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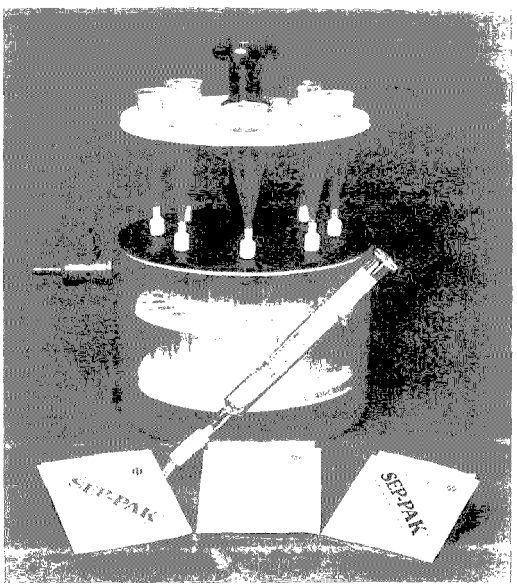

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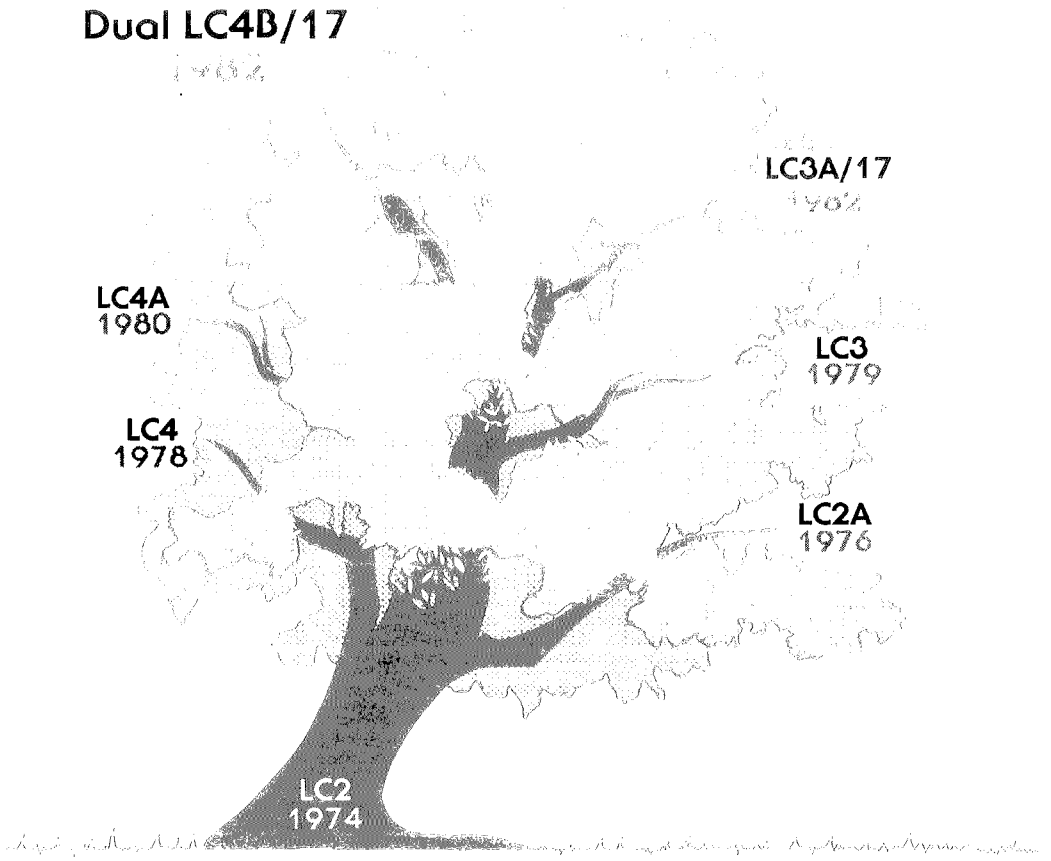

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DETERMINATION OF THE MONODISPERSE MARK-HOUWINK EQUATION OF HIGH-VINYL POLYBUTADIENE BY GEL CHROMATOGRAPHY AND LIGHT SCATTERING*

Shu-Qin Bo and Rong-Shi Cheng
Institute of Applied Chemistry
Academia Sinica
Changchun, Jilin, People's Republic of China

ABSTRACT

Gel chromatographic, light scattering, viscosimetric and osmotic pressure measurements have been made for high vinyl polybutadiene fractions and whole polymer. Three alternative methods for deducing a monodisperse Mark-Houwink equation from gel chromatographic, light scattering and viscosimetric data of samples with unequal distribution widths are suggested. The monodisperse Mark-Houwink equations obtained by these methods for high vinyl poly(butadiene) are all alike.

INTRODUCTION

The chain structure of polydiene has several possibilities due to difference of addition scheme for the monomer. For example, when butadiene is polymerized, two kinds of products might be obtained, by 1,2 or 1,4 addition, and for the latter the product might be classified again into cis and trans forms.

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Changing the catalytic system, polybutadienes with different compositional chain units might be obtained, and their solution properties might also be different in some respects. In recent years, high-vinyl polybutadiene has been prepared by some organometallic catalysts. The molecular comformation and the solution properties of vinyl polybutadiene may be influenced seriously by the large amount of pendant side groups existing. In the study of polymer solution properties, the monodisperse Mark-Houwink equation plays an important role. First, for measuring molecular weight of polymer, only the monodisperse Mark-Houwink equation can give the correct viscosity-average molecular weight. Secondly, in the study of the effect of the structure and polydispersity on various physical properties of polymer, a monodisperse relationship is frequently required. However, the Mark-Houwink equations given in the literature are almost always determined from the weight-or number-average molecular weights of fractions with uncertain molecular weight distributions. The relationships thus obtained are

$$[\eta] = K_w \cdot \langle M \rangle_w^{\alpha} , \qquad (1)$$

or

$$[\eta] = K_n \cdot \langle M \rangle_n^{\alpha} , \qquad (2)$$

both of them are not the monodisperse equation. Several methods have been suggested in the literatures (1-5) for deducing the parameters of the monodisperse Mark-Houwink equation. In all of these methods, a requirement must be satisfied, i.e. the relative width of the molecular weight distribution of the sample used must be the same, and known exactly. Under this condition the alpha value obtained is independent of the width of the MWD of the sample; therefore, it is only necessary to correct the K value for polydispersity. Since the width of the distribution may vary from sample to sample, the applicability of the suggested methods would be limited. For high-vinyl polybutadiene, the only data found in literature was that reported by Anderson et al., (6) in which the number-average

molecular weights were measured by osmotic pressure and the Mark-Houwink equation cited was treated as in Eq. 2.

In the present work, the molecular weight distribution of vinyl polybutadiene sample were measured by gel chromatography, and in connection with the light scattering and viscosity measurements, three alternative methods for deducing the monodisperse Mark-Houwink parameters from samples with unequal distribution widths were employed. The results obtained by these methods are all very much alike.

EXPERIMENTAL

Sample: The vinyl polybutadiene was prepared in toluene at 50°C with molybdenum naphthenate-triisobutylaluminium as catalyst. The product was deactivated and coagulated by addition of ethyl alcohol, and then dried in vacuum.

Fractionation: The sample was dissolved in hydrogenated gasoline, filtered through a G5 sintered glass funnel and precipitated with 1:1 methyl alcohol-acetone mixture for purification. The purified polymer was then fractionated by reverse fractional precipitation at 30°C in toluene by addition of methyl alcohol as precipitant.

Structure Analysis: The vinyl contents of the fractions were determined by infrared absorption spectra (PEKIN ELMER 599 B) and 13C nuclear magnetic resonance spectra (JEOL FX '00) respectively.

Gel Chromatography: The molecular weight distributions of the samples were determined with a ARL 950 Gel Permeation Chromatograph. The column was packed with deactivated porous-silica beads prepared in this laboratory. THF was used as the elution solvent with flow rate 1 ml/min at 42°C. The concentration of the eluted polymer solution was detected with a differential refractometer. The elution volume was measured with a siphon tube with a volume of 1.98 ml.

Light Scattering: The weight-average molecular weights of the samples were determined simultaneously with a Chromatix KMX-6 Low Angle Laser Light Scattering Photometer and a Shimadzu PG-21 Light Scattering Photometer, using cyclohexane as solvent at ambient temperature. The dn/dc of the vinyl polybutadiene is 0.0816 at 30°C determined by a Chromatix KMX-16 Differential Refractometer at 632.8 nm. The incident light of 436 nm and a dn/dc value of 0.094 were chosen for the conventional light scattering measurements.

Viscosity: Viscosities were measured for toluene and THF solutions using an Ubbelohde viscometer at 30°C. A kinetic energy correction was applied. The intrinsic viscosity of the sample was obtained by extrapolation to infinite dilution.

Osmotic Pressure: A Knauer Membrane Osmometer was used to determine the number-average molecular weight of arbitrarily selected samples for checking the gel chromatographic data; toluene was used as solvent.

RESULTS AND DISCUSSIONS

Experimental Results: The vinyl contents of the fractions and the whole polymer determined by 13C NMR and IR are listed in Table 1.

TABLE 1

Vinyl Contents of Polybutadiene Samples

Fraction	Vinyl Wt. %	
	IR	NMR
S1 - A	89.2	-
S1	93.8	93.5
S2	94.0	93.2
S3	93.1	94.7
S4	94.3	-
whole polymer	93.1	92.0

TABLE 2

Weight Average Molecular Weights and Intrinsic Viscosities of Vinyl Polybutadiene Samples

Fraction	$\langle M \rangle_w \times 10^{-5}$			[η] ml/g, 30°C	
	LALLS	LS	Average value	THF	Toluene
S1 - A	2.33	2.33	2.33	162	148
S1	6.85	6.06	6.46	388	330
S2	10.8	10.5	10.7	540	484
S3	11.8	14.9	13.4	670	571
S4	21.6	20.8	21.2	941	832
whole polymer	19.2	22.9	21.1	760	646

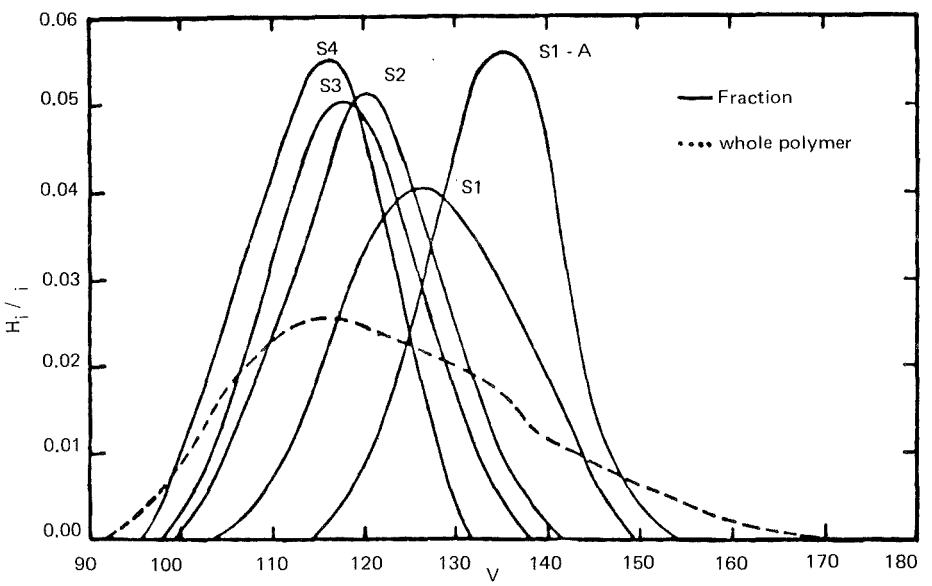

FIGURE 1. Normalized Gel Chromatograms of Vinyl Polybutadiene Samples.

TABLE 3

GPC Data in Elution Volume Count of Vinyl Polybutadiene Samples

Fraction	σ_T^2	$\bar{V}$	$V_R' \langle M \rangle_w$	$V_R' \cdot \langle [\eta] \rangle$
S1 - A	46.36	133.87	131.27	131.82
S1	79.17	127.28	123.11	123.93
S2	60.96	120.40	117.15	117.80
S3	55.45	117.91	114.96	115.55
S4	47.33	114.75	112.13	112.67
whole polymer	232.7	123.48	-	-

The vinyl contents are alike, independent of the molecular weight of the sample, except S1-A, which is a fraction of another sample with lower molecular weight. The light scattering and viscosity data are shown in Table 2. The weight-average molecular weights measured by conventional light scattering were very close to those measured by LALLS; therefore the average values were used to calibrate the gel chromatographic column. The normalized gel chromatogram for each fraction is shown in Figure 1; the mean elution volumes and variances calculated from experimental chromatograms are shown in Table 3.

