mechanism for a solute is the same whatever the mobile phase composition.

CONCLUSION

This new approach has enabled us to study the effect of temperature and eluent composition on the retention mechanism of ten benzodiazepines with a limited number of experiments. This procedure meant that 13 experiments instead of 49 were carried out (7 different mobile phase compositions x 7 different temperatures). The Van't Hoff plot curve shape was used to evaluate similitaries and differences between the compound retention mechanism when different mobile phases were used. Results of this work show the strong influence of solvant polarity on thermodynamic properties and this must be considered in order to accurately described the retention process.

REFERENCES

1) Martire D.E., Boehm R.E., J. Phys. Chem., 87, 1045, 1983.

2) Dill K.A., J. Phys. Chem., 91, 1980, 1987.

3) Dill K.A., Naghizadeh J., Marqusee J.A., Annu. Rev. Phys. Chem., 39, 425, 1988.

4) Dorsey J.G., Dill K. A., Chem. Rev., 89, 331, 1989.

5) Horvath C.S., Melander W., Molnar I., J.Chromatogr., 125, 129, 1976.

6) Melander W.R., Horvath C.S., In high performance liquid chromatography, Advances and perspectives, Horvath C.S., Ed., Academic press : New York, 2, 113, 1980.

7) Horvath C.S., Melander W.R., Am Lab., 17, 1978.

8) Knox John H., Vasvari Gabor., J. Chromatogr., 83, 181, 1973.

9) Marja S., Heikki V., Jaoko H., J. Chromatogr., 592, 127, 1992.

10) Sentell K.B., Dorsey J.G., J. Liq. Chromatogr., 11, 1875, 1988.

11) Guillaume Y., Guinchard C., J. Liq. Chromatogr., 16, 3457, 1993.

12) Boots H.M.J., De Bokx P.K., J. Phys. Chem., 93, 8240, 1989.

13) Tchapla A., Heron S., Colin H., Guiochon G., Anal. Chem., 60, 1443, 1988.

14) Yamamoto F.M., Rokushika S., Hatono H., J. Chromatogr. Sci., 27, 704, 1989.

15) Sander L.C., Field L.R., Anal. Chem., 52, 2009, 1980.

16) Kuchar M., Kraus E., Rejholec V., Miller V., J. Chromatogr., 449, 391, 1988.

17) Melander W.R., Campbell D.E., Horvath C.S., J. Chromatogr., 158, 215, 1978.

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TWO-DIMENSIONAL TLC AND FLUORESCENCE ANALYSIS WITH CCD VIDEO CAMERA USED TO DETERMINE THE DISSOCIATION OF DIPHENYLHEXATRIENE INCLUDED IN ß-CYCLODEXTRIN

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ABSTRACT

Two-dimensional TLC coupled to fluorescence analysis by CCD video camera is a sensitive, precise, and rapid method for the study of complexes which contain at least one fluorescent component. The method applied to the diphenylhexatriene·ß-cyclodextrin complex in water/acetonitrile system enables the separation of free and bound DPH and an accurate estimation of their respective quantities on the plate. Free DPH eluted by the hydrocarbon media was evaluated by the emitted fluorescence intensity by comparison with the linear 0 to 50 picomoles calibration scale. DPH bound to BCD was dissociated with a brief polar migration followed by elution with a non polar solvent mixture and evaluated as above. The balance of the free and/or dissociated fluorophore is maintained equal in every section of the TLC.

INTRODUCTION

The solubility of 1,6-diphenyl 1,3,5-hexatriene (DPH) and many other low polar molecules is increased in an essentially aqueous media when complexed with cyclodextrin (1,2). However, the inclusion compound is formed with a significant amount of the free form of DPH remaining in the preparation media. The advantages of studying DPH-BCD complexes with TLC are the possibilities to separate DPH into its free and complexed forms and to rapidly film the fluorescent emission data before the unbound form degradates (3). It has been shown that DPH's fluorescence quantum yield is sensitive to the polarity, polarisability and viscosity of the media (4). It has also been demonstrated that the fluorescence of polycyclic aromatics significantly increases in the presence of BCD (5). DPH included in BCD can be dissociated with a two-dimensional elution with the second migration perpendicular to the first one. This elution scheme places dissociated DPH at the same level as free DPH and ensures that their fluorescent quantum yield are identical. Both these DPH species can therefore be evaluated in the same conditions and be compared.

MATERIALS

Chemicals and supplies

Roquette ß-cyclodextrin crystals were recrystallized from water. The purity of Lancaster DPH was controlled by TLC, by the measure of its molar extinction coefficient with UV-absorption spectroscopy and by its fluorescence emission spectra in hexane and in acetonitrile. Diethyl ether, acetonitrile, acetic acid,

and 2-methyl propanol of analytical grade were used without further purification. Water and hexane were bidistilled.

The 0.2 mm thick TLC plates without fluorescent indicator were supplied by Merck (Art.5553).

Apparatus

The spots were deposited on TLC with a NICHIRYO capillary micropipette (0.05 μl precision).

Image recording of DPH's fluorescence on TLC was performed in a Camag illuminator cabinet under excitation at 366 nm.

A SONY CCD-TR705E video camera sensitive to 2 lux with two superimposed 370 nm (Corning 3-75) and 420 nm (Corning 3-73) cut-off filters and Hi-8 Metal-E magnetic bands were used to film the fluorescence emitted on TLC. The analogic images were numerized with a VITEC VideoMaker card on a 486-66 MHz compatible PC. The capture rate was 25 s^{-1} and the spatial definition equal to 768x576 pixels in 16 million colours.

A spectrofluorimeter assembled in the laboratory was used to record the emission spectra of DPH on TLC. The apparatus consists of a polychromatic light source (Xenon XBO 150 W) and two scanning Jobin Yvon (H 25) grating monochromators. The excitation monochromator has a series of diaphragms which permits the localization of the light beam on the spot in such a way as to reduce the background noise due to the TLC's silica gel. The signals were detected with a Hamamatsu R928 photomultiplier linked to a Keithley 610C electrometer.

Softwares

Imager^{TM} was used to capture the filmed images on computer, Photostyler^{TM} to transform the images, and a

program written in the laboratory to determine the density of the spots.

METHODS

Preparation of BCD-DPH complexes

0.08 g BCD was dissolved in 3 ml water/acetonitrile 62/38 (w/w) to obtain a final concentration of 0.02 $mol.l^{-1}$. 10 mg excess DPH was added to this mixture which was then sealed, protected from light and agitated for 48 hours in a thermally controlled shaker at 60°C. The non solubilized DPH was filtered without cooling with an Ederol n°11 filter paper. The filtrate containing solubilized DPH was then diluted with water/acetonitrile 62/38 (w/w) to obtain a 0.01 $mol.l^{-1}$ solution of BCD with DPH.

Two-dimensional TLC

The following steps in the procedure were carried out in a darkroom.

A 10x7 cm TLC plate was divided into four sections with two perpendicular lines which also indicated the final solvent front of each of the two developments. 1 μl of the 10 mM BCD/DPH system was spotted in each of the four sections labeled A, B, C and D. The different sections were eluted in the directions shown in figure 1.

Section A was not eluted by either eluent. In the first dimension, Sections B and C were eluted with hexane/ether 97/3 (v/v). Spots from section C, and section D were eluted in a dimension perpendicular to the first one. Two migration protocols were applied to the second dimension. The calibration scale consisting of 10, 20, 40 and 50 picomoles DPH in pure acetonitrile could be placed in section B or D.

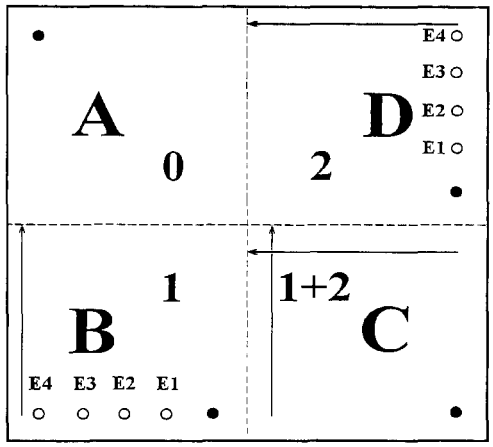

FIGURE 1. TLC spotting scheme. A: 0 dimension (no elution). B: elution in dimension 1 only. C: elution in 1+2 dimensions. D: elution in dimension 2 only. DPH calibration standards E1 to E4 can be placed in section B or C.

In the first method, the second migration was done in one step with the water/2-methyl propanol/acetic acid 5/12/3 (v/v/v) mixture. The calibration scale was placed in section D and eluted in the second dimension.

In the second protocol, the second migration was performed in two steps. The polar eluent, water/2-methyl propanol/acetic acid 5/12/3 (v/v/v) was used for an initial development over 3 mm. The plate was dried for one minute at 110°C and the migration was continued in the same direction with hexane/ether 97/3 (v/v). In this case, the DPH standards were placed in section B and eluted in the first dimension only.

Analysis of emission fluorescence data

1. Analysis by CCD camera.

The video camera was placed in automatic mode and the plate was immediately filmed upon excitation at 366 nm

for several seconds. The time lapse allowed the cell to adapt itself to the intensity of the emitted fluorescence. The images were numerized in 24 bits per pixel and transformed twice. The first transformation converted the images into 8 bits per pixel with a 256-grey scale. The images were then transformed a second time into 1-bit images with the Floyd-Steinberg algorithm (6,7). The last conversion increased the resolution of the image while completely conserving the light intensity data.

At this stage, the total emission of each spot was integrated over an area of 52500 pixels. The surrounding background light was also taken into account.

2. Analysis by spectrofluorimetry.

The emission spectra of free DPH and complexed DPH separated on TLC with the hexane/ether 97/3 (v/v) were analyzed with a 45° geometry. 12 μl of the 0.01 mol.l^{-1} BCD/DPH system was spotted on a 1.4x4 cm TLC plate. The resulting spots were excited at 380 nm and the emission spectra recorded from 540 nm to 390 nm.

RESULTS AND DISCUSSION

Spectrofluorimetric characteristics

The two spots on TLC resulting from the separation of the complex into bound and free DPH forms have similar emission spectra. The spectra are also analogous to DPH in hexane, cyclohexane and acetonitrile in solution with the emission maximum at around 430 nm (4). This data confirms that the fluorescent spots observed on the TLC plates are indeed DPH.

Linearity and sensibility precision

Figure 2 shows a chromatogram in which the free form of DPH was separated in the first dimension to R_f 0.5 with the low polar hexane/ether eluent in section B and C. The second dimension was eluted with the polar solvent only (first protocol). In this dimension dissociated and free DPH in section C both migrated to R_f 0.95. The densitometry study of the free samples in sections B and C show that both contain 16 picomoles DPH.

It must be noted that the calibration scale of standards E1 to E4 (10 to 40 pmoles) of DPH in pure acetonitrile does not allow the correct evaluation of all the spots after a polar elution. DPH's very high R_f value of 0.95 resulting from the polar eluent and a short migration distance, renders the differential of the emission intensity across spots E3 and E4 so high that the charge coupled devices saturate. The integration of the spots' density therefore underestimates their emission intensity. With this polar eluent, any calibration scale would be limited to 20 picomoles.

Placing the standards in the section (figure 3) where only the low polar eluent is used lowers the R_f value to 0.5 and avoids the under-evaluation obtained with the polar eluent. In this case, the regression for 0 to 50 picomoles DPH standards in pure acetonitrile is linear and runs through the origin ($R = 0.998$). The precision ranges from 0.4 at 10 picomoles (10 ± 0.4 picomoles) to 0.6 at 20 picomoles and increases to 1.4 at 50 picomoles. The detection limit is of the order of 3 picomoles.

Complex dissociation during the polar migration

To avoid the non linearity observed for the spots containing more than 20 picomoles free DPH, another elution dissociation method must be used.

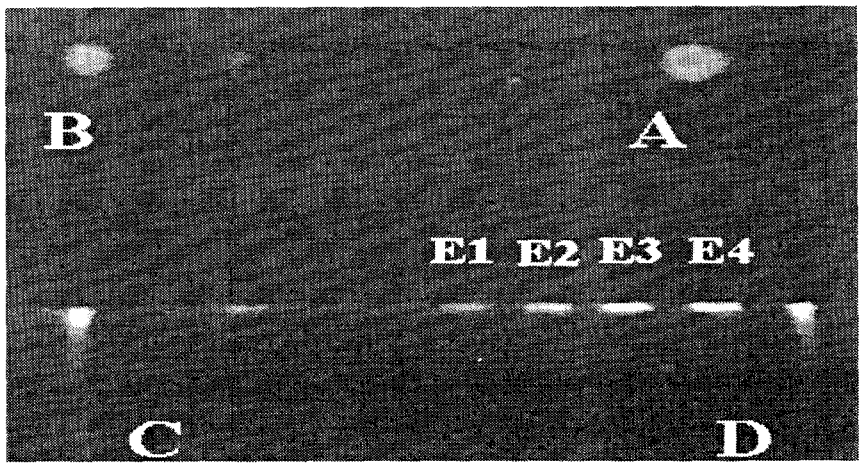

FIGURE 2. A CCD camera view of a two-dimensional TLC plate. The second migration was performed in one step following protocol 1.

Attempts to dissociate spotted samples of the 10 mM BCD/DPH/water/acetonitrile system with simple contact or by dipping the TLC plate in the polar mixture without elution did not work. However, DPH bound to the sugar was dissociated after a brief polar migration. The migration in the second dimension was therefore carried out in two steps (Figure 3).

After the short polar migration, the polyene was eluted with hexane/ether. The R_f value increased from 0.5 (characteristic of the low hexane/ether polar eluent used in the first dimension) to 0.8. This change indicated that either one or several of the polar agents remained on the TLC plate after it was dried.

Table 1 shows the balance of free and dissociated DPH after elution. Section B, eluted only once with the

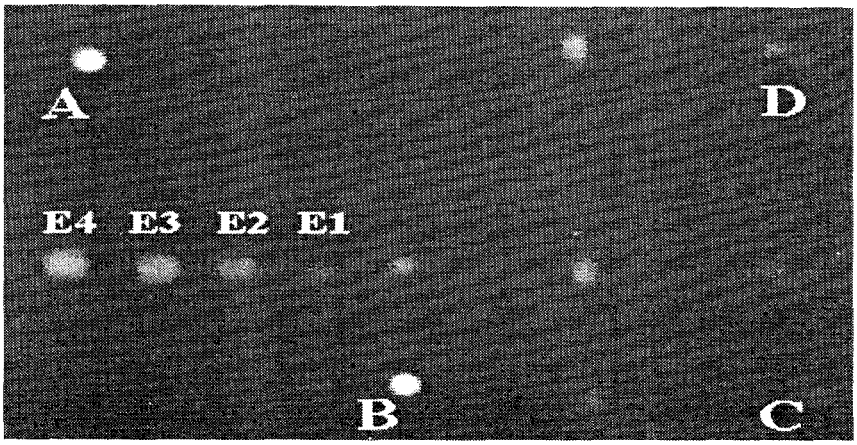

FIGURE 3. A CCD camera view of a two-dimensional TLC plate. The second migration was performed in two steps following protocol 2.

low polar eluent, resulted in 16.5 picomoles free DPH. Section C, eluted in two-dimensions, resulted in 17.2 picomoles free DPH and 12.4 picomoles dissociated DPH. The sum of these two unbound species gives a total of 29.6 picomoles DPH. In section D, eluted only in the second dimension, 28.8 picomoles free plus dissociated DPH were obtained. Despite the different treatments, the total quantities of free and dissociated DPH were conserved.

The good correlation between the value found for free DPH in section B and the value of free DPH in section C indicate that the quantum yield varies very little from one section to another.

It has been previously established (3) using U.V. absorption spectroscopy combined with TLC analysis that the total quantity of DPH bound to BCD (around

TABLE 1

Mass in picomoles of free and/or dissociated DPH separated from BCD in the various elution dimensions (data from Figure 3).

Sect.	elution*	free	dissociated	Total
B	1	16.5±0.6		
C	1+2	17.2±0.6	12.4±0.5	29.6±1.1(calculated)
D	2			28.8±0.9 (measured)

*elution dimension 1: with the low polar eluent, 2: brief polar dissociation followed by low polar elution.

22 picomoles) is greater than the free form. The dissociation is complete but not quantifiable when the elution is only performed with the polar eluent. However, with the double migration in the second dimension, the dissociation is incomplete. A certain quantity of the complex remained at the spotting position C. Supposing the emission quantum yield of this species is the same as that of free or dissociated DPH, then it would correspond to 9.7±0.4 picomoles. Therefore the two step protocol enables approximately a 50% dissociation of the complex.

CONCLUSION

In spite of technical problems associated specifically with the DPH-BCD system, this two-dimensional chromatography technique combined with CCD video analysis appears to be useful in the study of fluorescent cyclodextrin complexes. The method is sensitive, selective, and rapid. It may also be extended to the study of all complexes containing at least one fluorescent component.

REFERENCES

1 Connors, K.A., and Rosanske, T.W., J.Pharm.Sci., 68, 2, 1980.

2 Setzli, J., Cyclodextrins and their Inclusion Complexes, Akademiai Kiado, Budapest, 1982.

3 Guilleux, J.C., Barnouin, K.N., and Lerner, D.A., Anal.Chim.Acta, submitted.

4 Allen, M.T. and Whitten, D.G., Chem.Rev., 89, 1691, 1989.

5 Alak, A., Heilweil, E., Hinze, W.L., Oh, H., and Armstrong, D.W., J.Liq.Chromatogr., 7, 1273, 1984.

6 Floyd, R. and Steinberg, L., Int.Symp.Dig.Tech., Pap., 36, 1975.

7 Bayer, B.E., Int.Conf. Commun., Conf.Rec., 26, 11, 1973.

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OPTIMIZATION OF THE DETECTION WAVELENGTH APPLIED TO THE HPLC ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS

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ABSTRACT

Presented paper discuss a mathematical procedure which utilises the individual spectra of fifteen polyaromatic hydrocarbons for the optimization of the detection wavelength. Procedure is based upon use of weighting factors appropriate for the components under consideration (toxicological data). Two criteria have been used in the optimization procedure. If suggested criterion was used and all the components were of equal importance, then the optimum was at 220 and 254 nm respectively. When the weighting factors based on the toxicological data were employed, the optimum was found at 287 nm.

INTRODUCTION

The sensitivity of chromatographic method (observed peak height in a chromatogram) is an important aspect of the chromatographic process. It can be increased by the injection of larger volumes of samples, preconcentration on a pre-column in HPLC, by using a good column operated at the optimum flow rate [1] and selection of optimal

detection conditions. The use of programmable UV detectors which operate at wavelengths that are changed at pre-selected times [2-4] or the use of multiple wavelength detectors [5,7-9] represent an experimental way of increasing of the sensitivity of UV detection. The second way is mathematical optimization based on the optimization criteria [6,10,11].

The aim of this paper is to discuss the mathematical procedure which utilises the spectral data obtained by the UV photo diode array detector for the optimization of the detection wavelength from the toxicological point of view. The optimization has been applied to the chromatographic analysis of selected polycyclic aromatic hydrocarbons.

THEORY

Before any optimization, the goal of the process should be defined unambiguously. The goal of the optimization of detection wavelength of a group of components with different spectra is to obtain the areas of all peaks in a chromatogram as maximal as possible.

The area of Gaussian peak P_i can be related to the peak height at the maximum $h_{max,i}$ by the following equation [1]:

$$P_i = h_{\max,i} \cdot \sigma_i \cdot \sqrt{2 \cdot \pi} \qquad (1)$$

where σ_i is the standard deviation of a Gaussian peak i.

If the absorbance is the quantity measured by a detector, then $h_{max,i}$ can easily be expressed in terms of the absorptivity ε_i, concentration of the peak maximum $c_{max,i}$ and a width of a detection cell l.

$$h_{\max,i} = A_{\max,i} = \varepsilon_{\max,i} \cdot c_{\max,i} \cdot l \qquad (2)$$

where $A_{max,i}$ is the absorbance at the peak maximum.

Thus, equation (1) can be transformed to the following expression:

$$P_i = A_{\max,i} \cdot \sigma_i \cdot \sqrt{2 \cdot \pi} = \varepsilon_{\max,i} \cdot c_{\max,i} \cdot l \cdot \sqrt{2 \cdot \pi} \qquad (3)$$

As can be seen from equation (3), the area of the Gaussian peak depends linearly on the ε_i and therefore the area of a peak can be represented by the absorptivity ε or by the absorbance A.

Optimization criterion.

The elemental criterion *EC* can be defined by the following expression:

$$EC_i = \frac{\varepsilon_{i,\lambda_j}}{\varepsilon_{i,\lambda_{max}}} = \frac{A_{i,\lambda_j}}{A_{i,\lambda_{max}}} \quad (4)$$

where ε_i is the absorptivity of the component *i* at the wavelength λ_j which lies in the interval 200-400 nm and $\varepsilon_{i,\lambda max}$ is the absorptivity of component *i* at the wavelength of the main spectral maximum λ_{max} and $A_{i,\lambda j}$, $A_{i,\lambda max}$ are the absorbances in the spectrum. The criterion *C* can be written as:

$$C = \prod_{i=1}^{N} (EC_i)^{\alpha_i} \quad (5)$$

where *N* is the number of components and α_i is the weighting factor. By the proper choice of α_i one can optimize the detection wavelength for the peaks of interest. For the choice of the weighting factors, we have used the toxicological data of 15 polyaromatic hydrocarbons.

EXPERIMENTAL

Chromatographic system

Reversed phase HPLC was performed on a Waters Assoc. model 501 pumps with a Vydac - 5μm, C-18 column (length = 250 mm, inner diameter = 4.6 mm) and a Waters Assoc. photo diode array detector model 990. The composition of the gradient mobile phase is listed in Tab. 1.

Chemicals

The standards of 15 polycyclic aromatic hydrocarbons naphtalene (1), acenaphtylene (2), acenaphtene (3), fluorene (4), phenantrene (5), anthracene (6), fluoranthene (7), pyrene (8), benzo(a)anthracene (9), chrysene (10), benzo(b)fluoranthene (11), benzo(k)fluoranthene (12), benzo(a)pyrene (13), dibenz(a,h)anthracene (14),

Table 1. The Composition of the Gradient Mobile Phase

Time / min.	w(A) / %	w(B) / %
0	100	0
3	100	0
18	0	100
27	0	100
30	100	0

A = 50 % ^{V}/v acetonitrile in water, B = pure acetonitrile, flow rate = 1.4 ml / min..

benzo(g,h,i)perylene (15) were purchased from Supelco USA. The acetonitrile for gradient was from Merck, Germany. Standard solution was prepared by dissolving of the analytes in acetonitrile for gradient (0.1 mg.cm^{-3}).

Optimization procedure

One obvious advantage of the product criteria is that the result will be mainly determined by the smallest value of the elemental criterion EC_i. The value of EC_i lies in the interval (0-1) and it is zero if $A_{i,\lambda j}$ is equal to zero. All product criterion will be zero if any term EC_i is zero, hence the value of the absorptivity ε_i for the component i at λ_j is zero. During optimization process the maximum of criterion C has been found.

The second way is to maximize the minimal value of EC for the given set of compounds over whole range of wavelengths. The optimization method is shown in the next algorithm.

RESULTS AND DISCUSSION

In the first optimization procedure all peaks were considered to be of equal importance. The results of optimization are shown in Fig. 2-5. As can be seen from

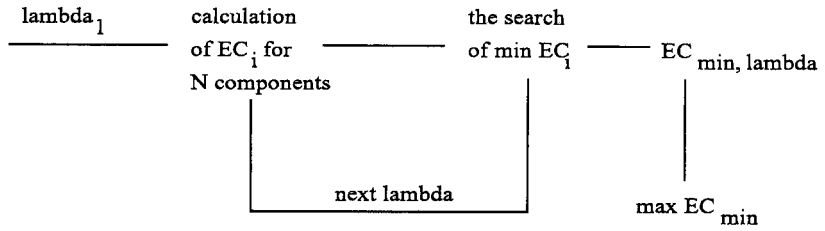
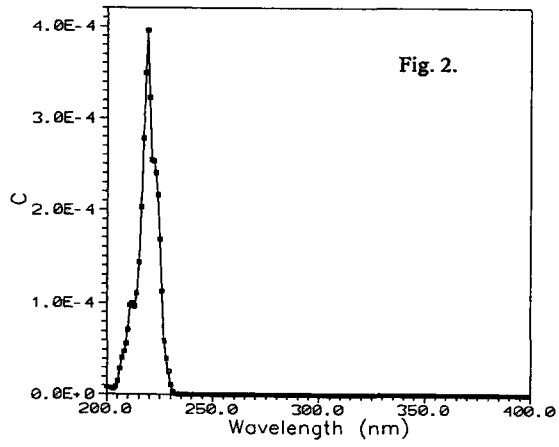


Figure 1. The algorithm of the optimization procedure.

Fig. 2, 4 the main maximum of criterion *C* resp. $maxEC_{min}$ was around 220 nm, but at this low wavelength some coeluting interferences could cause a reduction of selectivity. Therefore, we have considered the range of wavelengths over 250 nm. In Fig. 3 can be seen the detail of Fig.2 and the maximum of *C* was detected around 254 nm. This result is in agreement with the commonly used wavelength in HPLC for detection of polyaromatic hydrocarbons [13].

The toxicological characteristics of selected PAHs show that the health risks of individual PAHs are not at the same level, therefore in the optimization procedure they shouldn't be of equal importance. Many PAHs are known to be carcinogenic or cocarcinogenic as a result of oxidative reactions in the body [14]. In Table 2 the carcinogenities of 15 PAHs are summarised [15-17].

The polyaromatic hydrocarbons listed in Table 2 can be classified into 3 groups according to their carcinogenity. Naphtalene, acenaphtylene, acenaphtene, fluorene (group A) are relatively weak carcinogenic resp. non-carcinogenic (and also their carcinogenities are inadequately documented). Phenantrene, anthracene, fluoranthene, pyrene, chrysene and benzo(g,h,i)perylene (group B) are moderately active and benzo(a)anthracene, benzo(k) resp. benzo(b)fluoranthene, benzo(a)pyrene and dibenz(a,h)anthracene (group C) are the most dangerous carcinogens. We have assigned the following three levels of weighting factor α_i for the groups: A -0.01; B- 0.10; C- 1.00.



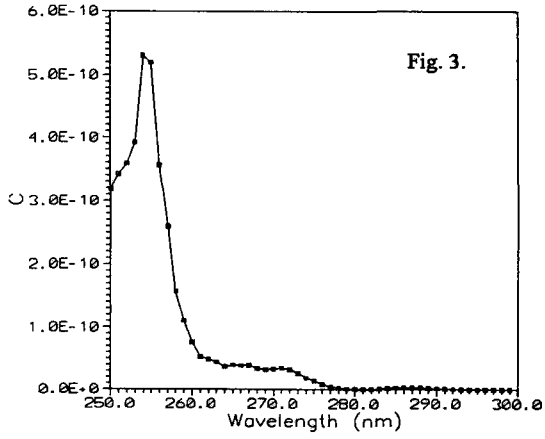


Fig. 2 - 5. The dependences of criterion C and EC_{min} on the wavelength.

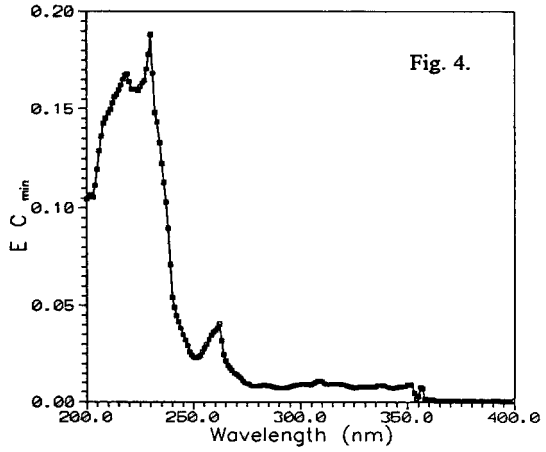

0.20
0.15
0.10
0.05
0.00
E C min
200.0
250.0
300.0
350.0
400.0
Wavelength (nm)

Fig. 4.

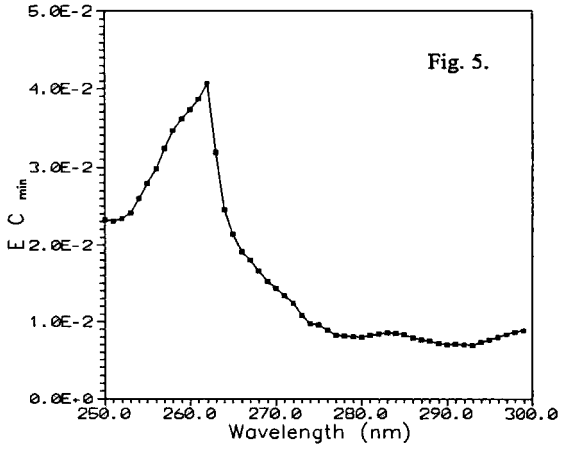

5.0E-2
4.0E-2
3.0E-2
2.0E-2
1.0E-2
0.0E+0
E C min
250.0
260.0
270.0
280.0
290.0
300.0
Wavelength (nm)

Fig. 5.

TABLE 2.The Toxicological characteristics of 15 PAHs

compound	classification according to p system	
	acute toxicity	carcinogenity
naphtalene	!C	?C
acenaphtylene	?B	??C
acenaphtene	?B	??C
fluorene	?B	?B
phenantrene	:C	?D
anthracene	:C	?D
fluoranthene	?C	?D
pyrene	?C	:D
benzo(a)anthracene	?C	!E
chrysene	?C	:D
benzo(b)fluoranthene	?C	:E
benzo(k)fluoranthene	?C	:E
benzo(a)pyrene	?C	!F
dibenz(a,h)anthracene	?C	!E
benzo(g,h,i)perylene	?C	:D

The reliability;!-strong, :-moderate, ?-weak, ??-very weak.

The activity; B - very weakly active,
C - weakly active,
D - active,
E - very active,
F - extremely active.

The results of the optimization procedure is shown in Fig 6. The maximum of *C* was detected around 287 nm. The significance of individual maxima (220, 254, 287 nm) are illustrated by the three chromatograms shown in Fig.7-9. Table 3 lists the values of normalized spectra (*EC*) of individual components at this maxima.

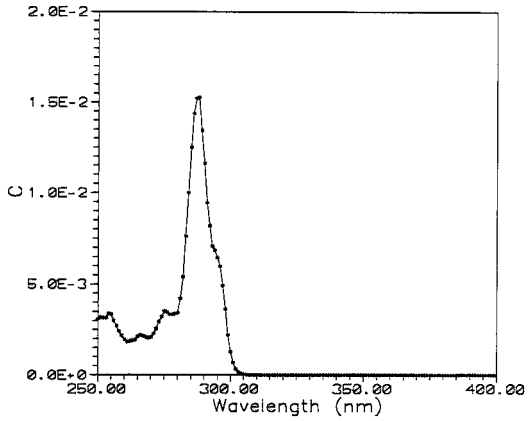


Fig. 6. The dependence of criterion C calculated with the toxicological weighting factors

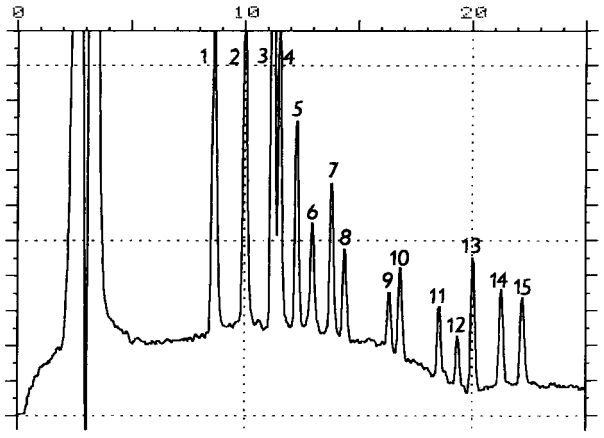


Fig. 7 The chromatogram of 15 PAHs corresponding to the maximum of C at 220 nm. (For the numbering of the peaks see experimental)

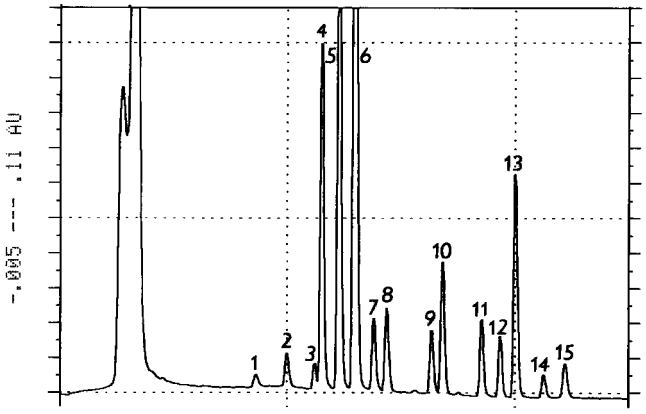


Fig. 8 The chromatogram of 15 PAHs recorded at 254 nm.

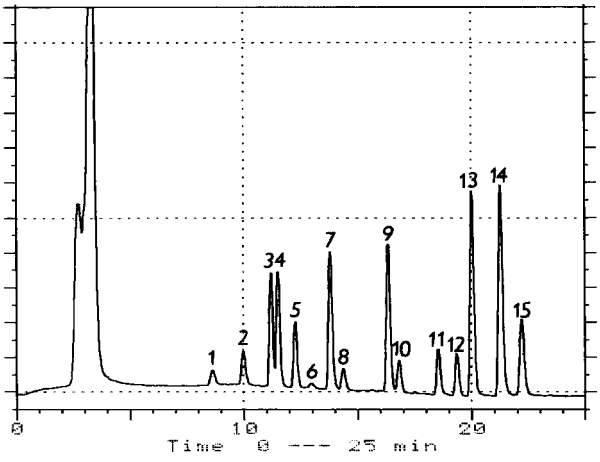


Fig. 9 The chromatogram of 15 PAHs recorded at 287 nm.

Table 3. The Normalized Spectra at 220, 254, 287 nm

compound	EC_{220}	EC_{254}	EC_{287}
naphtalene	1.00	0.04	0.06
acenaphtylene	0.62	0.06	0.06
acenaphtene	0.68	0.02	0.10
fluorene	0.47	0.37	0.13
phenantrene	0.52	0.95	0.12
anthracene	0.17	0.95	0.01
fluoranthene	0.85	0.26	0.50
pyrene	0.37	0.18	0.05
benzo(a)anthracene	0.85	0.41	0.96
chrysene	0.49	0.38	0.10
benzo(b)fluoranthene	0.99	0.57	0.34
benzo(k)fluoranthene	0.80	0.55	0.34
benzo(a)pyrene	0.60	0.78	0.78
dibenz(a,h)anthracene	0.47	0.06	0.74
benzo(g,h,i)perylene	0.75	0.18	0.43

CONCLUSION

We have used the two criteria for the optimization of the detection wavelength applied to the analysis of polyaromatic hydrocarbons. If EC_{min} is used as the function describing the sensitivity of the detection, then no attention is paid to all but one component in a chromatogram which is a disadvantage of this criterion. The criterion C evaluates the suitability of the detection wavelength more realistically. The obtained results confirmed the optimum commonly used wavelength used in HPLC of polyaromatic hydrocarbons (254 nm). If the weighting factors based on the toxicological characteristics of tested PAHs are used, then the optimum was found at 287 nm. The sensitivity of detection for selected polyaromatic hydrocarbons (group C) after optimization (287 nm) in compare with sensitivity at 254 nm was approximately the same for benzo(b),

benzo(k)fluoranthene and benzo(a)pyrene, two times higher for benzo(a)anthracene and ten times higher for dibenz(a,h)anthracene.

REFERENCES

1. P. J. Schoenmakers - OPTIMIZATION OF CHROMATOGRAPHIC SELECTIVITY, Elsevier, pp.305, 1986.
2. A. Marcomini, A. Sfriso, B. Pavoni, Mar.Chem., 21 (1): 15-23, 1987.
3. R. W. Roos, C.A. Lau-Cam, J.Chromatogr., 370:, 403-418, 1986.
4. R. Ohmacht, Z. Matus, J. Soos, Chromatographia, 32: 85-86, 1991.
5. I. Gagliardi, G. Cavazzutti, I. Montanarella, D. Tonelli, J.Chromatogr., 464: 428-443, 1989.
6. G. Wright, A.F. Fell, J.C. Berridge, Chromatographia, 24: 533-540, 1987.
7. H. G. Kicinski, A. Ketrupp, Vom Wasser, 71: 245-254, 1988
8. T. Hondo, M. Kuwajima, Y. Sato, K. Okamura, Y. Tanaka, H. Eda, Y. Okada, Anal.Sci., 7: 235-239, 1991.
9. L. Y. Lin, W.T. Cooper, J.Chromatogr., 390: 285-295, 1987.
10. Y. Hayashi, R. Matsuda, Chromatographia, 31: 374-380, 1991.
11. Y. Hayashi, R. Matsuda, Anal.Sci., 5: 459-464, 1989.
12. Y. Hayashi, R. Matsuda, Chromatographia, 30: 85-90, 1990.
13. W. Gerlich, G. Martin, H. Paming, Labor Praxis, 942, 1991.
14. POLYNUCLEAR AROMATIC HYDROCARBONS - 2 International symposium on analysis, chemistry and biology Vol.3., Raven Press, pp.139.
15. POLYNUCLEAR AROMATIC HYDROCARBONS - 2 International symposium on analysis, chemistry and biology Vol.3., Raven Press, pp.278.
16. J. Marhold, PŘEHLED PRŮMYSLOVÉ TOXIKOLOGIE, Svazek 1, Avicenum Praha, pp.48-73.
17. IARC monographs, Vol.54, 1987, Lyon.

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APPLICATION OF SOLVATOCHROMIC PARAMETERS TO SELECTIVITY TUNING IN CHROMATOGRAPHY FOR AROMATIC SOLUTES

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ABSTRACT

Solvatochromic parameters are the best descriptors of solute retention in RPLC. Accurate prediction of capacity factors for 26 solutes is obtained with binary mixtures of organic modifier/water in the vol/vol range 10-100%. Example of application is given.

INTRODUCTION

The exact mechanism of retention in reversed-phase liquid chromatography (RPLC) has been the subject of much controversy and debate. Ideally, knowledge of this mechanism would allow a priori prediction of retention times regardless of the column being used, as well as computer-based optimization of a given separation. The difficulty in elucidating the mechanism of retention lies in the numerous interactions that a solute may undergo in both stationary and mobile phases.

Retention is closely related to solute shape and ability to form weak complexes. In order to describe molecular properties of a solute, it is necessary that those parameters should exhibit additive and constitutive properties which can be obtained not only from experimental measurements but also from theoretical calculations. The descriptors which satisfy the above requirements were selected from many published sources and are as follows:

- **LogP**, the logarithm of the partition coefficient in 1-octanol-water, which is a measure of the hydrophobicity of the molecule [1,2]. LogP is determined from shake flask or slow stirring[3]. Some data are from RPLC and cannot be considered.

- **RI**, retention index of Burr and Smith[4-9]. The basis of the prediction system proposed in their study is that the retention index of an analyte in a selected eluent can be calculated by the summation of the retention index of a parent compoud(PI), substituent index values (SI) for each substituent plus terms required to describe interactions between substituents (interaction indices II, i.e., hydrogen bonding, steric and electronic interaction). The retention index of a compound can then be determined as

$$RI = PI + SI_R + \sum SI_{Ar\text{-}X} + \sum SI_{R\text{-}X} + \sum II_{YZ}$$

PI : Retention index value of a parent compound

SI_R : The retention index contribution from saturated aliphatic carbons

$\Sigma SI_{AR\text{-}X}$: Substituent index values for substituents on an aromatic ring

$\Sigma SI_{R\text{-}X}$: Substituent index values for subtituents on saturated aliphatic carbons

ΣII_{YZ} : Interaction index values between substituents to account for H-bonding, and electronic effects.

- Van der Waals volume, V_w, and Van der Waals surface area, A_w, calculated from the Van der Waals radii of the atoms of which the molecule is composed[10].

- Molecular connectivity index, χ, which measures the topological size of the molecule and its degree of branching[11].

- Correlation factor, **F**, calculated as (number of double bonds) + (number of primary and secondary carbon atoms) - 0.5 for a non-aromatic ring[12,13].

- Solvatochromic parameters proposed by Kamlet et al[14-16] which are :

. V_i the cavity term, is a measure of solute volume and may be V, the liquid molar volume, taken as the solute molecular weight divided by its liquid density at 20°C, or Vi, the intrinsic (Van der Waals) molar volume which can be either computer calculated or estimated by simple additivity methods like that of McGowan[17].

. π^* solvatochromic parameter is a measure of solute dipolarity/polarizability.

. β and α solvatochromic parameters are a measure of solute basicity (hydrogen bond acceptor) and acidity (hydrogen bond donor), respectively.

Recently the linear solvation energy relationship (LSER) based on the Kamlet Taft multiparameter scale has been successfuly used to model retention in RPHPLC[18,19].

In our aproach, described in previous works [20-22], we consider the phenomenon as a dark box (Fig. 1) and we try to correlate the relevant factors to the answer but no theoretical retention model is considered as the right one.

The purpose of this paper is to select those factors which can permit the determination of accurate answers.

CHOICE OF THE DESCRIPTORS

To avoid duplication of information that is provided by the variables, we must perform relevant choice of the solute descriptors from those enumerated above.

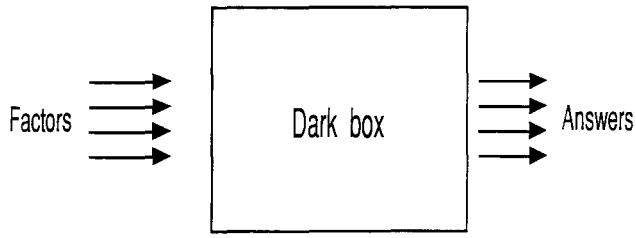


FIGURE 1. Adopted approach

logP and logk' are varying linearly with Vi/100, π^*, β and α according to:

$$\log P = b_0 + b_1 Vi/100 + b_2 \pi^* + b_3 \beta + b_4 \alpha \quad (16) \qquad (1)$$

$$\log k' = b'_0 + b'_1 Vi/100 + b'_2 \pi^* + b'_3 \beta + b'_4 \alpha \quad (19) \qquad (2)$$

with a fixed percentage of organic modifier which implies a similar equation for RI. Since $\log k' = a_0 + a_1 RI$ (9) (3)

we can write : $RI = b''_0 + b''_1 Vi/100 + b''_2 \pi^* + b''_3 \beta + b''_4 \alpha$ (4)

Subsequently we will discard logP and RI from the solute descriptors.

From a literature survey we could retrieve some relevant data which are compiled in Table 1. It must be pointed out that solutes are all aromatic compounds.

From Table 1 we can construct the following correlation matrix(24) (Table 2).

The scrutinizing of the off-diagonal elements of the correlation matrix, reveals the independence of the four descriptors (Vi/100, π^*, β and α) and the dependence between Vi/100 and the variables (χ , Vw, Aw and F). From these conclusions we can select the four variables Vi/100, π^*, β and α as solute descriptors only (Fig 2).

RESULTS AND DISCUSSION

From many experimental data it has been demonstrated(25-28) that lnk' varies quadratically with the percent of organic modifier :

$$\ln k' = A \Phi^2 + B \Phi + C \qquad (5)$$

Φ is the percent of organic modifier.

At a constant percentage of organic modifier many researchers found a linear relationship between lnk' and the four factors Vi/100, π^*, β, α (19,29,30) :

$$\ln k' = b_0 + b_1 (Vi/100) + b_2 \pi^* + b_3 \beta + b_4 \alpha. \qquad (6)$$

To take into account interactions between solute and mobile phase the general model can be obtained by forming the product of the two models proposed above:

$$\ln k' = (b_0 + b_1 (Vi/100) + b_2 \pi^* + b_3 \beta + b_4 \alpha) * (A \Phi^2 + B \Phi + C) \quad (7)$$

which is equivalent to :

TABLE 1
Selected Solute Descriptors in RPLC from Literature Data.

	Solute descriptors							
Solute	**F**	**χ**	**V_w**	**A_w**	**V_i/100**	**π***	**β**	**α**
Benzene	3	2.00	48.36	6.01	0.49	0.59	0.10	0.00
Toluene	4	2.41	59.51	7.45	0.59	0.55	0.11	0.00
Ethylbenzene	5	2.97	69.74	8.80	0.67	0.53	0.12	0.00
o-Xylene	5	2.83	70.66	8.89	0.67	0.51	0.12	0.00
m-Xylene	5	2.82	70.66	8.89	0.67	0.51	0.12	0.00
n-Propylbenzene	6	3.47	79.97	10.15	0.77	0.51	0.12	0.00
Isopropylbenzene	5	3.35	79.96	10.14	0.77	0.51	0.12	0.00
n-Butylbenzene	7	3.97	90.20	11.50	0.87	0.49	0.12	0.00
p-Cymene	6	3.77	91.11	11.58	0.87	0.47	0.13	0.00
1,2,4,5-Tetramethylbenzene	7	3.66	92.96	11.77	0.87	0.43	0.15	0.00
Naphtalene	5	3.41	73.96	8.42	0.75	0.70	0.15	0.00
Acenaphthene	6.5	4.45	87.88	9.54	0.92	0.66	0.17	0.00
Anthracene	7	4.81	99.56	10.84	1.02	0.80	0.20	0.00
Phenanthrene	7	4.82	99.56	10.84	1.02	0.80	0.20	0.00
Pyrene	8	5.56	109.04	11.26	1.16	0.90	0.25	0.00
Benz(a)anthracene	9	6.22	125.16	13.26	1.28	0.90	0.25	0.00
Aniline	3	2.20	56.38	7.07	0.56	0.73	0.50	0.26
N-Ethylaniline	5	3.22	77.82	9.79	0.76	0.82	0.47	0.17
Benzaldehyde	3	2.44	60.06	7.61	0.61	0.92	0.44	0.00
Benzonitrile	3	2.38	60.54	7.52	0.59	0.90	0.37	0.00
Nitrobenzene	3	2.45	62.64	7.88	0.63	1.01	0.30	0.00
Anisole	4	2.52	62.71	7.99	0.639	0.73	0.32	0.00
Acetophenone	4	2.87	71.21	9.05	0.69	0.90	0.49	0.04
Methyl benzoate	4	2.98	76.73	9.83	0.74	0.75	0.39	0.00
m-Chloroaniline	3	2.71	65.86	8.20	0.65	0.78	0.40	0.31

Data of χ , Vw, Aw and F are taken from ref 23 and data of Vi/100, π*, β and α are taken from ref 16.

TABLE 2
Correlation Matrix

	F	χ	V_w	A_w	$V_j/100$	π^*	β	α
F	1.000							
χ	0.937	1.000						
V_w	0.950	0.975	1.000					
A_w	0.904	0.859	0.947	1.000				
$V_j/100$	**0.934**	**0.994**	**0.989**	**0.891**	**1.000**			
π^*	-0.167	0.127	0.048	-0.127	0.141	1.000		
β	-0.441	-0.242	-0.235	-0.254	-0.201	0.723	1.000	
α	-0.357	-0.254	-0.257	-0.255	-0.246	-0.182	0.585	1.000

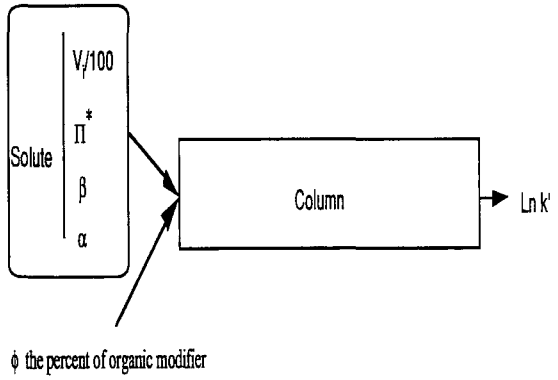


FIGURE 2. Selected factors

TABLE 3
Solvatochromic Parameters Values of 15 Solutes Taken from ref. 16

	Solute	$V_I/100$	π^*	β	α	Φ
1	Acetophenone	0.690	0.90	0.49	0.04	10
2	Aniline	0.562	0.73	0.50	0.26	10
3	Anisole	0.639	0.73	0.32	0.00	10
4	Anthracene	1.015	0.80	0.20	0.00	10
5	Benzaldehyde	0.606	0.92	0.44	0.00	10
6	Benzene	0.491	0.59	0.10	0.00	10
7	Benzonitrile	0.590	0.90	0.37	0.00	10
8	Benzyl alcohol	0.634	0.99	0.52	0.39	10
9	Chlorobenzene	0.581	0.71	0.07	0.00	10
10	Ethylbenzene	0.668	0.53	0.12	0.00	10
11	Nitrobenzene	0.631	1.01	0.30	0.00	10
12	Phenol	0.536	0.72	0.33	0.61	10
13	2-Phenylethanol	0.732	0.97	0.55	0.33	10
14	3-Phenylpropanol	0.830	0.95	0.55	0.33	10
15	Toluene	0.592	0.55	0.11	0.00	10
16	Acetophenone	0.690	0.90	0.49	0.04	32.5
17	Aniline	0.562	0.73	0.50	0.26	32.5
18	Anisole	0.639	0.73	0.32	0.00	32.5
19	Anthracene	1.015	0.80	0.20	0.00	32.5
20	Benzaldehyde	0.606	0.92	0.44	0.00	32.5
21	Benzene	0.491	0.59	0.10	0.00	32.5
22	Benzonitrile	0.590	0.90	0.37	0.00	32.5
23	Benzyl alcohol	0.634	0.99	0.52	0.39	32.5
24	Chlorobenzene	0.581	0.71	0.07	0.00	32.5
25	Ethylbenzene	0.668	0.53	0.12	0.00	32.5
26	Nitrobenzene	0.631	1.01	0.30	0.00	32.5
27	Phenol	0.536	0.72	0.33	0.61	32.5
28	2-Phenylethanol	0.732	0.97	0.55	0.33	32.5
29	3-Phenylpropanol	0.830	0.95	0.55	0.33	32.5
30	Toluene	0.592	0.55	0.11	0.00	32.5
31	Acetophenone	0.690	0.90	0.49	0.04	55
32	Aniline	0.562	0.73	0.50	0.26	55
33	Anisole	0.639	0.73	0.32	0.00	55
34	Anthracene	1.015	0.80	0.20	0.00	55
35	Benzaldehyde	0.606	0.92	0.44	0.00	55
36	Benzene	0.491	0.59	0.10	0.00	55
37	Benzonitrile	0.590	0.90	0.37	0.00	55
38	Benzyl alcohol	0.634	0.99	0.52	0.39	55
39	Chlorobenzene	0.581	0.71	0.07	0.00	55
40	Ethylbenzene	0.668	0.53	0.12	0.00	55
41	Nitrobenzene	0.631	1.01	0.30	0.00	55
42	Phenol	0.536	0.72	0.33	0.61	55
43	2-Phenylethanol	0.732	0.97	0.55	0.33	55
44	3-Phenylpropanol	0.830	0.95	0.55	0.33	55
45	Toluene	0.592	0.55	0.11	0.00	55

(continued)

TABLE 3 (Continued)
Solvatochromic Parameters Values of 15 Solutes Taken from ref. 16

	Solute	V_i /100	π^*	β	α	Φ
46	Acetophenone	0.690	0.90	0.49	0.04	77.5
47	Aniline	0.562	0.73	0.50	0.26	77.5
48	Anisole	0.639	0.73	0.32	0.00	77.5
49	Anthracene	1.015	0.80	0.20	0.00	77.5
50	Benzaldehyde	0.606	0.92	0.44	0.00	77.5
51	Benzene	0.491	0.59	0.10	0.00	77.5
52	Benzonitrile	0.590	0.90	0.37	0.00	77.5
53	Benzyl alcohol	0.634	0.99	0.52	0.39	77.5
54	Chlorobenzene	0.581	0.71	0.07	0.00	77.5
55	Ethylbenzene	0.668	0.53	0.12	0.00	77.5
56	Nitrobenzene	0.631	1.01	0.30	0.00	77.5
57	Phenol	0.536	0.72	0.33	0.61	77.5
58	2-Phenylethanol	0.732	0.97	0.55	0.33	77.5
59	3-Phenylpropanol	0.830	0.95	0.55	0.33	77.5
60	Toluene	0.592	0.55	0.11	0.00	77.5
61	Acetophenone	0.690	0.90	0.49	0.04	100
62	Aniline	0.562	0.73	0.50	0.26	100
63	Anisole	0.639	0.73	0.32	0.00	100
64	Anthracene	1.015	0.80	0.20	0.00	100
65	Benzaldehyde	0.606	0.92	0.44	0.00	100
66	Benzene	0.491	0.59	0.10	0.00	100
67	Benzonitrile	0.590	0.90	0.37	0.00	100
68	Benzyl alcohol	0.634	0.99	0.52	0.39	100
69	Chlorobenzene	0.581	0.71	0.07	0.00	100
70	Ethylbenzene	0.668	0.53	0.12	0.00	100
71	Nitrobenzene	0.631	1.01	0.30	0.00	100
72	Phenol	0.536	0.72	0.33	0.61	100
73	2-Phenylethanol	0.732	0.97	0.55	0.33	100
74	3-Phenylpropanol	0.830	0.95	0.55	0.33	100
75	Toluene	0.592	0.55	0.11	0.00	100

$$\ln k' = b_0' + b_1'\left(\frac{V_i}{100}\right) + b_2'\pi^* + b_3'\beta + b_4'\alpha + \left[b_0'' + b_1''\left(\frac{V_i}{100}\right) + b_2''\pi^* + b_3''\beta + b_4''\alpha\right]\Phi + \left[b_0''' + b_1'''\left(\frac{V_i}{100}\right) + b_2'''\pi^* + b_3'''\beta + b_4'''\alpha\right]\Phi^2. \quad (8)$$

From the data of Table 3 we have the following experimental domain :

TABLE 4
Experimental Domain

Natural variables	Center	Range of variation
$V_i/100$	0.753	0.262
π^*	0.770	0.240
β	0.310	0.240
α	0.305	0.305
Φ	0.550	0.450

$$\text{Center} = \frac{\text{Highest natural variable limit} + \text{Lowest natural variable limit}}{2}$$

$$\text{Range of variation} = \frac{\text{Highest natural variable limit} - \text{Lowest natural variable limit}}{2}$$

It must be pointed out that the lowest limit of Φ is 10%. Beyond this value retention is by far too high and our purpose is not to determine $\log K_w$ values. $V_i/100$, π^*, β, α and Φ are the natural variables which can be transformed into coded variables X_1, X_2, X_3, X_4, X_5 respectively by the relation:

$$X_i = \frac{\text{natural variable - center}}{\text{range of variation, } r_i}$$

which yield the following relationship of lnk' versus the five parameters:

$$\ln k' = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + [\, b_5 + b_{15}X_1 + b_{25}X_2 + b_{35}X_3 + b_{45}X_4] \; X_5 + [\, b_{55} + b_{155}X_1 + b_{255}X_2 + b_{355}X_3 + b_{455}X_4] \; X_5^2$$

TABLE 5
Matrix with coded variables

N° of experiment	Coded Variables					Answer values taken from ref. 26		
	X1	X2	X3	X4	X5	lnk' MeOH	lnk' MeCN	lnk' THF
1	-0.24	0.54	0.75	-0.87	-1	4.55	3.29	2.71
2	-0.73	-0.17	0.79	-0.15	-1	2.61	2.40	2.36
3	-0.44	-0.17	0.04	-1	-1	7.97	4.21	4.25
4	1	0.13	-0.46	-1	-1	11.58	6.51	6.56
5	-0.56	0.63	0.54	-1	-1	3.53	3.01	2.56
6	-1	-0.75	-0.87	-1	-1	4.50	4.16	4.38
7	-0.62	0.54	0.25	-1	-1	3.95	3.36	2.96
8	-0.45	0.92	0.87	0.28	-1	3.00	2.27	1.87
9	-0.66	-0.25	-1	-1	-1	7.28	5.33	5.48
10	-0.32	-1	-0.79	-1	-1	7.55	5.71	5.53
11	-0.47	1	-0.04	-1	-1	4.23	3.82	4.11
12	-0.83	-0.21	0.08	1	-1	2.82	2.52	2.94
13	-0.08	0.83	1	0.08	-1	3.90	3.06	2.51
14	0.29	0.75	1	0.08	-1	5.10	3.67	3.52
15	-0.61	-0.92	-0.83	-1	-1	6.80	5.19	5.28
16	-0.24	0.54	0.75	-0.87	-0.5	2.65	1.63	0.99
17	-0.73	-0.17	0.79	-0.15	-0.5	1.42	0.98	1.12
18	-0.44	-0.17	0.04	-1	-0.5	4.57	2.53	2.00
19	1	0.13	-0.46	-1	-0.5	8.75	4.61	3.36
20	-0.56	0.63	0.54	-1	-0.5	2.14	1.52	0.99
21	-1	-0.75	-0.87	-1	-0.5	3.19	2.48	2.21
22	-0.62	0.54	0.25	-1	-0.5	2.26	1.73	1.21
23	-0.45	0.92	0.87	0.28	-0.5	1.61	0.67	0.38
24	-0.66	-0.25	-1	-1	-0.5	4.82	3.24	2.73
25	-0.32	-1	-0.79	-1	-0.5	5.32	3.68	3.01
26	-0.47	1	-0.04	-1	-0.5	2.75	2.11	1.82
27	-0.83	-0.21	0.08	1	-0.5	1.52	0.91	1.16
28	-0.08	0.83	1	0.08	-0.5	2.24	1.08	0.66
29	0.29	0.75	1	0.08	-0.5	3.11	1.57	1.18
30	-0.61	-0.92	-0.83	-1	-0.5	4.60	3.21	2.75
31	-0.24	0.54	0.75	-0.87	0	1.13	0.40	-0.28
32	-0.73	-0.17	0.79	-0.15	0	0.41	-0.03	0.01
33	-0.44	-0.17	0.04	-1	0	2.05	1.21	0.32
34	1	0.13	-0.46	-1	0	5.91	2.95	0.96
35	-0.56	0.63	0.54	-1	0	0.94	0.37	-0.21
36	-1	-0.75	-0.87	-1	0	1.91	1.13	0.54
37	-0.62	0.54	0.25	-1	0	0.89	0.47	-0.13
38	-0.45	0.92	0.87	0.28	0	0.48	-0.39	-0.67
39	-0.66	-0.25	-1	-1	0	2.72	1.58	0.69

TABLE 5 (Continued)
Matrix with coded variables

N° of experiment	Coded Variables					Answer values taken from ref. 26		
	X1	X2	X3	X4	X5	lnk' MeOH	lnk' MeCN	lnk' THF
40	-0.32	-1	-0.79	-1	0	3.28	2.00	1.04
41	-0.47	1	-0.04	-1	0	1.41	0.75	0.14
42	-0.83	-0.21	0.08	1	0	0.43	-0.21	-0.19
43	-0.08	0.83	1	0.08	0	0.87	-0.22	-0.63
44	0.29	0.75	1	0.08	0	1.44	0.14	-0.43
45	-0.61	-0.92	-0.83	-1	0	2.68	1.61	0.82
46	-0.24	0.54	0.75	-0.87	0.5	-0.01	-0.40	-1.09
47	-0.73	-0.17	0.79	-0.15	0.5	-0.41	-0.63	-0.97
48	-0.44	-0.17	0.04	-1	0.5	0.39	0.25	-0.80
49	1	0.13	-0.46	-1	0.5	3.08	1.50	-0.64
50	-0.56	0.63	0.54	-1	0.5	-0.07	-0.42	-1.04
51	-1	-0.75	-0.87	-1	0.5	0.66	0.10	-0.62
52	-0.62	0.54	0.25	-1	0.5	-0.18	-0.41	-1.05
53	-0.45	0.92	0.87	0.28	0.5	-0.40	-0.91	-1.31
54	-0.66	-0.25	-1	-1	0.5	0.98	0.35	-0.65
55	-0.32	-1	-0.79	-1	0.5	1.42	0.68	-0.40
56	-0.47	1	-0.04	-1	0.5	0.22	-0.25	-0.94
57	-0.83	-0.21	0.08	1	0.5	-0.44	-0.84	-1.11
58	-0.08	0.83	1	0.08	0.5	-0.20	-0.83	-1.34
59	0.29	0.75	1	0.08	0.5	0.09	-0.61	-1.30
60	-0.61	-0.92	-0.83	-1	0.5	1.04	0.40	-0.49
61	-0.24	0.54	0.75	-0.87	1	-0.77	-0.77	-1.46
62	-0.73	-0.17	0.79	-0.15	1	-1.05	-0.83	-1.82
63	-0.44	-0.17	0.04	-1	1	-0.41	-0.33	-1.35
64	1	0.13	-0.46	-1	1	0.24	0.28	-1.43
65	-0.56	0.63	0.54	-1	1	-0.89	-0.86	-1.50
66	-1	-0.75	-0.87	-1	1	-0.56	-0.60	-1.27
67	-0.62	0.54	0.25	-1	1	-0.95	-0.91	-1.56
68	-0.45	0.92	0.87	0.28	1	-1.01	-0.88	-1.52
69	-0.66	-0.25	-1	-1	1	-0.40	-0.44	-1.28
70	-0.32	-1	-0.79	-1	1	-0.24	-0.30	-1.28
71	-0.47	1	-0.04	-1	1	-0.82	-0.90	-1.42
72	-0.83	-0.21	0.08	1	1	-1.09	-0.99	-1.61
73	-0.08	0.83	1	0.08	1	-0.97	-0.75	-1.48
74	0.29	0.75	1	0.08	1	-0.94	-0.68	-1.45
75	-0.61	-0.92	-0.83	-1	1	-0.33	-0.43	-1.20

According to the characteristics of the chosen matrix (see appendix n°1) this matrix cannot permit the calculation of the model coefficients with inflation factors greater than 7[31-34] and the term b_{355} should be discarded:

$$\begin{aligned} \ln k' = {} & b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 \\ & + [\, b_5 + b_{15}X_1 + b_{25}X_2 + b_{35}X_3 + b_{45}X_4] \;\; X_5 \\ & + [\, b_{55} + b_{155}X_1 + b_{255}X_2 + b_{455}X_4] \;\; X_5^2 \end{aligned} \qquad (10)$$

Since methanol, acetonitrile and tetrahydrofran represent the usual organic modifiers we calculated the relationship between lnk' and the 5 factors for these 3 solvents.

Equations are as follows :

- in methanol-water (see appendix n°2):

$$\begin{aligned} \ln k'_M = {} & 2.582 + 2.255\,X_1 - 0.478\,X_2 - 0.994\,X_3 - 0.342\,X_4 \\ & + [\, - 3.538 - 1.651\,X_1 + 0.359\,X_2 + 0.591\,X_3 + 0.305\,X_4] \;\; X_5 \\ & + [0.453 \;\; - 0.125\,X_1 + 0.067\,X_2 - 0.049\,X_4] \;\; X_5^2 \end{aligned} \qquad (11)$$

According to the results of residual plot and the normal plot (see appendix n°2) we observe high deviation between calculated and observed anisol capacity factor at methanol-water (10/90) vol/vol.

$$\ln k'_{exp} - \ln k'_{cal} = 7.96\text{-}5.68 = 2.28. \qquad (12)$$

In fact, this value can be discarded since we do not work with k'= exp(7.96) = 2864 because the analysis time is beyond usual range, and this experiment can be withdrawn from the matrix without introducing significant changes to the coefficient values of the postulated model (see appendix n°3).

- in acetonitrile-water (see appendix n°4):

$$\begin{aligned} \ln k'_A = {} & 1.01 + 1.05\,X_1 - 0.34\,X_2 - 0.67\,X_3 - 0.47\,X_4 \\ & + [\, - 2.41 - 0.50\,X_1 + 0.04\,X_2 + 0.51\,X_3 + 0.16\,X_4] \;\; X_5 \\ & + [\, 0.98 - 0.01\,X_1 + 0.11\,X_2 + 0.24\,X_4] \;\; X_5^2 \,. \end{aligned} \qquad (13)$$

- in tetrahydrofuran-water (see appendix n°5):

$$\ln k'_T = 0.28 + 0.33\,X_1 - 0.17\,X_2 - 0.64\,X_3 - 0.10\,X_4 + [\,-2.95 - 0.73\,X_1 + 0.12\,X_2 + 0.72\,X_3 + 0.04\,X_4]\;X_5 + [\,1.18 + 0.44\,X_1 - 0.09\,X_2 - 0.09\,X_4]\;X_5^2 . \quad (14)$$

To check the weight of the different coefficients we performed a Pareto analysis. Plots of the contribution of every term are displayed in Figure 3.

The percentage effect P_i of every term i, is calculated through[35] :

$$P_i = 100\left(\frac{b_i^2}{\sum b_j^2}\right) \quad (15)$$

We can conclude from these plots:

- that the effect of the percentage of the modifier increases from methanol-water (66%) to acetonitrile-water (74%) and from acetonitrile-water (74%) to tetrahydrofuran-water (84%) according to the eluting strength.

- the terms X_5, X_1, X_{15} explain 91 % (65.65+ 15.26 + 10.01) of the lnk' variation with methanol-water as mobile phase,

- the terms X_5, X_1, X_3, X_{55} explain 92 % of the lnk' variation with acetonitrile-water as mobile phase,

- the terms X_5, X_{55}, X_3 explain 92 % of the lnk' variation with tetrahydrofuran-water as mobile phase.

The cavity term $V_i/100$ (X_1) is less important with tetrahydrofuran as modifier than it is with methanol and acetonitrile.

EXAMPLE OF THE MODEL APPLICATION

Let take as an example the separation of ethylbenzene from benzene with a column Hibar 100 RP-18 (Merck) endcapped (packed with Lichrospher 5µm) (250*4mm). Although this separation is not very exciting data are readily available and methodology may successfully be extended to any other pair of solutes.

The resolution R_s[36] can be written as :

$$R_s = \frac{\alpha-1}{\alpha+1} \cdot \frac{\bar{k}}{1+\bar{k}} \cdot \frac{\sqrt{N}}{2} . \quad (16)$$

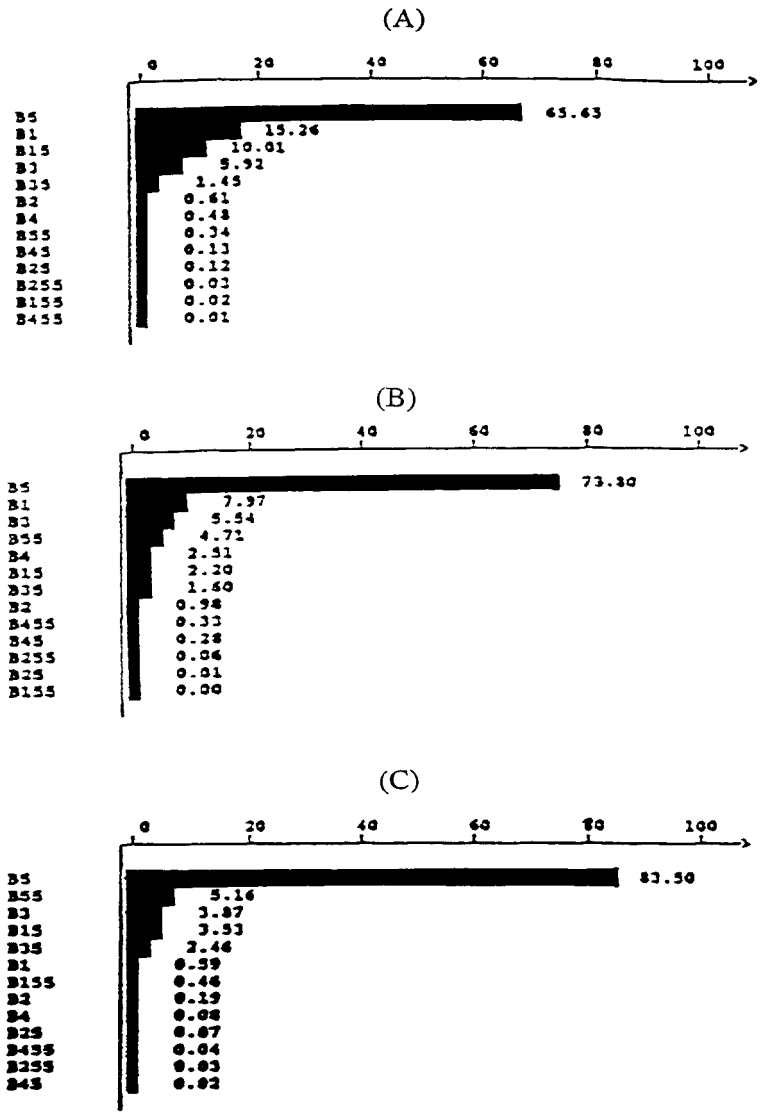


FIGURE 3. Pareto analysis

(A) methanol /water as eluent ; (B) acetonitrile /water as eluent ;

(C) tetrahydrofuran /water as eluent.

where :

α : the selectivity $\alpha_{12} = k_2/k_1$,

$\bar{k}$: the average capacity factor $\bar{k} = \frac{(k_1 + k_2)}{2}$,

N : the plate number.

If we decide to have an α value $\geq 2{,}2$ with an optimal analysis time then we have to separate the two components in a range of $1 < \bar{k} < 5$ [37]. The high value of α is selected according to the possibility of computer assisted sample pretreatment.

TABLE 6
Values of Natural Variables

		$V_i/100$	π^*	β	α
1	Benzene	0.491	0.59	0.10	0
2	Ethylbenzene	0.668	0.53	0.12	0

Table 6 can be converted as follows :

TABLE 7
Values of Coded Variables

		X_1	X_2	X_3	X_4
1	Benzene	-1	-0.75	-0.87	-1
2	Ethylbenzene	-0.32	-1	-0.79	-1

In the case of methanol-water :

Using equation (11) and values of Table (7) the lnk' of benzene in methanol-water binary mixture ($\ln k_{1M}$) becomes :

$\ln k_{1M}$ = 2.582 + (2.255*(-1)) - (0.478*(-0.75)) - (0.994*(-0.87)) - (0.342*(-1)) + [-3.538 - (1.651*(-1)) + (0.359*(-0.75))+ (0.591*(-0.87)) + (0.305*(-1))] * X_5 + [0.453 - (0.125*(-1)) + (0.067*(-0.75)) - (0.049*(-1))] * X_5*X_5 . (17)

then $\ln k_{1M} = 1.896 - 2.977\ X_5 + 0.576\ X_5^2$ (18)

and lnk' of ethylbenzene ($\ln k_{2M}$) becomes :

$$\ln k_{2M} = 3.456 - 4.133\ X_5 + 0.475\ X_5^2 .$$ (19)

As we look for value of $\alpha \geq 2.2$ then the following inequality must be fulfilled:

$$1.56 - 1.156\ X_5 - 0.101\ X_5^2 \geq \ln 2.2$$ (20)

this inequality comes from :

$\ln\alpha = \ln(k_{2M}/k_{1M}) = \ln k_{2M} - \ln k_{1M} = [\ 3.456 - 4.133\ X_5 + 0.475\ X_5^2] - [1.896 - 2.977\ X_5 + 0.576\ X_5^2] = 1.56 - 1.156\ X_5 - 0.101\ X_5^2$. (21)

To respect this inequality X_5 must be :

$$X_5 \leq 0.633$$

or $$X_5 \geq -12.055$$

As $-1 \leq X_5 \leq +1$, one single solution is valid, which means that we have to choose the percent of methanol lower or equal to (0.633*45+55) $\simeq$ 83 to get $\alpha \geq 2.2$.