Calibration of Gel Chromatographic Column: The calibration relationship of the gel chromatographic column represents the relationship between certain molecular size parameters of monodisperse polymer and its retention column under ideal working conditions. For a linear chromatographic column, the monodisperse calibration relationship may be written as

$$M(V_R): \quad \ln M = A_M - B_M V_R, \tag{3}$$

$$[\eta](V_R): \quad \ln[\eta] = A_\eta - B_\eta V_R, \tag{4}$$

The coefficients of the above relationships may be determined from the known experimental average values $\langle M \rangle$ or $\langle [\eta] \rangle$ of polydispersed polymer standards, and the corresponding retention volumes calculated by

$$V_{R,\langle M \rangle_w} = (-1/B_M) \ln \Sigma W(V_R) e^{-B_M V_R}, \quad (5)$$

$$V_{R,\langle M \rangle_n} = (1/B_M) \ln \Sigma W(V_R) e^{B_M V_R}, \quad (6)$$

$$V_{R,\langle [\eta] \rangle} = (-1/B) \ln \Sigma W(V_R) e^{-B_\eta V_R}, \quad (7)$$

where $W(V_R)$ is the true chromatogram of the polydispersed sample (7). If the experimental chromatogram $F(V)$ of the polydispersed polymer is used instead of $W(V_R)$, the calculated results are translated to corresponding retention volumes as

$$V'_{R,\langle M \rangle w} = (-1/B_M) \ln \Sigma F(V_R) e^{-B_M V_R} \quad (8)$$

$$V'_{R,\langle M \rangle n} = (1/B_M) \ln \Sigma F(V_R) e^{B_M V_R} \quad (9)$$

$$V'_{R,\langle [\eta] \rangle} = (-1/B_\eta) \ln \Sigma F(V_R) e^{-B_\eta V_R}, \quad (10)$$

due to the existence of the instrumental spreading effect. From Tung's integral equation

$$F(V) = \int_{V_R} W(V_R) G(V, V_R) dV_R \quad (11)$$

and if the spreading function $G(V,V_R)$ is Gaussian

$$G(V,V_R) = (1/\sigma\sqrt{2\pi}) \exp\left\{(1/2\sigma_0^2)(V-V_R)^2\right\}, \quad (12)$$

then (7)

$$V'_{R,\langle M \rangle w} = V_{R,\langle M \rangle w} - B_M \sigma_o^2 / 2, \quad (13)$$

$$V'_{R,\langle M\rangle n} = V_{R,\langle M\rangle n} + B_M \sigma_o^2 / 2 , \quad (14)$$

$$V'_{R,\langle[\eta]\rangle} = V_{R,\langle[\eta]\rangle} - B_\eta \sigma_o^2 / 2 , \quad (15)$$

Hence, the resultant calibration relationships are translated calibration relationships $M'(V_R)$ and $[\eta]'(V_R)$. Since the volume displacement directly depends upon the spreading factor σ_o^2 and the slope of the calibration curve, and also upon the initial calibration parameter chosen, the correction factors for the average molecular weight and intrinsic viscosity calculated from the experimental chromatogram F(V) using translated calibration relations are likewise related to the initial calibration parameter. If the calculated average and the initial calibration parameter are of the same type spreading correction is unnecessary. For example, for the translated calibration relation $M'_w(V_R)$ using weight-average molecular weight as the initial calibration parameter, the spreading correction factors for the average molecular weights calculated from experimental chromatogram F(V) are

$$\langle M\rangle_w, \text{ corr.} = \langle M\rangle_{w,\text{cal.}} , \quad (16)$$

$$\langle M\rangle_n, \text{ corr.} = e^{B_M^2 \sigma_o^2} \langle M\rangle_{n,\text{ cal.}} , \quad (17)$$

$$(\langle M\rangle_w / M_n)_{\text{corr.}} = e^{-B_M^2 \sigma_o^2} (\langle M\rangle_w / \langle M\rangle_n)_{\text{cal.}} , \quad (18)$$

$$\langle M\rangle_{\ , \text{ corr.}} = e^{(1/2)(1-\alpha) B_M^2 \sigma_o^2} \langle M\rangle_{\eta , \text{ cal.}} , \quad (19)$$

respectively.

The translated calibration relationships obtained from the corresponding retention volumes calculated from the experimental chromatograms with $\langle M\rangle_w$ and $\langle[\eta]\rangle$ as initial calibration parameters for vinyl polybutadiene are shown in Figure 2. The least square fit of the data gives

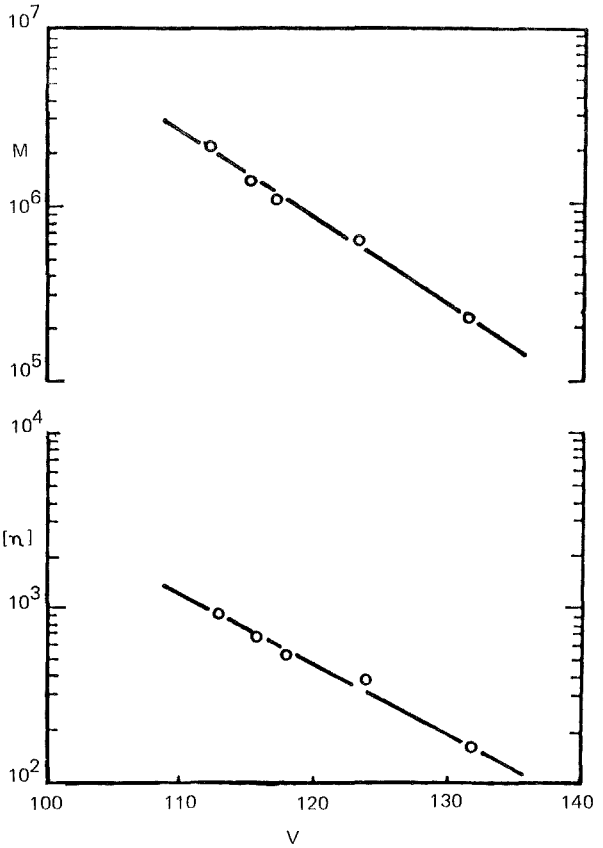

FIGURE 2. The Translated Calibration Curves of Vinyl Polybutadiene.

$$M'(V_R) : \ln M = 26.8697 - 0.1103\ V_R \quad (r^2=0.992), \tag{20}$$

$$[\eta]'(V_R) : \ln[\eta] = 16.6242 - 0.0871\ V_R \quad (r^2=0.984), \tag{21}$$

respectively. The weight average molecular weights and intrinsic viscosities of the samples studied, calculated from the experimental chromatogram using the above two relationships are listed in Table 4; both of them are very close to the experimentally determined values.

TABLE 4

The Experimental and GPC Calculated Average Molecular Weights and Instrinsic Viscosity of Vinyl Polybutadiene Samples

Fraction	$\langle M \rangle_w 10^{-5}$		$\langle M \rangle_n 10^{-5}$			$\langle [\eta] \rangle_{THF}$, ml/g		$\langle M \rangle_w / \langle M \rangle_n$
	LS	GPC	Osmotic	GPC Cal.	GPC Corr.	Exp.	GPC	Corr.
S1 - A	2.33	2.41	-	1.39	1.67	162	171	1.44
S1	6.46	5.92	3.00	2.36	2.83	388	340	2.09
S2	10.7	11.4	-	5.53	6.64	540	580	1.72
S3	13.4	14.5	8.69	7.51	9.01	670	706	1.61
S4	21.2	19.9	-	11.4	13.6	941	907	1.45
whole polymer	21.1	16.4	-	1.21	1.45	760	716	11.3

The number-average molecular weights, calculated from the experimental chromatograms using $M'_w(V_R)$ and the corrected values according to Equation (17) are also in Table 4. Two fractions were arbitrarily selected to take osmotic pressure measurement for checking the gel chromatographic data. The corrected number-average molecular weight from gel chromatography conforms to the experimentally determined value.

Monodisperse Mark-Houwink Equation:

The width of the molecular weight distribution of the polybutadiene sample studied varies considerably as seen in Table 4. It is difficult to determine the monodisperse Mark-Houwink parameters accurately for such cases using the literature-proposed methods. The parameters for the monodisperse Mark-Houwink equation

$$[\eta] = K M^{\alpha} \tag{22}$$

should be identical to those of the relationship between intrinsic viscosity and viscosity-average molecular weight for the polydispersed samples

$$\langle[\eta]\rangle = K \langle M\rangle_{\eta}^{\alpha} \tag{23}$$

where the viscosity average molecular weight is defined as

$$\langle M\rangle_{\eta} = \left\{\Sigma W(M)\ M^{\alpha}\right\}^{1/\alpha} \tag{24}$$

For deducing the monodisperse Mark-Houwink parameters, three alternative methods were employed in the present work. In method I and II the viscosity-average molecular weights are first calculated following different routes, while in method III they are deduced from the theoretical relationship between the monodisperse calibration curve $[\eta](V_R)$ and $M(V_R)$.

Method I, Assuming the molecular weight distribution fits the logarithmic normal distribution

$$W(M) = 1/\beta\sqrt{\pi} \cdot 1/M \cdot \exp\left\{-(1/\beta^2)\ln^2(M/M_o)\right\} \tag{25}$$

then $$\langle M\rangle_w = M_o e^{(1/4)\beta^2}, \tag{26}$$

$$\langle M\rangle_n = M_o e^{(-1/4)\beta^2}, \tag{27}$$

$$\langle M\rangle_{\eta} = M_o e^{(1/4)\alpha\beta^2}, \tag{28}$$

the polydispersity index

$$\langle M\rangle_w / \langle M\rangle_n = e^{(1/2)\beta^2} \tag{29}$$

Combine above equations, we see that

$$\langle M\rangle_{\eta} = (\langle M\rangle_w / \langle M\rangle_n)^{(1/2)(\alpha-1)} \langle M\rangle_w, \tag{30}$$

Thus, the viscosity average molecular weight can be calculated from the experimental weight-average molecular weight, polydispersity index and the parameter α to be determined. For this purpose, weight-

average molecular weight from light scattering measurement and polydispersity index from gel chromatographic data, after applying instrumental spreading correction, were used. The parameter α was determined by an iterative procedure. The initial value of α was taken from the least square fit of the experimentally determined $\langle[\eta]\rangle$ and $\langle M\rangle_w$, with which the viscosity average molecular weight was calculated according Eq.30. A new pair of parameters, K and α, was then deduced from $\langle[\eta]\rangle$ and $\langle M\rangle$ by the least square fit. This iterative procedure was repeated until the value no longer changed. A similar procedure for deducing the monodisperse Mark-Houwink equation for polyisbutene was employed by Mrkvickova and Lapour (8), in which the MWD of the sample was assumed to be a Schulz-Zimm distribution.

Method II, The same initial value of α as method I was used to calculate the viscosity-average molecular weight directly from F(V) and $M'_w(V_R)$ according to Equation 24. The calculated $\langle M\rangle_\eta$ was then corrected for instrumental spreading by Eq. 19. The new K and α values were deduced again from the experimentally determined intrinsic viscosity and the corrected viscosity-average molecular weight by a least square fit. The procedure is repeated until the value of α becomes constant.

Method III, If the coefficients of the monodisperse calibration relationships $M(V_R)$ and $[\eta]\ (V_R)$ as Eq. 3 and 4 are known, simple correlations between the Mark-Houwink parameters and the coefficients of the calibration relationships

$$\alpha = B_\eta / B_M \quad , \tag{31}$$

$$K = e^{A_\eta} - \alpha A_M \quad , \tag{32}$$

must be satisfied. Since the calibration relationship obtained from the initial calibration parameter, and corresponding retention volume calculated by F(V), is a translated calibration relationship, i.e.

$$M'(V_R) \quad : \quad \ln M = A'_M - B_M V_R \quad , \tag{33}$$

$$[\eta](V_R) \quad : \quad \ln[\eta] = A'_\eta - B_\eta V_R \quad , \tag{34}$$

it is necessary to apply a displacement correction on coefficients A'_M and A'_η for deducing the monodisperse relationship. From Eqs. 13 and 15, if the spreading factor σ_o^2 is considered as a constant equal to the middle value of the variable $\sigma_o^2(V_R)$ over the entire range of elution volume (9), then

$$A_M = A'_M + (1/2)B_M^2 \sigma_o^2 \quad , \tag{35}$$

$$A_\eta = A'_\eta + (1/2)B_\eta^2 \sigma_o^2 \quad , \tag{36}$$

Thus, the resultant monodisperse calibration relationships are

$$M(V_R) : \quad \ln M = 26.9609 - 0.1103 V_R \quad , \tag{37}$$

$$[\eta](V_R) : \ln[\eta] = 16.6811 - 0.0871 V_R \quad , \tag{38}$$

respectively, and the monodisperse Mark-Houwink parameters can be deduced.