At $X_5 = 0.633$, $\ln k_{1M} = 0.243$ and $\ln k_{2M} = 1.032$

then $\bar{k}_M = (k1M + k2M)/2 = 2.04.$ (22)

<u>with acetonitrile-water</u> :

- lnk' of benzene according to the equation (13) and Table (7) becomes :

$$\ln k_{1A} = 1.282 - 2.546\ X_5 + 0.660\ X_5^2.$$ (23)

- lnk' of ethylbenzene according to the equation (13) and Table(7) becomes:

$$\ln k_{2A} = 2.018 - 2.850\ X_5 + 0.628\ X_5^2. \qquad (24)$$

$\Delta \ln k' = \ln k_{1A} - \ln k_{2A} = 0.736 - 0.304\ X_5 - 0.032\ X_5^2 \geq \ln 2.2.$

then $X_5 \leq -0.175.$

At $X_5 = -0.175$, $\overline{k}_A = 9.19.$

with tetrahydrofuran-water :

- lnk' of benzene according to the equation (14) and Table(7) becomes :

$$\ln k_{1T} = 0.741 - 2.988\ X_5 + 0.888\ X_5^2. \qquad (25)$$

- lnk' of ethylbenzene according to the equation (14) and Table(7) becomes:

$$\ln k_{2T} = 0.951 - 3.448\ X_5 + 1.211\ X_5^2. \qquad (26)$$

$$\Delta \ln k' = \ln k_{1T} - \ln k_{2T} = 0.211 - 0.460\ X_5 + 0.322\ X_5^2 \geq \ln 2.2. \qquad (27)$$

then $X_5 \leq -0.804.$

At $X_5 = -0.804$, $\overline{k}_T = 65.69.$

TABLE 8.
Recapitulatory Table

$\alpha = 2.2$	Methanol/water (83/17) v/v	Acetonitrile/water (47/53) v/v	Tetrahydrofuran/water (19/81)v/v
$\overline{k}$	2.04	9.19	65.69

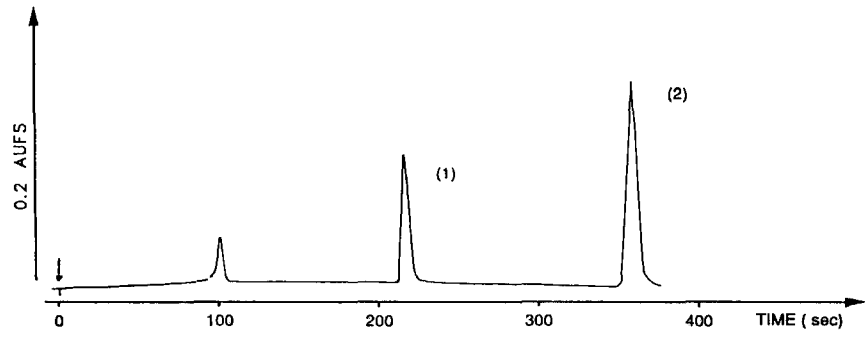


FIGURE 4. Separation of benzene from ethylbenzene

The separation of benzene from ethylbenzene was performed on :

- column : Hibar 100 RP-18 (Merck) endcapped (packed with Lichrospher 5µm) (250*4 mm)
- mobile phase : methanol/water (83/17) vol/vol
- injection volume : 20 µl
- flow-rate : 1 ml / min
- detection : UV detector - wavelength 254 nm.

(1) Benzene ; (2) Ethylbenzene.

Therefore, in order to obtain $\alpha \geq 2.2$ in minimal time, we have to select working with a 83/17 vol/vol methanol-water mixture. Chromatogram displayed in Fig 4 illustrates the validity of the approach since $\alpha_{exp} = 2.18$ and $\bar{k}_{exp} = 1.82$. These values are very close to those of Table 8.

EXPERIMENTAL

The liquid chromatography instrument was from Hitachi a 6000 A pump equipped with a Rheodyne 7125 sample loop (20 µl) and a fixed-wavelength detector (254 nm). Solvents were of Lichrosolv quality from Merck (Darmstadt, FRG). Water was distilled over potassium permanganate and purified by

percolation through a RP 18 Lobar type column (Darmstadt, FRG). Solutes were from Sigma and used without further purification, dissolved in the selected mobile phase.

REFERENCES

1- R. F. Rekker, The Hydrophobic Fragmental Constant, Elsevier Publishing Company, Amsterdam, 1977.

2- C. Hansch, A. Leo, Substituent Constants for Correlation Analysis in Chemistry and Biology, John Wiley & Sons., Inc., New York, 1979.

3- A. Noble, J. Chromatogr., 642: 3 (1993).

4- R. M. Smith, C. M. Burr, J. Chromatogr., 550: 335 (1991).

5- R. M. Smith, C. M. Burr, J. Chromatogr., 485: 325 (1989).

6- R. M. Smith, C. M. Burr, J. Chromatogr., 481: 85 (1989).

7- R. M. Smith, C. M. Burr, J. Chromatogr., 481: 71 (1989).

8- R. M. Smith, C. M. Burr, J. Chromatogr., 475: 75 (1989).

9- R. M. Smith, C. M. Burr, J. Chromatogr., 475: 57 (1989).

10- A. Bondi, J. Phys. Chem., 68: 441 (1964).

11- L. B. Kier, L. H. Hall, Molecular Connectivity in Chemistry and Drug Research, Academic Press, New York, 1976.

12- J. F. Schabron, R. J. Hurtubise, H. F. Silver, Anal. Chem., 49: 2253 (1977).

13- R. J. Hurtubise, T. W. Allen, H. F. Silver, J. Chromatogr., 235: 517 (1982).

14- R. W Taft, J. L. M Abboud, M. J. Kamlet, M. H. Abraham, J. Solution Chem., 14: 153 (1985).

15- M. J. Kamlet, R. M. Doherty, J. L. M. Abboud, R. W. Taft, M. H. Abraham, Chemtech., 16: 566 (1986).

16- M. J. Kamlet, R. M. Doherty, M. H. Abraham, Y. Marcus, R. W. Taft, J. Phys. Chem., 92: 5244 (1988).

17- J. C. McGowan, J. Appl. Chem. Biotechnol., 34A: 38 (1984).

18- P. C. Sadek, P. W. Carr, R. M. Doherty, M. J. Kamlet, R. W. Taft, M. H. Abraham, Anal. Chem., 57: 2971 (1985).

19- M. J. Kamlet, M. H. Abraham, P. W. Carr, R. M. Doherty, R. W. Taft, J. Chem. Soc. Perkin Trans. II., 2087 (1988).

20- R. Phan-Tan-Luu, D. Bazard, R. Flayeux, J. M. Lehuede, M. Parisot, T. Vinh, M. Moll, Industries Alimentaires et Agricoles, 441 (1979).

21- R. M. Claramunt, R. Gallo, J. Elguero, D. Mathieu, R. Phan-Tan-Luu, J. Chim. Phys., 78: 805 (1981).

22- E. Fargin, M. Sergent, D. Mathieu, R. Phan-Tan-Luu, Bio-sciences., 4 (1985).

23- K. Jinno, K. Kawasaki, J. Chromatogr., 316: 1 (1984).

24- R. F. Gunst, R. L. Mason, Regression Analysis and its Application, Marcel Dekker, Inc. New York and Basel, 1980.

25- P. J. Schoenmakers, H. A. H. Billiet, R. Tijssen, L. De Galan, J. Chromatogr., 149: 519 (1978).

26- P. J. Schoenmakers, H. A. H. Billiet, L. De Galan, J. Chromatogr., 185: 179 (1979).

27- K. A. Dill, J. Phys. Chem., 91: 1980 (1987).

28- P. Jandera, J. Chromatogr., 314: 13 (1984).

29- M. C. Pietrogrande, C. Bighi, P. A. Borea, F. Dondi, J. Chromatogr., 522: 37 (1990).

30- Hanfa Zou, Yukui Zhang, Peichang Lu, J. Chromatogr., 522: 49 (1990).

31- S. Chatterjee, B. Price, Regression Analysis by Example, John Wiley & Sons., Inc., New York, 1977.

32- J. G. Hahn, W. Q. Meeker, P. I. Feder, J. Qual. Techn., 8: 140 (1976).

33- D. W. Marquardt, Technometrics, 12: 591 (1970).

34- R. D. Snee, J. Qual. Techn., 5: 67 (1973).

35- P. D. Haaland, Experimental Design in Biotechnology, Marcel Dekker, Inc. New York and Basel, 1989.

36- P. J. Schoenmakers, Optimization of Chromatographic Selectivity, Journal of Chromatography Library - Volume 35, Elsevier, 1987.

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

APPENDIX n° 1

CHARACTERISTICS OF THE CHOSEN MATRIX

CHARACTERISTICS

Log Determinant (X'X)	=	15.813692
Log Determinant (M)	=	-12.312227
Log Determinant (M)** 1/NbrCoeff	=	-0.820815
Maximal variance fonction	=	0.657
Trace (X'X)-1	=	3.514
G (%) efficiency	=	30.451

Name	Variance coefficient	Inflation factor	Name	Variance coefficient	Inflation factor
b0	0.1124	0.000			
b1	0.1607	2.746	b2	0.2124	6.819
b3	0.2275	8.531	b4	0.1283	3.826
b5	0.0926	3.471	b51	0.1324	1.852
b52	0.1749	2.910	b53	0.1874	3.568
b54	0.1056	2.870	b55	0.2645	3.471
b551	0.3782	3.467	b552	0.4998	6.922
b553	0.5353	8.587	b554	0.3018	5.120

APPENDIX n°2

CHARACTERISTICS OF THE MATRIX AFTER REDUCING THE MODEL

CHARACTERISTICS

Log Determinant (X'X)	=	15.542320
Log Determinant (M)	=	-10.708538
Log Determinant (M)** 1/NbrCoeff	=	-0.764896
Maximal variance fonction	=	0.642
Trace (X'X)-1	=	2.338
G (%) efficiency	=	29.065

Name	Variance coefficient	Inflation factor	Name	Variance coefficient	Inflation factor
b0	0.1057	0.000			
b1	0.1606	2.744	b2	0.1412	4.532
b3	0.0937	3.513	b4	0.1051	3.135
b5	0.0926	3.471	b51	0.1324	1.852
b52	0.1749	2.910	b53	0.1874	3.568
b54	0.1056	2.870	b55	0.2375	3.117
b551	0.3778	3.463	b552	0.2149	2.976
b554	0.2091	3.548			

(continued)

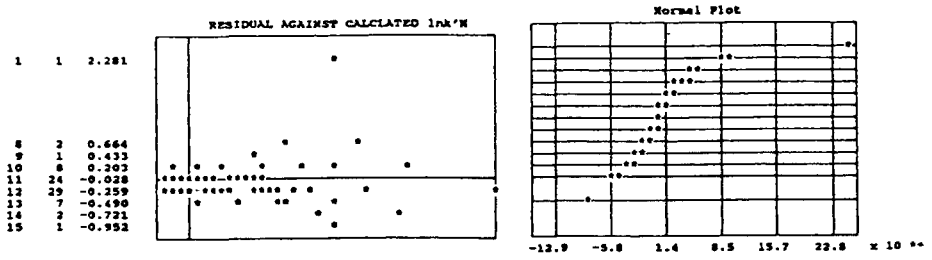
APPENDIX n°2 (continued)

LNK' VERSUS THE FIVE FACTORS : RELATIONSHIP WITH METHANOL-WATER

Source	Sums of squares	Degrees of freedom	Mean sum of squares	Ratio	SIGNIF
REGRESSION	510.08116	13	39.23701	227.72	***
RESIDUAL	10.51065	61	0.17231		
TOTAL	520.59181	74			

Estimation of standard deviation: 0.4151
Multiple Regression Coeff.(R2) : 0.9798
Multiple Regression Coeff.(R2A): 0.9755
Degrees of Freedom ..... : 61

VAR	COEFFICIENT	INFLATION FACTOR	STANDARD DEVIATION	t Exp.	Signif
b0	2.58154		0.13492	19.133	***
b1	2.25531	2.7443	0.16637	13.556	***
b2	-0.47752	4.5324	0.15598	-3.061	**
b3	-0.99405	3.5129	0.12705	-7.824	***
b4	-0.34201	3.1354	0.13456	-2.542	**
b5	-3.53797	3.4713	0.12629	-28.014	***
b51	-1.65112	1.8520	0.15103	-10.933	***
b52	0.35862	2.9104	0.17362	2.066	*
b53	0.59075	3.5685	0.17968	3.288	***
b54	0.30468	2.8696	0.13490	2.259	*
b55	0.45298	3.1169	0.20228	2.239	*
b551	-0.12505	3.4634	0.25514	-0.490	31.6%
b552	0.06684	2.9757	0.19242	0.347	36.5%
b554	-0.04856	3.5485	0.18982	-0.256	39.7%



$$\ln k'_M = 2.582 + 2.255\,X_1 - 0.478\,X_2 - 0.994\,X_3 - 0.342\,X_4 + [-3.538 - 1.651\,X_1 + 0.359\,X_2 + 0.591\,X_3 + 0.305\,X_4]\,X_5 + [0.453 - 0.125\,X_1 + 0.067\,X_2 - 0.049\,X_4]\,X_5^2 \quad (11)$$

APPENDIX n° 3

Source	Sums of squares	Degrees of freedom	Mean sum of squares	Ratio	SIGNIF
REGRESSION	480.40736	13	36.95441	484.96	***
RESIDUAL	4.57207	60	0.07620		
TOTAL	484.97943	73			

Estimation of standard deviation: 0.2760
Multiple Regression Coeff.(R2) : 0.9906
Multiple Regression Coeff.(R2A): 0.9885
Degrees of Freedom ..... : 60

VAR	COEFFICIENT	INFLATION FACTOR	STANDARD DEVIATION	t Exp.	Signif
b0	2.60701		0.08977	29.040	***
b1	2.25115	2.7439	0.11064	20.347	***
b2	-0.42275	4.5364	0.10391	-4.068	***
b3	-1.07753	3.5567	0.08502	-12.674	***
b4	-0.32173	3.1180	0.08952	-3.594	***
b5	-3.54414	3.3777	0.08399	-42.197	***
b51	-1.65467	1.8266	0.10044	-16.475	***
b52	0.20811	2.9688	0.11671	1.783	*
b53	0.75772	3.6575	0.12098	6.263	***
b54	0.16782	2.8451	0.09104	1.843	*
b55	0.40823	3.0610	0.13462	3.033	**
b551	-0.11319	3.4407	0.16967	-0.667	25.7%
b552	0.10783	2.9681	0.12805	0.842	20.4%
b554	0.04772	3.4660	0.12671	0.377	35.4%

APPENDIX n° 4

LNK' VERSUS THE FIVE FACTORS : RELATIONSHIP WITH ACETONITRILE-WATER:

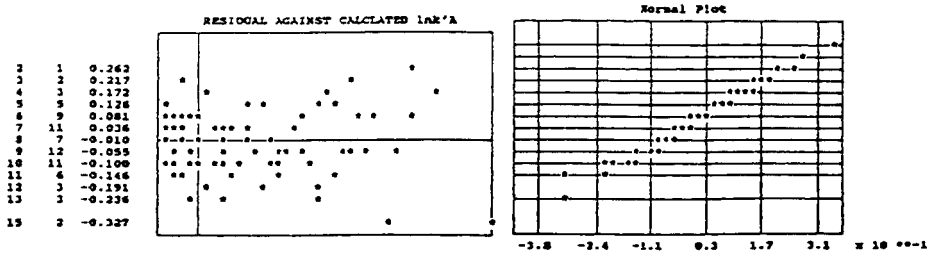
Source	Sums of squares	Degrees of freedom	Mean sum of squares	Ratio	SIGNIF
REGRESSION	262.53885	13	20.19530	1051.85	***
RESIDUAL	1.17119	61	0.01920		
TOTAL	263.71004	74			

Estimation of standard deviation: 0.1386
Multiple Regression Coeff.(R2) : 0.9956
Multiple Regression Coeff.(R2A): 0.9946
Degrees of Freedom ..... : 61

(continued)

APPENDIX n° 4 (continued)

VAR	COEFFICIENT	INFLATION FACTOR	STANDARD DEVIATION	t Exp.	Signif
b0	1.01319		0.04504	22.496	***
b1	1.04463	2.7443	0.05554	18.810	***
b2	-0.34323	4.5324	0.05207	-6.592	***
b3	-0.66513	3.5129	0.04241	-15.683	***
b4	-0.47404	3.1354	0.04492	-10.553	***
b5	-2.41250	3.4713	0.04216	-57.225	***
b51	-0.49802	1.8520	0.05041	-9.879	***
b52	0.04003	2.9104	0.05796	0.691	25.0%
b53	0.50568	3.5685	0.05998	8.431	***
b54	0.15913	2.8696	0.04503	3.534	***
b55	0.97626	3.1169	0.06752	14.458	***
b551	-0.00699	3.4634	0.08517	-0.082	46.6%
b552	0.10783	2.9757	0.06423	1.679	*
b554	0.24260	3.5485	0.06337	3.829	***



$$\ln k'_A = 1.01 + 1.05\,X_1 - 0.34\,X_2 - 0.67\,X_3 - 0.47\,X_4 + [-2.41 - 0.50\,X_1 + 0.04\,X_2 + 0.51\,X_3 + 0.16\,X_4]\,X_5 + [0.98 - 0.01\,X_1 + 0.11\,X_2 + 0.24\,X_4]\,X_5^2 . \quad (13)$$

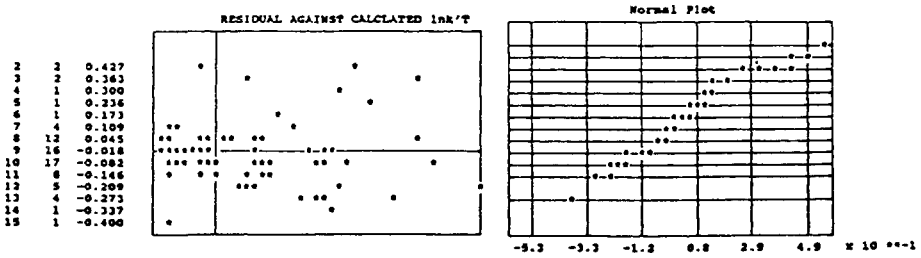
APPENDIX n°5
LNK' VERSUS THE FIVE FACTORS : RELATIONSHIP WITH THF-WATER :

Source	Sums of squares	Degrees of freedom	Mean sum of squares	Ratio	SIGNIF
REGRESSION	316.32670	13	24.33282	777.37	***
RESIDUAL	1.90938	61	0.03130		
TOTAL	318.23607	74			

Estimation of standard deviation:	0.1769
Multiple Regression Coeff.(R2) :	0.9940
Multiple Regression Coeff.(R2A):	0.9937
Degrees of Freedom ..... :	61

APPENDIX n°5 (continued)

VAR	COEFFICIENT	INFLATION FACTOR	STANDARD DEVIATION	t Exp.	Signif
b0	0.28013		0.05751	4.871	***
b1	0.32648	2.7443	0.07091	4.604	***
b2	-0.17399	4.5324	0.06648	-2.617	**
b3	-0.63949	3.5129	0.05415	-11.809	***
b4	-0.09687	3.1354	0.05735	-1.689	*
b5	-2.95239	3.4713	0.05383	-54.848	***
b51	-0.72632	1.8520	0.06437	-11.283	***
b52	0.11690	2.9104	0.07400	1.580	5.8%
b53	0.72102	3.5685	0.07658	9.415	***
b54	0.04318	2.8696	0.05750	0.751	23.1%
b55	1.17525	3.1169	0.08622	13.631	***
b551	0.44470	3.4634	0.10874	4.089	***
b552	-0.08820	2.9757	0.08201	-1.075	14.3%
b554	-0.09157	3.5485	0.08091	-1.132	13.1%



$$\ln k'_T = 0.28 + 0.33\,X_1 - 0.17\,X_2 - 0.64\,X_3 - 0.10\,X_4 + [\,-2.95 - 0.73\,X_1 + 0.12\,X_2 + 0.72\,X_3 + 0.04\,X_4]\;X_5 + [\,1.18 + 0.44\,X_1 - 0.09\,X_2 - 0.09\,X_4]\;X_5^2 . \qquad (14)$$

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A METHOD FOR DETERMINATION OF LIPID-BOUND SIALIC ACID AFTER CHROMATOGRAPHIC ISOLATION OF BRAIN GANGLIOSIDES

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ABSTRACT

The lipid-bound sialic acid determination gives an indirect idea of the ganglioside composition. An attempt at adapting the 'resorcinol method' of Svennerholm using thin-layer chromatography is described in this article.An aliquot of a lipid extract from bovine brain was chromatographed and the ganglioside carrying area was visualised. The sorbent was scrapped and eluted. The sialic acid was hydrolysed with a mixture of resorcinol/hydrochloric acid and the color product formed was extracted with butanol/butylacetate. The color intensity was measured at 580 nm. It was found that the lipid-bound sialic acid in bovine brain was about 780 μg/g fresh tissue.The recovery data calculated by means of a standart solution were 97-102%. This method is particularly useful for some routine diagnostic studies.

INTRODUCTION

Gangliosides are presented in relatively small amounts in brain and other tissues. Chromatographic methods are widely used for their qantitation. Another indirect method is based on defining the sialic acid (N-acetylneuraminic acid) content as a specific molecular component. Two colorimetric procedures are mainly in use: the method of Svennerholm [1,2] with some modifications [3] and the 'thiobarbituric method' [4,5]. By the former the free sialic acid is treated with resorcinol/hydrochloric acid at high temperature while the latter approach rests on an oxidising cleavage of the side chain of the free sialic acid after reaction with thiobarbituric acid. The color compounds formed by both methods are extracted with organic solvents and their intensity measured spectrophotometrically. Some authors claim that the 'thiobarbituric method' is more sensitive while other prefer the Svennerholm's procedure because of its rapidity, facility and cheapness.

The ganglioside isolation from a total lipid extract is a preliminary procedure and various techniques such as liquid-liquid partition, ion exchange chromatography, gel filtration could be applied. A method for preparative thin-layer chromatographic isolation of gangliosides was published earlier [6]. This approach was adapted for quantitative determination of gangliosides according to their sialic acid content.

MATERIAL AND METHODS

A sample of whole bovine brain was used. The extraction procedure was similar to the one published earlier [6]. The tissue was subjected to three successive extractions with: a) cyclohexane (1:5 w/v) ; b) chloroform/methanol = 1:3 (1:15 w/v) mixture ; c) chloroform/methanol = 1:1 (1:10 w/v) mixture. The combined extracts were evaporated at 35-40^0 C using a rotary vacuum evaporator. The residue formed was dispersed by ultrasonication after addition of portions of a chloroform/methanol = 1:1 mixture to form a brain tissue weight solvent volume ratio 1:5 or 1:7. The lipid solution thus obtained was divided in vials (0.5-1.0 ml) and stored frozen for the next procedures.

Preparative Thin-Layer Chromatography

The sample was submitted to preparative TLC fractionation (10x10 cm glass plates; 0.75 mm silica gel G layer thickness (Merck, Darmstadt,Germany)). The solution from one vial was applied bandwise by hand or automatically (Camag Linomat IV, Muttenz, Switzerland). Mobile phase: chloroform/methanol/0.3% KCl = 30:18:4 (v/v/v). After 8 cm run distance the plate was dried and the zones were detected by spraying with reagent, prepared by dissolving of 0.5 g 3,5-dihydroxytoluene in 100 ml of water followed by addition of 12 ml concentrated sulfuric acid. A small path at the edge of the plate was sprayed followed by local heating until color spots on a white background could be seen on the plate. The sorbent of the corresponding ganglioside carrying zones was scrapped (Fig. 1) and placed into a centrifuge tube. The whole ganglioside carrying area scrapped area ratio was measured (Q = 1.90-1.95). Five ml of a mixture of 96 ml methanol/4 ml concentrated hydrochloric acid was added and, after shaking, was centrifuged at 4000 min^{-1} for 10 min. Two ml supernatant were transferred into a distillation flask and the solvent was evaporated at 40°C under nitrogen. Two ml bidistilled water and 2 ml reagent (a mixture of 1 ml 3% resorcine, 8 ml hydrochloric acid, 0.03 ml 0.1M copper sulfate) were added to the residue. After 20-25 min heating in a boiling water bath the flask was cooled to 5-10°C and the chromogen was extracted with 3 ml of butanol/butylacetate = 15:85 (v/v). The solvent mixture was then centrifuged and the absorbance of the organic phase was read at 580 nm (Carl Zeiss Jena VSU-2P spectrophotometer, 1 cm pathlength). Standard and blank samples were prepared separately.

The sialic acid content was calculated according to the following equations:

$$R = \frac{A.St}{A_{St}} \cdot \frac{V_t.Q.V}{V_1.Vp.g} \; [\,\mu g/g \text{ fresh tissue }] \qquad (1)$$

where: A - absorbance at 580 nm (sample)

St - sialic acid content (μg) in the standard sample

A_{St} - absorbance of the standard sample at 580 nm

V_t - total volume of eluate obtained from the scrapped zones

Vp - volume of eluate taken for analysis

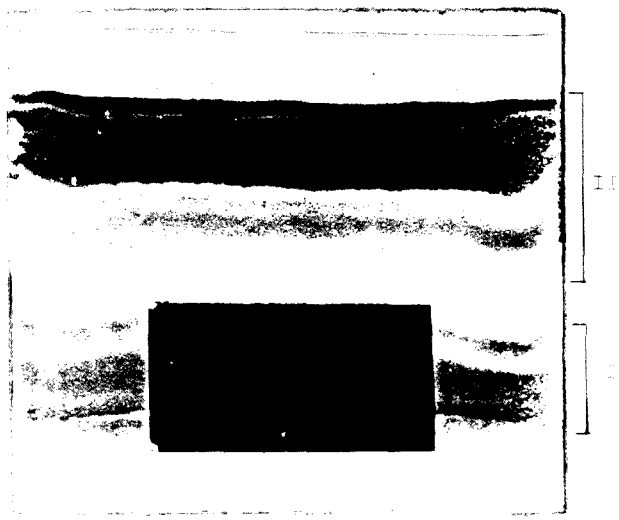

Figure 1. Preparative thin-layer chromatogram of total brain lipids (conditions - see text). I - gangliosides; II - other lipid fractions; III - scrapped sorbent area

Q - whole ganglioside carrying area scrapped area ratio (see Fig. 1)
V - total lipid extract volume
V_1 - volume applied on the TLC plate
g - sample weight in grams

$$R_1 = \frac{R}{0.01.d} \text{ [} \mu g/g \text{ dry tissue] } (2)$$

where: d - dry tissue in %

$$R_2 = \frac{R_1}{M_W} \text{ [} \mu mol/g \text{ dry tissue]} \quad (3)$$

where: M_W - sialic acid molecular weight (309.28)

RESULTS AND DISCUSSION

A standard curve obtained by a series of dilutions of a water solution of sialic acid (Sigma, St. Louis, Missouri, U.S.A.) is given in Figure 2. The average St / A_{St} ratio (see equation 1) is 68±6 (n=19) using 13620 as a molar extinction coefficient.

We have accomplished an additional experiment in order to determine the overall recovery. Aliquot volumes containing (30 μg) standard solution of pure sialic acid were processed as already described. The calculated recovery data were 97.5-102.4% (n=5). Fifteen samples of brain tissue have been analysed by this procedure and the following values were found:

$R = 780 \pm 68$ μg sialic acid/g fresh tissue

$R_1 = 3760 \pm 334$ μg sialic acid/g dry tissue

$R_2 = 12.2 \pm 1.08$ μmol sialic acid/g dry tissue

Using these data the sialic acid distribution for the main ganglioside fractions could be presented according to the corresponding rel.% from the densitogram according to the following equation (Table 1):

$$S_{Gi} = \frac{R_2}{\Sigma \frac{(Pi.Mw)}{M_{WGi}}} \cdot \frac{Pi.Mw}{M_{WGi}} \quad [\mu mol] \qquad (4)$$

where:

- S_{Gi} - sialic acid content (μmol)
- R_2 - μmol sialic acid/g dry tissue (12.2) (see text)
- Mw - sialic acid molecular weight (309.28).(1, 2, ...)
- Pi - ganglioside part from the densitogram in rel. % (see Table 1) (densitometry conditions are described in [6])
- M_{WGi} - ganglioside molecular weight (monosialo - 1545; disialo - 1836; trisialo - 2127)

The ganglioside fractions are colorless (both in UV and VIS region) and should be visualised by spraying with reagents. The mixture of resorcinol/HCl reacts with the lipid-bound sialic acid and the intensity of the color compound formed is proportional to its quantity and could be expressed in rel. % from the densitogram.

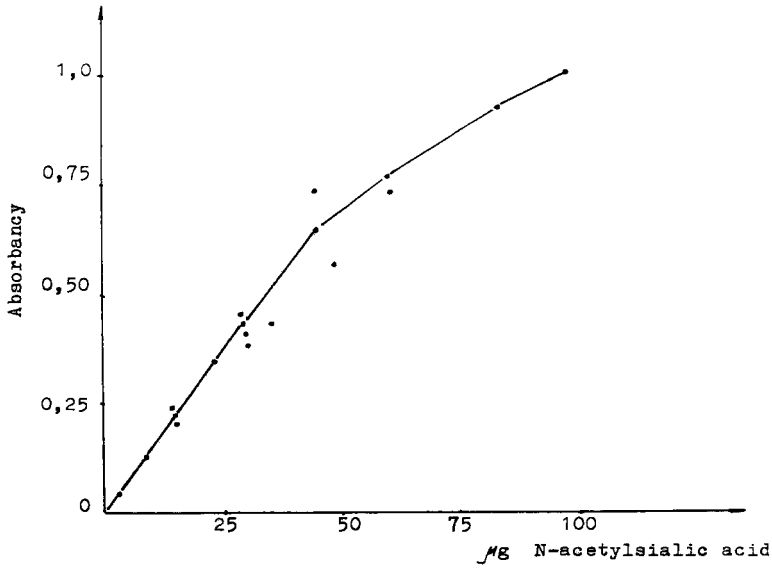

Figure 2. Standard curve of a series of dilutions of pure sialic acid

TABLE 1

Sialic Acid Distribution

Gangliosides	GT1b	GD1b	GD1a	GM1
part from the densitogram (rel. %) n=9	16.1±3.7	12.3±2.4	38.2±3.6	33.4± 2.6
sialic acid distribution (R2) (µmol)	2.80	1.64	5.10	2.67

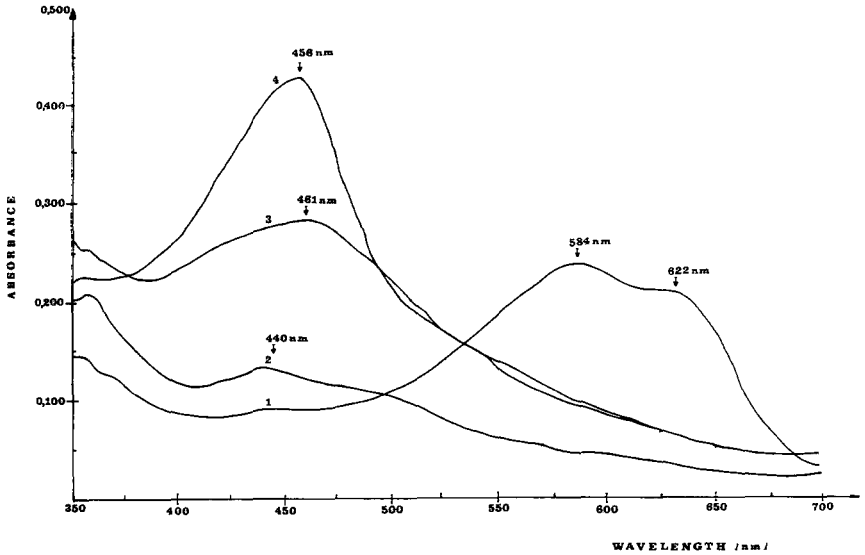

Figure 3. Absorption spectra: I-pure sialic acid; II- phospholipids and cholesterol (eluate-see Fig. 1-II); III- pure glucose; IV- pure fructose

TABLE 2

Comparative Data

Human and mammalian brain	Grey matter	White matter	Whole brain	References
μmol/g fresh tissue	2.85 - 3.55	0.90 - 1.57		[8]
μg/g fresh tissue	992	205		[9]
Our data:				
μmol/g fresh tissue	-	-	2.52	
μg/g fresh tissue	-	-	780	

There is a linear part in the standard curve covering concentrations from 0 to 50 mg sialic acid (Fig. 2). Experimental conditions ensuring hit this part could be chosen.

Some authors [7] suggest the color intensity to be measured at the second absorption maximum (620 nm) in order to avoid interference of the impurities (carbohydrates, phospholipids). Absorption spectra of such a possible impurities (ca. 100 μg of each) are given in Figure 3. In the course of time their color is changing with a shift to the lower wavelengths. The amount of impurities could affect the results and precautions (preliminary purification of the ganglioside fractions) should be taken into account. The sensitivity of the method proposed in this article has a lower limit of 3 nmol. Comparisons of data for sialic acid content in bovine brain presented by other authors [8, 9] are shown in Table 2. They correlate well with those described in this article.

ACKNOWLEDGEMENTS

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REFERENCES

1. Svennerholm L., Quantitative Estimation of Sialic Acids. A Colorimetric Resorcinol-Hydrochloric Acid Method, *Biochim. Biophys. Acta*, **24**, 604, 1957.

2. Svennerholm L., Fredman P., A Procedure for the Quantitative Isolation of Brain Gangliosides, *Biochim. Biophys. Acta*, **617**, 97, 1980.

3. Miettinen T., Takki-Luukkainen J. T., Use of Butylacetate in Determination of Sialic Acid, *Acta Chem. Scand.*, **13**, 856, 1959.

4. Hahn H., Hellman B., Lernmark A., Sehlin J., Taljedal I., The Pancreatic b-Cell Recognition of Insulin Secretagogues, *J. Biol. Chem.*, **249**, 5257, 1974.

5. Yavin E., Yavin Z., Ganglioside Profiles during Neural Tissue Development, *Dev. Neurosci.*, **2**, 25, 1979.

6. Ilinov P., Katzarova E., Dimov S., Zaprianova E., Direct Thin-Layer Chromatographic Method for Isolation of Gangliosides, *J. Liquid Chromatogr.*, **13** (10), 1921, 1990.

7. Chigorno V., Sonnino S., Ghidoni R., Tettamanti G., Changes in Rabbit Cerebrum and Cerebellum Gangliosides during Postnatal Life. A Study Especially Referring to Alkali Labile Gangliosides, *Neurochem. Int.*, **4** (5), 397, 1982.

8. Ledeen R. W., *Handbook of Neurochemistry*, A. Laytha (Ed.), Second Edition, Plenum Press, New York and London, (1983), 41.

9. Yu R. K., Macala L. J., Farooq M., Sbasching-Agler M., Ledeen W. T., Ganglioside and Lipid Composition of Bulk-Isolated Rat and Bovine Oligodendroglia, *J. Neurochem. Res.*, **23**, 136, 1989.

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CHARACTERIZATION OF TRYPTIC PEPTIDES OF A POTENT GROWTH HORMONE RELEASING HORMONE ANALOG BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-IONSPRAY MASS SPECTROMETRY

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ABSTRACT

A highly reproducible tryptic digestion procedure was developed for the characterization of a potent growth hormone releasing hormone (GHRH) analog, para-methylhippuroyl derivative of the N-terminus of a precursor porcine-GHRH (2-76) (pMHpGHRH) derived by recombinant DNA technology. Tryptic digestion can be completed within one hour at room temperature. All tryptic peptides can be separated by reversed phase high performance liquid chromatography(RPHPLC) on a Vydac C18 protein column with trifluoroacetic acid(TFA)-acetonitrile(ACN) gradient elution. Eleven single fragments obtained in tryptic peptide mapping have been identified by coupled Ionspray mass spectrometry. Combined fragments T2-3 and T4-5 were also observed and

verified by Ionspray mass spectrometry and microsequencing analysis. A stability study indicated that the digestion solution will remain stable for 9 days at 5 °C. The retention time variations for the tryptic peptides found to be 1.9% relative standard deviation(RSD).

INTRODUCTION

Growth hormone releasing hormones (GHRH) are a series of hypothalamic peptides that stimulate the synthesis and release of growth hormone (1). Native GHRH and its analogs have been reported to have long term effects on increasing growth rate in growth hormone deficient children (2) and lactation in dairy cows (3). In practical use, however, stable analogs were required in the use of long term processes. Several hybrid GHRH produced by recombinant DNA technology have been reported (4). A para-methylhippuroyl derivative of the N-terminus of a recombinant precusor porcine-GHRH(2-76) (pMHpGHRH) has been developed(5). This new modified recombinant GHRH which is composed of 76 amino acids, results in an increase in production of growth hormone in mammals. pMHpGHRH may be used to increase lean muscle mass, to foster wound healing and to counter the effects of aging (5).

Peptide mapping is a powerful technique to characterize the primary structure of proteins. Reversed-phase high performance liquid chromatography (RPHPLC) is commonly used for the separation of enzymatic digest peptides. In a previous paper, a free solution capillary electrophoresis (FSCE) method and RPHPLC have been used for the characterization of tryptic peptides of pGHRH 2-76, the parent peptide of pMHpGHRH (6). However, the recognition of digest peptide fragments was carried out by adding synthesized peptide fragments in the tryptic digestion solution. The weakness of this procedure is the lack of selectivity since two or more different peptide fragments can have identical retention times.

The combination of high performance liquid chromatography and mass spectrometry (HPLC-MS) offers one of the most powerful techniques in modern time for the characterization of biomolecules. Several ionization interfaces such as thermospray (7) and particle beam (8) have been used in LC-MS. These interfaces, however, have been less successful for the analysis of biopolymers with relatively large molecular weights. A technique using the combination of high-performance displacement chromatography with continuous flow fast atom bombardment (FAB)-mass spectrometry has been used for the characterization of tryptic peptides of recombinant human growth hormone (9),but this method has had only limited success in the use of routine analysis of proteins. Ionspray (IS) is a very gentle ionization process and operates without the input of heat into the spray-ionization step. As a consequence, sample molecules are ionized without thermal degradation (10, 11) and generally little or no fragmentation for proteins and peptides. A particularly significant aspect of this technique is the multiple charging observed for large biomolecules, such as proteins, peptides and nucleic acids, that can be used for highly accurate molecular weight determination of biomolecules.

In this paper, based upon the studies of effects of digestion temperature and incubation time on peptide separation, a highly reproducible tryptic digestion procedure was developed for the characterization of pMHpGHRH. The combination of HPLC-ionspray MS was employed to identify tryptic fragments of pMHpGHRH.

EXPERIMENTAL

Materials

pMHpGHRH samples were obtained from Eli Lilly & Co (Indianapolis, IN, USA). TPCK-treated trypsin was purchased from Fluka Chemie AG (Switzerland). HPLC grade tri-

fluoroacetic acid (TFA) (Fischer, NJ, USA), Trizma base (Sigma, St. Louis, MO, USA), HPLC-grade acetonitrile (ACN) (Mallinckrodt, St. Louis, MO, USA), all analytical grade reagent and Millipore Milli Q water were used in this work.

Methods

Tryptic digestion. Tryptic digestions of pMHpGHRH were performed at room temperature in a Tris-acetate buffer system. One milligram of the pMHpGHRH sample was dissolved in 0.5 mL of water and mixed with 0.5 mL of 100 mM Tris-acetate buffer(pH 8.5). In this case, the solution became cloudy because of the poor solubility of pMHpGHRH at this pH condition but digestion results were not affected. A 40 µL aliquot of TPCK treated trypsin solution (1 mg/mL in 100 mM Tris-acetate buffer, pH 8.5) was added and vortexed for 2 minutes. The digest mixture was incubated for 1 hour at room temperature. After digestion, the mixture was maintained under refrigeration throughout the chromatographic analysis. Aliquots of the digest mixture were stored frozen at - 20 ^{o} C for use at a later time.

Reversed-phase HPLC. Reversed-phase chromatographic analyses of tryptic digest peptides were performed on a Waters 625 LC-system equipped with a Waters 991+ photodiode array detector (Waters, Milford, MA, USA) and a Vydac protein and peptide C18 column (4.6 x 250 mm) (Vydac, Hesperia, CA, USA). Mobile phase consisted of 0.1% aqueous TFA(Solution A) and (v/v) 0.1% TFA in 50% ACN/H2O (Solution B). The flow rate was 1.0 mL/min. A 50 µL aliguot of the tryptic digest mixture was injected onto the column at room temperature. A linear gradient from 0% to 100% solution B over 50 min was carried out upon injection. Peptides were detected at 214 nm. A Beckman System-Gold instrument consisting of a model 126 programmable solvent delivery system, a Model 168 photodiode array detector and a Beckman manual injector with a 100µL sample loop was used for the LC-MS experiments.

Mass Spectrometry . Ionspray mass spectrometry(ISMS) was conducted using a Sciex API III triple quadrupole mass spectrometer equipped with a pneumatically assisted ionspray interface. The mass calibration of the instrument was performed according to manufacturer's instuctions using a mixture of polypropylene glycols. A post-column flow split tee was used to maintain a split flow from HPLC eluant in a ratio of 50:1. A second tee was set up downstream of the flow split tee into which a 10 μL/min post column addition of a solution of 1% (v/v) acetic acid in acetonitrile was introduced using a Harvard syringe pump (model #22) for increasing the sensitivity in Ionspray MS. The instrument was operated in the positive ion detection mode with an inlet orifice potential of 50V (+20 volts relative to RO). Spectra were collected over a range of 150-2400 amu at 0.33 amu intervals with a dwell time of 0.7 msec. per interval. The duration of a single scan was 5.10 sec.

N-terminal peptide sequencing. Sequencing of pMHpGHRH tryptic peptides was performed using the Edman degradation technique on an Applied Biosystems Model 477A Protein Sequencer and analyzed using an Applied biosystems Model 120A Analyzer.

RESULTS AND DISCUSSION

The structure of pMHpGHRH is shown in Fig. 1. Complete tryptic digestion of pMHpGHRH should generate 12 tryptic peptide fragments. Table 1 lists the amino acid sequences and masses of the peptide fragments expected from the digestion mapping and two combined peptide fragments. A chromatogram of the tryptic digestion mixture of pMHpGHRH under linear gradient elution conditions is shown in Fig. 2. The tryptic peptide mapping of pMHpGHRH is very similar to that of pGHRH 2-76 previously reported (7) except the T1 peak of pMHpGHRH appears at a later retention time in the chromatographic profile because of the more hydrophobicity of this peptide.

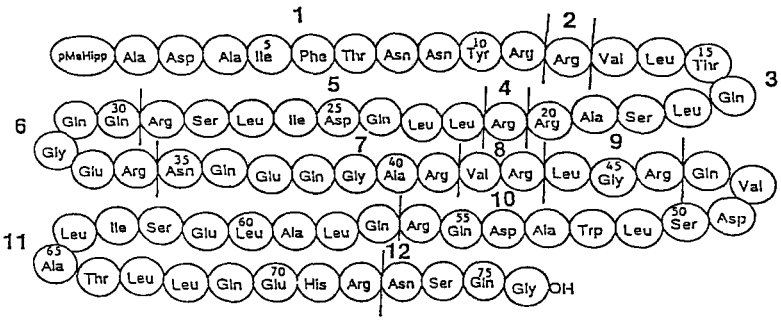

FIG. 1
Sequence structure of pMHpGHRH and predicted tryptic cleavage sites.

TABLE 1. TRYPTIC PEPTIDE FRAGMENTS OF pMHpGHRH

Num.	Fragment	MH+(mass)	Sequence
T1	1 - 11	1359.6	pMH-ADAIFTNNYR
T2	12 - 12	175.1	R
T2-3	12 - 20	1043.6	RVLTQLSAR
T3	13 - 20	887.5	VLTQLSAR
T4	21 - 21	175.1	R
T4-5	21 - 29	1113.7	RLLQDILSR
T5	22 - 29	957.6	LLQDILSR
T6	30 - 34	617.3	QQGER
T7	35 - 41	802.4	NQEQGAR
T8	42 - 43	274.2	VR
T9	44 - 46	345.2	LGR
T10	47 - 56	1217.6	QVDSLWADQR
T11	57 - 72	1835.0	QLALESILATLLQEHR
T12	73 - 76	405.2	NSQG

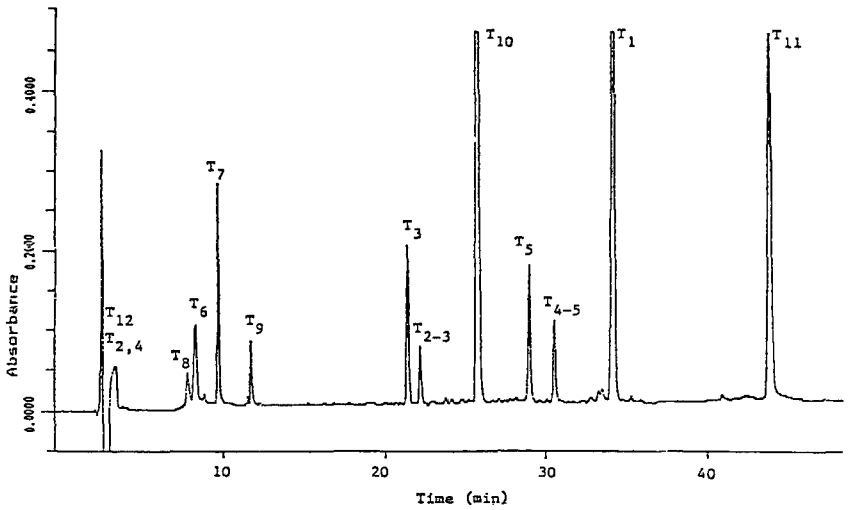

Fig. 2
RPHPLC tryptic mapping obtained from the digest mixture of pMHpGHRH. Tryptic fragments are numbered sequencially from N-terminus of the peptide and given in Table 1.

Peak identification

Peak identification of the tryptic peptide fragments of pMHpGHRH was performed on a HPLC system interfaced with an ionspray triple quadrupole mass spectrometer (Fig. 3). All tryptic peptides found in the digest mixture have been identified and are listed in Table 2. The sequence assignment of fragments was made by comparison of the observed mass with that calculated for the most abundant isotopes of each peptide. It can be seen that two tryptic peptides comprising T2(or T4), arginine, and T12, a tetrapeptide were found in the void peak by plotting selected ion chromatogram (SIC). Fig. 4a and 4b show the reconstructed SIC of fragment T2(or T4) and T12 which are dominated by ions corresponding to a $[M + H]^+$ of 175.3 amu for T2(or T4) and 405.4 amu for T12. The

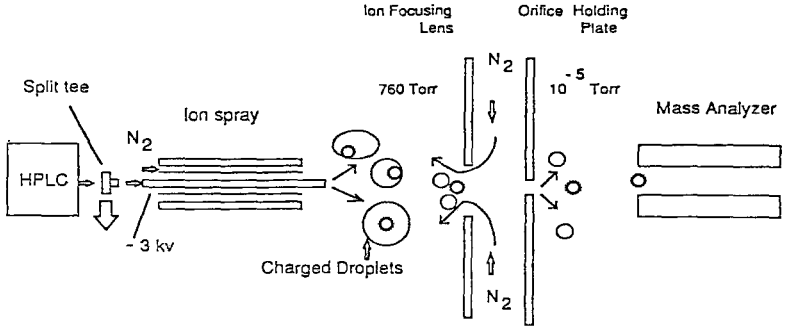

Fig. 3
Schematic diagram of the HPLC-ISMS.

TABLE 2. MOLECULAR IONS OBSERVED IN RPHPLC - ISMS

Fragment	Observed[M+H]			Calculated[M+H]		
	Z=1	Z=2	Z=3	Z=1	Z=2	Z=3
T1		680.8		1359.6	680.8	
T2,T4	175.3			175.1		
T3		444.4		887.5	444.8	
T2+T3		522.6		1043.6	522.8	
T4+T5		557.6		1113.7	557.9	
T5		479.7		957.6	479.8	
T6	617.5	309.2		617.3	309.7	
T7		402.1		802.4	402.2	
T8	274.2			274.2		
T9	345.2	173.0		346.2	173.6	
T10		609.5		1217.6	610.3	
T11		918.6	612.5	1836.0	918.5	612.7
T12	405.4			406.2		

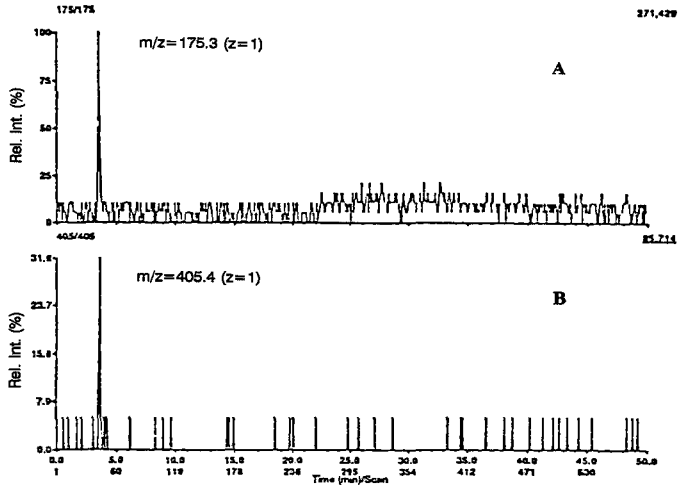

Fig. 4
Reconstructed single ion current chromatograms.
A: $[M + H]^+$ of 175.3 amu.(T2 or T4)
B.: $[M + H]^+$ = 405.4.(T12)

observed masses are identical with the predicted masses. In fact, a slightly longer retention time for T12 peak was observed in SIC that is due to the slightly stronger hydrophobicity of T12 than that of T2(or T4).

Incomplete digestion was also observed at two sites as indicated in Table 2. The mass spectral analysis assigned the observed molecular weight of these peptides to the sequences comprising residues T2-3 or T3-4 ($[M + 2H]^{2+}$ = 522.8)(Fig. 5a) and T4-5 ($[M + 2H]^{2+}$ = 557.9)(Fig. 5b) in which additional arginine is retained at the N-terminus bound of the fragments. It is not possible to identify the sequence of the combined fragment, that is T2-3 or T3-4, by MS because both fragments have the same molecular weight. In order to

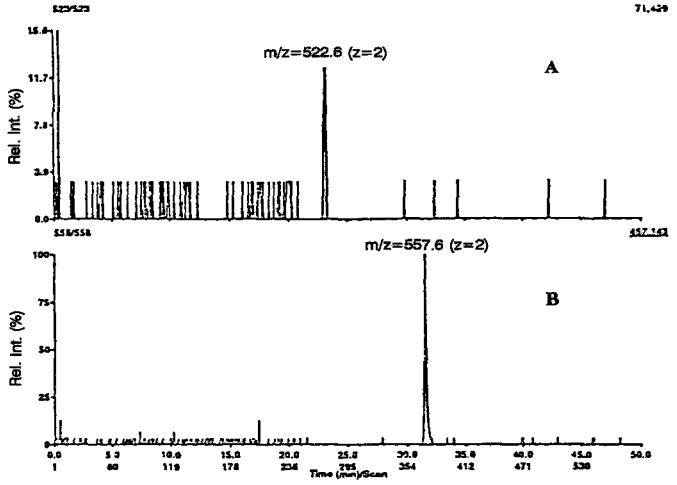

Fig.5
Reconstructed single ion current chromatogram for the combined peptides.
A: $[M + H]^+ = 522.6$ (T2-T3)
B: $[M + H]^+ = 557.6$ (T4-T5)

identify the sequence of this fragment, the collected fraction of this fragment was examined by micro-sequence analysis. The result obtained indicated that the combined fragment is T2-3.

RPHPLC of tryptic peptide mapping

In RPHPLC, the retention order depends on the molecular size and hydrophobicity of the peptide. In general, the larger the more hydrophobic peptides the stronger the retention. In Fig.2, we can see that the retention order basically follows the molecular size of the peptides. For example, T2, and T4, monopeptide, eluted at void volume and T8, dipeptide, T6, pentapeptide, and T7, heptapeptide eluted

successively afterwards. In some cases, however, hydrophobicity of the peptide strongly effects the retention order. The tripeptide T9 exhibits a stronger hydrophobicity because of its two hydrophobic amino acid, glycine and isoleucine. Consequently, T9 elutes at a retention time later than that of T6. Peptide T12 eluted at the void volume owing to three strong polar amino acids in this tetrapeptide. In addition, T5 containing four hydrophobic amino acids exhibits stronger retention although the molecular size of this peptide is smaller than T10. The largest peptide T11 presents the strongest retention in the peptide mapping.

Validation of the tryptic digestion of pMHpGHRH

The tryptic digestion procedure has been validated for temperature effects, incubation time, stability, and reproducibility.

The temperature effect on the tryptic digestion of pMHpGHRH is shown in Fig.6. The maps obtained from the peptide mixtures digested at 22°C and 37°C demonstrate identical chromatographic profiles. The slight variation in retention time for some peaks is due to slight fluctuation in the gradient elution profile.

The effect of incubation time on tryptic digestion has been examined. Chromatograms in Fig.7 demonstrated that when the incubation time varied from 1 to 7 hours identical peptide maps were obtained. This result indicated the tryptic digestion of pMHpGHRH can be completed within one hour.

In the stability study, the same profiles of digest peptide mapping were obtained after the peptide digestion solutions were stored in a refrigerator at 5 °C for 9 days.

Reproducibility of tryptic mapping has also been determined by using 4 different lots with duplicate or

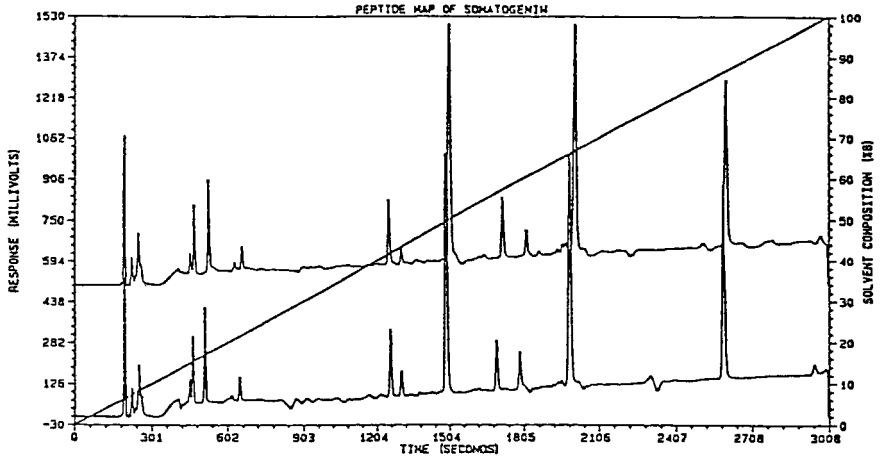

Fig. 6
Effect of digestion temperature on the tryptic peptide map of pMHpGHRH. A: 22°C. B: 37°C.

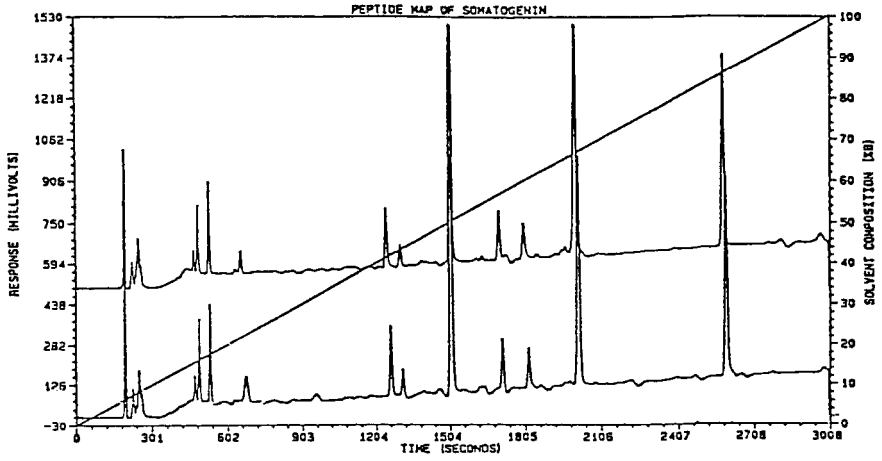

Fig.7
Effect of digestion time on the tryptic peptide map of pMHpGHRH.
A: 1 hour incubation. B: 7 hours incubation.

TABLE 3. REPRODUCIBILITY OF THE RETENTION TIME OF FRAGMENT PEAKS

Fragment	Sample No.	Run No.	tr	RSD%
T6	4	10	7.78	4.32
T7	4	10	8.95	3.49
T9	4	10	11.04	3.90
T3	4	10	21.04	2.02
T2-3	4	10	21.94	2.11
T10	4	10	25.33	1.11
T5	4	10	28.56	0.86
T4-5	4	10	30.18	0.57
T1	4	10	33.57	0.39
T11	4	10	42.63	0.49

triplicate analyses. Results listed in Table 3 indicated that the variability in the retention time of the tryptic fragments appears to be quite small. Peaks with less retention have larger relative standard deviation (RSD).

CONCLUSION

RPHPLC-ionspray MS is a fast, sensitive method for the identification of tryptic digest peptides. Analysis of tryptic peptides of pMHpGHRH by this method demonstrates the presence of all the expected peptide fragments. High-quality mass spectra were obtained for the tryptic peptides which co-eluted at the void volume. Two combined peptides were also observed in the analysis. Those have been comfirmed to be T2-3 and T4-5.

The tryptic digestion procedure developed in this paper is a simple and highly reproducible method for the characterization of pMHpGHRH.

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REFERENCES

1. L. A. Frohman and J. O. Jansson, Endocrine Rev. 7: 223(1986)

2. P. E. Rochiccioli, M. T. Tauber, F. X. Coude, M. Arnone, M. Moore, F. Uboldi and C. Barbeau, J. Clin. Endocrinol. Metab. 85: 268(1987).

3. W. J. Enright, L. T. Chapin, W. M. Moseley and H. A. Tucker, J. Dairy Sci., 71: 99(1988).

4. D. S. Smith, M. L. Heiman, J. F. Wagner, R. L. Jackson, R. A. Bimm and H. M. Hsiung, Biotechnology 10: 315(1992).

5. R. D. Dimarchi, M. L. Heiman, J. E. Shields, D. L. Smiley, European Patent Application 511003 (1992)

6. J. P. Chang, D. L. Smiley and M. R. Coleman, M.R. J. Liquid Chromatog., In press.

7. K. Stachowiak, C. Wilder, M. L. Vestal, D. F. Dyckes, J. Am. Chem. Soc. 110: 1758(1988).

8. Winkler, P.C.; Perkins, D.D.; Williams, D.K.; Browner, R. F. Anal. Chem. 60: 489(1988).

9. J. Frenz, J. Bourell, W. S. Hancock, J. Chromatogr. 512: 299(1990).

10. J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse, Science, 246: 64(1989).

11. S. A. McLuckey, G. J. Van Berkel, G. L. Glish, E. C. Huand, and J. D. Henion, Anal. Chem. 1991, 63: 375(1991).

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A SIMULTANEOUS ASSAY OF THE DIFFERENTIATING AGENTS, PHENYLACETIC ACID AND PHENYLBUTYRIC ACID, AND ONE OF THEIR METABOLITES, PHENYLACETYL-GLUTAMINE, BY REVERSED-PHASE, HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

This paper describes a reversed-phase, high performance liquid chromatographic (HPLC) method for the isolation, detection and quantification of phenylacetic acid, phenylbutyric acid and one of their metabolites, phenylacetylglutamine, from serum. The compounds are initially extracted from serum after protein precipitation with perchloric acid. The solution is neutralized with potassium bicarbonate and injected onto a C-18 column. The compounds are then differentially eluted using an increasing gradient of acetonitrile. Ionization of the two acids is suppressed by adding phosphoric acid to the mobile phase. The compounds are detected by UV absorbance at 208 nm. The assay yields a lower limit of detection of 2 μg/ml and is linear to concentrations as high as 2,000 μg/ml. Between 20 and 1,000 μg/ml the interassay percent coefficient of variation is less than 10%.

INTRODUCTION

Phenylacetic acid (Figure 1a), a product of phenylalanine metabolism, is a small molecule (MW =136) normally present in the mammalian circulation in low concentrations (1). It has been administered as the sodium salt of phenylacetate to children with hyperammonemia due to inborn errors of urea synthesis and to adults with hyperammonemia resulting from the chemotherapy of leukemias or from portal systemic encephalopathy (2 - 5). In humans, phenylacetate is conjugated

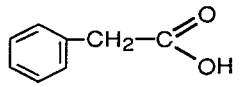

Figure 1 a: Molecular structure of phenylacetic acid.

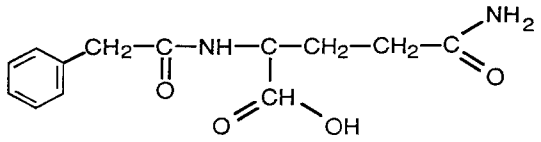

Figure 1 b: Molecular structure of phenylacetylglutamine.

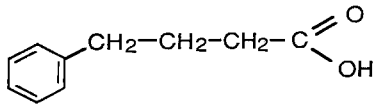

Figure 1 c: Molecular structure of phenylbutyric acid.

with glutamine by the hepatic enzyme phenylacetyl Coenzyme A: glutamine acyltransferase to yield phenylacetylglutamine (Figure 1b), which is then excreted in the urine (6). Phenylbutyric acid (Figure 1c), a structurally related compound which is thought to act as the prodrug of phenylacetate, undergoes beta-oxidation in the liver to yield phenylacetic acid. Both compounds have recently received attention for their ability to induce differentiation of human leukemic cells *in vitro* and to increase the synthesis of fetal hemoglobin in patients with hemoglobinopathies (1,7,8). In addition, phenylacetic acid has demonstrated growth-inhibitory activity against prostatic carcinoma and glioblastoma in humans (9). To support the National Cancer Institute's clinical trials of phenylacetate and phenylbutyrate, we have developed a simultaneous HPLC assay for both compounds and one of their common metabolites, phenylacetylglutamine, in serum.

METHODS

Materials and Reagents

Sodium phenylacetate and sodium phenylbutyrate were supplied as sterile powders by Elan Pharmaceutical Research Corporation (Gainesville, GA, USA). Both phenylacetate and phenylbutyrate were stored at room temperature in light-protective containers. Phenylacetylglutamine was a gift of Dr. Saul Brusilow (John Hopkins University, Baltimore, MD, USA). Perchloric acid, phosphoric acid and potassium bicarbonate were purchased from Aldrich Chemical Co., Milwaukee, WI. HPLC grade acetonitrile from J.T. Baker Chemical Co., Phillipsburg, NJ was used as a component of the mobile phase in the chromatographic analysis. HPLC grade water was obtained with an in-house reverse osmosis system whose product was further processed through a Milli-Q UV Plus polishing unit (Millipore Co., Marlborough, MA). All other reagents were reagent grade or better.

Preparation of Standards

Powder of phenylacetate, phenylbutyrate and phenylacetylglutamine was weighed on a Mettler AE 240 analytical balance (Mettler Instrument Co., Highstown, NJ) and dissolved in water to yield a stock solution containing 10 mg/ml of each compound. Solutions containing 0.1 and 1.0 mg/ml of each compound were then generated by serial dilution of the 10 mg/ml stock solution. These three solutions were then aliquoted into 6 ml polystyrene test tubes (Becton Dickinson and Co., Lincoln Park, NJ) and stored at - 4°C.

On the day of analysis, a standard curve for each of the compounds was prepared by thawing one aliquot of each of the three stock solutions and adding appropriate amounts of those solutions to a commercial preparation of pooled serum (Baxter Healthcare Co., Deerfield, IL) to generate the following serum concentrations: 1, 2, 3, 4, 5, 10, 20, 50, 100, 250, 500, 750, 1,000, 1,250, 1,500, 1,750, and 2,000 µg/ml. The preparation of the standard curve itself did not involve serial dilution. This spiked serum then underwent the same procedures as the patient samples described below.

Determination of Assay Precision

Quality control samples were prepared separately from the standard curve. The samples were generated with sodium phenylacetate, sodium phenylbutyrate and phenylacetylglutamine concentrations of 20, 250 and 1,000 µg/ml. These concentrations were chosen to span the range of expected drug concentrations during the conduct of clinical trials. For each concentration, sufficient sample was prepared to aliquot it in 20 separate vials. With each day's assay, a vial at each concentration was assayed along with patient samples, allowing a determination of the assay's precision.

Sample Preparation and Extraction

Blood for the determination of circulating levels of phenylacetate, phenylbutyrate and phenylacetylglutamine was drawn by venipuncture into red top (no additive) Vacutainer™ collection tubes which were immediately refrigerated and then centrifuged at 1,202 g at 4°C for 5 minutes in a Sorvall™ RT 6000D centrifuge (DuPont Co., Wilmington, DE). Serum samples were then stored in cryotubes (Nunc Co., Denmark) at -85°C until assayed.

Two hundred (200) µl of thawed serum were pipetted into a 1.7 ml Eppendorf tube (PGC Scientifics, Gaithersburg, MD). Protein precipitation was carried out by adding 100 µl of a 10% (v/v) solution of perchloric acid (Aldrich Chemical Co., Milwaukee, WI). The tube was vortexed and then centrifuged at 4,500 g for 10 minutes. One hundred and fifty (150) µl of supernatant were transferred to a new 1.7 ml Eppendorf tube and 25 µl of 20% $KHCO_3$ (w/v) were added to neutralize the solution. This was centrifuged at 4,500 g for 10 minutes and 125 µl of supernatant were transferred into borosilicate autosampler vials (Hewlett Packard, Co., Germany) and maintained at 10°C pending injection onto the liquid chromatographic system described below.

HPLC of Phenylacetic acid, Phenylbutyric acid and Phenylacetylglutamine

The chromatographic apparatus consisted of a Hewlett Packard 1090 series II liquid chromatograph (Hewlett Packard Co., Avondale, PA) (LC) equipped with a refrigerated autosampler compartment and a diode-array ultraviolet absorbance (UV) detector. The column used was a Nova-Pak™ C-18 (Waters Inc., Milford, MA). 3.9 mm x 300 mm, maintained at 60°C and protected by a Nova Pak™ C-18 Guard-Pak (Waters, Milford, MA). After injecting 25 µl of the 125 µl sample, phenylacetic acid, phenylbutyric acid and phenylacetylglutamine were separated from one another using a gradient of water/H_3PO_4 0.005M (mobile phase A) and acetonitrile/H_3PO_4 0.005M (mobile phase B) at a flow rate of 1 ml /minute. The gradient of mobile phase B increased from 5% to 40% over 30 minutes. The total run time was 45 minutes. The column effluent was monitored for its UV absorbance at 208 nm.

RESULTS

Recovery of Phenylacetic acid, Phenylbutyric acid and Phenylacetylglutamine from serum

Following protein precipitation and neutralization of the supernatant with $KHCO_3$ the recoveries of phenylacetic acid, phenylbutyric acid, and phenylacetylglutamine (all at 1,000 µg/ml) were 81%, 58% and 82%, respectively.

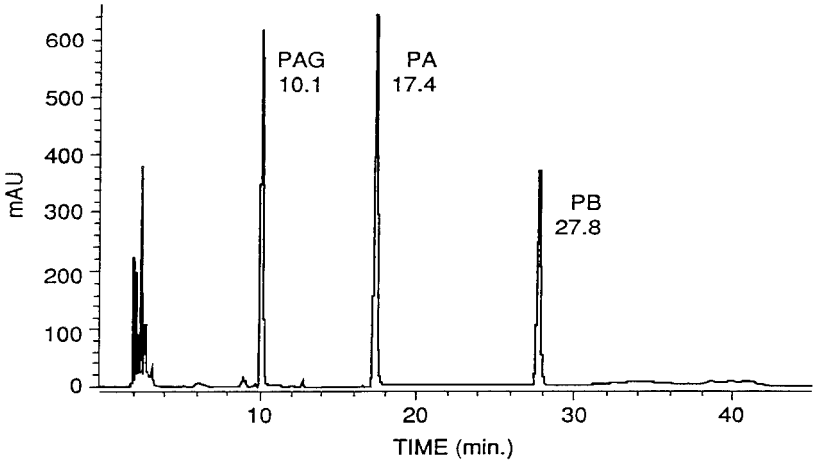

Figure 2: Typical chromatogram of phenylacetylglutamine (PAG) (10.1 min), phenylacetic acid (PA) (17.4 min) and phenylbutyric acid (PB) (27.8 min). Serum concentration of 500 μg/ml for the three compounds.

Chromatography of Phenylacetic acid, Phenylbutyric acid and Phenylacetylglutamine

Figure 2 is a representative chromatogram of a serum sample containing 500 μg/ml each of sodium phenylacetate, sodium phenylbutyrate and phenylacetylglutamine. The compounds elute in an order consistent with their decreasing water solubility: phenylacetylglutamine, 10.1 min; phenylacetate, 17.4 min and phenylbutyrate, 27.8 min .

Assay Performance

The lower limit of quantitation of this assay was 2 μg/ml for all three compounds, based upon a signal to noise ratio of 5:1. The assay was linear between concentrations of 2 and 2,000 μg/ml. The inter-assay coefficient of variations for all three compounds were less than 10% and the intra-assay coefficients of variations were less than 5% (determined by five repeat samples) (concentration range: 20 to 1,000 μg/ml, see Table 1). Figure 3 shows a representative plasma concentration versus time course of phenylacetic acid, phenylbutyric acid and phenylacetylglutamine from a single patient who received a 30 minute infusion of phenylbutyric acid at a dose of 1,200 mg/m^2.

Discussion

Several chromatographic methods have been described in the past for assaying phenylacetate, phenylbutyrate and phenylacetylglutamine in serum, urine or the cerebrospinal fluid (10-13). None describes the simultaneous measurement of all three compounds in a single analytical run. Simplicity is the main advantage of the method we are now reporting: a single-step extraction method followed by sample neutralization, a simple gradient elution and no reliance on compound derivatization to increase detectability. Since the three compounds display widely varying octanol:water partition coefficients, associated with correspondingly different retention times, eluting the compounds with a gradient in addition to heating of the column was beneficial to achieve a simultaneous analysis.

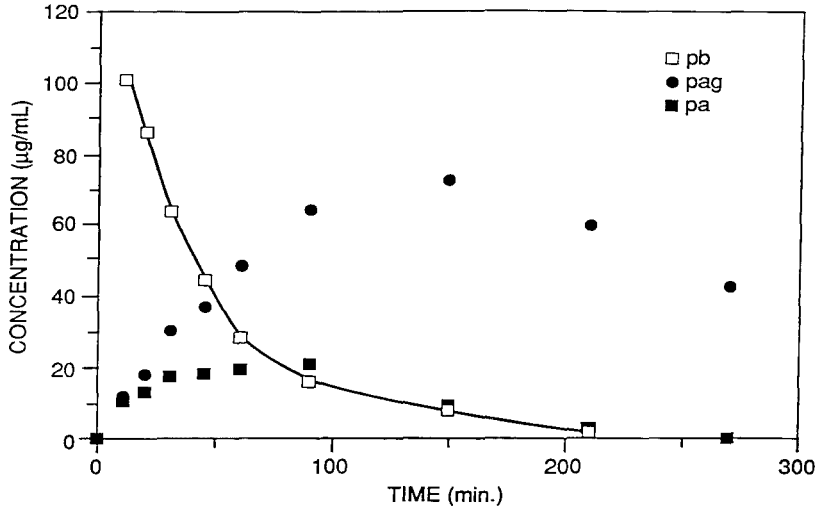

Figure 3: Concentration versus time graph of phenylbutyric acid (pb), phenylacetic acid (pa) and phenylacetylglutamine (pag), following the administration of a 1,200 mg/m2 bolus of sodium phenylbutyrate in a 53 year old man with cancer.