The α value became constant simply after two iterations for methods I and II; the calculated viscosity-average molecular weights are listed in Table 5. The parameters for the monodisperse Mark-Houwink equation thus obtained for vinyl polybutadiene in THF are listed in Table 6, in which the linear correlation coefficient r^2, for the least square fit, and the results of method III are also listed.

The data in Table 6 indicate that the results obtained by the three alternative methods are very close to each other and nearly indistinguishable in the investigated molecular weight range as illustrated by Figure 3. That is to say, the monodisperse Mark-Houwink equation could be deduced by any one of the above three

TABLE 5

Viscosity-Average Molecular Weight for Vinyl Polybutadiene Samples

Fraction	$\langle M \rangle_\eta \ 10^{-5}$		
	THF		Toluene
	Method I	Method II	Method I
S1 - A	2.24	2.31	2.24
S1	5.98	5.52	5.95
S2	10.1	10.8	10.1
S3	12.8	13.9	12.7
S4	20.4	19.1	20.3
whole polymer	16.3	14.1	-

methods if the polydispersity of the polymer spreading effect in gel chromatography are considered. The small difference in the values of the parameters shown in Table 6 originated from the slight difference of the calculated viscosity-average molecular weights by methods I and II. If the average value of $\langle M \rangle_\eta$ was considered, the

TABLE 6

The Monodisperse Mark-Houwink Parameters for Vinyl Polybutadiene in THF at 30°C

Methods	$K \ 10^3$	α	r^2
I	9.65	0.79	0.997
II	9.84	0.79	0.986
III	9.87	0.79	-

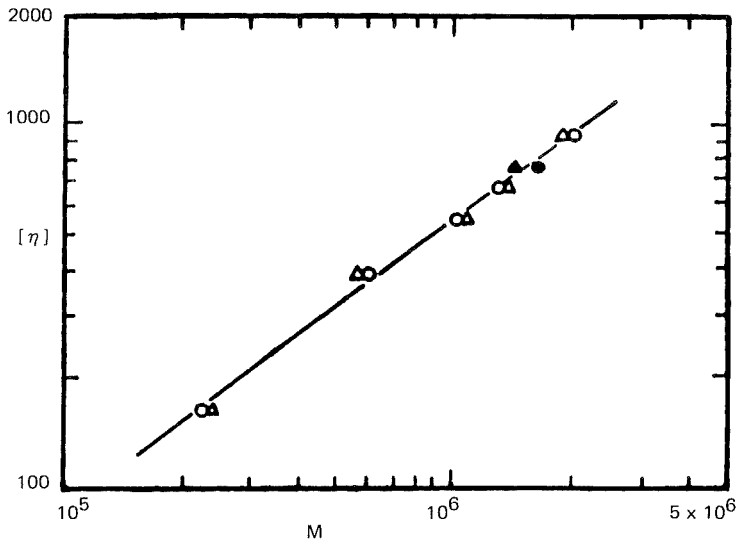

FIGURE 3. The Intrinsic Viscosity-Viscosity Average Molecular Weight Log-Log Plots of Vinyl Polybutadiene in THF at 30°C.
○ Fractions ● Whole Polymer/Method I
△ Fractions ▲ Whole Polymer/Method II
— For Method III

monodisperse Mark-Houwink equation of vinyl polybutadiene in THF 30°C could be expressed as

$$[\eta] = 9.97 \times 10^{-3} \cdot M^{0.79} \quad \text{THF, 30°C} \quad , \qquad (39)$$

The experimental data for the toluene solution were treated with method I; the resultant monodisperse Mark-Houwink equation is

$$[\eta] = 1.04 \times 10^{-2} \cdot M^{0.78} \quad \text{toluene, 30°C, } r^2=0.999 \qquad (40)$$

Anderson et al. (6) have reported some data including number-average molecular weight, polydispersity index, and intrinsic viscosity of high 1,2-polybutadiene samples in toluene, but the

deduced Mark-Houwink equation was not the monodisperse equation. Suppose the molecular weight distribution of their sample fits the logarithmic normal distribution; the relationship between the viscosity-average and number-average molecular weight should be

$$\langle M\rangle_\eta = \left(\langle M\rangle_w / \langle M\rangle_n \right)^{(1+\alpha)/2} \langle M\rangle_n , \qquad (41)$$

Their data were retreated by method I, using Eq. 41, to calculate $\langle M\rangle_\eta$. The monodisperse Mark-Houwink equation obtained by the least square fit was

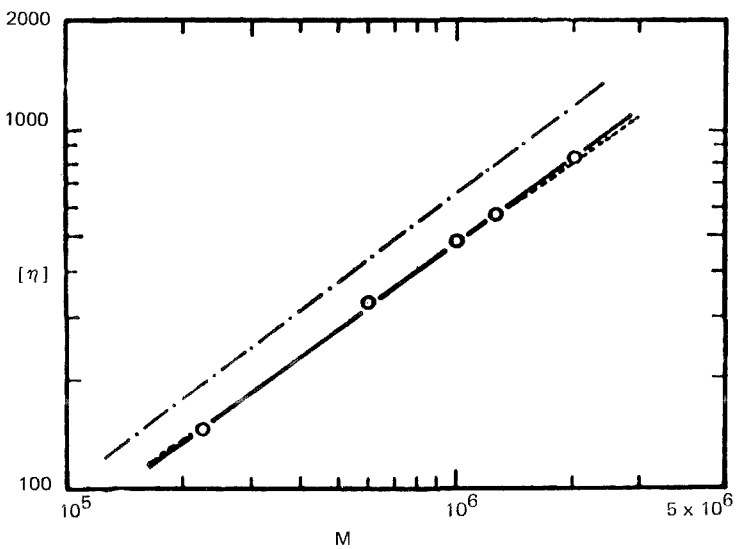

FIGURE 4. The Intrinsic Viscosity-Viscosity Average Molecular Weight Log-Log Plots of Vinyl Polybutadiene in Toluene at 30°C.

— The Present Work: $[\eta] = 1.04\times10^{-2}M^{0.78}$

•— Anderson, et al.(6): $[\eta] = 9.01\times10^{-3}M_n$

•••• Anderson's Data, Corrected for Polydispersity: $[\eta] = 1.24\times10^{-2}M^{0.78}$

$$[\eta] = 1.24 \times 10^{-2} \cdot M^{0.76} \quad \text{toluene, } 30°C,\ r^2=0.996 \quad , \quad (42)$$

Comparing it with our results, there are no distinct differences, as shown in Figure 4. For the three alternative methods employed in the present work, there are no restrictions on the molecular weight distribution of the sample used. Thus, some conveniences are realized for planning experiments and selecting samples. For samples with broad molecular weight distribution, as for the whole polymer used in the present work (the polydispersity index is as high as 11.3) the data points of $\langle[\eta]\rangle$ against $\langle M\rangle_\eta$ calculated by method I or II also are located near the determined monodisperse line as shown in Figure 3. This fact indicates that the monodisperse Mark-Houwink equation obtained is valid.

ACKNOWLEDGEMENT

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REFERENCES

1. Kodera, Akira, Science (Japan) 27, 38-9 (1957).
2. Koningsveld, R. and Tuiznman, C.A.F., Makromol. Chem. 38, 39 (1960).
3. Qian Renyuan and Ying Qizong, Scientia Sinica, 1962(1), 66.
4. Kurata, M., Tsunashima, Y., Iwama, M. and Kamada, K., Polymer Handbook, Second Edition, Brandrup J., Immergut E.H., eds. Wiley and Sons, New York, 1975, IV-1.

5. Bareiss, R.E., Polymer Handbook, Second Edition, Brandrup J., Immergut E.H., eds. Wiley and Sons, New York, 1975, IV-115.
6. Anderson, J.N., Barzan, M.L. and Adams, H.E., Rubber Chem. Technol., 45, 1270-82 (1972)
7. Cheng Rongshi, J.Liq.Chromatogr., in press
8. Mrkvickovà, L. and Lopour, P., Angew. Makromol. Chem. 69, 117 (1978)
9. He Zhiduan, Zhang Xuanqi and Cgeng Rongshi, J. Liq. Chromatogr., in press.

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CHARACTERIZATION OF MACROCYCLES FORMED FROM CATIONIC COPOLYMERIZATION OF TETRAHYDROFURAN WITH PROPYLENE OXIDE BY GPC AND GC/MS *

Ming-hui Hu, Jia-Ji Zhang, Yong-Zhen Xu
Shanghai Institute of Organic Chemistry
Shanghai, People's Republic of China

and

Ying-Song Yang
Laboratory of Chemistry
Shanghai Precision Machinery Corporation
Shanghai, People's Republic of China

ABSTRACT

The copolymers obtained from a THF/PO-$BF_3O(C_2H_5)_2$-glycerin-ethylene chloride cationic copolymerization system were analyzed by GPC. The chromatograms show two groups of peaks, one located in the high molecular weight (MW) region and the other in the low MW region. In order to examine the low MW region in detail, the oligomers were separated from the copolymer by distillation under high vacuum, and further identified by GC/MS, NMR. It was found that these were an expected series of cyclic oligomers, i.e., PO:THF crown ethers: 1:2, 3:1, 2:2, 1:3, 3:2, 2:3, 1:4, 4:2, 3:3, 2:4, 4:3. Through the study of the elution behavior of these crown ethers in toluene with GPC, a linear relationship of the elution time against the logarithm of their MW's was obtained. Under the same GPC conditions, the relationship between the elution time and MW of n-alkanes was also determined. It was found that the hydrodynamic volume of the crown ethers is less than that of the n-alkanes with same MW, and they can be treated as homologous series as in the case of n-alkanes on the study of GPC elution behavior ignoring the influence of PO:THF composition ratios in crown ether rings.

INTRODUCTION

Previous reports(1-4) have shown that in the cationic copolymerization of tetrahydrofuran(THF) and propylene oxide (PO) with boron fluoride-ethyl ether ($BF_3O(C_2H_5)_2$) as catalyst, low MW by-products formed simultaneously with the copolymer appeared clearly in the GPC chromatogram of the total polymerization product. From the total product, Hammond and co-workers (2) have isolated cyclic cotetramers, which are apparently mixtures of cyclic entities containing o,1, and 2 THF units, and inferred the existence of pentamer and hexamer by

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GPC. Similar results were obtained by us (3). Recently, Li Pengfei and co-workers (4) identified some oligomers from the total product by GC/MS, indicating that they are composed of cyclic cotetramers, i.e., PO:THF crown ethers: 3:1, 2:2, 1:3.

To further clarify the oligomer contents in the total product and to examine GPC elution behavior of these oligomers in detail, it seems interesting to identify the oligomer species which were isolated by high vacuum distillation. This paper describes the characterization of these oligomers by GC/MS, NMR, and their elution behavior of GPC.

EXPERIMENTAL

Materials

The cationic copolymerization of THF and PO was carried out as previously described (3,4). $BF_3O(C_2H_5)_2$, glycerin (GLYC) and ethylene chloride (EC) were used as catalyst—co-catalyst—solvent system. Molar ratios of PO:THF:$BF_3O(C_2H_5)_2$: GLYC:EC are 1:2:0.03:0.03:6. Total conversion to polymer was about 75% after 3 hours at 0°C. The number average molecular weight, $\bar{M}n$ of total product determined by vapor-pressure osmometry is 920(3,4). $\bar{M}n$ of linear copolymer calculated by GPC is 1900. The THF:PO mole composition ratio of total product is 1.33:1. The content of the oligomers was about 25% of total product.

The total product was distilled and redistilled under high vacuum. The oligomer fractions collected were identified by GC, MS, NMR.

Apparatus

GC chromatograms of various fractions were obtained on a Model SP-501 gas chromatography with flame ionization detector. (South-Santong Chemical Instru. Co. China) A column packed with 5% OV-101 on Chromsorb W AW-DMCS (60-80 mesh) with 200 cm in length was used. Column temperature was maintained at 200, 230, or 270°C for different fractions, respectively. For total polymerization product, a temperature program, 150-280°C (10°C/min), was used.

Chemical ionization mass spectra were obtained with a Finnigan 4021 model gas chromatograph-mass spectroscopy system with methane as the reagent gas.

For GPC study, the chromatograms were carried out on Waters Model 244 Liquid Chromatograph with a differential refractometer and a Model 730 Data Module. Waters μ-STYRAGEL-100Å Column (30 cm x 7.8 mm I.D.) was used. The instrument was operated at ambient temperature and a flow rate of 1 ml/min with toluene as solvent.

^1H NMR spectra were determined in carbon tetrachloride solution and tetramethylsilane (TMS) as internal reference on a Varian EM-360L-60MHz NMR spectrometer.

RESULTS AND DISCUSSION

The total polymerization product and seven oligomer fractions obtained from high vacuum distillation were analyzed by GC as shown in Figure 1,2 and Table 1, respectively. They indicate that total product contains more than eleven components and that each oligomer fraction is composed of a major component and several minor components, except Fraction G.

The ^{1}H NMR spectra of seven fractions all show the same result as follows: doublet δ 1.06, C-CH_3; δ 1.60, C-CH_2; multiplet δ 3.38, O-CH and O-CH_2 protons. To compare with the results of NMR obtained for homopolymer of PO and THF (1-3), It seems that these seven fractions all comsist of THF and PO units.

In order to confirm the nonhydroxylic structure for these oligomers, the total product was separated into non-, mono-, di-, and tri-hydroxyl-functional fractions by liquid-solid silica gel column chromatography (5). The GC chromatogram of the nonfunctional fraction was found to coincide with

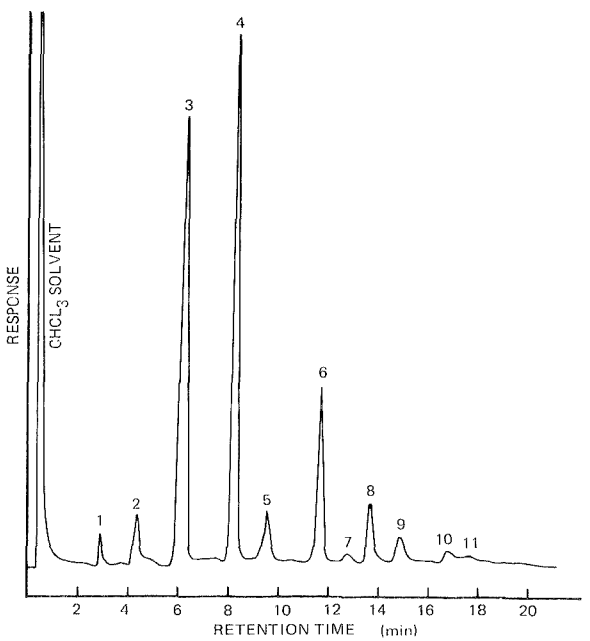

Figure 1. GC Chromatogram of Total Polymerization Product. Oligomer components were expressed with peak numbers.

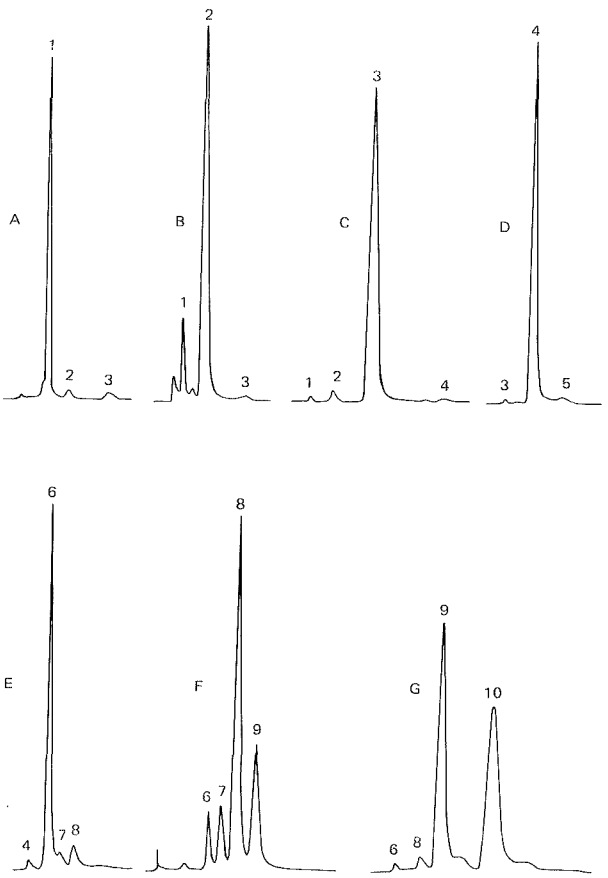

Figure 2. GC Chromatograms of Seven Oligomer Fractions A to G. The bp of seven fractions were listed in Table 1.

Figure 1, while GPC chromatograms of nono-, di-, and trifunctional fractions show absence of the low MW component as expected. The number average functionality (6) measured on the nonfunctional fraction is 0.037-0.91. Hence, the cyclic structure was assumed for all oligomer fractions.

Moreover, the oligomers evaporated from total product were identified by chemical ionization mass spectroscopy. It was observed that these oligomers are an expected series of cyclic oligomers, i.e., PO:THF crown ethers. Their

TABLE 1

The Major Component of the Oligomer Fractions in GC

Sample	bp °C/mm	Major Component*
Fraction A	48/0.01	1
B	58/0.01	2
C	68/0.007	3
D	80/0.01	4
E	117/0.01	6
F	125/0.0003	8
G	143/0.0003	9,10

*The major component of each fraction was expressed with the peak number shown in Figure 2.

MW and corresponding ring structure are correlated in Table 2, which shows that, at the comonomer ratios studied, only mixed PO:THF crown ether will exist and that the largest ring indentified is the PO:THF crown ether 4:3, i.e., cyclic coheptamer. Relatively larger rings may also be present, as shown by GC peaks at longer retention times, but these were not unambiguously assigned yet.

TABLE 2

Crown Ethers Identified by Chemical Ionization Mass Spectroscopy in Total Product

Peak Number[a]	Mol. Wt. (m/e-1)	Crown Ethers Composition (PO:THF)
1	202	1:2
2	246	3:1
3	260	2:2
4	274	1:3
5	318	3:2
6	332	2:3
7	376	4:2
8	346	1:4
9	390	3:3
10	404	2:4
11	448	4:3

a. The oligomer components in total product were expressed with peak number in Figure 1.

b. The Robinson's mixed macrocycle crown ether nomenclature (7) is used in this paper.

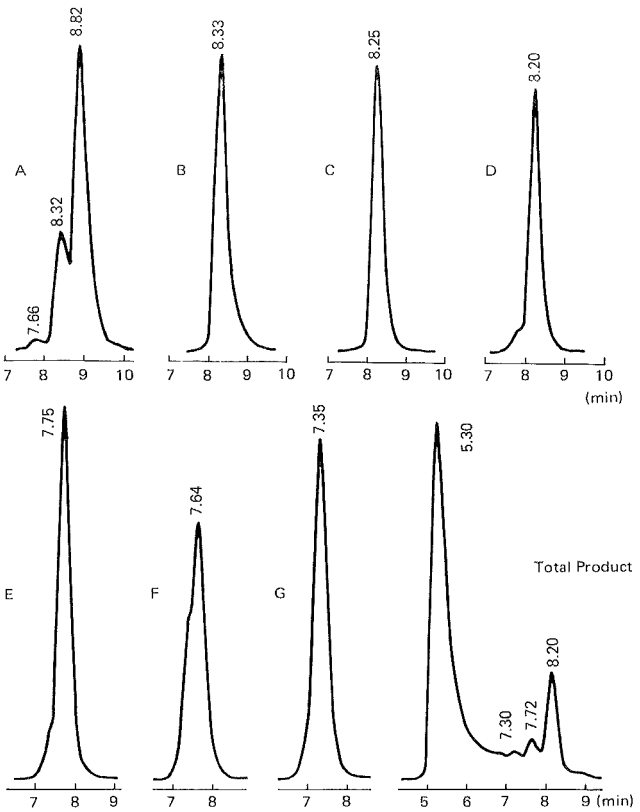

Figure 3. GPC Chromatograms of Seven Oligomer Fractions A to G, and Total Polymerization Product.

*The main peak represents high MW copolymer in the total product, 5.30 min. is exclusion limit time of this column.

Then, by using the seven oligomer fractions with known MW and composition, the elution behavior of the PO:THF crown ethers in GPC was studied.

Figure 3 shows the GPC chromatograms of seven fractions and total polymerization product. The μ-STYRAGEL-100Å column can provide efficient separation for these macrocycles (MW 200-400) in short time.

A linear relationship between the elution time of these macrocycles and logarithm of their MW was obtained (see Figure 4.). Under the same GPC con-

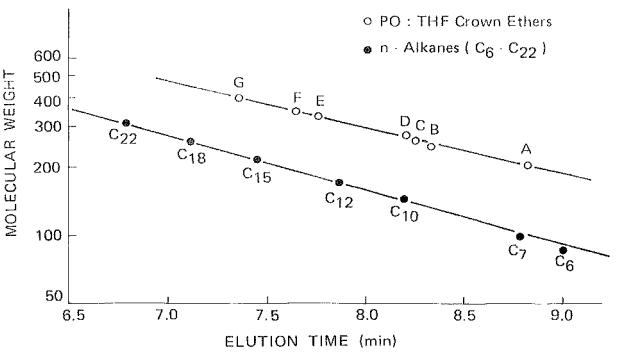

Figure 4. Correlation between GPC Elution Time and Molecular Weight of the PO:THF Crown Ethers and n-Alkanes.

ditions, correlation between the elution time and log MW for n-alkanes was also determined. (also see Figure 4.) The calibration curve in Figure 4 shows that the line for the macrocycles was located above that of n-alkanes, indicating that their hydrodynamic volumes were less than that of n-alkanes with the same MW's. The PO:THF crown ethers can be treated as homologous series as in the case of n-alkanes on the study of GPC elution behavior (8), mo matter what the composition ratios is.

The GPC chromatogram of total product in Figure 3 shows three small peaks corresponding to the cyclic cotetramer, cyclic copentamer and cohexamer mixtures, respectively, and clearly indicates that the cyclic cotetramer mixtures predominates, as is frequently observed in other cyclization systems (7). This agrees with that shown from GC determination of total product in Figure 1. In order to understand the factors affecting formation of cyclic oligomers under different polymerization conditions, the content of cyclic oligomers in total product can be estimated from the GPC chromatogram. For example, the content of oligomers is reduced from 10.4% to 1.0% when glycerin concentration is increased from 0 to 8.0% during the bulk copolymerization process (9).

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REFERENCES

1. a Blanchard,L.P. and Baijal, M.D., J.Polym.Sci., Part A-1, 5, 2045, 1967; b Blanchard, L.P., Kondo, S., Moinard, J., Pierson, J. and Tahiani, F. ibid, 10, 399, 1972; c Blanchard, L.P., Gabra, G.G. and Malhotra, S.L., J.Polym.Sci., Polym.Chem.Ed., 13, 1619, 1975.

2. Hammond, J.H., Hooper, J.F. and Robertson, W.G.P., J.Polym.Sci., Part A-1, 9, a 265; b 281, 1971.
3. Zhang Jiaji, Wang Zhiqin, Yang Yingsong, Tang Guoliang, Organic Chemistry (Shanghai, China) No.1, 34, 1981.
4. Li Pengfei, Zhang Zhenhe, unpublished.
5. Zhang Jiaji, Wang Zhiqin, Yang Yingsong, Shen Ximei, to be published.
6. Law, R.D., J.Polym.Sci., Part A-1, 9, 589, 1971.
7. Robison, I.M. and Pruckmary, G., Macromolecules, 12, 1043, 1979.
8. Krishen, A., J.Chromatogr.Sci., 15, 434, 1977.
9. Zhang Jiaji, Hu Minghui, Yang Yingsong, GPC Symposium of the Chinese Chemical Society, Guilin, June, 1981.