Table 1: Assay Precision

Concentration (µg/ml)	% Coefficient of Variation Inter-assay PA	PB	PAG	Intra-assay PA	PB	PAG
1,000	2.5%	3.0%	3.1%	0.5%	3.9%	3.3%
250	3.7%	2.8%	4.3%	2.2%	4.9%	4.3%
20	9.1%	2.7%	4.6%	0%	0%	0%

PA=phenylacetic acid, PB=phenylbutyric acid, and PAG=phenylacetylglutamine
For both the inter-assay and intra-assay determination, each concentration was assessed by five repeat samples.

A choice had to be made between an extraction method that relied upon the water solubility of these compounds and one that involved an organic solvent. The lower recovery of phenylbutyric acid from serum may reflect that it is the least water soluble of the three compounds. This, nonetheless, did not adversely affect the quality of the assay, as shown by the low coefficient of variation reported in Table 1.

The development of an analytical assay for phenylacetic acid, phenylbutyric acid, and for their common metabolite, phenylacetylglutamine, answered the need for close monitoring of the drugs' concentrations in patients in order to correlate toxicity and efficacy data with pharmacokinetic behavior. The sensitivity of our assay is inferior to methods involving gas chromatography (12), but provides a rapid, accurate and clinically useful means of monitoring the course of therapy in patients. In this respect, the method can conceivably be applied to plasma, urine or CSF, which broadens its clinical use.

REFERENCES

1. D. Samid, S. Shack, and L.T. Sherman. Phenylacetate: a novel nontoxic inducer of tumor cell differentiation. Cancer Res., 52: 1988-1992 (1992).

2. S.W. Brusilow, M. Danney, L.J. Waber, M. Batshaw, B. Burton, L. Levitzky, K. Roth, C. Mckeethren, and J. Ward. Treatment of episodic hyperammonemia in children with inborn errors of urea synthesis. N. Engl. J. Med., 310: 1630-1634 (1984).

3. O. Simell, I. Sipila, J. Rajantie, D.L. Valle, and S.W. Brusilow. Waste nitrogen excretion via amino acid acylation: benzoate and phenylacetate in lysinuric protein intolerance. Pediatr. Res., 20: 1117-1121 (1986).

4. A.J. Watson, J.E. Karp, W.G. Walker, T. Chambers, V.R. Risch, and S.W. Brusilow. Transient idiopathic hyperammonemia in adults. Lancet, ii: 1271-1274 (1985).

5. C.L. Mendenhall, S. Rouster, L. Marshall, R.A. Weesner. A new therapy for portal systemic encephalopathy. Am. J. Gastroenterol., 81: 540-543 (1986).

6. M.O. James, R.L. Smith, F.R.S. Williams, and M. Reidenberg. The conjugation of phenylacetic acid in man, sub-human primates and some non-primate species. Proc. R. Soc. Lond. B., 182: 25-35 (1972).

7. D. Samid, A. Yeh, and P. Prasana. Induction of erythroid differentiation and fetal hemoglobin production in human leukemic cells treated with phenylacetate. Blood, 80: 1576-1581 (1992).

8. G.J. Dover, S. Brusilow, and D. Samid. Increased fetal hemoglobin in patients receiving sodium 4-phenylbutyrate. N. Engl. J.Med., 327: 569-570 (1992).

9. D. Samid, S. Shack, and C.E. Myers. Selective growth arrest and phenotypic reversion of prostate cancer cells in vitro by nontoxic pharmacological concentrations of phenylacetate. J. Clin. Invest., 91: 2288-2295 (1993).

10. S.W. Brusilow. Phenylacetylglutamine may replace urea as a vehicle for waste nitrogen excretion. Pediatr. Res., 29: 147-150 (1991).

11. W.H. Stein. A chromatographic investigation of the amino acid constituents of normal urine. J. Biol. Chem. 201: 45-47 (1953).

12. M. Sandler, C.R.J. Ruthven, B.L. Goodwin, A. Lees, and G.M. Stern. Phenylacetic acid in human body fluids: high correlation between plasma and cerebrospinal fluid concentrations values. J. Neurol. Neurosurg. Psych. 45: 366-368 (1982).

13. M.E. Martin, F. Karoum, and R.J. Wyatt. Phenylacetic acid excretion in man. Analyt. Chem. 99: 283-287 (1979).

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SIMULTANEOUS EXTRACTION AND DETERMINATION OF OXOLINIC ACID AND FLUMEQUINE IN FISH SILAGE BY HPLC

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ABSTRACT

A simple and rapid method for the simultaneous extraction and determination of residues of oxolinic acid (OX) and flumequine (FQ) in fish silage, is presented. The samples were extracted with acetone and ammonia. When applying traditional liquid-liquid extraction, clean extracts were obtained, the recovery being 95.3 - 96.2 % for OX and 96.9 - 99.2 % for FQ. The detection limits were 40 ng/g for OX and 50 ng/g for FQ.

INTRODUCTION

In recent years, the quinolones oxolinic acid (OX) and flumequine (FQ) have been the most used drugs for treatment of infectious diseases in Norwegian fish farming.

In 1992 for instance, the total consumption of OX and FQ was 7687 kg and 9833 kg, respectively (Statistics provided by the Norwegian Medicinal Depot, Oslo).

The annual production of farmed fish in Norway is about 160.000 tons, a substantial proportion of the fish being treated with drugs during the growing period.

The slaughter of fish for human consumption is not allowed until the fish have been screened for residues of drugs in liver and muscle.

Residues of OX and FQ have appeared to be especially bound to bone and skin (1) and can be detected in these organs even though edible tissue like muscle and liver from the same fish are free of drugs.

A lot of fish waste, such as guts, skin and bone arise from the fish processing industry. In Norway, in order to utilize this waste, it is ensilaged. This silage can then further be mixed into animal feed.

According to Norwegian regulations, the addition of antibiotics to feed for food-producing animals is not allowed . An official method of analysis for OX and FQ in silage is therefore needed.

Several analytical methods have been developed for the determination of OX in biological materials using microbiological and high performance liquid chromatographic procedures (2, 3, 4, 5, 6). Analytical methods have also been developed for the determination of FQ in plasma, urine and tissues (7, 8, 9, 10). Tao et al. (11) published a microbiological method for residue analysis of FQ in fish tissues.

Rogstad et al. (12), Steffenak et al. (13) and Rasmussen et al. (14) have published methods for the simultaneous determination of OX and FQ in fish tissues.

However, none of the published methods appeared to be applicable to such a complex material as fish silage, because of problems with interfering peaks in the HPLC chromatograms, or unspecific inhibition zones in microbiological test-systems.

The purpose of the present study was thus to develop a rapid and efficient HPLC method for routine analysis of OX acid and FQ in fish silage.

MATERIALS AND METHODS

Materials and Reagents

The fish silage which served as sample material, was supplied by Stormøllen A/S (Vaksdal, Norway).

All chemicals were of analytical or HPLC grade. OX was supplied by Sigma Co. (St. Louis, MO, USA.), and FQ by Solchem Italiana s.p.a. (Mulazzano, Italy).

Solvents were of analytical and HPLC grade. Stock solutions (1 mg/ml) of OX and FQ were prepared in 0.03 M sodium hydroxide, and working standards were prepared by dilution with 0.002 M phosphoric acid-acetonitrile-tetrahydrofurane (68:17:15).

The solutions were stored in the refrigerator in dark stoppered flasks.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system consisting of a Series 410 Bio solvent delivery system, an ISS 100 sampling system equipped with a cooler (14°C) Lauda RMT6 from Messgeräte Werk Lauda, (Lauda-Königshafen, Germany), and a LS 240 fluorescence detector (Perkin-Elmer, Norwalk, Conn., USA). The detector was operated at an excitation wavelength of 325 nm and emission wavelength of 360 nm, with a Resp. 5 and a Factr. 256. The analytical column (stainless steel, 150 X 4.6 mm I.D.) and guard column (stainless steel, 5.0 X 3.0 mm I.D.) were packed with 5 µm particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA, USA). The mobile phase was 0.02 M phosphoric acid-acetonitrile-tetrahydrofurane (68:17:15). The flow rate was 0.7 ml/min. for 6 min. followed by 0.8 ml/min. for 3 min. The samples were injected at intervals of 10 min.

Aliquots of 20 µl and 30 µl were injected onto the column for the determination of OX and FQ respectively.

Sample Preparation and Clean-up (Figure 1)

The tissue sample, 0.5 g of ground fish silage, was weighed into a 50 ml centrifuge tube with screw cap (NUNC); 1.5 ml H_2O,

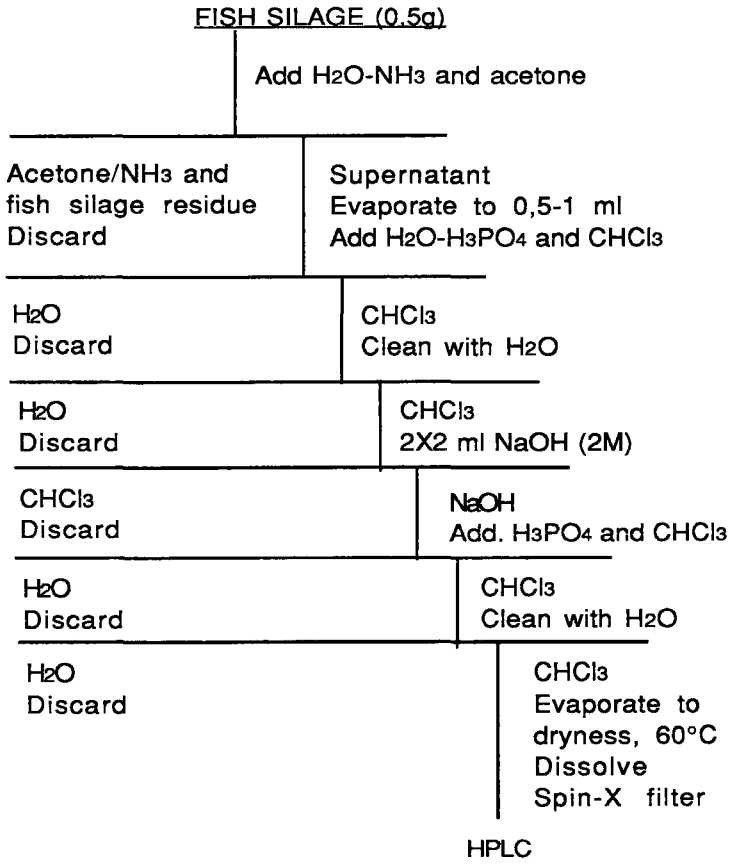

FIGURE 1

Extraction and Clean-up procedure for OX and FQ from Fish Silage

1 ml NH_3 (25%), and 7 ml acetone were added. The sample was mixed for 5 s., and then left with the extraction fluid for 5 min. before again being whirlimixed for 5 s. The homogenate was then centrifuged for 3 min. at 5000 rpm.

Five ml of the supernatant was pipetted into a graduated glass-stoppered centrifuge tube, (Rep. 0.250 g). The acetone phase was evaporated to 0.5 -1 ml under a stream of nitrogen (60°C), and 0.5 ml 85% H_3PO_4, 3 ml H_2O and 4 ml $CHCl_3$ were added. The sample was shaken vigorously for 10 s. followed by centrifugation for approximately 1 min. (3000 rpm). The upper aqueous layer and all solid residues between the two phases were discarded. Two ml H_2O were added (no mixing). The water layer was discarded. Two ml of sodium hydroxide (2M) were added. The sample was then mixed for 10 s. After centrifugation (4000 rpm, 5 min.), the water phase was collected. The chloroform was blended twice more with 2 ml sodium hydroxide and centrifuged. The collected water phases were acidified (1 ml 85% H_3PO_4) and extracted once more with 4 ml chloroform. The water layer was discarded, 2 ml H_2O were added (no mixing), and the water layer was again discarded. The chloroform was evaporated to dryness under a stream of nitrogen (60°C), after which the residue was dissolved in 2 ml 0.002 M phosphoric acid-acetonitrile-tetrahydrofurane (68:17:15), and approximately 0.5 ml filtered through a Costar Spin-X centrifuge filter (lowtype) with 0.22 µm cellulose acetate binding by centrifugation for 5 min. at 10.000 rpm. Aliquots of the filtrate were injected onto the HPLC.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for OX and FQ were determined by spiking silage samples with standard solutions to yield 40, 50, 75, 100, 150, 200, 500 and 1000 ng OX and FQ per gram of sample, respectively. The samples were extracted using the above procedures. Duplicate samples were used. The recovery rates were determined by comparing the results of analysis of spiked fish silage to those of pure standard solutions.

The linearity of the standard curves for OX and FQ in fish silage was calculated using peak height measurements.

RESULTS AND DISCUSSION

Chromatograms of clean fish silage samples, spiked samples and real samples are shown in Figure 2.

The standard curves were linear in the investigated area 40-1000 ng/g for both OX and FQ in fish silage, while the corresponding correlation coefficients were r=0.9994 and r=0.9987, respectively.

The recovery of OX and FQ varied from 95.3 - 96.2 % and from 96.9 - 99.2 %, respectively, with a standard deviation ranging from 1.56 to 6.70 and from 1.58 to 8.10, respectively (Table 1).

The limit of determination for OX and FQ was 40 ng/g and 75 ng/g tissue, respectively, when aliquots of 20 µl were injected onto the column. The limit of determination for FQ was 50 ng/g when aliquots of 30 µl were injected. However, when injecting 30 µl aliquots of OX, an interfering peak was sometimes observed close to the OX peak (Figure 2B). When 20 µl was injected onto the HPLC, for the determination of OX, no such interference was seen (Figure 2A).

The method presented in this paper should be useful for most work on residues of OX and FQ in fish silage. The method is

TABLE 1

Recovery of Oxolinic Acid and Flumequine from Spiked Samples of Fish Silage.

Sample	No. of samples	Amount (µg/g)	Recovery % OX		FQ	
			Mean	SD*	Mean	SD*
Silage 0.5 g	8	0.15	95.3	6.70		
	8	0.50	96.2	1.56		
	8	0.15			96.9	8.10
	8	0.50			99.2	1.58

SD* = standard deviation

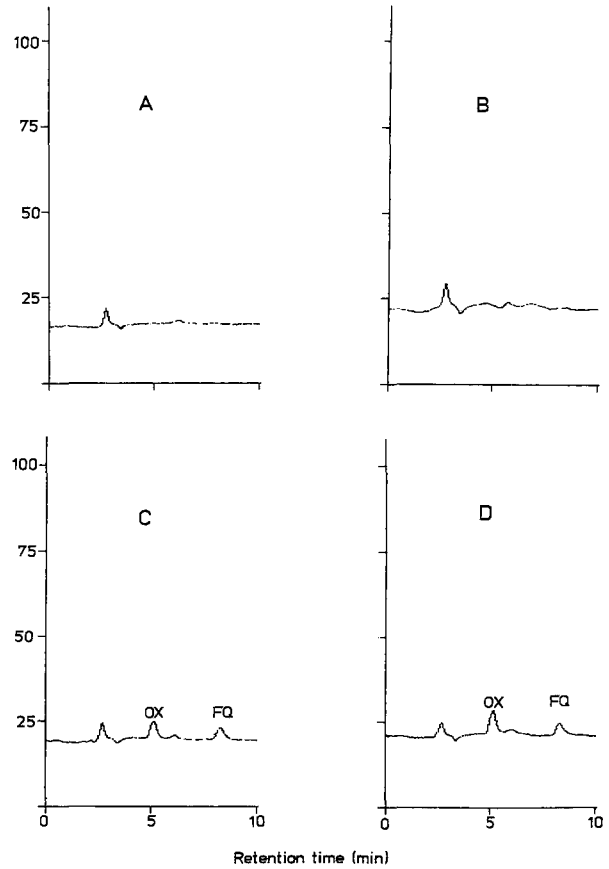

FIGURE 2

Chromatograms of extracts from 0,5 g fish silage for the determination of oxolinic acid and flumequine.

A - Unspiked sample (20 µl injected onto the HPLC)

B - Unspiked sample (30 µl injected onto the HPLC)

C - Fish silage spiked with 200 ng OX and 200 ng FQ per gram sample (20 µl injected onto the HPLC)

D - Chromatograms of real samples. The samples contained 259 ng/g OX and 190 ng/g FQ (20 µl injected onto the HPLC).

selective, and robust, and should be generally applicable for monitoring drug levels in fish silage. The method is also very rapid, a technician easily managing to analyse 18 samples a day. Moreover, the consumption of reagents is low.

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REFERENCES

1. Steffenak, I., Hormazábal, V. & Yndestad M. Reservoir of quinolone residues in fish. Food Additi and Contami.,1991, 8, 777, 1991.

2. Ringel, S. M., Turner, F. J., Roemer, S., Daly, J. M., Zlatanoff, R & Schwartz, B. S. Oxolinic acid, a new synthetic antimicrobial agent. III. Concentrations in serum, urine, and renal tissue. Antimicrob. Agents Chemother, 7, 486, 1967.

3. Ziv, G. Clinical pharmacology of oxolinic acid in young dairy calves. Am. J. Vet. Res. 37, 513, 1976.

4. Kasuga, Y., Sugitani, A. & Yamada, F. Simultaneous determination of oxolinic acid, nalidixic and piromidic acids in fishes by high performance liquid chromatography. J. Food Hyg. Soc. Jpn., 23, 344, 1982.

5. Kasuga, Y., Sugitani, A. & Yamada, F. Identification of oxolinic acid and two major metabolites of piromidic acid in fishes J. Food Hyg. Soc. Jpn., 24, 484, 1983.

6. Hamamoto, K. Rapid high performance liquid chromatographic method for the determination of oxolinic acid in chicken plasma. J. Chromatogr. Biomed. Appl., 381, 453, 1986.

7. Harrison, L. I., Schuppan, D., Rohlfing, S. R., Hansen, A. R., Hansen, C. S., Funk, M. L, Collins, S. M. & Ober, R. E. Determination of flumequine and a hydroxy metabolite in biological fluids by high-pressure liquid chromatographic, fluorometric, and microbiological methods. Antimicrob. Agents Chemother., 25, 301, 1984.

8. Decolin, D., Nicolas, A. & Siest, G. Determination of flumequine and its 7-hydroxy metabolite by reversed-phase high performance liquid chromatography. J. Chromatogr. Biomed. Appl., 414, 499, 1987.

9. Ellerbroek, L. & Bruhn, M. Determination of flumequine in biological fluids and meat by high-performance liquid chromatography. J. Chromatogr. Biomed. Appl., 49, 314, 1989.

10. Samuelsen, O. B. Determination of flumequine in fish by high-performance liquid chromatography and fluorescence detection. J. Chromatogr. Biomed. Appl., 497, 355, 1989.

11. Tao, S. H., Nicolas, M. & Bonnot, M. Méthode de recherche de résidus de la flumequine dans la chair des truites. Rev. Méd. Vét., 136, 623,1985.

12. Rogstad, A., Hormazábal, V., & Yndestad, M. Simultaneous extraction and determination of oxolinic acid and flumequine in fish tissues by high performance liquid chromatography. J. Liq. Chromatogr., 12, 3073,1989.

13. Steffenak, I., Hormazábal, V., & Yndestad, M. Rapid assay for the simultaneous determination of oxolinic acid and flumequine in fish tissues by high-performance liquid chromatography. J. Liq. Chromatogr., 14 (1), 61, 1991.

14. Rasmussen, K. E, Tonnesen, F., Thanh, H. H., Rogstad, A. & Aanesrud, A. Solid-phase extraction and high performance liquid chromatographic determination of flumequine and oxolinic acid in salmon plasma. J. Chromatogr. Biomed. Appl., 496, 355, 1989.

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A RAPID AND TIME-EFFECTIVE ASSAY FOR DETERMINATION OF OXOLINIC ACID AND FLUMEQUINE IN FISH TISSUES BY HPLC

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ABSTRACT

A simple method for analysis of oxolinic acid and flumequine in fish muscle and liver is described. The samples were extracted with trichloroacetic acid, neutralized and analysed by HPLC, with minimal sample manipulation. The limit of quantification was 30 µg/kg for oxolinic acid and 35µg/kg for flumequine.The simplified extraction and clean-up procedure makes it possible for one person to monitor the concentration of the drugs in approximately 80 samples per day.

INTRODUCTION

The extensive use of oxolinic acid (OX) and flumequine (FQ) by the fish farming industry for treatment of bacterial infections in fish, has created a demand for a rapid and simple analytical method for residue control of these drugs in fish.

Several methods based on high-performance liquid chromatography (HPLC) for the determination of OX and FQ in fish tissues have been published (1-8). All methods involve extraction of the compounds with organic solvents, and the manual work-up procedures include steps such as liquid-liquid extraction (1-7) or solid phase extraction (6-8). Microbiological methods for residue analysis of flumequine in fish tissues, with a sensitivity of 250ng/g, have been published (9). The purpose of the present study was to develop a rapid, simple and sufficiently sensitive method, for the simultaneous determination of OX acid and FQ, which required minimal sample manipulation and only small quantities of chemical reagents.

MATERIALS AND METHODS

Materials and Reagents

Samples of muscle and liver tissue of salmon and rainbow trout were used.
All chemicals and solvents were of analytical or HPLC grade. OX and FQ were supplied by Sigma Co. (St. Louis, MO, USA). Stock solutions (1 mg/ml) of OX and FQ were prepared in 0.03 M sodium hydroxide, and working standards were prepared by dilution with mobile phase. The solutions were stored in a refrigerator.
Trichloroacetic acid (TCA) was supplied by Ferax (Laborat GMBH Berlin-Germany), and ortho-phosphoric acid 85% by Merk, Germany.
Spin-X centrifuge filter units from Costar (Cambridge, MA, USA) were also used.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, an ISS 100 sampling system equipped with a Lauda RMT6 cooler (14°C) from Messgeräte Werk Lauda, (Lauda Königshafen, Germany), and a LS 240 fluorescence detector (Perkin-Elmer, Norwalk, Conn., USA). The detector was operated at an excitation wavelength of 325 nm and emission wavelength of 360 nm, and with a Resp. of 5 and a Fctr. of 1024. The analytical column (stainless steel, 150 x 4.6 mm I.D.) and guard column (stainless steel, 5.0 x 3 mm I.D.), were packed with 5 µm particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA, USA).

The mobile phase was 0.02 M phosphoric acid-acetonitrile-tetrahydrofurane (65:20:15) at a flow rate of 0.7 ml/min. The samples were injected at intervals of 10 min. Aliquots of 25 µl were injected onto the column for the determination of OX and FQ.

Sample pretreatment

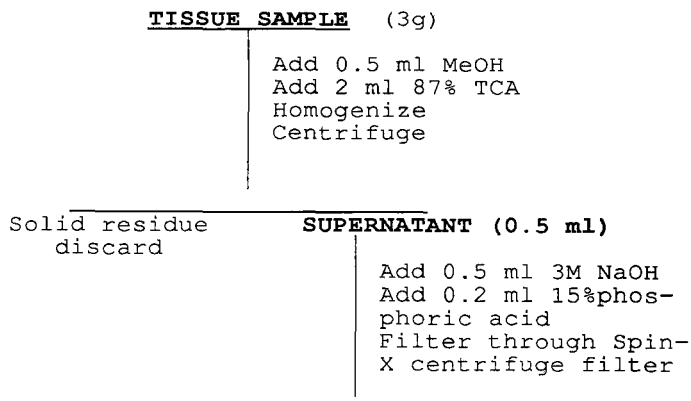
The sample pretreatment of tissues is shown in Fig. 1. Spiked samples (3g) of muscle and liver were mixed with 500 µl MeOH (or standard) and 2 ml 87% TCA in water. The mixture was homogenized for approx. 1 min. in an Ultra-Turrax TP 18/2 (Janke & Kunkel KG, Ika Werk, Staufen,F.R.G.). The mixture was then centrifuged for 3 min. (5000 rpm.). 500 µl of the supernatant were transferred to a centrifuge tube and 500 µl 3 M NaOH added. The sample was mixed for approx. 1 sec. and 200 µl 15% phosphoric acid in methanol then added. The sample was again mixed for 1 sec. Approximatly 500 µl were then filtered through a Costar Spin-X centrifuge filter unit (low type) with 0.22 µm cellulose acetate binding and centrifuged for 5 min. (10000 rpm.). Aliquots of the filtrate were injected onto the HPLC.

Calibration Curves and Recovery Studies

The calibration curves for OX were established by spiking muscle and liver tissue samples with standard solutions to yield 20,50,100,200,400 and 500 ng OX pr. gram, the calibration curves for FQ being similarly determined by spiking tissue samples with FQ standard solution to yield 25,50,100,200,400 and 500 ng FQ pr.gram. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked tissues, muscle and liver, with those of standard solutions. The linearity of the standard curves for OX and FQ was calculated using peak-heigth measurements.

RESULTS AND DISCUSSION

Chromatograms of clean muscle and liver samples, and spiked samples are shown in Figure 2. The standard curves were linear in the investigated areas; 20-500 ng/g for OX and 25-500 ng/g for FQ, in muscle and liver. The correlation coefficients for both OX and FQ in muscle and liver were r=0.999. Table 1 shows the recovery and repeatabilities for OX and FQ from muscle and liver.



F I G U R E 1
Extraction and Clean-up Procedure for Oxolinic acid and Flumequine from Fish Tissue.

The recovery of OX acid and FQ from muscle tissue varied from 67 to 71%, and from 61 to 64%, respectively. The corresponding figures for liver tissue were 56%, and from 51 to 53%, respectively. OX and FQ are acids with low solubility in water. Their solubility increases in alkaline solutions, and in TCA. The results presented in this paper show that OX and FQ can be extracted from samples of muscle and liver tissue with TCA. The extraction procedure appeared applicable to tissues of both Atlantic salmon and rainbow trout. The simplified extraction and clean-up procedure makes it possible to monitor drug concentrations in approximately 80 samples per day. The linearity of the standard curve for both OX and FQ in muscle and liver were 0.999, when using the external standard method. The limit of quantification was 30 ng/g for OX and 35 ng/g for FQ in muscle and liver. When 25 µl was injected onto the HPLC, no interfering peaks were observed. However, the sensitivity may be enhanced by using a larger sample amount. The cost of chemicals and the manual work-up procedures is also reduced compared to previously published methods. This method is specific and robust. The method is demonstrated to be efficient for screening and quantification of residues of drug of OX and FQ, and should replace the less specific microbiological

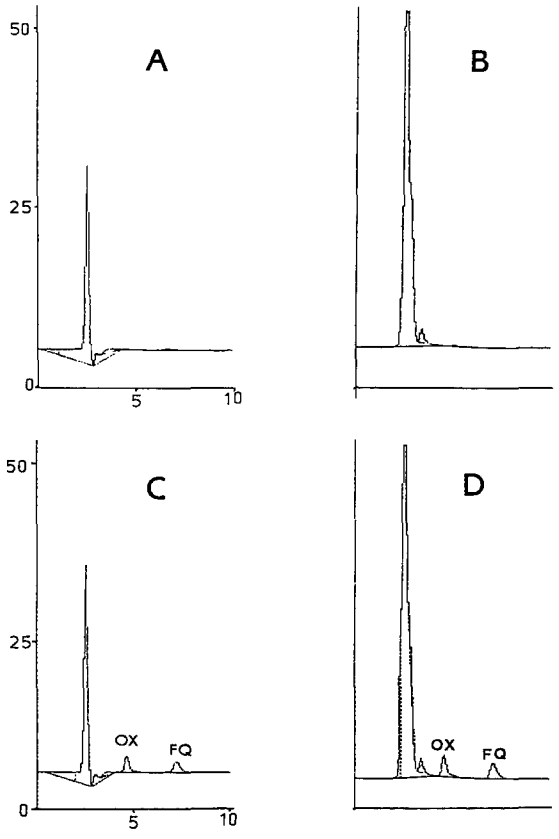


FIGURE 2

Chromatograms of extracts from fish muscle and liver. A: drug-free muscle, B: drug-free liver, C: muscle spiked with oxolinic acid and flumequine (300 ng/g respectively), D: liver spiked with oxolinic acid and flumequine (400 ng/g respectively).

T A B L E 1.

Recovery and Repeatability for Oxolinic acid and Flumequine from Spiked Samples of Fish Muscle and Liver Tissue.

Tissue	No. of samples	Amount in spiked samples (µg/g)	Recovery % OX Mean	OX SD	FQ Mean	FQ SD
	8	0.1	71	3.0		
Muscle	8	0.4	67	1.3		
(3g)	8	0.1			64	1.3
	8	0.4			61	1.0
	8	0.1	56	1.9		
Liver	8	0.4	56	1.3		
(3g)	8	0.1			51	1.7
	8	0.4			53	1.1

SD = relative standard deviation

T A B L E 2.

Concentration of FQ in muscle of salmon monitored using two different methods

Sample no.	**1**	**2**	**3**	**4**	**5**	**6**
This method conc. FQ (ng/g)	79	366	774	501	502	588
Method ref.5 conc. FQ (ng/g)	68	340	786	492	514	564

methods for residue control of these drugs in fish tissue.
The method was correlated to that (5) published previously by analysing muscle of salmon treated with FQ. The results were compared by using linear regression and the correlation coefficient was 0.998. The individual concentration values are given in Table 2.

CONCLUSION

This study has shown that residues of the two antibacterial compounds oxolinic acid and flumequine in tissues of Atlantic salmon and rainbow trout can be analysed after samples have been subjected to some very simple clean-up steps. The method can replace the less sensitive microbiological method.

ACKNOWLEDGEMENT

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REFERENCES

1 N.Nose, Y.Hoshino, Y. Kikuchi, M. Horie, K. Saito, T.Kawachi and H. Nakazawa, J. Assoc. Off. Anal. Chem., 70 (1987) 714.
2 S. Horii, C. Yasuoka and M.Matsumoto.,J.Chromatogr., 338 (1987) 459.
3 L. Ellerbroek and M. Bruhn, J. Chromatogr., 495 (1989) 314.
4 O. B. Samuelsen, J. Chromatogr., 497 (1989) 355.
5 I. Steffenak, V. Hormazábal and M.Yndestad, J. Liquid Chromatogr., 14(1), 61-70 (1991).
6 A. Rogstad, V. Hormazábal and M. Yndestad, J. Liquid Chromatogr., 12 (1989) 3073.
7 M. Horie, K. Saito, Y. Hoshino, N. Nose, E.Mochizuki,H. Nakasawa, J. Chromatogr., 402 (1987) 301.
8 Y. Ikai, H. Oka, N. Kawamura, M. Yamada, K.-I. Harada, M. Suzuki and H. Nakazawa, J. Chromatogr., 477 (1989) 397.
9 Tao,S. H., Nicolas, M. & Bonnot, M. Méthode de recherche de résidus de la flumequine dans la chair des truites. Rev. Méd. Vét., 136: 623, 1985.

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HPLC ANALYSIS OF OXIDATION HAIR DYES IN PERMANENT HAIR COLORANTS

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ABSTRACT

The reversed-phase liquid chromatographic separation of several hair dyes (diamines, aminophenols, phenols, etc.), using mobile phases containing 1,8-diaminoctane as new amine modifier and sodium heptansulfonate, is described. The combined effect of the amine and of the alkylsulfonate enabled very good separation of all the dyes studied. The proposed chromatographic system was found to be selective, rugged and therefore suitable for the reliable quality control of commercial permanent colorants.

A preliminary solid phase extraction (SPE) procedure using SCX sorbents, was found useful to enhance detectability of low concentrated dyes and to isolate phenol compounds from the basic dyes.

INTRODUCTION

Oxidation hair dyes are the precursors of permanent colours developed on the hair by their oxidation [1]. In general, dye formation involves slow oxidation of a primary intermediate (generally aromatic p-diamines and p-aminophenols) by hydrogen peroxide to give p-benzoquinone diimine [2,3] which reacts with the

couplers (generally m-diamines, m-aminophenols, resorcinol etc.) to indophenols, indamine or indaniline or for further reaction to trinuclear or polinuclear dyes.

Semipermanent products incorporate amino or hydroxy- nitrobenzenes which readily diffuse into hair, but also diffuse out again over the course of several shampooings.

Temporary dye products generally consist of high molecular weight acid dyes which are deposited on the hair surface to give a coloring effect removable by a single shampoo .

Many of these compounds may present a risk to users, expecially with regard to sensitization [4,14]. In annex III of European Directive (76/768) limitations on the use of several oxidation hair dyes are reported ; besides in annexe II, a list of these compounds are banned for the cosmetic use.

Despite the fact that TLC [5,6], GC [7] and electrophoretic [8] methods have been used to determine oxidation hair dyes, most reports in the literature concern HPLC methods [9-12]. More recently an ion-pairing method for the determination of p-phenylenediamine in hair dyes [13] was developed, besides a cation exchange HPLC column with electrochemical detection was used to determine p-phenylenediamine in the vapour of haidressing salons [14] and a polymeric reversed phase was used to separate o-,m- and p- isomers of aminophenol [15]. Nevertheless, there is a lack of a systematic analysis of all the most common oxidation hair dyes as raw materials or cosmetic preparations. The reported methods deal just with particular aspects of the whole analytical problems connected with this class of compounds and there exists a need for versatile HPLC methods of general application.

The aim of the present work was to offer a selective and reliable RP-HPLC method with photodiode array detection capable of identifing and determining

most of the oxidation dyes in commercial formulations with improved resolution, reproducibility and sensitivity. To this end an isocratic ion-pairing method, involving the use of a new amine modifier to the mobile phase (1,8-diamminoctane), was developed. When compared to the other amine modifiers (triethylamine, octylamine) 1,8- diaminoctane led to a better performance in terms of resolution, selectivity and reproducibility. The method allowed several dyes to be separated under the same chromatographic system just by modifying the percentage of the organic modifier in the mobile phase, according to the lipophilicity of the analysed dyes. Furthermore, to increase detection sensitivity for hair dyes in cosmetic samples, a solid phase extraction (SPE) method with strong cation exchanger (SCX) sorbent was performed . Through this extraction step, basic dyes can be selectively extracted and separated from phenolic compounds (resorcinol, 1-naphtol, pyrogallol, 4-hydroxy-1-naphtalenesulfonic acid sodium salt) and concentrated with satisfactory recovery and precision.

The proposed method was therefore successfully applied to the analysis of commercial professional hair dye products of complex composition (paste), containing different association of oxidation hair dyes.

EXPERIMENTAL

Materials

All the oxidation dyes (TABLE 1) were obtained from Intercosmo Spa (Bologna , Italy) and used as received.

1,8-diaminoctane (DAO), n-octylamine and triethylamine were from Aldrich (USA); heptansulfonic acid sodium salt was purchased from Janssen (Belgium); urea and sodium dihydrogen phosphate were obtained from Fluka (Switzerland); sodium chloride, ortophosphoric acid and sodium sulfite were from Merck (FRG).

TABLE 1 - Oxidation hair dyes examined by the described RP-HPLC method

Compounds	Abr.	Peak
2-Aminophenol	o-AP	1
3-Aminophenol	m-AP	2
4-Aminophenol	p-AP	3
4-Amino-o-cresol	p-AOC	4
2-Amino-4,6-dinitrophenol (picramic acid)	PA	5
N,N-Dimethyl-1,4-phenylen-diamine	DMPDA	6
2-Ethoxy-1,4-phenylen-diamine	ETPDA	7
4-Methylamino-phenol	p-MAP	8
2-Nitro-1,4-phenylendiamine	NPDA	9
1,4-Phenylendiamine	p-PDA	10
1,3-Phenylendiamine	m-PDA	11
1,2-Phenylendiamine	o-PDA	12
1-Naphtol		
Resorcinol		13
2-Methylresorcinol		
4-Hydroxy-1-naphtalenesulfonic acid, sodium salt	HNSA	14

All the reagents were of analytical grade. Acetonitrile was HPLC grade from Promochem (FRG); water double distilled and filtered through 0.45 μm filter was used to prepare all solutions and buffers .