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SPHERICAL VERSUS IRREGULAR-SHAPED SILICA GEL PARTICLES IN HPLC

M. Verzele*, J. Van Dijck, P. Mussche and C. Dewaele
Laboratory for Organic Chemistry,
State University of Ghent,
Krijgslaan, 281 (S.4), B-9000 GENT (Belgium)

ABSTRACT

With optimised packing procedures, spherical shaped silica gel particles produce 1.5 to 2 times more plates in HPLC than irregular shaped silica gel particles. The lowest reduced plate height obtained by us so far is for 5 µm ROSiL-C_{18}-HL-D and is h : 1.62 for k' : 4.5. It is suggested to transform h into 100/h % and to name this the "Chromatographic efficiency", or a % of the ideal 100 % limit. This limit would be an h value equal to the mean particle diameter. Spherical and irregular silica gel particles of 5 and 10 µm particle diameter and with similar physical characteristics have the same permeability in HPLC columns.
Whether a correct column packing procedure is used can be shown by the constancy of plate number and column permeability in function of different packing pressures.

INTRODUCTION

In the present study we evaluate the chromatographic characteristics of spherical silica gel particles in HPLC and compare these with irregular-shaped silica gel particles. The case for or against spherical silica gel in HPLC is indeed not yet settled. Asshauer and Halasz obtain better reproducibility in packing spherical silica gel (1). Manius and Tscherne claim that spherical silica gel gives a tighter bed (2),

which would suggest better column life expectancy. Kirkland (3) finds that spherical particles produce more efficient columns. Laird contradicts this (4) and states "little if any advantage arises from the use of spherical particles rather than broken chips". Unger (5,6) and Halasz (7), as recently as 1981, agree with this last opinion. We are sure many more contributions to these contradictory views can be found in the literature of the last years.
Today, many commercial brochures claim and show by actual chromatograms, that spherical silica gel is superior to irregular-shaped particle silica gel. The general commercial introduction of small sized spherical particles is even one of the most important advances in HPLC of the last years. We report now on our efforts in the field using some reversed phase silica gels, the most used HPLC stationary phases today.

Materials and instruments

All irregular-shaped silica gels tested were of the RSiL series (RSL-Alltech, Eke - B-9731 Belgium). This material is available as such and in most derivatised forms in 5 and 10 µm mean particle size. As spherical silica gel, we used, mainly, ROSiL (RSL-Alltech, Eke - B-9731 Belgium) and, for a restricted number of experiments, Spherisorb (Phase Sep, Queensferry, Clwyd, UK) and Nucleosil (Macherey Nagel, Düren - Germany). Most experiments were carried out with 5 µm ROSiL-C_{18}-HL-D, a spherical silica gel with mean particle diameter of 5 µm, derivatised with octadecyl chains in as high a concentration as possible without polymerisation, and capped with trimethylsilyl functions.

For all chromatographic runs, a Varian 5020 LC instrument (Varian, Walnut Creek - California) was used with a Varichrom variable wave length detector.

Column tubing was Lichroma with ID of 4.6 mm, injection unless otherwise stated, was with a 7000 psi Valco (Houston, Texas) 10 µl sample loop injector. The recorder was a Varian A25 recorder. All chromatographic experiments were carried out isocratically and mostly with acetonitrile-water mixtures as eluent.

Except for the column filling materials, the chromatographic conditions were, therefore, very simple and routine in character.

Chromatographic Efficiency (CE value)

Important points for comparison of column packing materials are the plate number at optimum flow and the permeability of the system. Much used in HPLC is the concept of reduced plate height or reduced parameters in general as introduced by Giddings (8) and in various papers by Bristow and Knox (9). Bristow and Knox (9) recommend as major comparison properties : the reduced plate height (h), the reduced eluent linear velocity (V) and the dimensionless permeability measure (Ø) which is called the column flow resistance. Some years after these recommendations, it turns out that the reduced plate height has found acceptance, permeability is also mentioned sometimes, but the reduced eluent velocity is rarely used. Today, the efficiency of systems is often expressed in plate number per meter although this does not account for the influence of particle diameter. Reduction of the figures seems essential, however, since this allows better comparison of the performance of particles of different sizes and shapes. Unger, in his excellent book on porous silica (6) summarising literature data, mentions that the lowest reduced plate height is about 2 for an unretained solute but that this is easily much higher for retained solutes. This was so in 1979 when Unger's book was published and still is true for

irregular silica gel; but it is not applicable anymore to spherical silica gel as shown in the present paper. Our lowest reduced plate value obtained so far for 5 µm ROSiL-C_{18}-HL-D, is only 1.62 and that is for k' ∿ 4.5 (table 1).

In capillary GC, the theoretical limit of the plate height can be calculated as shown by Ettre (10). In our paper on the static coating technique for glass capillaries (11), we have called this yield actually obtained at optimum flow rate, the "coating efficiency". Expressing the quality of a GC capillary column as a percent of the theoretically obtainable plates has been adopted by the workers in this field, and the notion of coating efficiency has been found quite useful in practice. Ettre (12) has criticised this "coating efficiency" nomenclature, mainly on the ground that factors other than

TABLE 1

	Silica gel	Column cm	Plates	h	CE in %	ΔP (Atm)
1.	5 µ ROSiL-C_{18}-D	25xO.46	25.5OO	1.96	51	96
2.	5 µ ROSiL-C_{18}-D	"	24.15O	2.O7	48	98
3.	5 µ ROSiL-C_{18}-D	"	25.8OO	1.93	52	96
4.	5 µ Spherisorb ODS	"	22.OOO	2.27	44	75
5.	5 µ RSiL-C_{18}-D	"	14.OOO	3.57	28	96
6.	5 µ ROSiL-C_{18}-D	"	3O.8OO	1.62	61	96

All columns except n° 6 tested on a standard Varian LC 5O2O at 1 ml/min of CH_3CN/H_2O - 75/25. Mixture of polycyclic aromatic hydrocarbons injected with a 1O µl Valco sample loop injector. Column 6 coupled to a self made 1.7 µl small volume detector cell. Plate calculation for the pyrene peak with k' ∿ 4.5.

column qualities affect the plate number. This is, of course, correct in principle. In practice however, it is obvious that the column far outweighs all other factors. Still, it would be better to avoid such criticism and to introduce e.g. a "chromatographic efficiency" or CE value. This is the same as Ettre's utilized fraction of theoretically best performance %, but at optimum flow rate. The CE value can be used as a measure of the system quality.

Something similar could be done with HPLC columns. Bristow and Knox state in their paper (9) that the "test procedure ... should enable one to see at a glance how near the performance of any particular column approaches the theoretical ideal ...". They do not mention what the "theoretical ideal" is. It is, indeed, not clear what the limit of HETP could be in HPLC, but the mean particle diameter of the packing material seems to be an acceptable limit. Halasz, indeed, mentions this for small particles like the ones mostly used with spherical silica gel (13). It is, however, easier to measure the internal diameter of capillary GC columns than to pin point the mean particle diameter of a heterogenous mixture of HPLC packing particles. Still, expressing the quality of a column by a % of the attainable and calling this the Chromatographic Efficiency (CE value) would be useful. A name such as "Packing Efficiency" could again be criticised because it excludes extra-column effects. This Chromatographic Efficiency then is defined as $CE = 100 \frac{dp}{H} = 100/h$. Expressing column plate height or plate number in % of an idealised limit enables one to see at a glance how close the system approaches that ideal. A reduced plate height of 2 is equivalent to a CE value of 50 %. In the above example of h = 1.62 the CE value is then of course 61 %. The CE concept has the

advantage that it automatically measures the quality of an LC system against a 100 % limit. It is a reduced value as recommended by Knox (9) but then, of course, it is only 100 times the inverse of h.

Column permeability

Column permeability is related to the mean particle diameter (dp) solvent viscosity (η) column length (L) flow rate of the solvent (u) and the pressure drop (ΔP). For comparison of different materials, Bristow and Knox (9) proposed the column resistance factor (Φ) which is sort of a reduced permeability value.

$$\Phi = \left(\frac{dp}{L}\right)^2 \frac{\Delta P . to}{\eta}$$

For a given column, solvent and solvent rate, Φ is proportional to ΔP. Unger (6) mentions that Φ is about twice as high for irregular silica gel particles than for spherical silica gel particles. Unger rightly states however that these "differences cannot be discussed in terms of particle shape because both types of packings also differ in porosity and mean pore diameter". This point of permeability differences between spherical and non-spherical material is not at all clear.
A point hardly mentioned in the literature is that Φ or, therefore, ΔP for otherwise fixed conditions, can be strongly influenced by the packing pressure. We feel that this should not be so. If the densest packing is achieved, additional pressure should not increase the amount of material in the column or should increase it only very slightly and, therefore, Φ and ΔP should be constant regardless of packing pressure; unless the material is compressible and this is an undesirable situation.

This is, indeed, what is observed when the material is good and/or when the packing method is adapted to the material (table 2).

Table 2 shows that 200-250 Bar is insufficient as packing pressure for all ROSiL materials. Bristow mentions that underivatised Spherisorb can be packed at lower pressure (14). Above 250 Bar, permeability and plate number are practically constant for 5 and 10 µm ROSiL-C_{18} and for 10 µm RSiL-C_{18}. Why this is not so for the other materials is unclear. The 3 µm experimental material was probably not hard enough and was crushed under packing pressure. For 5 µ RSiL we do not understand yet why the back pressure goes up as it does with increasing packing pressure. The most obvious reasons could also be crushing under pressure (why then is this absent with 10 µ RSiL ?) or obstruction of leads or filters. Anyway, we believe now that constancy of back pressure and plate number for various packing pressures are indications that good procedures are being used. The inverse situation proves that different approaches should be attempted for that particular packing material.

Amazingly enough, a 15 x 0.46 cm column with 3 µm irregular silica gel phase, RSiL-C_{18} packed upwards at 600 kg gave only a back pressure of 85 kg under the standard conditions of our test. This was reproducible. Together with the data of tables 2 and 4, this indicates that the permeability of spherical silica gel can be either higher or lower than that of a comparable irregular shaped silica gel. Generally, the difference is minor. The same 3 µ RSiL-C_{18} mentioned above, packed downwards in a CCl_4 slurry produces very high back pressures or even totally impermeable columns. We find it very surprising that the packing technique so strongly influences permeability.

TABLE 2

ΔP and N for octadecylated and end-capped materials

Packing pressure in bar	3 μ spher.	3 μ ROSiL	5 μ ROSiL	5 μ RSiL	10 μ ROSiL	10 μ RSiL
200-250	- ( )	- ( )	86(20.300)	86(14.200)	-	20(6.500)
350-400	160(18.000)	160(19.000)	98(25.000)	96(14.000)	28(13.000)	30(8.700)
650-700	- (-)	- (-)	100(23.400)	128(13.420)	-	30(8.500)
750-800	220(13.000)	- (-)	- (-)	- (- -	-(-)	-(-)
950-1.000	230(10.500)	- (-)	96(26.000)	160(8.240)	-(-)	34(7.030)

Figures for 15 x 0.46 cm (3 μ materials) and for 25 x 0.46 cm (others) octadecylated and end-capped materials. Acetonitrile/water 75/25.
Plates for pyrene with k' = 4.5. Back pressure ΔP for 1 ml/min.

In comparing columns and packings, the most difficult point is to be sure that the packing procedure is the best and cannot be further optimised. Comparing performance (which should be very similar) under different packing pressure is one of the few confidence criteria that we know of in this respect.

Packing HPLC columns

Martin and Guiochon (15) have extensively reviewed the literature on packing procedures. A discussion on general terms of packing procedures is also due to Unger (6) and more recently to Majors (16). We tried most of these techniques with irregular silica gels and found that acceptable-to-good results were obtainable with all of them. With a variety of derivatised irregular-shaped silica gels we obtained good results with a simple slurry in dry CCl_4, packing downward at 600-900 kg/cm^2 pressure with a Haskel air-driven pump. With 5 µ RSiL-C_{18}-HL-D, for example, (irregular shaped octadecylated silica gel heavely substituted and end-capped with trimethylsilyl-groups) we obtain, in this way, a maximum of 13.600 plates for k' : 5-8 with polycyclic aromatic hydrocarbons on a 25 x 0.46 cm column. The reduced plate height is, therefore, below 4 for retained sol tes; this is good for irregular-shaped silica gel. Packing 5 µ ROSiL-C_{18}-HL-D with the CCl_4 slurry technique gave essentially the same plate number. The reproducibility is also acceptable. Using a CCl_4 slurry and downward packing, therefore, reveals no difference between irregular and spherical silica gel. Whether a result is "good" or not depends on comparison with earlier figures. It suffices to obtain a higher efficiency once in order to classify all earlier "good" results in a lower category. This happened to us with the ROSiL-C_{18} packing. With

the dry acetone upwards packing method of Phase Sep (17), we obtain higher plate numbers. Occasionally a column has more than 20.000 plates. With 5 µm Spherisorb ODS, the plate numbers obtained showed exactly the same tendency : for a 25 x 0.46 cm column, about 12-14.000 with a CCl_4 slurry and downward packing, but above 20.000 with the dry acetone upward packing technique. Having obtained these very high efficiencies a few times, we wanted, of course, to achieve this reproducibly. The striking points in the dry acetone slurry packing technique are the relatively low packing pressure and the speed of packing with the low viscosity solvent. Assuming that this last point was important, we tried systems with even lower viscosity than acetone, such as ether or pentane. In the same line of approach, we packed at elevated temperatures to reduce viscosity. This sometimes leads to a positive effect, but not always ! We also packed columns by blowing in the packing with a nitrogen gas cylinder at 150 kg/cm^2. Reasonable columns are obtained but liquid slurries produce better results. We also tried the latest contributions to the field (18) with precolumns, postcolumns, both pre- and postcolumns to equalise, regulate pressure. We used pressure programmers, different pumps etc. etc. Our conclusion is that every different packing material requires its optimised individualised packing procedure. By different we do not only mean different in brand, but also differently derivatised and even different in particle size. If applicable, forcing the slurry up into columns is better than packing downward. With some packing materials, upwards packing does not work; the columns are only partially filled, and downward packing is the only possibility. With larger ROSiL-C_{18} particles, acetone as slurry solvent gives good results.

TABLE 3

n°	ROSiL-C_{18}-D	Plates	h	CE	ΔP(Atm)
1	8 µm	14.360	2.17	46	50
2	"	13.600	2.29	43	50
3	"	15.300	2.04	41	50
4	"	13.100	2.38	42	50
5	"	14.000	2.23	49	50
6	10 µm	13.240	1.89	53	28
7	"	13.000	1.92	52	30

Performance data for 25 x 0.46 cm columns. Acetonitrile-water 75-25 at 1 ml/min. Varian 5020 LC with Varichrom and 10 µl Valco injector. Calculations on pyrene with k' : 4.5.

With smaller ROSiL-C_{18} particle sizes, pentane is a better slurrying solvent. With an optimised packing procedure the slurry concentration does not seem to be very important. We used mostly 20 % slurry concentrations. The data for some columns packed with larger particle sizes with the upwards acetone slurry packing technique are shown in table 3. The data are again obtained on an unmodified Varian 5020 LC instrument.

Concluding this section on column packing, we feel that this part of HPLC leaves still much to be desired. We understand practically nothing of the mechanism of the procedures. Many methods presented as optimal, work only for one particular stationary phase. Points that are certain about column packing are :

- slurry packing is better than dry packing for very small particles
- the packing has to be dried (for reversed phase at 70-80° because in some cases higher temperature may

cause ignition of the materials) except, of course, when water is used in the slurry medium
- the dimensions and form of the packing vessel are important and have to be adapted to the packing material
- taking off the column from the packing vessel is a most important and delicate step in the procedure
- packing upwards is better if applicable (in this we agree with the reasons given by Bristow (14))
- ultrasonication of the slurry is beneficial.

Points that maybe are beneficial but of which we are not so sure that this indeed is so, are :
- dilute or high (< 10-25 % <) concentration slurry packing
- the use of dried solvents
- the use of very low viscosity solvents
- changing from slurry solvent to another solvent to follow up in the packing procedure

Points which have been claimed to be important but which are overrated or unimportant :
- the use of high viscosity slurries
- the use of balanced density slurries
- the use of very high pressures

H-u curves with ROSiLs

Graphs of the plate height against the eluent rate for various ROSiLs in C_{18} form are shown in Figure 1. The data are for pyrene with k' : 4.5 in acetonitrile-water 75/25 on 25 x 0.46 cm columns for the 5 µm material and on 15 x 0.46 cm columns for the 3 µm material. Chromatographic conditions are standard as explained elsewhere in this text. Band widths are measured at half-peak-height for chart speeds of 2 m/h or with the VISTA 401 - Chromatography Data System of Varian leading to good precision of the needed measurements.

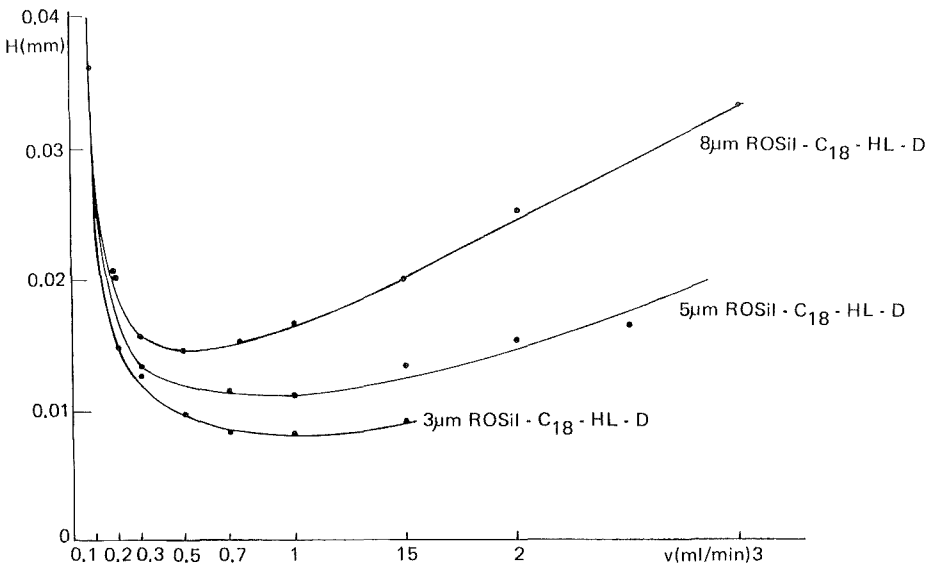

FIG. 1

Van Deemter plots for octadecylated spherical silica gels of different mean particle size. Varian LC 5020 chromatograph with Varichrom detector at 254 nm. Acetonitrile-water, 75-25 at 1 ml/min. Data for pyrene peak with k' : 4.5. 25 x 0.46 cm columns for 5 and 8 µm, 15 x 0.46 cm columns for 3 µm material. Injection through 10 µl Valco sample loop.

The behaviour of the ROSiLs in this context is classical. With the small particle sizes, the solvent speed can be increased by a factor 2 to 3 above the optimum, without adversely affecting column efficiency. This is a most important point for high speed analysis. For plate number measurements of these high performance columns, it is essential to use fast electronic detection. With an older fixed 254 nm wave length detector having a time constant of 3 sec, plate numbers were halved only because of the slow detector response. Even

the "fast" time constant of 0.5 sec of the Varichrom proved to be too slow. Therefore we modified the 2 sec "slow" time constant of the detector to an ultra fast 0.25 sec time constant. Some results are shown in Figure 2.

Figure 2 was reproduced for several other polar and non-polar compounds with similar results. For early, hardly retained peaks, the plate number can be drastically different, depending upon the speed of the electronics of the detector. Some contradictory literature statements concerning this point can probably be ascribed to this effect. Even at higher k' values, around k' : 4.5, differences can easily be 10-20 % in favour of faster detection.

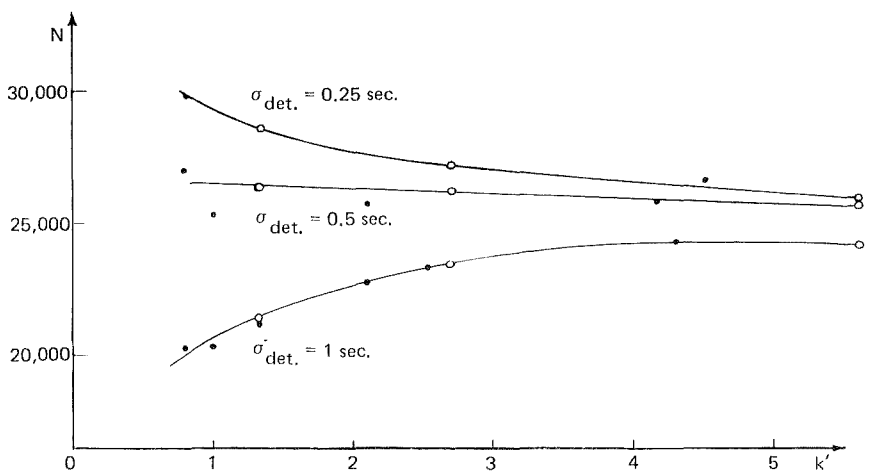

FIG. 2

Efficiencies as a function of detector time constant for the same 5 μ ROSiL-C_{18} column, 25 x 0.46 cm at 1 ml/min with variable mixtures of acetonitrile-water. Open circles : pyrene, shaded circles : naphtalene or anthracene.

Conclusions concerning spherical versus irregular silica gel

A direct comparison of spherical and irregular silica gel is shown in the next table.
Table 4 mentions values for the column resistance factor Ø and the Separation Impedance E of Bristow and Knox (9). It is important to note that these values are for k' : 4.5. For unretained peaks the literature mentions mostly lower h values and therefore more favourable E values. We feel that h values for unretained peaks should not be used as comparison criteria, if only because of their dependence of detector electronics speed as shown in the present paper. This situation is very similar to what has been experienced in GC where it now is customary only to calculate efficiencies for decidedly retained peaks e.g. k' > 3.

TABLE 4

Comparison of spherical and irregular silica gel

	Material	N	ΔP	h	Ø	E
1.	5 μ ROSiL-C_{18}-D	25.000	98	2.00	818	3.272 (2.250)
2.	5 μ RSiL-C_{18}-D	14.000	96	3.57	815	10.387
3.	10 μ ROSiL-C_{18}-C	13.000	28	1.92	1.022	3.779
4.	10 μ RSiL-C_{18}-D	8.700	30	2.87	1.029	9.041

All columns packed upwards at 350-450 kg/cm^2.
Data for pyrene with k' : 4.5 in acetonitrile-water 75/25. All chromatographic conditions standard as explained in the text and exactly equal. Ø is the column resistance factor and E is the Separation Impedance as described by Bristow and Knox (9). $E = h^2.Ø$. The value 2.250 was for optimised conditions using a small volume detector cell.

It has been suggested that further improvement through more efficient injection should be possible. We find, however, that coaxial flow injection, stopped-flow hand-injection or small-loop-volume injection do not improve the performance of a top quality column. A positive effect is only found for these approaches when column performance is not the best attainable to start with.

It should also be mentioned that the efficiency of these high performance columns is decidedly lower when methanol-water mixtures are used as eluent and/or when relative large molecular weight polyfunctional polar compounds are chromatographed. For hop α- and β-acids (humulone etc.) with methanol-water as eluent, the efficiency can be more than halved. A chromatogram of a synthetic mixture is shown in Figure 3.

The back pressure of spherical versus irregular silica gel columns can be higher or lower without obvious reason. Astonishingly enough, permeability can be strongly influenced by the packing technique. In most cases the permeability is about the same for comparable phases. The higher efficiency of spherical silica gel allows to use shorter columns while still achieving sufficient efficiency. This in turn leads to reduced pressure drops, allowing to use smaller particle sizes leading again to better efficiency and further reduced column length. Smaller particle sizes can be effectively used at higher relative eluent rates (flat H-u curve). Thus we come to very fast and very efficient HPLC. This is the most important aspect of spherical silica gel as HPLC packing material.

Because of this point it is possible to replace systematically, classic 25 cm long 10 µm irregular silica gel columns (∿ 5-10.000 plates) by 10 to 15 cm long 5 µm spherical silica gel columns (10-15.000 plates) with

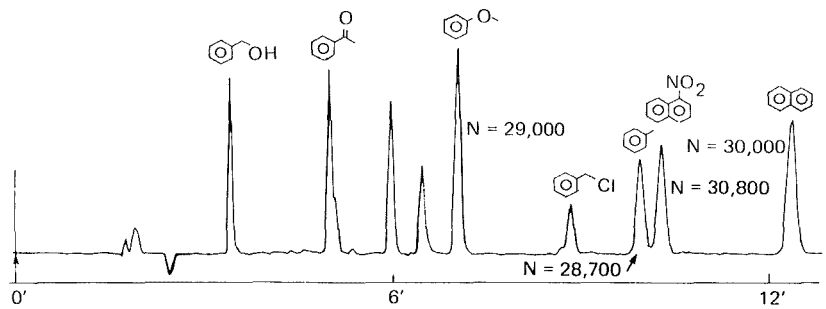

FIG. 3
Polarity mixture on 5 µ ROSiL-C_{18}-HL-D Varian 5020 LC instrument with Varichrom detector and 10 µl Valco sample loop injector. 1 ml/min of acetonitrile-water 60-40 at 120 Bar.

better resolution, better sensitivity and less than half the time of analysis.
Spherical silica gel will not agglomerate on standing for longer periods like can be the case with 3-5 µm irregular silica gel.
We have no experimental evidence about lifetime expectancy differences. Spherical silica gel columns seem to loose fairly rapidly (after 20-30 injections) about 10-20 % of their efficiency.