For the solid phase extraction (SPE) 2.8 ml (500 mg) SCX Analitichem Bond-Elut cartridges were used ; the extraction was carried out using the Baker 10-SPE system connected to a water aspirator. The cartridge was previously conditioned with 3 ml of methanol, followed by 3 ml of 0.85% orthophosphoric acid solution with 0.5% sodium sulfite.

Solutions

Diammoniumoctane heptansulfonate solution (pH 4.5) was prepared by dissolving 1,8-diaminoctane (DAO) (5 mmoles) and heptansulfonic acid sodium salt (20 mmoles) in bidistilled water (1000 ml), adjusting the pH to 4.5 with 8.5% orthophosphoric acid. The dye standard and stock solutions, as well as the sample solutions, were prepared in 0.85% orthophosphoric acid solution containing sodium sulfite (0.5%).

The standard solutions were prepared in 0.025 M phosphate buffer (pH 3.0) containing 1M sodium chloride , 0.5 % sodium sulfite and 5M urea, when the HPLC analysis involved the SPE step. The analyte elution from the SPE column was then performed with the same solvent system but a pH 8.0 was used.

The internal standard(NPDA and DMPDA) solutions were prepared in the same solvent system used for the sample preparation.

Apparatus

The solvent delivery system was a quaternary HP 1050 Ti series Pump, equipped with a Reodyne Model 7125 injector with a 20 μl sample loop . The

eluents were monitored by a Multiwavelenght HP 1050 Detector connected to a 3396 series HP integrator . A photodiode array detector 1040 A(HP) was also used. For routine analyses the detector wavelenght was set at 220-240-275-290 nm, with the integrator attenuation at 0-1-3-8, depending on the dye nature and concentration in the analysed commercial sample.

Chromatographic conditions

Routine analyses were carried out isocratically at ambient temperature on a 10 μm reversed phase Phenomenex Bondclone C18 (300x3.9 I.D.). As mobile phase binary mixtures of acetonitrile with a buffer solution (pH 4.5) containing DAO (0.005 M) and sodium heptansulfonate (0.020 M), were used. The acetonitrile content was comprised between 5 and 30%. The flow rate varied between 1-1.3 ml min^{-1} .

Calibration curves

Standard solutions of the pure dyes (conc. in Table 2) in 8.5% orthophosphoric acid with 0.5% sodium sulfite , containing 15 μg ml^{-1} of nitro p-phenylendiamine as internal standard, were injected in triplicate in the chromatograph. The ratios of analyte area to internal standard area were plotted against the corresponding analyte concentration to obtain the calibration graphs.

Analysis of commercial hair dyes

Four commercial professional cosmetic samples (paste) representative of the various dye compositions were analysed .

Direct Analysis. A 0.2 g aliquot of commercial paste was dispersed with magnetic stirring in 0.85% orthophosphoric acid, containing 0.5% sodium sulfite,

TABLE 2 - Data for the calibration graphs (n=6) in the HPLC determination of selected oxidation hair dyes.

Compounds	Slope	Intercept	Correlation coefficient	Concentration range($\mu g\ ml^{-1}$)
m-AP	0.0692	-0.0108	0.9992	1.0-20.0
p-AP	0.0758	0.0230	0.9990	1.0-23.0
p-PDA	0.0481	-0.0110	0.9990	1.5-25.0
p-AOC	0.0704	0.0180	0.9980	1.5-12.0
ETPDA	0.1045	-0.0210	0.9990	0.5-10.0
p-MAP	0.0832	-0.0153	0.9985	1.5-15.0
mPDA	0.0704	0.0120	0.9980	1.5-15.0

in a 100 ml volumetric flask. Sample 1 and 2 solutions were directly analysed; therefore, 5 ml of the internal standard solution was added before adjusting the volume with 0.85% orthophosphoric acid. NPDA (18 $\mu g\ ml^{-1}$) was the internal standard for the analysis of sample 2; DMPDA (30 $\mu g\ ml^{-1}$) was utilised for the sample 1. The sample suspensions were all filtered through a 0.45 μm Cameo filter and injected into the chromatograph.

Solid phase extraction. Clear solutions from the sample 3 and 4 were prepared, without the internal standard, as above described (filtration through 0.45 mm Cameo filter) and aliquots (12 and 15 ml respectively for the samples 3 and 4) were applied to a SPE SCX cartridge . The subsequent wash step consisted of 3 ml

of water. The dyes retained on the SPE column were then eluted with a solution consisting of 0.025 M potassium phosphate buffer (pH 8.0), 1M NaCl ,5M urea and 0.5 % sodium sulfite, using 3.8 ml for the sample 3 and 2.8 ml for the sample 4.

The eluates from the SPE cartridge were acidified with 200 µl of 17% orthophosphoric acid ; internal standard was added (200 µl of a 140 µg ml^{-1} solution of NPDA for sample 3; 400 µl of a 16 µg ml^{-1} solution of NPDA for sample 4) and 20 µl of each solution were injected in triplicate into the chromatograph . The basic dye contents in each cosmetic sample was calculated by comparison to an appropriate standard solution.

In order to determine the phenolic compounds, after being applied through the cartridge, the sample solutions and the washings were collected together in a 20 ml volumetric flask, the volume adjusted with 0.85% orthphosphoric acid and the solution direcly injected in triplicate into the chromatograph .The phenolic compounds content in each cosmetic sample was calculated by comparison to an appropriate standard solution.

RESULTS AND DISCUSSION

Chromatographic conditions.

According to the basic properties of the hair dyes examined, a reversed-phase liquid chromatographic (RP-HPLC) method, involving the use of an amine modifier in the mobile phase was chosen. Amine modifiers play an important role in RP-HPLC of basic compounds to suppress the adverse silanol effect responsible for severe peak tailing and band broadening [16-21]. In the present work 1,8-diaminoctane (DAO) proved to be a new , useful amine modifier, able to improve the peak shape and the resolution of the basic hair dyes.

The chromatographic analyses were performed on a Bondclone 10 ODS column (300x3.9 mm I.D.) at ambient temperature ,using, as mobile phase, a binary mixture consisting of acetonitrile - DAO heptansulfonate buffer (pH 4.5). The organic phase percentage ranged between 5-30% depending on the lipophilicity of the dyes to be analysed. In order properly to condition the chromatographic system , the mobile phase was allowed to flow through the column for about one hour.

Figures 1 and 2 show two typical chromatographic separations of basic dyes under isocratic conditions. p-PDA, p-AP, m-AP, m-PDA, p-MAP, o-PDA, oAP, DMPDA were separated using a mobile phase consisting of acetonitrile-pH 4.5 DAO Heptansulfonate buffer (5+95), while p-PDA, NPDA, PMAP, DMPDA, ETPDA, PA, p-AOC were separated with lower retention times, using a mobile phase with an increased acetonitrile content (15+85). When compared to other reported methods, the chromatographic system described appears to supply a better selectivity and resolution . Isocratic elution had to be used, because a too long riequilibration time was necessary to perform gradient elutions.

Various DAO\heptansulfonate ratios were experimented on a rapresentative mixture of 4 dyes . As shown in Figures 3 , 5 mM DAO and 20 mM heptansulfonate were found to be the best conditions for a good resolution and peak symmetry. Higher concentrations of heptansulfonate did not increase the analyte retention and therefore did not improve their resolution.

The influence of other amine modifiers, was investigated : octylamine and TEA were used at the same concentration as DAO, with the same amount of heptansulfonate and at the same pH but worse separations were obtained . Higher contents of aqueous phase resulted in longer analysis times without significant resolution improvements.

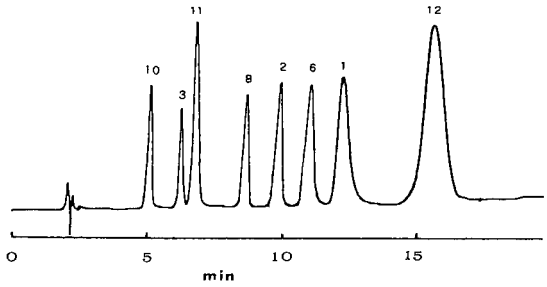

Figure 1-Representative HPLC separation of oxidation hair dyes. Column: Phenomenex Bondclone C-18 (10 μm); mobile phase:acetonitrile-aqueous 5mM 1,8-diaminoctane and 20 mM sodium heptansulfonate solution (pH 4.5) (5+95) at a flow rate of 1,3 ml $min.^{-1}$. Detection at 275 nm. Peaks: Table 1.

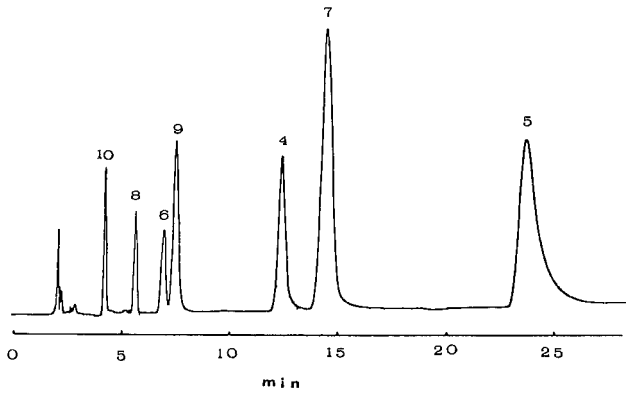

Figure 2- Representative HPLC separation of oxidation hair dyes. Column: Phenomenex Bondclone C-18 (10 μm); mobile phase: acetonitrile-aqueous 5mM 1,8-diaminoctane and 20mM sodium heptansulfonate (pH 4.5), (15+85) at a flow rate of 1.3 ml $min.^{-1}$. Detection at 275 nm. Peaks: Table 1.

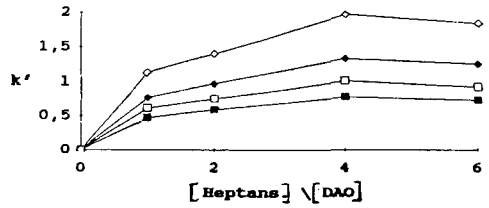

Figure 3- Effect of [heptansulfonate]\[1,8 diaminoctane] molar ratio on the capacity factors (k') for p-PDA(■), m-PDA(□), p-MAP (♦) and ETPDA (◊).

When this study first began, a buffer containing just DAO was employed to suppress the adverse silanol interactions; symmetric peaks were obtained , but an increasing loss of resolution was noticed over time. Therefore, heptansulfonate was added to the mobile phase: increased retention times with improved resolution were obtained and better reproducibility of the chromatographic parameters was attained. This effect was consistent with previously reported studies [21]. More than 1000 injections were performed on the same column under the described conditions without considerable reduction in the column efficiency .On account of the good results obtained with the selected column (Bondclone 10 ODS), a limited number of stationary phases were investigated; among these a Supelco pkb-100 (50x4.5 mm I.D.) column for basic compounds was also used, but, in all the various experimental conditions tried, unsatisfactory separations were obtained.

Sodium Dodecyl sulfate was also assessed as an ion pairing agent, but it was found to be not compatible with DAO because of the poor solubility of the resulting ion pair. On the other hand sodium dodecyl sulfate alone , without amine modifier, provided a useful, but less selective chromatographic system .

Concerning the effect of the pH on the resolution, experiments showed that the range between pH 3.0 and 4.5 is appropriate. Besides, changing from pH 4.5 to

pH 3.0, the system selectivity is slightly modified, with a resulting retention time inversion of some analytes (e.g. p-PDA, p-AP).

Analysis of commercial formulations

The sample pretreatment constituted an important and critical part of the analysis . Because of the poor stability of the basic hair dyes in neutral and basic media, a rapid extractive procedure in acidic medium was developed.

The extraction of the dyes from the cosmetic matrix was performed with 0.85% orthophosphoric acid containing 0.5% sodium sulfite as antioxidant . The resulting suspension was then filtered through a 0.45 mm Cameo filter which is supplied with a prefilter to avoid pores blocking. Care has to be taken in the weighed sample amount, because samples exceeding 0.3 g\100 ml were found to be difficult to filter. The filtered solution was then directly injected into the chromatograph . In Figures 4 and 5, the chromatograms of two representative sample solutions are reported .

When the dyes content in the sample solution was very low (<0.05 mg ml^{-1}), the sample solution was submitted to a solid phase extraction (SPE) procedure which allowed the dyes to be concentrated , enhancing their detectability. Moreover, the SPE step enabled the basic dyes to be separated from the neutral and acidic components, providing simplified chromatograms. In fact, the basic dyes in their protonated form are selectively extracted from the sample solution and retained on the strong cation exchanger, while the phenolic compounds (Resorcinol, 1-naphtol, HNSA) unretained are collected in the filtrate. The subsequent wash step allowed the complete recovery of the phenolic compounds and the washing was therefore added to the filtrate in the same volumetric flask. The volume was adjusted with 0.85% orthophosphoric acid solution and the resulting solution was subjected to the HPLC analysis.

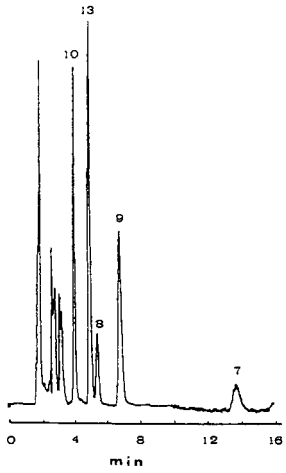

Figure 4- HPLC chromatogram obtained from a commercial hair colorant (sample n°1). Chromatographic conditions as in Fig. 2. UV detection at 275-290 nm. Peaks : Table 1.

The basic dyes retained on the SPE column were eluted with a phosphate buffer (pH 8.0) solution consisting of 1 M sodium chloride and 5 M Urea; this buffer solution, capable of deprotonising and desorpting the basic dyes from the matrix provided an enhanced analyte concentration The eluate obtained was quickly acidified with 0.85 % orthophosphoric acid and, after addition of the internal standard, was subjected to the HPLC analysis. In Figure 6 the chromatograms from sample 3 before and after the SPE procedure are reported .

The same solvent system (acetonitrile-DAO heptansulfonate) proved to be suitable for the HPLC analysis of basic dyes and phenolic compounds. HNSA and Resorcinol (sample 3) were separated using a mobile phase consisting of acetonitrile-pH 4.5 DAO heptansulfonate (5+95) , at a flow rate of 1.3 ml min^{-1}

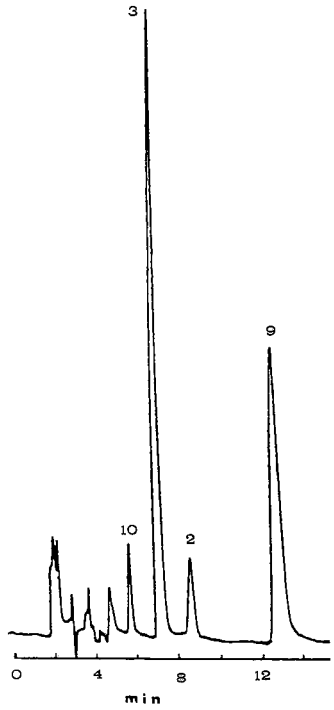

Figure 5- HPLC chromatogram obtained from a commercial hair colorant (sample 2) .Chromatographic conditions and detection as in Figure 1. Peaks: Table 1.

(UV detection at 275 nm)(Figure 6 c), while 1- naphtol required higher percentage of acetonitrile (30%) to obtain a convenient retention time (25 min.)

The identity of the analyte peaks was confirmed by the UV spectra registered by a photo diode array detector.

For quantitative applications linear relationships between the peak area ratios (analyte to internal standard) and analyte concentration were obtained (Table 2)

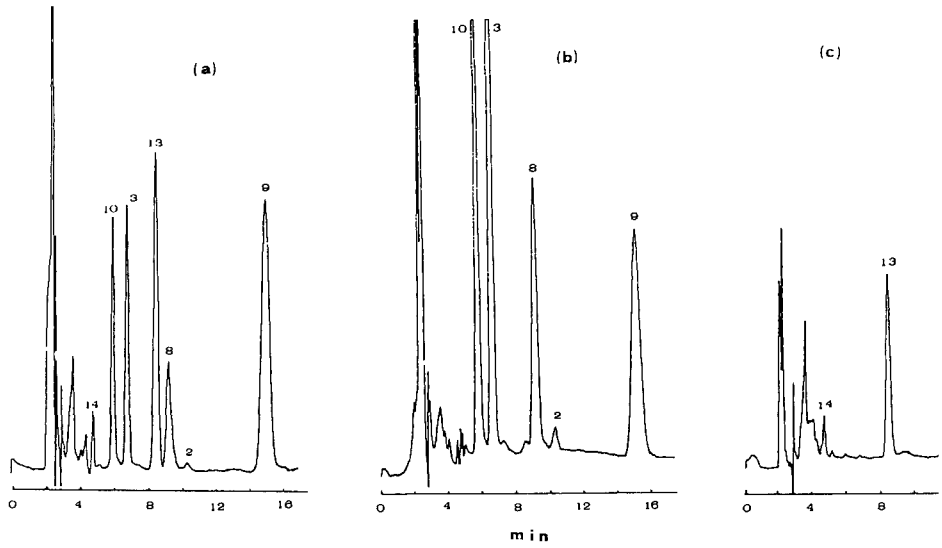

Figure 6-HPLC chromatogram of a) solution of sample n°3 before SPE procedure b) same solution after SPE and c) filtrate through the SCX cartridge . Chromatographic conditions and detection as in Figure 1. Peaks: Table I.

with good precision (RSD% = 0.53-0.96). In the present study our attention was mainly directed to the determination of basic dyes, while just preliminary trials, confirming the suitability of the proposed method, were performed for the assay of phenolic compounds. Four commercial professional products were analysed selecting an appropriate measurement wavelength for each analyte in order to properly modulate the sensitivity. The results obtained are summarised in Table 3. As can be seen, the assay results were in satisfactory agreement with the content declared; the Relative Standard Deviation was between 2-5.6%, with the higher values for m-AP, p-AOC and ETPDA present in the commercial formulations at very low levels (0.0024%,0.0078%,0.034%).

TABLE 3 - Assay results for the HPLC determination of oxidation hair dyes in commercial hair colorants. The results are the average of five determinations and are expressed as a percentage of the content declared by the manufacturer.

Sample	analytes	λ	Found	RSD%	Recovery	RSD%
1[a)]	p-PDA	275	105.50	5.15	100.44	5.09
	p-MAP	275	98.38	2.00	101.80	2.00
	ETPDA	290	95.22	2.53	98.39	3.10
2[a)]	p-PDA	275	99.57	2.31		
	p-AP	275	101.18	2.34	100.60	1.80
	m-AP	275	98.60	5.33	99.50	3.20
3[b)]	p-PDA	275	99.06	4.64	80.08	4.24
	p-AP	275	97.47	4.90	86.82	4.88
	p-MAP	275	94.55	3.84	82.58	1.47
	m-AP	275	91.13	5.60	103.30	5.50
4[b)]	p-PDA	240	103.46	4.23	82.00	3.50
	p-MAP	275	100.20	4.50	80.20	2.40
	p-AOC	220	99.67	4.91	98.00	2.15
	p-PDA[a)]	275	102.25	3.59		

a) The direct method of analysis was used.

b) The method involved the SPE step. The results are related to the effective recovery value

The accuracy of the method was verified by analysing the blank spiked with known quantities of the examined hair dyes . As shown in Table 3, good recovery values were obtained when the direct analysis was performed , while a lower recovery was found when SPE step was necessary to increase the analysis sensitivity. In any case, however, the precision of the method was satisfactory.

CONCLUSION

The proposed reversed phase (C18) HPLC method, involving the use of 1,8-diaminoctane as amine modifier in association with sodium heptansulfonate, provided adequate selectivity for the separation of all the most important oxidation hair dyes. The chromatographic system (isocratic conditions) proved to be rugged enough for routine quality control, because no particular decay of the column (Phenomenex Bondclone 10 mm C18) life was observed . A simplified sample pretreatment can usually be adopted; if necessary, a preliminary SPE on SCX material offers the opportunity for the analyte concentration and the separation of the basic dyes from the other formulation components (phenols).

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REFERENCES

1. M.S. Balsam & E. Sagarin , in Cosmetic, Science and Technology, John Wiley § Sons, New York, 1974,vol. II, p.300.

2. J.F. Corbett, J.Soc.Cosmet.Chem., **35**, 297 (1984).

3. K.C.Brown, J.Soc.Cosmet.Chem., **33**, 375 (1982).

4. K.P.N. Viswanathan, V.Gupta, V.Misra, Int. J. Cosmet. Sc., 7, 213 (1985).

5. H.Gottschalck, R. Machens, J.Soc.Cosmet.Chem., **33**, 97 (1982).

6. Italian Gazz. Uff. Suppl. No 7, Jan. 23rd, 1987, 32.

7. N.Goetz, P.Lasserre, P.Borè, G.Kalopissis, Int. J. Cosmet. Sc., **10**, 63 (1988).

8. S.Fanali, J. Chromatogr., **470**, 123 (1989).

9. N.Goetz, J.Mavro, L.Bouleau, A.de Labbey, in Cosmetic Analysis, Cosmetic Science and Tecnology Series, Vol.4, ed. Borè, P., Dekker, M., Inc. New York, 1985, p.245.

10. D.H.Liemand, J.Rooselaar, Mitt.Geb.Lebensmittelunters Hyg., **72**,164 (1981).

11. K.Tashiro, A.Kojima, Y.Okuda, J.Soc.Cosmet.Chem.,**16**, 125 (1983) (Chem.Abstr. 98:221605 b).

12. G.Hoogewiss, D.L. Massart, J.Pharm.Belg., **38**, 76 (1983).

13. M.C.Gennaro, P.L.Bertolo, E.Marengo, J. Chromatogr., **518**, 149 (1990).

14. L.Gagliardi, M. Ambroso, J. Mavro, F.Furno, G.Discalzi. Int.J.Cosmet.Sc., **14,** 19 (1992).

15. C.J.Dowleand, A.P.Malyan, A.M.Matheson, Analyst, **115**, 105 (1990)

16. D.C.Leach, M.A.Stadelius, J.S.Berns, L.R.Snyder, LC-GC Int., **1**, 22 (1988).

17. R.W.Ross, C.A. Lan Cam, J.Chromatogr., **370**, 406 (1986).

18. R.K.Gilpin, S.S.Yang, G.Werner, J.Chromatogr.Sci., **26**, 388 (1988).

19. A.M.Di Pietra , V.Cavrini, V. Andrisano, R.Gatti, J. Pharm. Biomed. Anal., **10**, 873 (1992).

20. G.Stoev,D. Uzunov, J.Liq.Chromatogr., **15**, 3097 (1992).

21. J.A.De Schutter, W. Van Den Bossche, P. De Moerloose, J. Pharm. Biomed. Anal., **4**, 559 (1987).

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SIMULTANEOUS HPLC ANALYSIS OF TRYPTOPHAN HYDROXYLASE ACTIVITY AND SEROTONIN METABOLISM IN RAT PINEAL GLAND: DETERMINATION OF ITS KINETIC PROPERTIES

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ABSTRACT

The determination of tryptophan hydroxylase activity (TPH) in the pineal gland was based upon the separation and detection of 5-hydroxy tryptophan (5OH-TRP) formed from L-Tryptophan (TRP) by high performance-liquid chromatography (HPLC) with fluorimetric detection. Tryptophan hydroxylase activity and TRP metabolites content show circadian variation in the rat pineal gland. The enzyme exhibited a Km value of 53 ± 15 μM and a Vmax of 243 ± 23 pmol 5OH-TRP/min/mg. prot. for L-TRP, and a Km value of 27 ± 4.54 μM and a Vmax of 90.2 ± 4.35 pmol 5OH-TRP/min/ mg. prot. for tetrahydrobiopterine. The present assay is accurate, simple and sensitive, allowing the determination of TPH activity in a variety of enzyme sources. The combination of simultaneous measurements of serotonin, as well as serotonin precursors and metabolites, from a single tissue sample makes it extremely useful for physiological and pharmacological studies.

INTRODUCTION

Tryptophan 5-monooxygenase (hydroxylase) [L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4], catalyzes the conversion of L-tryptophan (L-TRP) to 5-hydroxytryptophan (5OH-TRP) using tetrahydrobiopterine as a reducing agent and molecular oxygen as a oxidizing agent, and is widely accepted as the rate-limiting step in the biosynthesis of the neurotransmitter serotonin (5HT) (1). A typical circadian rhythm of TPH has been previously described in the rat pineal gland (2, 3), and since it is blocked by propanolol and the enzyme activity increased by isoproterenol and $_cAMP$ 4-6), it seems to be under sympathetic regulation as it occurs, *in vitro* and *in vivo,* with serotonin-N-acetyltransferase (NAT) (7, 8). Current methods for measuring TPH activity are based upon the determination of the reaction product, 5OH-TRP, by either fluorimetric (9), radioisotopic (2, 10), or high pressure-liquid chromatographic (HPLC) procedures coupled with fluorimetric or electrochemical detection (11, 12). In the present study we have modified previous separations, in order to be capable of simultaneously measure both TPH activity and tryptophan metabolites within the same pineal throughout a 24 hour cycle. In addition, we have applied the present assay to characterize the kinetic properties of the enzyme in the rat pineal gland.

MATERIAL AND METHODS

Chemicals

L-tryptophan (L-TRP), dithiothreitol, 3 hydroxybencylhydrazine (NSD 1055), catalase, and 5-hydroxytryptophan (5OH-TRP) were obtained from Sigma Chemical Co. (Fancy Road, Poole, England).

Preparation of tissues

Pineal glands were obtained from male Sprague-Dawley rats (300-400 g) maintained in 12:12 h light-dark cycle (lights on, 02:00), with *ad libitum* access to rat chow and tap water. Animals were killed by decapitation at different times and glands were rapidly removed, frozen on liquid nitrogen, and kept at -80°C until assayed. Individual pineals were

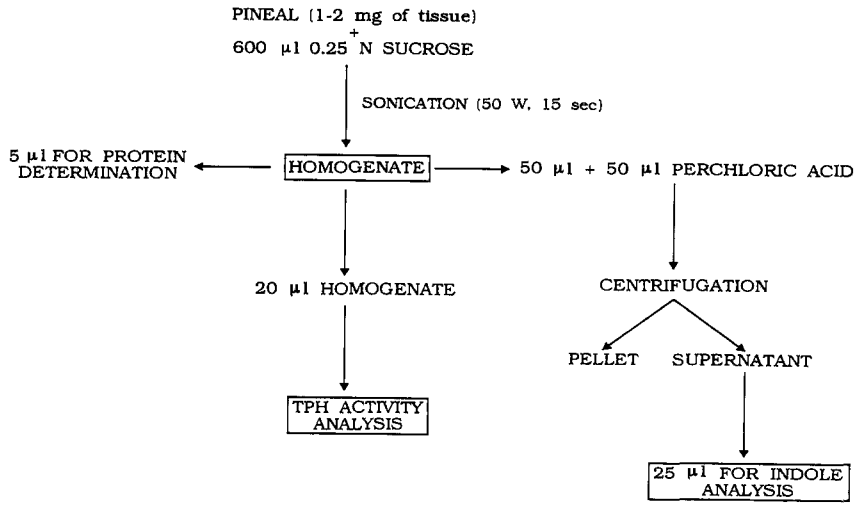

Figure 1.- General sample processing for the analysis, in the same pineal gland, of TPH activity and L-TRP metabolites.

homogenized by sonication (50 W, 15 sec) in 600 µl of 0.25 N sucrose, and different aliquots were used for protein determination, TPH assay, and indoleamine analysis (Fig. 1).

Assay of TPH activity

TPH activity was assayed by measuring the amount of 5OH-TRP formed from L-TRP by high performance-liquid chromatography (HPLC) with fluorimetric detection (FD) (12). Aliquots of 20 µl homogenate were mixed with 18 µl of 1 M tris-acetate buffer (pH = 7.5), 7.5 µl of 6 mM DL-6-methyl-5,6,7,8 tetrahydrobiopterine (THB), 15 µl of 10 mM dithiothreitol, 15 µl of 10 mM NSD 1055, 50 µl of catalase (1462 U), 10 µl of 16 mM ferrous ammonium sulphate, 10 µl of 9 mM L-TRP and 5 µl of destilated water. The general analytical protocol is summarized in figure 2. Briefly, samples were incubated at 37°C for 30 min and the enzymatic reaction was stopped by addition of 20 µl of ice-cold 70% perchloric acid. Following centrifugation at 11.000 rpm for 5 min, 150 µl of 0.05 M carbonate buffer (pH=10.25) and 500 µl of chloroform were added. After vigorous shaking, samples

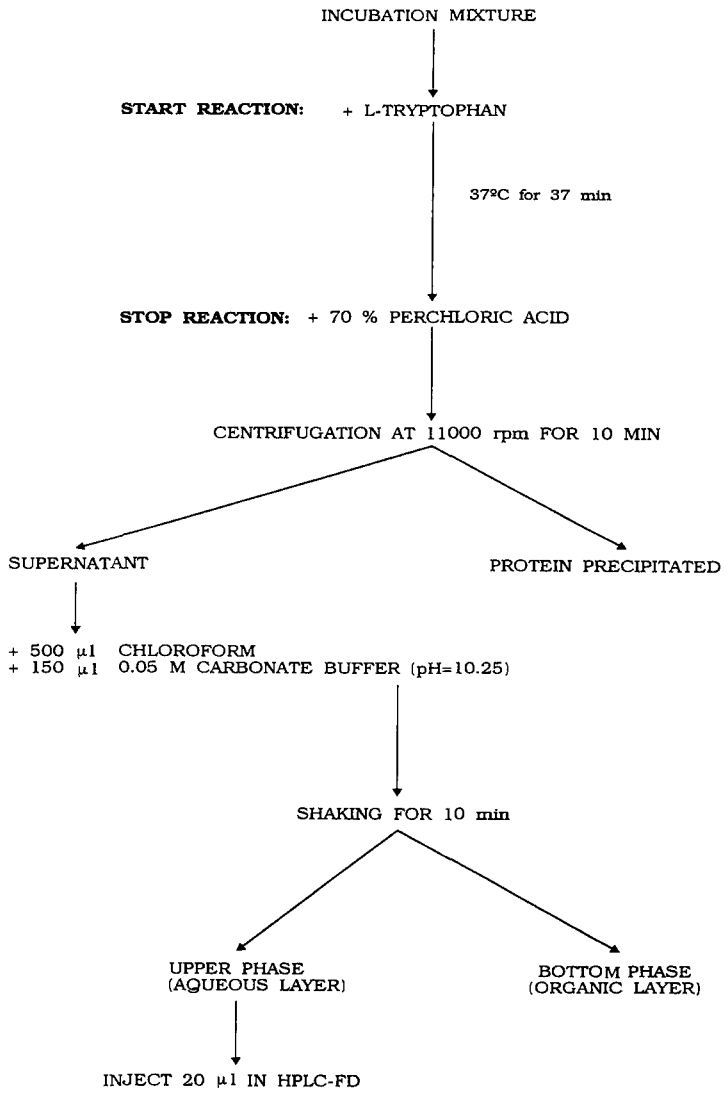

Figure 2.- Diagram summarizing the analytical protocol for TPH activity assay by HPLC-FD.

were centrifuged and 25 µl of aqueous layer (upper phase) were injected into the chromatographic system. The protein content was determined in 10 µl aliquots of the homogenate by the method of Bradford (13), using bovine serum albumin as standard.

In order to carry out the kinetic studies, the final concentration of L-TRP was varied up to 400 µM, while keeping the concentration of THB at a constant level of 400 µM. Conversely, in a different serie of experiments the final concentration of THB varied up to 500 µM, while keeping the concentration of L-TRP at a constant level of 400 µM.

Indoleamine analysis

Fifty µl of the sucrose homogenate were taken into 1.5 ml polypropilene tubes, and 50 µl of 0.4 M perchloric acid/0.025% sodium metabisulfite were added. Samples were then centrifuged at 15,000 rpm for 5 min, and 50 µl aliquots from the clear supernatant were injected into the chromatographic system.

Chromatographic procedure

For both TPH activity and indoleamine content, aliquots prepared as described above were injected into a C_{18} Nucleosil ODS reversed-phase column (particle size 5 µm, Scharlau S.A, Barcelona, Spain). The mobile phase consisted of 0.1 M sodium dihydrogenphosphate, 0.1 mM EDTA, 300 mg/l sodium heptanesulphonate (PIC B7), 5% acetonitrile (vol/vol) in deionized water (Millipore Q System, Millipore Ibérica S.A, Barcelona, Spain), which was adjusted to pH 4.17, and filtered and degassed immediately before used. The system was run at a flow rate of 0.8 ml/min (Waters 510 pump, Millipore Ibérica, Barcelona, Spain), and the fluorescence detector (LS 40, Perkin Elmer Ltd, Buckinghamshire., U.K.) was set at 294 nm and 345 nm as excitation and emission wavelengths, respectively. The identification of peaks by retention time and quantification of 5OH-TRP by peak height was determined using a HP 3396A integrator (Hewlett Packard Co., Avondale, U.S.A.). Under the conditions described above, 5OH-TRP displayed a retention time of 4.2 minutes (Fig. 3), and was completely separated from other potentially interfering substances.

Data analysis

The kinetic analysis of TPH activity was performed by using a nonlinear regression data analysis program (Enzfitter) (14).

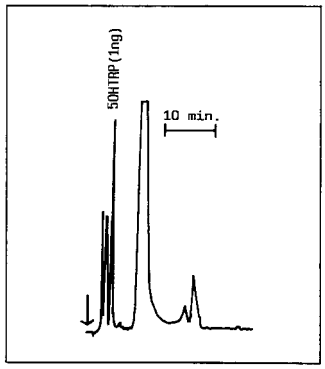

Figure 3.- Representative HPLC-FD elution pattern of 5OH-TRP enzymatically formed from L-TRP in the rat pineal gland. The chromatographic conditions are described in the "Material and Methods" section.

RESULTS AND DISCUSSION

The HPLC-fluorescence method described in this paper provides a rapid, selective, and sensitive approach for the separation and quantitation of the product enzymatically formed from L-TRP, 5OH-TRP, from the rest of compounds present in the enzymatic mixture. The clean up step performed by the addition of organic solvent after stopping the reaction, avoids the presence of interfering substances into analysis matrix, resulting in a complete separation of the reaction product (Fig 3). The correlation between detector response and the amount of authentic 5OH-TRP injected, with and without an organic clean up step, showed a satisfactory coefficient in both cases (0.9886 and 0.9897, respectively) (Fig. 4). The recovery of 5OH-TRP formed enzymatically was 95-97%, and the difference between retention time for 5OH-TRP samples and that of authentic 5OH-TRP was less than 2%. The detection limit for 5OH-TRP, based on a peak height versus background noise ratio of 3:1, was obtained by measuring the native fluorescence and displayed a value of 30 pg/injection.

As shown in figure 5, 5OH-TRP formation increased in a linear manner with increasing amounts of tissue up to 140 μg/tube (Fig. 5A). On the other hand, the rate of

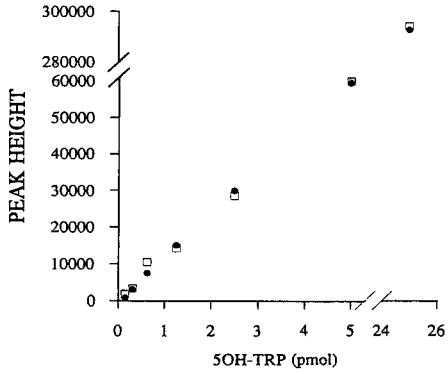

Figure 4.- Linear relationship between detector response and different amounts of authentic 5OH-TRP with (clear squares, r = 0.9886) and without (solid circles, r = 0.9897) an organic extraction with chlorofom.