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REFERENCES

1. J. Asshauer and I. Halasz, J. Chromatogr. Sci., 12, 139 (1974).

2. G. Manius and R. Tscherne, International Laboratory, vol. -1, (2), 38 (1981).
3. J. Kirkland, J. Chromatographic Science, 10, 593 (1972).
4. G. Laird, J. Jurand and J. Knox, Proc. Soc. Anal. Chem., 11, 311 (1974).
5. K. Unger, W. Messner and K. Krebs, J. Chromatogr., 149, 1 (1978).
6. K. Unger, p. 184 in Porous Silica, Elsevier, (Amsterdam), 1979.
7. R. Ohmacht and I. Halasz, Chromatographia, 14(4), 216 (1981).
8. J. Giddings, Dynamics of Chromatography, Part I, Marcel Dekker, New York, 1965.
9. P. Bristow and J. Knox, Chromatographia, 10, 279 (1976).
10. L. Ettre, "Open Tubular Columns in GC", Plenum Press, New York, 1965.
11. J. Bouche and M. Verzele, J. Chrom. Sci. (J.GC), 5, 501 (1968).
12. L. Ettre, in Applications of Glass Capillary Gas Chromatography, p. 33, Ed. W. Jennings, Marcel Dekker, New York, 1981.
13. I. Halasz, R. Endele and J. Asshauer, J. Chromatogr., 112, 37 (1975).
14. P. Bristow, J. Chromatogr., 149, 13 (1978).
15. M. Martin and G. Guiochon, Chromatographia, 10, 194 (1977).
16. R. Majors, J. Chrom. Sci., 18 (10), 488 (1980).
17. Phase Sep., Commercial brochure on Spherisorb - Queensferry, Clwyd, U.K.
18. I. Halasz, International Symposium on Column Liquid Chromatography - Avignon, May 1981 (oral presentation).

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APPLICATIONS OF A MODIFIED "ISOHYDRIC SOLVENT SYSTEM" IN HPLC ON SILICA GEL FOR THE ANALYSIS OF THE MACROLIDE ANTIBIOTICS TURIMYCINS AND SPIRAMYCINS

G.A. Bens, E. Crombez and P. De Moerloose
Department of Pharmaceutical Chemistry
and Drug Quality Control
State University of Ghent,
Harelbekestraat 72, B-9000-Ghent, Belgium

ABSTRACT

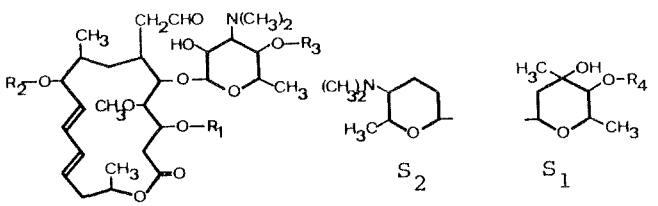
The behaviour of some macrolide antibiotics in high-performance liquid chromatography on silica gel using an isohydric eluent system and its modifications are described. These modifications involve water content and diethylamine content variations in the eluent, and also column temperature variations, using column packing material previously described. Extention to other types of silica gel packing materials was also performed. The experiments showed excellent results for the separations of the macrolide antibiotic complexes Turimycins and Spiramycins and proved that the chromatographic process is not only an adsorption one but that partitioning effects also play an important role.

INTRODUCTION

The problem of separation of various macrolide antibiotics is frequently encountered. In several instances, the efficiencies of the separations by high-performance thin-layer chromatography (H.P.T.L.C.) and high-performance liquid chromatography (H.P.L.C.) are not satisfactory due to the similar structures and large molecular weights of these components.

Table I

Structure of Spiramycin and Turimycin Macrolide Antibiotics



Name	Abbreviated name	R_1	R_2	R_3	R_4
Spiramycin 1	Sp 1	H	S_2	S_1	H
2	Sp 2	$COCH_3$	S_2	S_1	H
3	Sp 3	$COCH_2CH_3$	S_2	S_1	H
Turimycin H_2	H_2	H	H	S_1	$COCH_3$
H_3	H_3	H	H	S_1	$COCH_2CH_3$
H_4	H_4	H	H	S_1	$COCH_2CH_2CH_3$
H_5	H_5	H	H	S_1	$COCH_2CH(CH_3)_2$

The introduction of the isohydric solvent system theory by Thomas et al. (1-4) as an all-round eluent system on silica gel, prompted us to investigate the application of this theory to the separation of two macrolide antibiotic complexes; Turimycins and Spiramycins (5-10) (Table I).

During these investigations it became clear that several chromatographic parameters could be improved to a great extent by modifying the original isohydric solvent system.

EXPERIMENTAL

High-Performance Liquid Chromatography

A Varian 4100 liquid chromatograph or a Spectra Physics SP 8000 liquid chromatograph was used, equipped with a variable wavelength detector (Varichrom, Varian, Palo Alto, Ca, USA), set at 232 nm.

The temperature of the column was controlled using a waterbath (Varian) or a heated air oven (Spectra-Physics). Samples, dissolved in the eluent, were injected using a six way Valco valve with a sample loop of 20 mm^3.

Reagents and Materials

- Di-isopropylether, methanol and iso-octane were of analytical grade (Merck, Darmstadt, G.F.R.)
- Diethylamine (U.C.B., Brussels, Belgium) and water were destilled in an all-glass apparatus prior to use
- Column packing materials :
  - Spherosil XOA-600 (5 µm)(Prolabo, Paris, France), spherical porous silica gel, specific surface area, 580 m^2/g, mean pore diameter 90 Å
  - Lichrosorb Si 60 (5 µm)(Merck, Darmstadt, G.F.R.) irregular silica gel, specific surface area, 500 m^2/g, mean pore diameter 60 Å
  - Lichrospher Si 100 (5 µm)(Merck, Darmstadt, G.F.R.) spherical porous silica gel, specific surface area 250 m^2/g, mean pore diameter 100 Å
  - Spherisorb S 5W (5 µm)(Phase Separations, Queensferry, U.K.) spherical porous silica gel, specific surface area 220 m^2/g, mean pore diameter 80 Å
- Columns (150 mm x 4.6 mm I.D., stainless-steel tubing with 2 µm porosity frits) were filled by means of a slurry technique (slurry : 2 g silica gel in 15 ml carbontetrachloride:methanol (80:20), methanol as pressuri-

zingsolvent, upward filling).

- Spiramycins : bulk powders and pure components were obtained from S.P.E.C.I.A. (Paris, France)
- Turimycins : bulk powders and pure components were obtained from Dr. Fricke, Forschungszentrum für Molekularbiologie und Medizin, Jena,(G.D.R.)

RESULTS AND DISCUSSION

A normal isohydric eluent system is a combination of two isohydric solvent mixtures, one apolar and the other polar in nature (1,3). The original isohydric eluent used in our experiments consisted of the apolar mixture A[Di-isopropylether:iso-octane (500:500)] and the polar mixture B [Di-isopropylether:methanol:water (500:474:26)]. To suppress the ionization of the basic functions in the macrolide molecules, 0.2 % diethylamine was added to each combination of A and B.

A first trial for the separation of the Turimycin and the Spiramycin group was made by plotting the capacity factors of the various antibiotics as a function of the inverse of the molar water fraction, for different concentrations of mixture B in the eluent. As pointed out by Thomas et al., there was a linear relationship between the capacity factors and the inverse of the molar water fraction in the eluent. A representation of this phenomenon for the Spiramycins is given in figure I . A mixture of about 70 to 80 per cent A and 30 to 20 per cent B showed the best results for the separation of Spiramycins and Turimycins. Figure II gives as an example, the separation of Turimycins on 80A-20B . To improve the resolution between Turimycins H_4 and H_5 and the Spiramycins 2 and 3, the effects of water content and diethylamine concentration of the eluent were studied and also the effect of column temperature variation, as these showed to be important to improve the column efficiency.

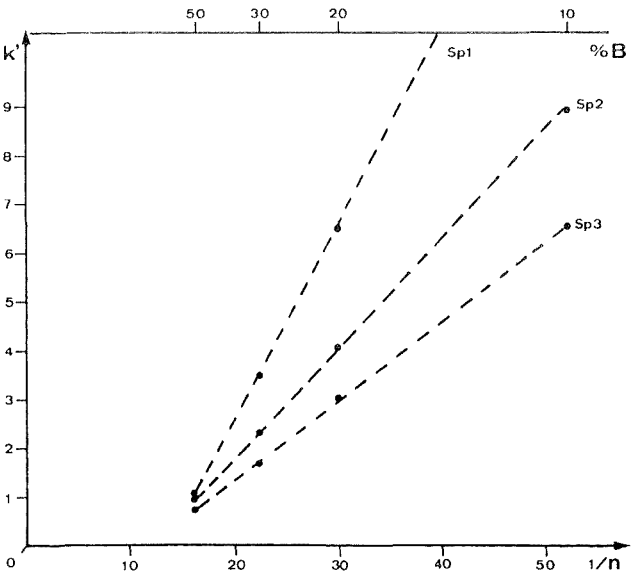


FIGURE I

Capacity factors of Spiramycins as a function of the inverse of the molar water fraction in the eluent (mixture B with 2.6% H_2O).

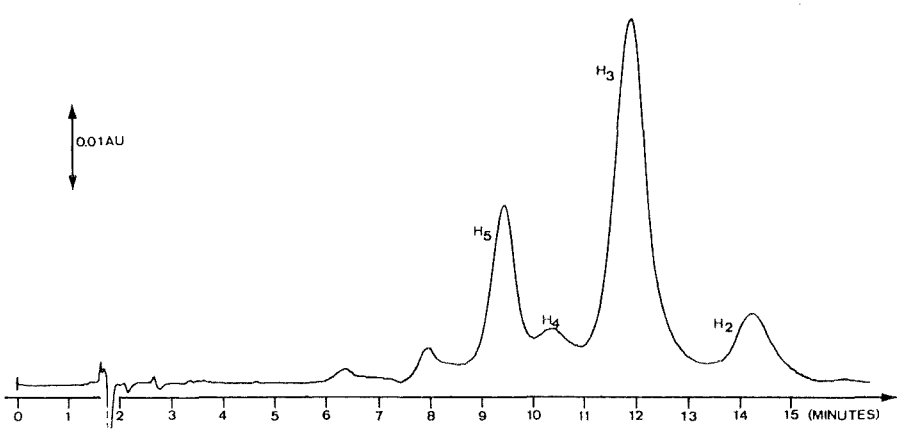


FIGURE II

Separation of components of a Turimycin bulk powder (H_5, H_4, H_3 and H_2, X : unknowns)

Column : Spherosil XOA 600, 5 µm, 150 mm x 4.6 I.D.
Eluent : 80A-20B with 0.2 % diethylamine (B with 2.6 % H_2O)
Flow : 1.0 $ml.min^{-1}$
Detection : 232 nm
Temperature : ambient

Variation of the water content of mixture B

When using an isohydric eluent system as described (1), the activity of the adsorbent is equal to one (α_a = 1) by definition. Normally, to be isohydric, mixture B should contain 2.604 % of water and mixture A a negligable amount of 0.004 % of water. By altering the amount of water in mixture B, the activity of the adsorbent is also changed. To study this effect on the different macrolide antibiotics, the water content of mixture B was changed in following steps : 0 %-0.65 %-1.3 %-2.6 % (α_a = 1)-3.9 % and 4.55 % water in mixture B. At 5.2. % water in mixture B, demixing was noticed when adding A and B together at a ratio of 70 A and 30 B.