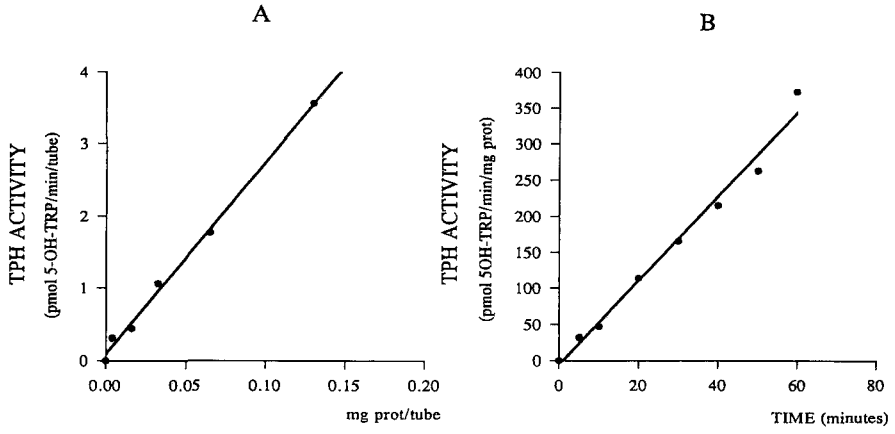

Figure 5.- Linearity of TPH activity with respect to either enzyme concentration or incubation time. Assay was done with incubation time fixed at 30 min, as a function on enzyme concentration (A), or by fixing that variable and then modifying incubation time (B). Standard assay conditions were as described in "Material and Methods". In this and the next figure each point represents the mean of six determinations.

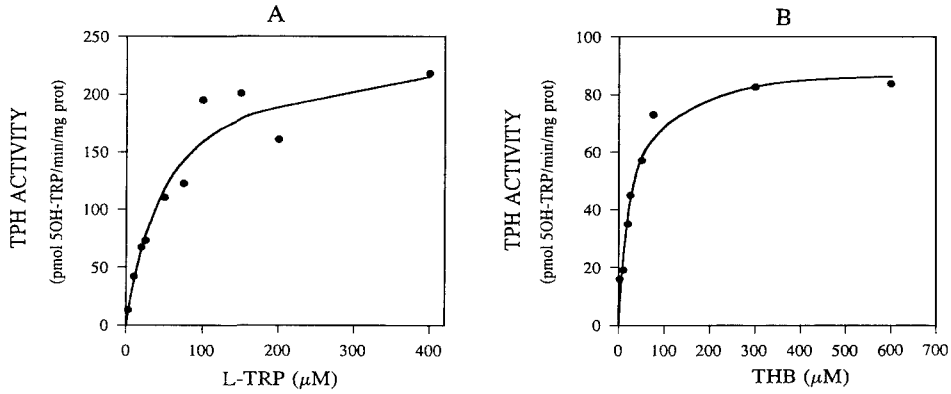

Figure 6.- Michaelis-Menten plots of TPH activity in the rat pineal gland. These studies were carried out while varying the concentration of L-TRP from 2.5 to 400 μM, with a fixed concentration of 300 μM THB (A), or by varying the concentration of THB from 2.5 to 400 μM, with a fixed concentration of 300 μM L-TRP (B).

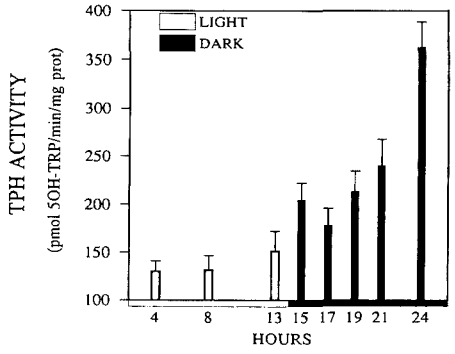

Figure 7.- Circadian rhythm of TPH activity in the rat pineal gland. Groups of animals were sacrifized at the times indicated. Each bar represents the mean ± SEM of eight animals.

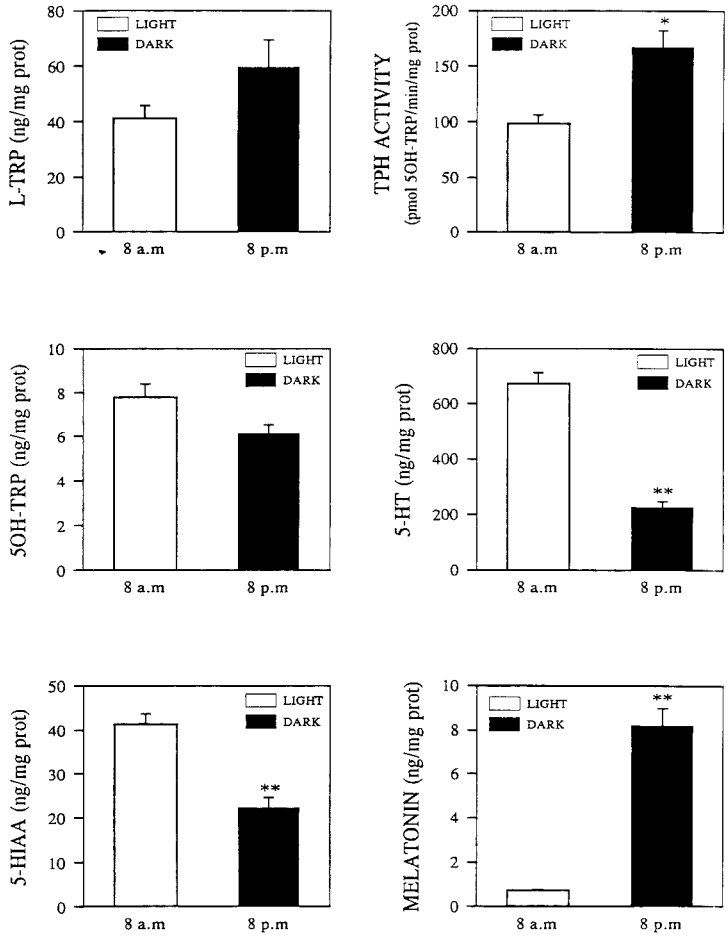

Figure 8.- Pineal levels of L-TRP, TPH activity, 5OH-TRP, 5-HT, 5-HIAA, and melatonin of male rats. The glands were obtained during light (08:00) or dark period (20:00) of a 12:12 hr cycle (lights on, 02:00). Vertical bars represent the mean ± SEM of eight animals.

(*), $p<0.005$ vs Light; (**), $p<0.0001$ vs Light.

product formation was also linear up to 70 min when using a fixed amount of enzyme (Fig. 5B). This allows the use of a wide range of incubation times, as well as the application of the assay to different tissues containing various protein quantities.

The kinetic analysis revealed a Km value of 53.4 ± 15 μM and a Vmax of 245 ± 23 pmol 5OH-TRP/min/mg. prot. for L-TRP (Fig. 6A), a Km value of 27 ± 4.54 μM and a Vmax of 90.2 ± 4.35 pmol 5OH-TRP/min/ mg. prot. for tetrahydrobiopterine (Fig. 6B). These results are consistent with previous data using crude enzyme preparation from rabbit brain (9). We can conclude that the affinity of L-TRP (substrate) and tetrahydrobiopterine (cofactor) for the enzyme is similar. However, the maximum initial velocity, for the same protein concentration, is greater for L-TRP.

Figure 7 shows the variation of TPH activity from pineal glands of male rats collected at different times during a 24 hr period. TPH activity increased gradually from dark onset, displaying highest values at the end of the dark period. This finding confirms a circadian rhythmicity of TPH activity previously reported by others (2, 3, 11), as opposed to early data (16), and supports the view of this enzyme being nocturnally regulated as it occurs with other components of the melatonin synthesizing pathway.

When plotting day and night values of different variables involved in melatonin metabolism, a coherent picture is viewed (Fig. 8). Pineal L-TRP and TPH activity presented a rhythmic pattern similar to that of melatonin, and opposite to that of 5OH-TRP, serotonin (5HT), or 5-hydroxyindole acetic acid (5HIAA). However, as shown in the figure, day/night differences in the content of L-TRP or 5OH-TRP were not significant at the times used in this study, which is in agreement with previous reports in rats (17) and rabbits (18).

In summary, the present assay offers a simple and reliable method for measuring TPH activity in homogenates from rat pineal glands and other tissues. The assay is accurate and highly sensitive, allowing the determination of TPH activity as well as several TRP metabolites within individual pineals with the same chromatographic system. This approach also allows us to reduce the number of animals needed for physiological or pharmacological studies, and provides a way to correlate circadian or experimentally induced changes in various components of the serotonin metabolic pathway. In addition, this method has been proved useful to detect low levels of TPH activity, not only in homogenates from rat pineal glands, but also in other enzymatic sources, such as rat dorsal raphe and retina (data not shown), which contain small amounts of neural tissue.

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REFERENCES

1. E. Jéquier, N. Lovenberg, A. Sjöerdsme, **Mol. Pharmacol.**, 3: 274-278 (1967).

2. H. Shibuya, M. Toru, S. Watanabe, **Brain Res.**, 138: 364-368 (1978).

3. B.R. Siratam, G.J. Lees, **J. Neurochem.**, 31: 1021-1026 (1978).

4. M. Toru, S. Watanabe, T. Nishikama, J. Semba, H. Shibuya, "Physiological and pharmacological properties of circadian rhythm of tryptophan hydroxylase in rat pineals", in Advances in Biosciences, Vol. 21: Pharmacology of the state of alertness, P. Passouant, I. Oswald, eds., Pergamon Press, Oxford, 1979, pp 253-255.

5. B.R. Siratam, G.J. Lees, **J. Neurochem.**, 42: 1183-1185 (1984).

6. M. Ehret, P. Pevet, M. Maitre, **J. Neurochem.**, 57: 1516-1521 (1991).

7. T. Deguchi, **Mol. Cell. Biochem.**, 27: 57-66 (1979).

8. J.J. Morrissey, **Essays Neurochem. Neuropharmacol.**, 5: 25-45 (1981).

9. P.A. Friedman, A.H. Kappelman, S. Kaufman, **J. Biol. Chem.**, 247: 4165-4173, (1972).

10. A. Ichiyama, S. Nakamura, Y. Nishizuka, O. Hayaishi, **J. Biol. Chem.**, 245: 1699-1709 (1970).

11. D. Sugden, R. Grady, I.N. Mefford, **J. Pineal Res.**, 6: 285-292 (1989).

12. T. Nagatsu, **J. Chromatogr.**, 566: 287-307, (1991).

13. M.M. Bradford, **Anal. Biochem.**, 72: 248-254 (1976).

14. R.J. Leatherbarrow, Enzfitter. A non-linear regression data analysis program for the IBM PC, Elsevier Science Publisher, Amsterdam, 1987.

15. A. Carlsson, W. Kehr, M. Lindqvist, T. Magnusson, C.V. Atach, **Pharmac. Rev.**, 24: 371-384, (1972).

16. T. Deguchi, **J. Neurochem.**, 28: 667-668, (1977).

17. I.N. Mefford, P. Chang, D.C. Klein, M.A.A. Nambodiri, D. Sugden, J. Barchas, **Endocrinology**, 113: 1582-1586, (1983).

18. G.C. Brainard, S.A. Matthews, R.W. Steger, R.J. Reiter, R.H. Asch, **Life Sci.**, 35: 1615-1622, (1984).

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DETERMINATION OF HARPAGIDE, 8-PARA-COUMAROYL HARPAGIDE, AND HARPAGOSIDE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN HARPAGOPHYTUM PROCUMBENS DRUGS AND IN A COMMERCIAL EXTRACT

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ABSTRACT

A selective method for the determination of HARPAGIDE (HG), 8-para-coumaroyl HARPAGIDE (8PCHG) and HARPAGOSIDE (HS) is described. HS and 8PCHG are the two main compounds extracted from commercial dry extract of Harpagophytum secondary roots.

Analysis of iridoids was performed using a linear gradient system of methanol / water as mobile phase, on RP 18 column. For HG Light-Scattering and UV detection were used for the quantification. Assays were linear in the concentration ranges studied. Statistic evaluation of the method was also realised to improve its security. In a total run time of 60 mn this procedure permitted to quantify simultaneously these three iridoids in the drug including HG for the first time in HPLC.

INTRODUCTION

Harpagophytum procumbens is a medicinal plant [1] which grows in Kalaharia desert (Namibia, South Africa) and presents anti-inflammatory properties [2]. Main active compounds have been isolated and identified: They are glucoiridoids [3](fig. 1),

HARPAGOSIDE (HS) and 8 PARA-COUMAROYL HARPAGIDE (8PCHG) were found in large proportion in the drug and HARPAGIDE (HG) is not a negligible

* Corresponding author

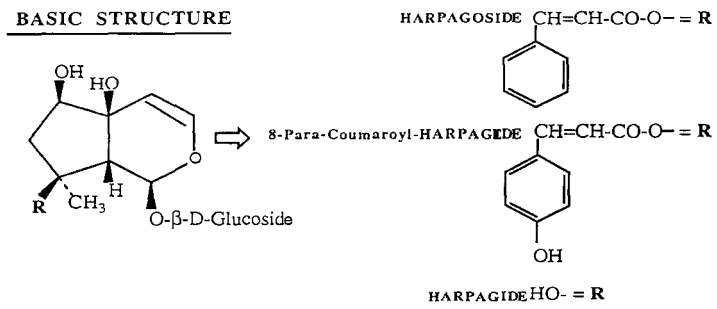

FIGURE 1 : Glucoiridoïds of Harpagophytum procumbens [3]

iridoid, so its quantification is very important because it is a direct metabolite of HS and 8 PCHG.

In order to use Harpagophytum for new pharmaceutical applications, it was necessary to know a sensitive and selective method for the quantification of drug's active components and also their main metabolite : HG.

A few methods using HPLC are described [4] [5] for the assay of HS but until now, there was not any procedure reported for the analysis of 8PCHG and HG.The very low UV absorbtion of HG did not permit quantitative assay by HPLC. The use of Light-Scattering detector and UV detector allows the simultaneous assay of all the components.

The aim of our study was to establish a sensitive and rapid method to appreciate quality and stability of the drug.

MATERIALS

Standards :

8 PCHG and HS (for preparation of HG) were obtained by preparative liquid chromatography from dry commercial extract of Harpagophytum secondary roots (RES PHARMA ®).
HG for HPLC was prepared by hydrolysis of HS isolated by preparative liquid chromatography.
HS was purchased from Extrasynthèse ® Genay France

Samples

Drug :

Drugs were purchased from Alban Muller ® (Marseille, France) (little discs) and Technic Arome ® (Allauch, France)(peaces 1 cm3). Both were grinded and placed into a dessicator before preparation.

Extract :
Extract was purchased from RES PHARMA ® (Paris, France) (fine water soluble powder)

Solvents for isolation :

Solvents were from Farmitalia Carlo Erba ® Chemicals (Milano, Italy) Puro grade for isolation.

Solvents for HPLC :

We used special solvents grade for residute analysis to prepare HPLC mobile phases.
Methylic Alcohol RS 414917 RSE for electronic use ERBATRON, Farmitalia Carlo Erba ® Milano, Italy.
Purified and desionised Water was from Milli-Ro system Millipore (USA).
All solvents were filtered throught a Millipore filter (0,45 µm for methanol, and 0,2 µm for water). A solvent in line filter 0,2 µm Wathman ® (Maidstone, England) was installed before HPLC pump for the water.

Stationary preparative phases :

- Aluminium-oxyd, activity I, II Merck (Paris, France)
- Silica-gel, kieselgel 60 Merck
- Preparative HPLC RP 18 column Merck Lichroprep 15 - 25 µm, 5 X 14 cm.
- Preparative HPLC RP 18 pre-column Jones Chromatography (Hengoed, Austria), 15 - 35 µm, 5 X 5 cm.
- Sep-pak ® Waters C18 cartridges for solid phase extraction (Milford, Massachusetts, USA)

Apparatus :

Preparative HPLC system consisted of one C.E.D.I. (Lannemesan, France) equipped with two pumps.

Analytic HPLC system consisted of :
2 pumps : Waters ® Model 501.
1 20 µl rheodyne injector.
1 Waters ® programmable multiwavelenght detector Model 490.
1 Light-Scattering detector (Cunow DDL 21, Waters, St Quentin en Yveline France)
All the system was monitored by a Waters 840 data and chromatographic control station installed on a digital professional 380 computer.

As stationary phase, a Phenomenex ® RP 18 (Interchim France) Bondclone 10 µm 300 X 3,9 mm column was used with a precolumn Guard pak ® µBondapak C18 Waters.

Composition of the mobile phase as well as the UV detector wavelenght and Light Scattering Detector settings are described in the following.

METHOD

Isolation of standards :

Dry extract was extracted with methanol, then, after evaporation under vacuo, the residue was chromatographied on Aluminium-oxyd (Ethanol 80v : H2O 20v) and on Silica-gel (CHCl3 : Methanol) preparative open column. A final purification was realised by HPLC (preparative column RP 18 with solvent Methanol : H2O). Hydrolysis of HS was performed with NaOH 0,2% and isolation of HG was achieved by HPLC (preparative column RP 18, solvent Methanol : H2O). Identification was performed by NMR C^{13} spectroscopy by comparison of experimental data with references [3].

Chromatographic conditions

The analytical column was maintained at room temperature, the mobile phase consisted of Methanol / Water gradient (fig 2) delivered at a flow rate of 1 ml/min, the UV multichanel detector was set at 278, 305 and 312 nm to monitor HS and 8PCHG respectively on a large range of concentrations.

Under these conditions, the retention times for HS and 8PCHG were 36,3 min and 30,8 min respectively. The total run time was 60 min.

In serie after UV detector with a 500 nm lenght capillar, was connected a Light Scattering Detector (DDL). For evaporative, the nebulization of the eluent was provided by a stream of pressurized air at 1,6 bars. The nebulized solvent was evapored at 63 ± 3°C. The pressurized air was filtered through a millex FG 50 (0,2 µm, Millipore).

Under these conditions the retention times for HG, 8PCHG and HS were 8 min, 31 min and 36,5 min, respectively.

Preparation of standards :

Solutions of HS, 8PCHG, HG were prepared at 10 mg/ml in water and cold stored at -20°C until use.
Appropriate dilutions of the stock were made just before analysis with water to prepare standards. Solutions containing HS and 8PCHG were stable for one week. HG in solution at ambient temperature was stable during no more one hour. Peak areas were processed to establish a relation with concentrations.

Preparation of assays :

Drugs :
5 g of drug were placed into 70 ml of methylic alcohol and warmed during 15 minutes. Preparation was filtered and completed to 100 ml of methylic alcohol and evaporated in vacuo. The residue was dissolved into 50 ml of water, 2 ml of this preparation were concentred on SEP PACK ®. Elution was performed with 10 ml of water and 10 ml of methanol, the methanolic fraction was evapored under vacuo and dissolved with 500 µl of water. The final solution was filtered (0,2 µm Dynagard ® Merck, Paris) and injected.

FIGURE 2a

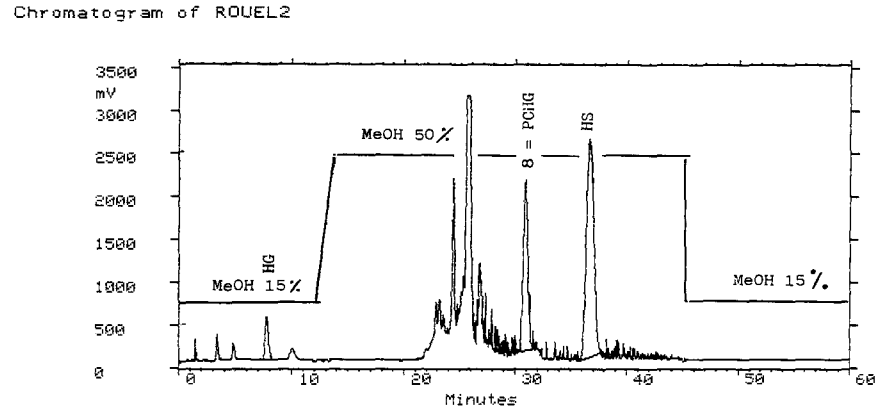

FIGURE 2b

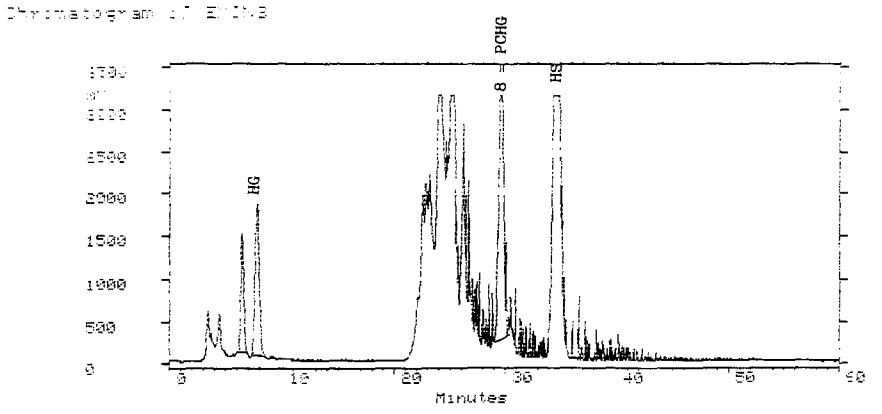

FIGURE 2 : Chromatogram of a drug (a)
Chromatogram of an extract (b)

Extract :
5 g of dry extract were dispersed in 100 ml of water. 2 ml of the solution were concentrated on SEP PACK ®, with the same method than described previously.

RESULTS

Assay of standards

Linearity
Calibration curves were plotted by correlating the peak areas against the corresponding concentrations, the responses were linear in the concentration range studied; the square regression lines and the corresponding coefficients of correlation are described in the following table.

With light scattering detection the relation between Concentrations and Areas is an exponentiel curve. We took [Log (Areas) - 5] and [Log (Concentrations) + 2] in order to propose a linear curve of calibration. So, coefficients a and b were calculated with the Log method above described.

$Y = a X + b$.
Y = Response (Area or Log (Area)); X = Concentration (mg/ml).
r = Coefficient of correlation.
n = number of calibration curves from which the data were calculated

Corresponding calibration curves are described by Fig 3.

Injection reproducibility of references samples

One synthetic solution of the 3 references samples was injected 10 times. Concentration of each sample and coefficient of variation observed are described in the following TABLE 2.

Preparation reproducibility of references samples

Five solutions of HS, 8PCHG were prepared and injected twice. Coefficient of variation observed for the five analysis and correspondent concentrations are displayed in the TABLE 3.

TABLE 1

Compounds	Detection	a	b	r	n
HS	UV 278 nm	50407674	-89452	0,9998	3
	UV 305 nm	18801766	-1154236	0,9993	3
	DDL	1,9468	-1,1398	0,9981	3
8PCHG	UV 278 nm	28193498	-724201	0,9992	3
	UV 312 nm	42225630	22390	0,9994	3
	DDL	1,8879	-0,831564	0,9995	3
HG	DDL	1,9404	-1,44357	0,9982	3

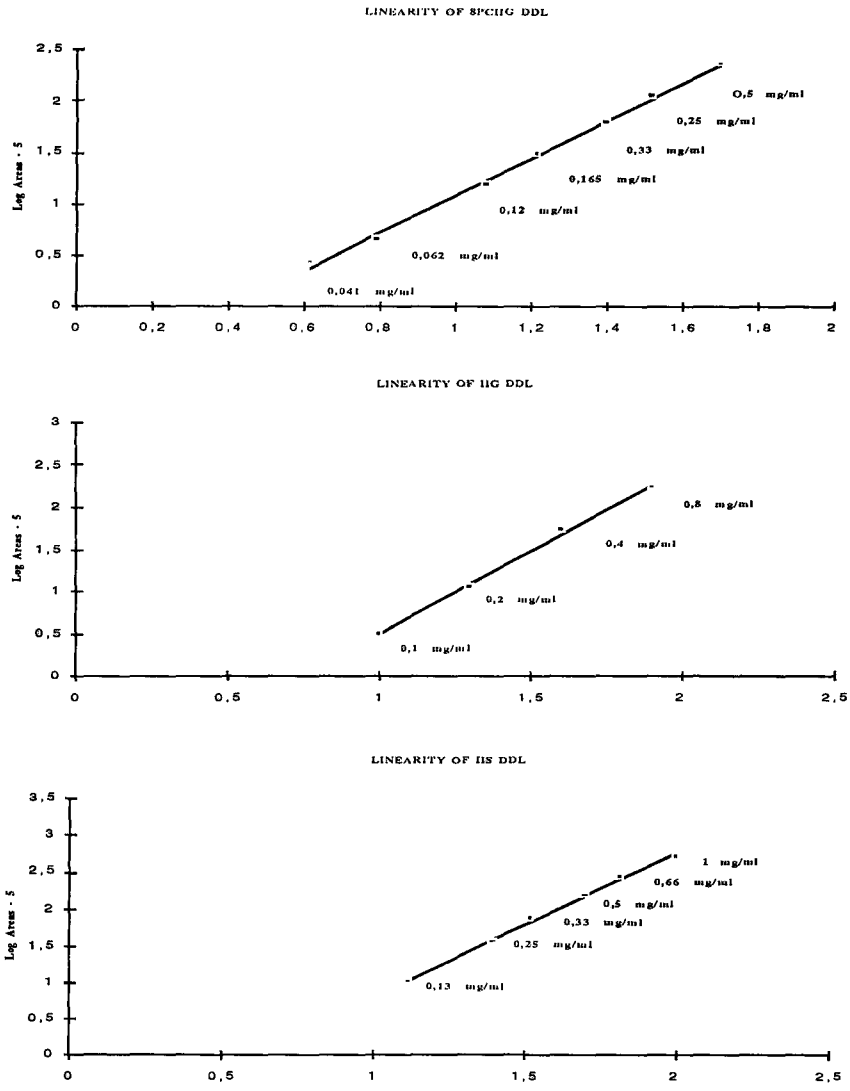

FIGURE 3 : Calibration curves with DDL

TABLE 2

Compounds	HG (0,8 mg/ml)	8PCHG (1 mg/ml)		HS (2 mg/ml)	
	DDL	DDL	UV 278 nm	DDL	UV 305 nm
Average	17747330	28215629	27284507	90517456	36178026
$\sigma 2$	461209	1931394	430856	1948255	605520
Coef of Variation	2,60%	6,85%	1,58%	2,15%	1,67%

Compounds	HS 0,2 mg/ml UV 278 nm	8 PCHG 0,06 mg/ml UV 312 nm
Average	10828267	2747117
$\sigma 2$	166323	40121
Coef of variation	1,54%	1,46%

TABLE 3

Compounds	HG (0,8 mg/ml)	8PCHG (1 mg/ml)		HS (2 mg/ml)	
	DDL	DDL	UV 278 nm	DDL	UV 305 nm
Areas' average	18225698	33338323	25698286	91077167	36288233
$\sigma 2$	760229	894877	243775	4016324	349475
Coef of Variation	4,17%	2,68%	0,95%	4,41%	0,96%

Compounds	HS 0,2 mg/ml UV 278 nm	8PCHG 0,06 mg/ml UV 312 nm
Average	*10655909*	1737040,4
$\sigma 2$	247851,17	22831,64
Coef of Variation	*1,54%*	1,31%

Detection limits

Limits of detection were observed with a minimum signal-to-noise ratio of 2:1.

8PCHG with : UV 312 nm detection = 2 µg/ml,
DDL = 50 µg/ml,
HS with : UV 278 nm detection = 4 µg/ml,
DDL = 50 µg/ml,
HG with DDL detection = 60 µg/ml.

Quantification limits

Quantification limits were for HS : 8 µg/ml and for 8PCHG : 4 µg/ml. For ten injections, coefficients of variation were 2,44 % and 3,05 % respectively. These results were obtained with UV detection (respectively : 278 and 312 nm). With the DDL we found, in order to titrate simultaneously the three compounds, 100 µg/ml and the following coefficients of variation : HG = 7,54 %, 8PCHG = 4,98 %, HS = 7,56 % for ten consecutive injections.

Assay of samples

Assays were realised on two samples of drugs (Alban Muller ® and Technic Aromes ®) and a commercial extract : RES PHARMA ®.
A 1/20 dilution has been performed for injections realised at 278 nm and 312 nm wavelenghts to avoid saturation of the detector.
Injections and methods of preparation are reproducible.
A part of results are summarized in the TABLES 4 and 5

Quantification of samples :

The final concentration in drugs and extract was (in g /100 g of sample).

The difference between each detection mode was 5 % max.

DISCUSSION

Our method enables quantification of the main iridoids of Harpagophytum. Even if Light-Scattering Detection is less sensitive than UV detection for the HS and 8PCHG, it permits to titrate drugs or extracts with a quantification limit of 0,05 %.
The concentration of the iridoids are similar in the different samples studied.
This method presents a great interest for Harpagide. It was until now impossible to

TABLE 4
Injection reproducibility of extracts

Compounds n=10	HG DDL	8PCHG UV 278 nm	8 PCHG UV 312 nm	HS UV 278 nm	HS UV 305 nm
Areas' average	26161233	38532201	2747117	9840648	58428109
σ2	945422	443739	40121	153932	581160
Coef of variation	3,61%	1,15%	1,46%	1,56%	0,99%

TABLE 5
Preparation reproducibility of extracts

Compounds n=10	HG DDL	8 PCHG UV 312 nm	8PCHG DDL	HS UV 278 nm	HS DDL
Areas' average	24942936	3600447	37339706	10105709	57372130
σ2	307501	55650	755635	295357	820691
Coef of variation	1,23%	1,46%	2,02%	2,92%	1,43%

TABLE 6

Compounds	HG	8PCHG	HS
Drugs			
Technic Aromes	0,3	0,98	1,64
Alban Muller	0,37	1,07	1,64
Extract			
Res Pharma	0,56	0,7	1,61

titrate it by traditionnal HPLC detection method, combination of the two detection modes allows the assay of the three compounds simultaneously.

For UV detection the different wavelenghts employed permit quantification on a large range of concentrations :
- For low concentrations, 278 nm for Harpagoside and 312 nm for 8 Para Coumaroyl Harpagide, will be used (with a quantification limit in extracts of 0,004 % for HS and 0,002 % for 8 PCHG).
- For high concentrations, 305 nm for HS and 278 nm for 8PCHG will be chosen to ovoid a dilution and so titrate the three products simultaneously. (Run time is 60 min for each analysis).
- For medium concentrations (no more than 0,8 %) it is possible to use the Light-Scattering Detector for HS, 8PCHG and HG without dilution of the sample prepared with the method described previously.

Comparison between both detections was very important to do an evalution of Light Scattering Detector performances. For the HG there is not any solution with UV detection, so this detector is interesting. But for the other compounds, it is less sensitive and less reproducible than UV detector.

Therefore, the complete separation and the simultaneous determination of Harpagoside, 8 para-coumaroyl Harpagide and Harpagide is possible by the proposed method in one hour. It is the first HPLC method described for 8PCHG and HG using an original kind of detection : the Light Scattering Detector for HG.

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REFERENCES

[1] O. STICHER, Deutshe Apotheker Zeitung, 117 :1279 - 1284 (1980)

[2] C. CIRCOSTA, F. OCCHIUTO, S. RAGUSA, A. TROVATO, G. TUMINO, F. BRIGUGLIO and A. DE PASQUALE, Journal of Ethnopharmacology, 11 : 259 - 274 (1984)

[3] T. KIKUCHI, S. MATSUDA, Y. KUBO, T. NAMBA, Chem. Pharm. Bull., 31 (7) : 2296 - 2301 (1983)

[4] 0. STICHER and B. MEIER, Deutshe Apotheker Zeitung, 120 : 1592 - 1594 (1980)

[5] B. MEIER and 0. STICHER, Journal of Chromatography, 138 : 453 - 457 (1977)

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EXTRACTION AND HPLC ANALYSIS OF HALOFUGINONE IN CHICKEN SERUM*

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ABSTRACT

Extraction and HPLC methods are described for the analysis of the anticoccidial quinazolinone drug, halofuginone (Hal), in chicken serum. Serum samples were diluted with acetic acid and Hal was adsorbed onto a Bond Elut® C8 column followed by elution and HPLC analysis. Recoveries of Hal from chicken serum spiked with 97 ng/ml Hal after 18 and 48 hours incubation were 97.6 and 96.2%, respectively, and recoveries of a 10 ng/ml spike after 18 and 48 hours incubation were 99.7 and 96.7%, respectively, with a detection limit of 1.5 ng/ml. Hal-HBr fed to chickens at 3 ppm for 10 days resulted in 3.75 ng/ml Hal in the serum.

*Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

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INTRODUCTION

Halofuginone [55837–2–2] (Hal, Fig. 1) is used worldwide as an antiprotozoal drug to prevent coccidiosis in commercial poultry production [1]. The drug is the hydrobromide (HBr) salt [64924–67–0] [2] of a halogenated analog of the naturally-occurring quinazolinone alkaloid, febrifugine [3], and was among the top five coccidiostats used in poultry production in the U.S. during 1991 [4]. Hal-HBr is an FDA approved feed additive at 3 ppm for the prevention of coccidiosis in broiler chickens, and has a tolerance in chicken liver of 0.16 ppm and a required withdrawal of 4 days [5,6,7].

Both GLC and HPLC methods for determination of Hal in chicken feed were investigated for the purpose of registering the drug [8]. It was shown that Hal decomposed when chromatographed by GLC, and that the use of HPLC resulted in a 95.6% recovery of Hal from feed. It was also demonstrated that Hal degrades in alkaline solution [8]. Hal appears to be susceptible to methanolysis; during modifications of the HPLC method [8] used for analysis of Hal in feed, an Analytical Methods Committee [9] found that recoveries ranged from 58–91% when Hal was chromatographed with methanolic solutions, and that increasing the solvent flow rate improved recoveries. When acetonitrile was substituted for methanol in the mobile phase, recoveries of 93.4–97.4% were obtained. We have noted while synthetically converting Hal to various derivatives that the use of methanol in the reactions results in degradation of Hal to a number of decomposition products [10].

An extraction and HPLC method was published for the analysis of Hal in feeds and chicken tissues, including liver, kidney, muscle, skin, and fat [11]. The recovery obtained from feed was 92.7%, while the recoveries from chicken tissues

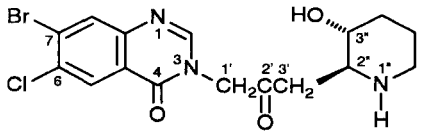

Figure 1. The chemical structure of halofuginone.

varied from 68–117%. Analytical methods accepted by the Food and Drug Administration (FDA) must have recoveries of 60–110% [12]. The Analytical Methods Committee accepted, with minor changes, the published method [11] for determining Hal in chicken tissues [13]. The recovery of Hal in a collaborative study from different chicken tissues ranged from 36–93% [13]. Extracting Hal from chicken tissues with a methanol:acetate buffer (1:1) followed by HPLC analysis resulted in recoveries that varied from 64–87.4% [14].

The Food Safety Inspection Service (FSIS) currently monitors chicken livers for the presence of Hal. The official method [15] used is a composite of a number of methods described above, and is complex and tedious. FSIS accepts recoveries of 60–114% for this method.

We have begun a systematic study of the levels of Hal found in chicken liver versus the levels found in a more workable tissue, chicken serum. The use of an immunochemical method based on monoclonal antibodies to Hal [16] would provide good sensitivity and a high through-put of samples. To verify the accuracy of the monoclonal technology, comparisons of data obtained by a competition enzyme-linked immunosorbent assay (ELISA) with data obtained from a standard HPLC analysis method is required.

A simple method for the determination of Hal in bovine plasma by solid phase extraction and HPLC analysis resulted in an average recovery of 75% [17]. This method for analysis of Hal in bovine serum was very simple, straight forward, but lacked an acceptable recovery rate.

This paper presents an improved method of extraction of Hal from chicken serum followed by HPLC analysis which provides consistent, high recoveries. The serum dilution and acidification step used during the extraction process was extensively investigated to determine its impact on the potential loss of Hal. The extraction and HPLC analysis of Hal as described here was used to determine the concentration of Hal in Leghorn chickens fed a ration containing 3 ppm Hal-HBr.

MATERIALS AND METHODS

Chemicals

Halofuginone-HBr (99.4%, Lot #0A3081B) and Stenorol® (2.72 g halofuginone/lb., Lot #42307062) were provided by Hoechst-Roussel Agri-Vet

Co., Somerville, NJ. Instrumental grade acetic acid [64–19–7] Tracepur® Plus, OmniSolv HPLC grade methanol [67–56–1] and acetonitrile [75–05–8] were obtained from EM Science, Gibbstown, NJ. HPLC grade ammonium acetate [631–61–8] was obtained from Fisher Scientific, Fair Lawn, NJ. Decylamine [2016–57–1] was obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. Water was prepared on a Millipore RO system, Marlborough, MA.

Materials and Instrumentation

All surfaces that came in contact with Hal when preparing and storing standards or analyzing samples were either polypropylene or silylated glass. Glass active sites were exposed by cleaning the surfaces thoroughly with a solution of 5% potassium hydroxide in 95% ethanol followed by drying in an oven. The glass surfaces were then deactivated by reacting them in an atmosphere of dimethyldichlorosilane [75–78–5] (Pierce Chemical Co., Rockford, IL) overnight followed by rinsing with dry methanol.

Chicken serum (Sigma Cell Culture Reagents®) was used for Hal spiking experiments and methods development (Sigma Chemical Co., St. Louis, MO). Bond Elut® (1cc) C8 bonded phase columns, Lot #060956, were obtained from Varian, Harbor City, CA.

A Tracor model 951 LC Pump (Tracor, Inc., Austin, TX) delivered the solvent system through a Whatman guard column (Pierce Chemical Co., Rockford, IL) filled with CO:PELL ODS (octadecyl groups chemically bonded to 30–38 μm glass beads) (Whatman, Inc., Clifton, NJ), and then through a 4.6 mm ID x 25 cm 5 μm Supelcosil™ LC-18-DB column (Supelco, Inc., Bellefonte, PA).

Samples were introduced via a 50 μl loop injector, Model 7125 (Rheodyne, Inc., Cotati, CA). The column's effluent was monitored by a LDC/Milton Roy spectroMonitor® D, variable wavelength detector, at 243 nm and recorded with a HP 3390A integrator (Hewlett-Packard, Palo Alto, CA).

Solutions

Ammonium acetate buffer (AA buffer) was prepared at 0.25 M, pH 4.3. Elution solvent used with the C8 Bond Elut® columns consisted of 79.9:20:0.1

v/v/v water-acetonitrile-acetic acid plus 210.4 µl decylamine per 100 ml batch, pH approximately 4.3. The HPLC solvent system consisted of 22:15:63 v/v/v acetonitrile-AA buffer-water plus 210.4 µl decylamine per 1 liter batch, pH approximately 4.75. A stock standard solution of Hal-HBr was prepared by dissolving the compound in AA buffer. Fortification standards used for additions to serum were made by diluting the stock standard with water. HPLC standards were prepared by diluting the stock standard with the HPLC solvent system.

Extraction and HPLC Analysis

Extraction and HPLC analysis of chicken serum was similar to that reported for bovine serum [17]; however, a number of changes were required to improve recoveries of Hal. Hal eluted from HPLC with a uniform peak shape and consistently when both decylamine and a buffer system were used.

The C8 Bond Elut® columns were conditioned by passing methanol (2 ml) followed by acidified water (8 ml, adjusted to pH 4.3 with acetic acid) through the columns prior to use. Ten percent acetic acid (8 ml) was added to serum (4 ml) spiked with Hal-HBr and serum obtained from Hal-HBr treated chickens. The diluted acidified serum was immediately passed through a Bond Elut® column. The column was washed with acidified water (5 ml). The column was then washed with 35% methanol (1 ml, adjusted to pH 4.3 with acetic acid), and finally washed again with 1 ml of acidified water. Hal was then eluted from the column with the elution solvent (1 ml). The column eluent was injected (50 µl) on HPLC.

A standard curve was generated by the HPLC analysis of solutions that contained from 0.01 ng/µl to 0.6 ng/µl of Hal-HBr; the correlation coefficient for the standard curve was 0.9998.

Incurred Residues of Hal in Chicken Serum

Broilers (White Rock Cross), Hubbard x Pettersen, were obtained at the age of 1 day old and fed unmedicated chicken feed for 3 weeks at which time they were placed on feed consisting of unmedicated chicken feed to which Stenorol® had been added with mixing to provide a level of 3 ppm Hal-HBr. Blood was taken from 8 broilers that were fed *ad libitum* for 10 days on Hal-HBr treated feed; serum was collected after centrifugation following clot formation. The serum was pooled and frozen (-70°C) within two hours of collection.

RESULTS AND DISCUSSION

Initial attempts at using the extraction and HPLC analysis method for Hal in bovine serum [17] for the detection of Hal in chicken serum gave recoveries that ranged from 65–85%. These recoveries were in good agreement with those published with the method [17]. However, these low and variable recoveries prompted further investigation aimed at improving the method. It was discovered that adjusting all solvents used for washing the Bond Elut® C8 column to pH 4.3 with acetic acid, and collecting 1 ml of the eluted Hal rather than 0.5 ml drastically improved the recoveries of the method. However, variability in recoveries of Hal remained unacceptable.

We subsequently observed that serum samples diluted with 10% acetic acid but not processed immediately had lower recoveries than others that were processed immediately. An experiment was carried out to evaluate the apparent loss of Hal in the acidified serum. Chicken serum samples were spiked with 97 ng/ml Hal-HBr and allowed to sit for 48 hours at 13°C. Samples were diluted with the 10% acetic acid and processed at different time intervals. Fig. 2 shows the recovery results of the timed study of Hal in acidified serum. A linear loss of

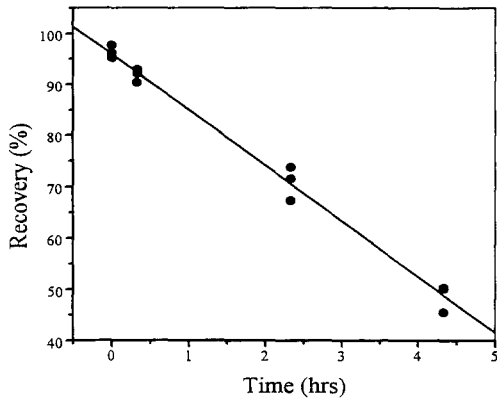

Figure 2. Recovery of Hal from serum at different time intervals after acidification, at room temperature.

available Hal at the rate of 11% per hour was observed. Upon dilution of the serum with 10% acetic acid, the resultant solution had a pH of ≈ 2.8. Since Hal is stable for considerable periods of time in acid solutions, FSIS makes their stock standard reagent of Hal-HBr in an ammonium acetate buffer at pH 4.3 [15]; therefore, it seems most likely that the diminished levels of Hal is the result of Hal binding to serum components under acid conditions.

The HPLC system containing both ammonium acetate buffer and decylamine gave very good peak shape for Hal as seen in Fig. 3. Frame A is a tracing of an

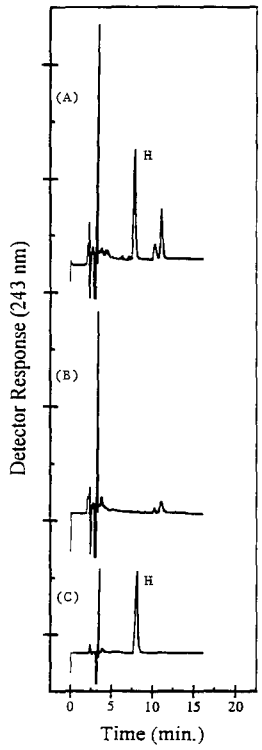

Figure 3. HPLC chromatograms of a 48 hour Hal spiked serum extract (A), of a serum control extract (B), and a Hal standard (C). The Hal peak is marked with an H.

Table 1

Observed Recoveries of Hal After Incubation in Chicken Serum at Different Time Intervals.

High Spike Level[1]			
Time (hours)[2]	Recovery	SD[3]	CV[4]
18	97.6%	± 1.75	1.79%
48	96.2%	± 1.12	1.16%
Low Spike Level[5]			
18	99.7%	± 3.30	3.31%
48	96.7%	± 3.16	3.26%

[1]97 ng/ml serum.
[2]Incubation period prior to acidification and imediate HPLC analysis.
[3]SD = standard deviation, n = 3 or 4.
[4]CV = coefficient of variation.
[5]10 ng/ml serum.

extract of a 48 hour spiked chicken serum sample. Frame B is a tracing of an extract of control chicken serum. Frame C is a tracing of a Hal standard. Not only was the peak shape for Hal symmetrical, but there also were no interfering peaks in chicken serum using this system. This method can quantitate as low as 1.5 ppb Hal in chicken serum.

The recoveries of Hal spiked at a high and low level from chicken serum after different times of incubation are shown in Table 1. After 18 or 48 hours of incubation, high and consistent recoveries were observed with both high and low spiked levels of Hal. The standard deviations bear out the results showing that the recoveries observed are consistent.

Finally, we determined the quantity of Hal that occurred in chicken serum after administration of Hal-HBr in the feed at 3 ppm for 10 days. The frozen pooled chicken serum was thawed and processed after reaching room temperature. The analysis showed Hal residues of 3.75 ng/ml (SD = ± 0.36, CV = 9.6), and the chromatography was as clean as that of the spiked chicken serum (Fig. 3, A).

The extraction and analysis method developed here for Hal in chicken serum provides an HPLC method that is moderately fast with consistently high recoveries. The confirmation that Hal decomposes readily in methanolic solutions,

and the knowledge that it binds to serum components under acid conditions will be successful work with Hal in future studies.

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REFERENCES

1. L. R. McDougald, "Control of Coccidiosis: Chemotherapy," in Coccidiosis of Man and Domestic Animals, P. L. Long, ed., CRC Press, Boca Raton, FL, 1990, pp. 307–320.

2. "Stenorol™ (Hoechst-Rousell Agri-Vet, Somerville, NJ)," in Handbook of Approved New Animal Drug Applications in the United States, M. L. Shepard, ed., R12–1–1991, Shotwell and Carr, Inc., Dallas, TX, 1991, p. 3.9.

3. H. T. Openshaw, "The Quinazoline Alkaloids," in The Alkaloids, Vol. III, R. H. F. Manske, H. L. Holmes, eds., Academic Press, Inc., New York, NY, 1953, pp. 101–118.

4. Dialog. Chemical Economics Handbook, Files 359, 959. SRI International, Menlo Park, CA.

5. S. F. Sundlof, J. E. Riviere, A. L. Craigmill, Food Animal Residue Avoidance Databank Trade Name File: A Comprehensive Compendium of Food Animal Drugs, 8th Ed., University of Florida, Gainesville, FL, 1992.

6. Anon, Federal Register, Vol. 50, No. 162, 1985, pp. 33718-9.

7. Anon, "Food Additive Tolerance for Halofuginone Revised," in Food Chemical News, March 4, 1991, p.54.

8. A. Anderson, D. H. Christopher, R. N. Woodhouse, J. Chromatogr., 168: 471–480 (1979).

9. Analytical Methods Committee, Analyst, 108: 1252–1256 (1983).

10. L. D. Rowe, R. C. Beier, M. H. Elissalde, L. H. Stanker, R. D. Stipanovic, Synth. Commun., 23: 2191–2197 (1993).

11. A. Anderson, E. Goodall, G. W. Bliss, R. N. Woodhouse, J. Chromatogr., 212: 347–355 (1981).

12. M. K. Perez, J. Assoc. Off. Anal. Chem., 61: 1183–1191 (1978).

13. Analytical Methods Committee, Analyst, 109: 171–174 (1984).

14. Y. Yamamoto, R. Hashiguchi, K. Araki, H. Kushima, J. Food Hyg. Soc. Japan, 32: 444–447 (1991).

15. USDA, FSIS, Science and Technology, Analytical Chemistry Laboratory Guide Book: Residue Chemistry, R. L. Ellis, Director, Chemistry Division, Room 302, Annex Bldg., 300 12th St., Washington, DC 20250, Winter 1991, pp. HLF-0–HLF-20.

16. L. D. Rowe, R. C. Beier, M. H. Elissalde, L. H. Stanker, J. Agric. Food Chem., (In press) (1994).

17. L. D. B. Kinabo, Q. A. McKellar, M. Murray, Biomed. Chromatogr., 3: 156–158 (1989).

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION FOLLOWING MICROWAVE ASSISTED EXTRACTION OF 3-NITRO-4-HYDROXYPHENYLARSONIC ACID FROM SWINE LIVER, KIDNEY, AND MUSCLE

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ABSTRACT

A simple and rapid analytical method is described for the determination of 3-nitro-4-hydroxyphenylarsonic acid (roxarsone, 3-nitro) in tissues. It involves extraction of 3-nitro from swine tissues by microwave-assisted process (MAP™) followed by high performance liquid chromatography using a PRP-1 column. The compound is detected in visible region at 410 nm after post-column reaction with 0.15 N sodium hydroxide. The detection

*Author to whom correspondence and reprint requests should be directed, CFAR contribution number 2199.

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limit is estimated to be 0.25 $\mu g\ g^{-1}$ for 3-nitro, equivalent to about 0.2 $\mu g\ g^{-1}$ of arsenic. Official tolerances for arsenic in liver and muscle are set at 2.0 $\mu g\ g^{-1}$ and 0.5 $\mu g\ g^{-1}$, respectively. The method was employed to detect 3-nitro residues in incurred tissue samples from pigs fed 150 mg kg^{-1} of 3-nitro for a 28-d period.

INTRODUCTION

3-Nitro (3-nitro-4-hydroxyphenylarsonic acid; roxarsone) is recommended at a feed level of 25-50 mg kg^{-1} as a general growth promotant for swine (1). The compound has been used in Canada in swine production for over 25 years and is considered one of the "old" drugs which was registered without sufficient nutritional and toxicological data.

Edmonds and Baker noted an improvement in the growth of young pigs (2) when raised on diet supplemented with 3-nitro. However, Akhtar et al. (3,4) did not observe any beneficial effect of 3-nitro on the growth of young as well as growing-finishing pigs.

It is possible that swine fed a 3-nitro medicated diet may accumulate the arsenical compound as well as its metabolites in liver, kidney and muscle. A 5-day withdrawal is regulated prior to slaughter of treated animals. Official tolerances for arsenic in liver and muscles are set at 2.0 $\mu g\ g^{-1}$ and 0.5 $\mu g\ g^{-1}$, respectively (5).

There are two official methods for determination of 3-nitro in feeds and premixes. One method involves extraction of feed with 2% K_2HPO_4, precipitation of proteins at isoelectric point followed by treatment with activated charcoal at pH 12. The resultant colored solution is analyzed by visible spectrophotometry at 410 nm (6). The second method, on the other hand, is not specific for 3-nitro, but determines the total arsenic. The method consists of extraction of samples with aqueous

ammonium carbonate solution followed by direct analysis on a graphite furnace atomic absorption spectrometry (AAS) for total arsenic (7). Recently, a high performance liquid chromatography (HPLC) method using ultraviolet detection at 243 nm without post-column reaction has appeared in the literature for roxarsone (3-nitro) in poultry feed (8).

There is no method to detect intact 3-nitro in tissues. Currently, the total residues of arsenic are determined by atomic absorption method following a wet digestion step (9). This paper describes a simple, rapid, reproducible and cost effective extraction technique involving microwave assisted process (MAP™) (10, 11), and detection by visible spectrometry at 410 nm for determination of 3-nitro in swine tissues (liver, kidney and muscle). The method was also applied to detect intact 3-nitro in incurred samples derived by feeding the compound for 28 days.

MATERIALS

Chemicals and reagents

3-Nitro (3-nitro-4-hydroxyphenylarsonic acid; roxarsone) was obtained from Sigma (St.Louis, MO.). Trifluoroacetic acid was HPLC/Spectro Grade, 99.5% pure from Chromatographic Specialties (Brockville, ON.). Glacial acetic acid (aldehyde free) was 'Baker Analyzed' reagent (J.T. Baker, Toronto, ON.). Isopropyl alcohol and ethanol were HPLC grade obtained from Caledon (Georgetown, ON.).

Instrumentation

The liquid chromatography system consisted of two model 302 solvent delivery pumps, a model 803C manometric module, and an auto-sampling injector model

231-401 with a 50 µL loop (all from Gilson). The detector was a Waters 486 Tunable Absorbance Detector set at 410 nm. The temperature of the column was kept at 30°C using a Waters Temperature Control Module. The column used was a PRP-1 (Hamilton), 10 µm, 250 x 4.1 mm, 75 Å and a PRP-1 guard column of 2 µm, 25 x 2.3 mm i.d. A Hewlett-Packard HP3394 integrator collected the chromatographic data.

For the extraction of tissue samples a Panasonic microwave oven model NE-7850C was used as well as a sonicator (Heat Systems) and an homogenizer (Brinkmann). Extracts were centrifuged in a refrigerated centrifuge (Mistral 3000*i* ,rotor head 43124-129, Johns Scientific Inc.)at -5°C. Extracts were evaporated to dryness in vacuo with a rotary evaporator Büchi, model RE121 (Brinkmann).

METHOD

Biological Samples

Weanling pigs were fed 0, 50 and 150 mg kg^{-1} of 3-nitro for 28 days. At the end of the experiment, animals were killed, the whole-liver, both kidneys and a portion of hind-quarters muscle were passed through a meat grinder, frozen and stored at -20°C until analyzed. Details on animal experimental procedure are reported elsewhere (4).

Standards and spiked samples

3-Nitro working standard solutions of 10, 20, 30, 40 and 60 µg mL^{-1} were prepared using mobile phase (water-isopropyl alcohol- trifluoroacetic acid 94:6:0.1 v/v/v) and were kept at room temperature.

One gram of previously frozen ground tissue was weighed into a 125 mL screw-capped specimen container.

Individual tissue samples were spiked with 100 μL of the appropriate 3-nitro working standard solutions to produce samples with tissue equivalencies of 1.0, 2.0, 3.0, 4.0 and 6.0 μg g^{-1}, respectively.

Extraction of 3-nitro from tissues

Spiked tissue samples were left to stand at room temperature for about 30 minutes prior to extraction by MAP™, homogenization or sonication. Twenty-five mL of absolute ethanol and 500 μL glacial acetic acid were added to each container and mixed gently. The samples were then homogenized or sonicated for 1.5 minutes or individually irradiated in a microwave oven for 9 seconds at maximum power but the content was not allowed to boil. The supernatant from the microwave extraction was immediately decanted into a 50 mL screw-capped polypropylene centrifuge tube. Samples from the three extraction techniques were then centrifuged at 1380 x g for 20 minutes at 0°C. The clear supernatant was decanted into a 250 mL round-bottom flask and evaporated to dryness under vacuum with a waterbath temperature of 69°C. Two mL mobile phase was added to dissolve all residue adhering to the glass and was transferred into a microfuge tube. The extract was centrifuged at 16000 x g for 8 minutes. The sample was transferred into a glass sample vial for HPLC analysis. An aliquot of 50 μL was injected onto the column. Standards of 3-nitro were subjected to the identical irradiation and extraction procedures prior to analyses. Incurred samples were also treated similarly to determine 3-nitro residues.

HPLC procedure

The mobile phase, water-isopropyl alcohol-trifluoroacetic acid (94:6:0.1 v/v/v), was filtered

through a 0.45 μm filter (Nylaflo®, Gelman Sciences), degassed under vacuum and delivered at a flow rate of 1.0 mL min^{-1}. Sodium hydroxide solution, 0.15 N, was provided at a flow rate of 1.0 mL min^{-1} by a second pump and mixed through a low pressure tee with the column eluate before detection at 410 nm. The purpose of the post-column reaction with sodium hydroxide was to generate a chromophore of 3-nitro to be visualized at 410 nm.

RESULTS AND DISCUSSION

Existing methods for the extraction of 3-nitro from feed involve laborious, lengthy and expensive clean-up steps before samples can be analyzed (6-9). We evaluated the classical extraction techniques of homogenization and sonication with the recently patented microwave-assisted process (MAP™) as a rapid, cost-effective and reproducible alternate method (10,11). Recoveries of spiked 3-nitro from liver, kidney and muscle using different extraction methods are listed in Table 1. Table 1 shows that recoveries of 3-nitro from spiked tissue samples were superior with the MAP™ technique than with the other two techniques.

Chromatograms of standard 3-nitro and extracts of spiked liver samples by MAP™, homogenization and sonication, shown in Figure 1, show that 3-nitro elutes in a reasonable time of under 10 minutes. The blank samples did not exhibit significant interferences in that region. The extracts from homogenization and sonication methods contained more impurities than that from MAP™ (Fig 1). The same extraction procedures were repeated for kidney and muscle tissues. Again, no interferences due to endogenous substances were found in either kidney or muscle extracts. Further studies were, then, carried out with MAP™ extraction technique.

TABLE 1

Recoveries of 3-nitro from spiked tissues by different extraction techniques[1]

Tissues	Techniques	%Recoveries
liver	homogenization	50.6 ± 6.4
	sonication	60.4 ± 7.3
	MAP™	82.4 ± 2.5
kidney	homogenization	37.6 ± 1.4
	sonication	42.7 ± 4.8
	MAP™	74.7 ± 1.8
muscle	homogenization	40.5 ± 7.8
	sonication	44.3 ± 4.5
	MAP™	80.8 ± 1.8

1) n=5, spiked at 4 $\mu g\ g^{-1}$.

Recovery studies at five different levels of fortification of 3-nitro in liver, kidneys and muscle were carried out using extraction by MAP™ and results are shown graphically in Figure 2. The average percentage recoveries from liver were 82.4 ± 2.5; muscle 80.8 ± 1.8; and 74.7 ± 1.8 for kidney. The calibration curves, representing mean peak areas versus concentration, show excellent linearity for concentrations from 0 to 6.0 $\mu g\ g^{-1}$. The correlation coefficients (r) were 0.9999, 0.9997, 0.9999 and 0.9998 for standard 3-nitro, liver, muscle and kidney, respectively.

The described method was successfully applied to swine liver, muscle tissue fortified with roxarsone in

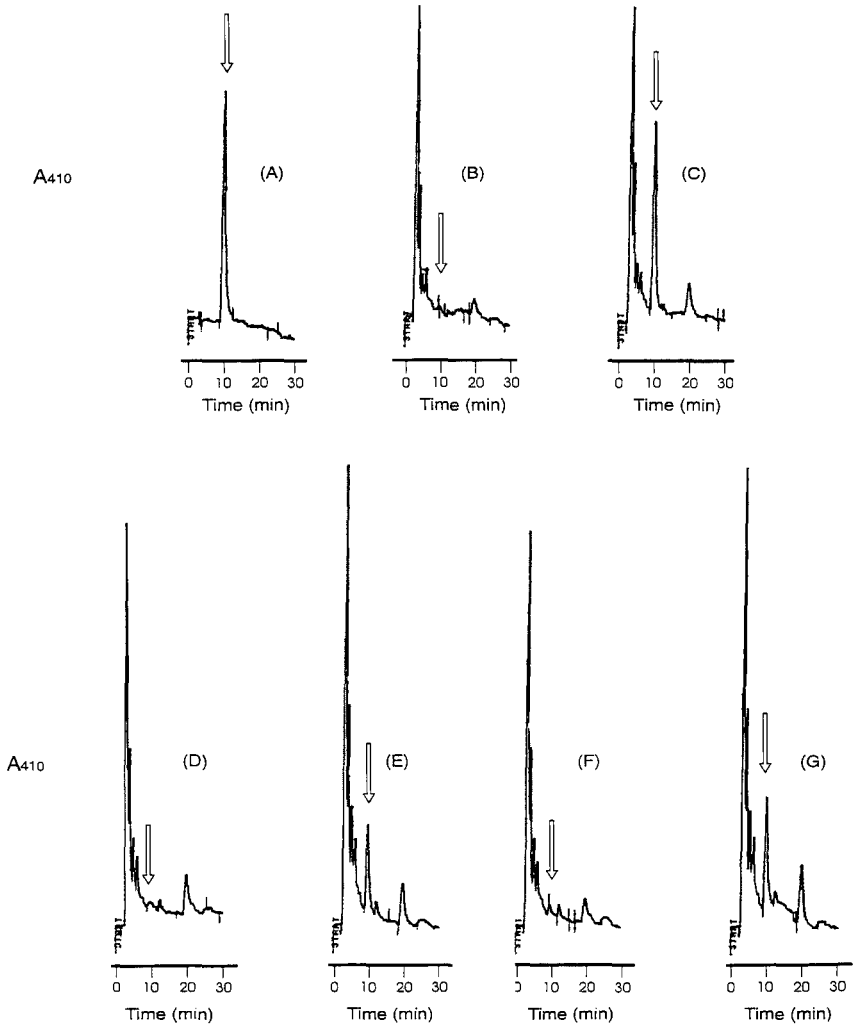

Figure 1. Chromatograms of (A) 3-nitro standard 100 ng, (B) blank liver MAP™ sample, (C) spiked liver MAP™ sample at a concentration of 4 $\mu g\ g^{-1}$, (D) homogenized blank liver sample, (E) spiked homogenized liver sample at a concentration of 4 $\mu g\ g^{-1}$, (F) blank sonicated liver sample and (G) spiked sonicated liver sample at a concentration of 4 $\mu g\ g^{-1}$. Arrow indicates 3-nitro.

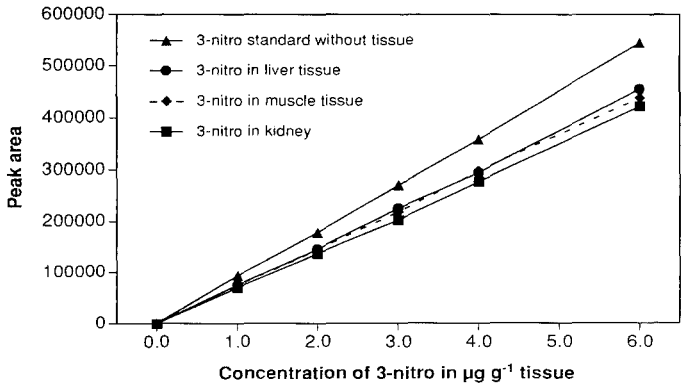

Figure 2. Recovery of 3-nitro in liver, muscle and kidney tissues spiked in the range of 1.0 to 6.0 $\mu g\ g^{-1}$.

the range of 1.0 to 6.0 $\mu g\ g^{-1}$ with excellent linearity and a limit of quantitation of 0.25 $\mu g\ g^{-1}$ (S/N >3:1). Consistent recoveries of roxarsone were obtained in the tissues (75-82%). The MAP™ method enabled us to process a large number of samples in a short time since no clean-up was required.

The tolerance for total arsenic residues in tissues is set at 0.5 $\mu g\ g^{-1}$ for muscle and kidney and 2.0 $\mu g\ g^{-1}$ for liver. These values are equivalent to about 1.75 $\mu g\ g^{-1}$ and 7.02 $\mu g\ g^{-1}$ of 3-nitro, respectively. The calibration curves in Figure 2 indicate the suitability of the method for detection of 3-nitro well below the lower established tolerance level.

Arsenic residues were determined by the atomic absorption technique in the incurred samples from feeding trials (4). Data showed that arsenic residues were the highest in liver, closely followed in kidney and considerably less in muscle. The arsenic residues in liver at 150 mg kg^{-1} supplementation level were 2.04 μg

g^{-1} for liver, 1.76 $\mu g\ g^{-1}$ for kidney and 0.14 $\mu g\ g^{-1}$ for muscle at 0 withdrawal period. The value reached 0.87 $\mu g\ g^{-1}$, 0.46 $\mu g\ g^{-1}$ and 0.1 $\mu g\ g^{-1}$ following the 5-d withdrawal period.

Liver samples containing the highest arsenic residues (2.04 $\mu g\ g^{-1}$) were subjected to the developed extraction method (MAP™) followed by analysis on HPLC. It was surprising to note that no 3-nitro was detected in any of the tissue samples of pigs fed the compound at 150 mg kg^{-1} for 28 days. Since 3-nitro was not detected in samples containing the highest arsenic residues, no further attempts were made to analyze the samples containing the lower amounts of arsenic.

The total absence of 3-nitro in incurred samples clearly suggest that the arsenic residues found in the sample are due to compounds other than 3-nitro. However, the nature of the arsenic moieties is not known. One can speculate that the compound has undergone metabolism to produce compounds including inorganic arsenicals which could not be detected by methods other than the atomic absorption method for total arsenic.

On the basis of data presented here, we see MAP™ as an alternate to classical extraction techniques including solvent extraction and supercritical fluid extraction for its versatility, simplicity, environmental safety and cost effectiveness. The technique has the potential for incorporation in routine analysis by regulatory agencies. The simplicity and cost-effectiveness features dictates further exploration with a variety of organic residues in various media including vegetables and fish.

REFERENCES

1. Compendium of Medicating Ingredients. Agriculture Canada. 1990. 6th edition.

2. Edmonds, M.S., Baker, D.H. Toxic effects of supplemental copper and roxarsone when fed alone or in combination to young pigs. J. Anim. Sci., 63:553-557, 1986.

3. Akhtar, M.H., Patterson, J.R., Salisbury, C.D.C., Ho, S.K., Jui, P.Y., Hartin, K.E. Effects of feeding 3-nitro-4-hydroxyphenylarsonic acid on growing-finishing pigs. Can. J. Anim. Sci., 72:389-394, 1992.

4. Akhtar, M.H., Croteau, L.G., Ho, S.K., Salisbury, C.D.C. and Patterson, J.R. Further assessment of 3-nitro-4-hydroxyphenylarsonic acid as growth promotant for young pigs. Can. J. Anim. Sci. (Unpublished results).

5. Canada Gazette, 1990. Part I. Sept.29: 3539-3551.

6. Association of Official Analytical Chemists, "Roxarsone in Feeds and Premixes, Spectrophotometric method," in Official Methods of Analysis, Arlington, VA., 15th Edition, 1990.

7. Association of Official Analytical Chemists, "Roxarsone in Feeds, Atomic Absorption Spectrophotometric Method," in Official Methods of Analysis, Arlington, VA., 15th Edition, 1990.

8. Sapp R.E. and Davidson S. Determination of roxarsone in feeds using solid phase extraction and liquid chromatography with ultraviolet detection. J. Assoc. Off. Anal. Chem. Int., 76:956-961, 1993.

9. Salisbury, C.D. and Chan, W. Simple automated wet digestion of animal tissues for determination of seven elements by atomic absorption spectrophotometry. J. Assoc. Off. Anal. Chem., 68:218-219, 1985.

10. Paré, J.R., Sigouin, M., Lapointe, J. Microwave-assisted natural products extraction. US Patent 5002784, 1991.

11. Raloff J. Microwaves accelerate chemical extractions. Science News, Vol.144:118, 1993.

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LIQUID CHROMATOGRAPHY CALENDAR

1994

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 3 - 4: Course on Capillary Electrophoresis, Budapest, Hungary. Contact: Dr. F. Kilar, Central Research Laboratory, Medical School, University of Pecs, Szigeti ut 12, H-7643 Pecs, Hungary.

OCTOBER 5 - 7: 9th Inetrnational Symposium on Capillary Electrophoresis, Budapest, Hungary. Contact: Dr. F. Kilar, Central Research Laboratory, Medical School, University of Pecs, Szigeti ut 12, H-7643 Pecs, Hungary.

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 3: Anachem Symposium, Dearborn, Michigan. Contact: Paul Beckwith, Program Chairman, Detroit Edison Co., 6100 W. warren, Detroit, MI 48210, 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 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

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.

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One typewritten manuscript, suitable for direct reproduction, and two (2) clear copies with figures must be submitted. Since the Journal is produced by direct photography of the manuscripts, typing and format instructions must be strictly followed. Non-compliance will result in return of the manuscript to the author and will delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards before mailing.

Manuscripts may also be submitted on **computer diskettes**. A printed manuscript must also be submitted with diskettes because, at the present time, we are experimenting with manuscripts on diskettes. Diskettes must be readable with an IBM-compatible computer (Macintosh or other type not acceptable) and must be formatted with MS-DOS 3.1 or greater. Be sure to indicate the word processing software that was used to prepare the manuscript diskette.

Manuscripts and computer diskettes should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 2180
Cherry Hill, NJ 08034

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Due to the short production time for papers in this journal, it is essential to order reprints immediately upon receiving notification of acceptance of the manuscript. A reprint order form will be sent to the author with the letter of acceptance for the manuscript. Reprints are available in quantities of 100 and multiples thereof. Twenty (20) free reprints will be included with orders of 100 or more reprints.

Format of the Manuscript

1. The general format of the manuscript should be:
   Title
   Author(s)' names and full addresses
   Abstract
   Text Discussion
   References

2. **Title & Authors:** The entire title should be in capital letters and centered within the width of the typing area, located at least 2 inches (5.1 cm) from the top of the page. This should be followed by 3 lines of space, then by the names and addresses of the authors, also centered, in the following manner:

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN WATER BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

3. **Abstract:** The title **ABSTRACT** should be typed, capitalized and centered, 3 lines below the addresses. This should be followed by a **single-spaced**, concise abstract. Allow 3 lines of space below the abstract before beginning the text of the manuscript.

4. **Text Discussion:** Whenever possible, the text discussion should be divided into major sections such as

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGEMENTS
REFERENCES

These **major headings** should be separated from the text by two lines of space above and one line of space below. Each major heading should be typed in capital letters, centered and underlined.

Secondary headings, if any, should be placed flush with the left margin, underlined and have the first letter of main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. The first word of each **paragraph** within the body of the text should be indented five spaces.

6. **Acknowledgements**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

7. **References** should be numbered consecutively and placed in a separate section at the end of the manuscript. They should be typed single-spaced, with one line space between each reference. Each reference should contain names of all authors (with initials of their first and middle names); do not use *et al.* for a list of authors. Abbreviations of journal titles will follow the American Chemical Society's Chemical Abstracts List of Periodicals. The word **REFERENCES** should be capitalized and centered above the reference list.

Following are acceptable reference formats:

Journal:

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

Book:

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

2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

Gooding, E. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used.

10. Material that cannot be typed, such as Greek symbols, script letters and structural formulae, should be drawn carefully with dark black India ink. Do not use any other color ink.

Additional Typing Instructions

1. The manuscript must be prepared on good quality **white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). The typing area of the first page, including the title and authors, should be 5½ inches wide by 7 inches high (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches high (14 cm x 21.6 cm).

2. The **title, abstract, tables and references** are typed single-spaced. All other text should be typed 1½-line spaced or double line spaced.

3. It is essential to use **dark black** typewriter or printer ribbon so that clean, clear, **solid characters** are produced. Characters produced with a dot/matrix printer are not acceptable, even if they are "near letter quality" or "letter quality." Erasure marks, smudges, hand-drawn corrections and creases are not acceptable.

4. **Tables** should be typed on separate pages, one table to a page. A table may not be longer than one page. If a table is larger than one page, it should be divided into more than one table. The word **TABLE** (capitalized and followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

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

Captions for figures should be typed single-spaced on a separate sheet of white paper, along the full width of the type page, and should be preceded with the word FIGURE and an Arabic numeral. All figures and lettering must be of a size that will remain legible after a 20% reduction from the original size. Figure numbers, name of senior author and an arrow indicating "top" should be written in light blue pencil on the back of the figure. Indicate the approximate placement for each figure in the text with a note written with a light blue pencil in the margin of the manuscript page.

6. The **reference list** should be typed single-spaced. A single line space should be inserted after each reference. The format for references should be as given above.