In contradiction with the adsorption theory of Thomas et al., the capacity factors of the Turimycins and Spiramycins did not decrease, but they showed an increase with increasing water content of mixture B. This effect can be seen in figure III,where capacity factors of

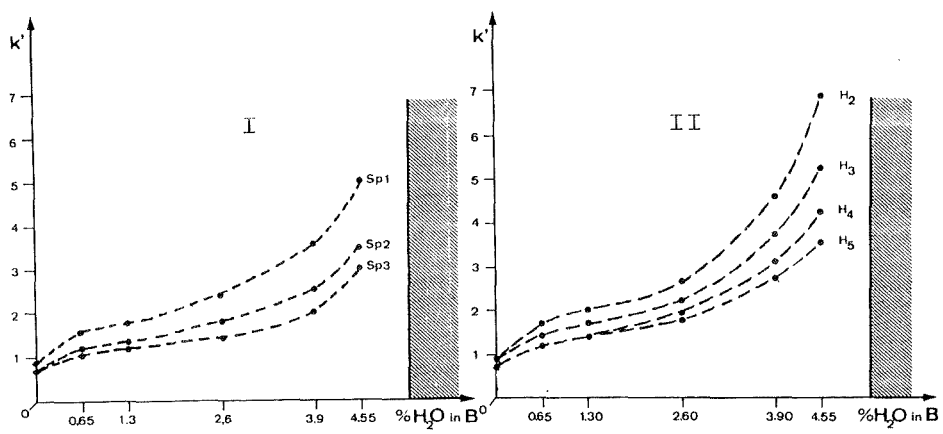


FIGURE III

Capacity factors (k') of Spiramycins (I) and Turimycins (II) as a function of the water content of mixture B (Eluent: 70A-30B, with 0.2% diethylamine)

TABLE II

Column-efficiencies (N) and Resolutions (R_S) for Spiramycins and Turimycins as a Function of the Percentage of Water in Mixture B

% Water in mixture B	N Sp 1	R_S Sp 2 - Sp 3	N H_3	R_S H_4 - H_5
0 %	-	-	-	-
0.65 %	1496	0.77	1491	-
1.30 %	2561	1.25	2567	-
2.60 %	2127	1.38	3570	0.56
3.90 %	1702	1.53	2976	1.27
4.55 %	1364	1.71	2376	1.58

Turimycins and Spiramycins are given as a function of the water content in mixture B at the same proportion (70:30) of mixtures A and B and with the same amount of organic base (0.2 % diethylamine) in the eluent. From table II one may also notice that the efficiency of the column was maximal for 2.6 % water in mixture B for the Turimycins (calculated on Turimycin H_3) and for 1.3 % water in mixture B for the Spiramycins (calculated on Spiramycin 1). Nevertheless, the resolutions between Turimycins H_4 and H_5 and Spiramycins 2 and 3 were optimal for 4.55 % water in mixture B. This improvement in resolution with less efficiency is the result of the important contribution of the capacity factor and selectivity terms in the equation of the resolution:

$$R_S = \frac{1}{4} \sqrt{N} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{k'}{k' + 1}\right)$$

An example of this effect is shown in figure IV where chromatograms are given of the separations of Turimycins at 2.6 % water and 4.55 % water in mixture B.

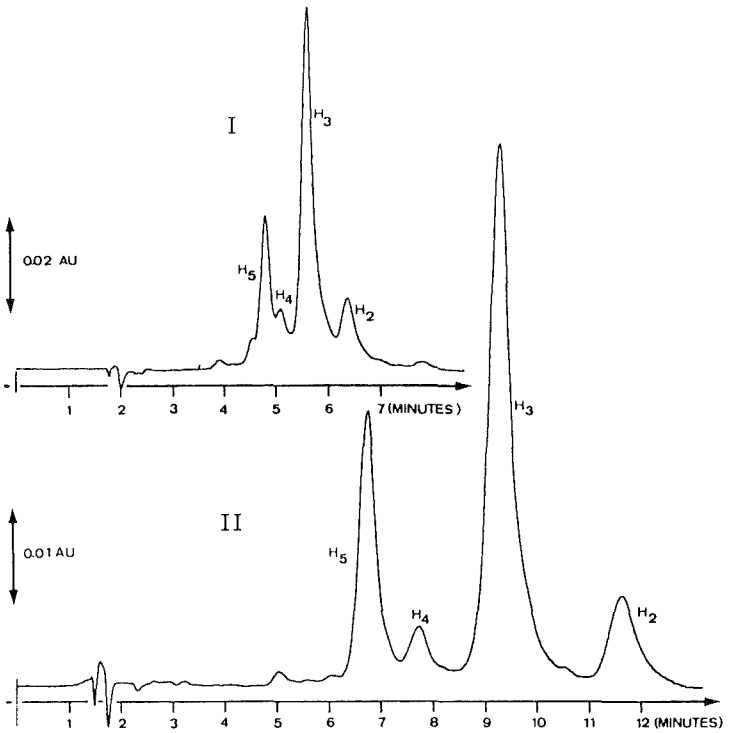


FIGURE IV

Influence of the water content of mixture B on the separation of Turimycins.
Column : Spherosil XOA 600, 5 µm, 150 mm x 4.6 mm I.D.
Eluent : 70A-30B (B with 2.60 % H_2O (I) and with 4.55 % H_2O (II)), with 0.2 % diethylamine
Flow : 1.0 $ml.min^{-1}$
Detection : 232 nm
Temperature : ambient

Variation of the diethylamine concentration in the eluent

The influence of the concentration of organic base in the eluent on capacity factors, efficiency and resolution was studied, by adding different amounts of diethylamine to the original isohydric mixture (70A-30B; 2.6 % water in B). The effect on the capacity factors of Turi-

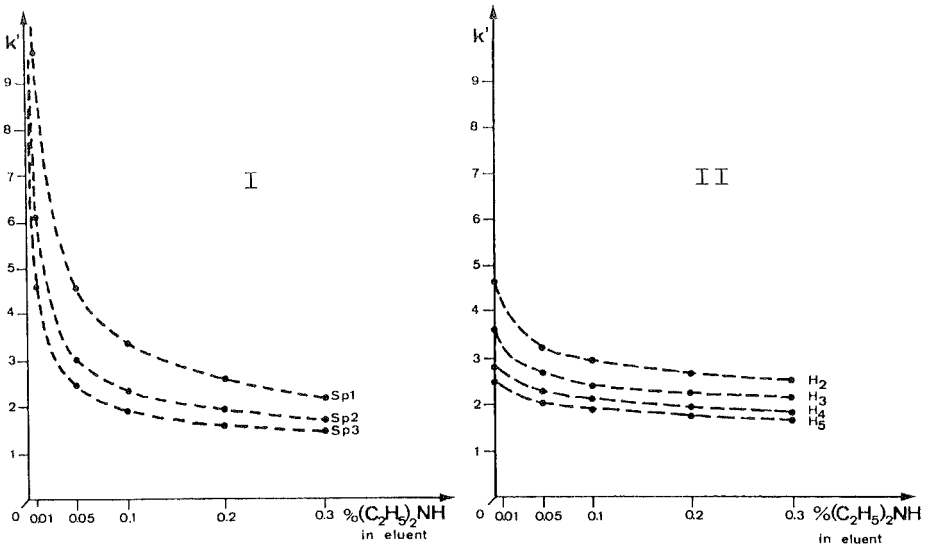


FIGURE V

Influence of diethylamine concentration in the eluent on the capacity factors (k') of Spiramycins (I) and Turimycins (II) (Eluent : 70A:30B, with 2.6 % H_2O in B)

mycins and Spiramycins are given in figure V. From this figure one can see that the Spiramycins do not elute from the column when there is no diethylamine in the eluent, due to their supplementary basic function in the forosamine sugar moiety (S_2; see table I), while Turimycins elute within a reasonable time. In table III it can be seen that the resolution is not maximal for concentrations of diethylamine for which column efficiency is maximal (0.05 % diethylamine in the eluent), due to a great contribution of the capacity factors in the equation of the resolution. Figure VI represents the separations of Spiramycins at 0.3 % and 0.05 % diethylamine in the eluent, showing extreme differences in efficiencies for slight differences in base concentrations.

TABLE III

Column-efficiencies (N) and Resolutions (R_S) for Spiramycins and Turimycins as a Function of the Percentage of Diethylamine in the Eluent

% Diethylamine in the eluent	N Sp 1	R_S Sp 2 - Sp 3	N H_3	R_S H_4 - H_5
0 %	-	-	3414	-
0.01 %	2920	2.75	3677	1.35
0.05 %	3830	2.54	4820	1.29
0.1 %	3292	2.05	3910	1.06
0.2 %	2328	1.46	3011	0.83
0.3 %	1072	0.95	2228	0.57

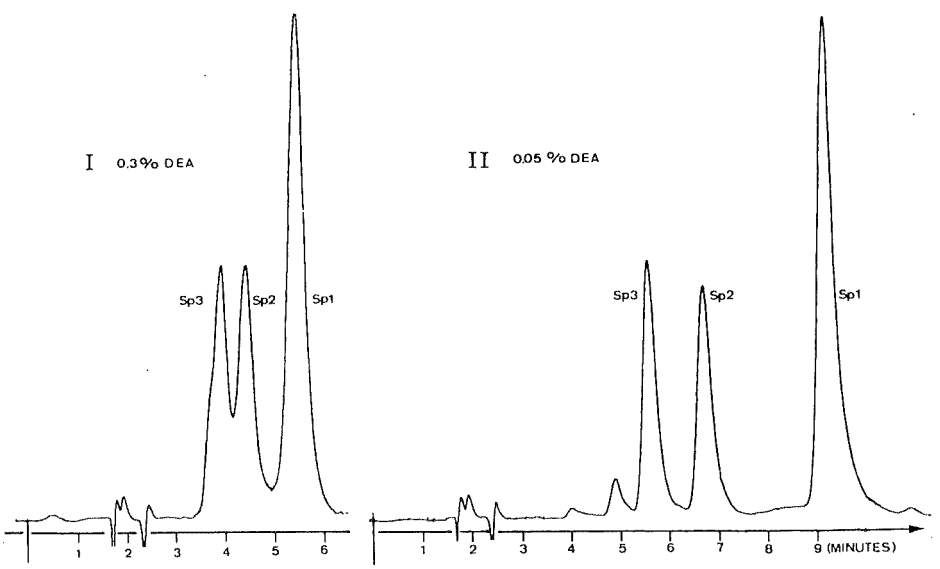


FIGURE VI

Influence of the diethylamine concentration of the eluent on the separation of Spiramycin antibiotics (Eluent 70A:30B; B with 2.6 % H_2O)

I : 0.3 % D.E.A.
II : 0.05 % D.E.A.
Flow : 1.0 $ml.min^{-1}$
Detection : 232 nm
Temperature : ambient

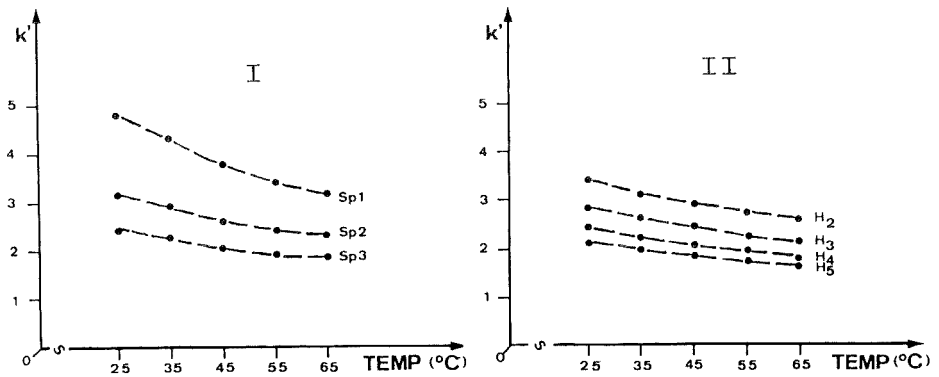


FIGURE VII

Influence of column temperature on the capacity factors (k') of Spiramycins (I) and Turimycins (II). Eluent : 70A:30B, with 0.05 % diethylamine (B with 2.6 % H_2O)

Variation of column temperature

The effect of column temperature variation on the separation of the macrolide antibiotic complexes was investigated using the original isohydric eluent system, i.e. the combination 70 A - 30 B, whereas B contained 2.6 % water, but with 0.05 % diethylamine. As this is already a partially optimized system (0.05 % diethylamine instead of 0.2 % originally), the results of temperature variation were not as spectacular as for water- and diethylamine content variation (Figure VII). Capacity factors slightly decreased with increasing column temperatures but the selectivities for the pairs :

Sp 2 - Sp 3 ($\alpha^{Sp\ 2}/_{Sp3} = \frac{k'\ Sp\ 2}{k'\ Sp\ 3}$) and

H_5-H_4 ($\alpha^{H5}/_{H4} = \frac{k'\ H5}{k'\ H4}$) remained practically unchanged.

In table IV it can be seen that there is an optimum at 35°C for Turimycins and for Spiramycins at higher tem-

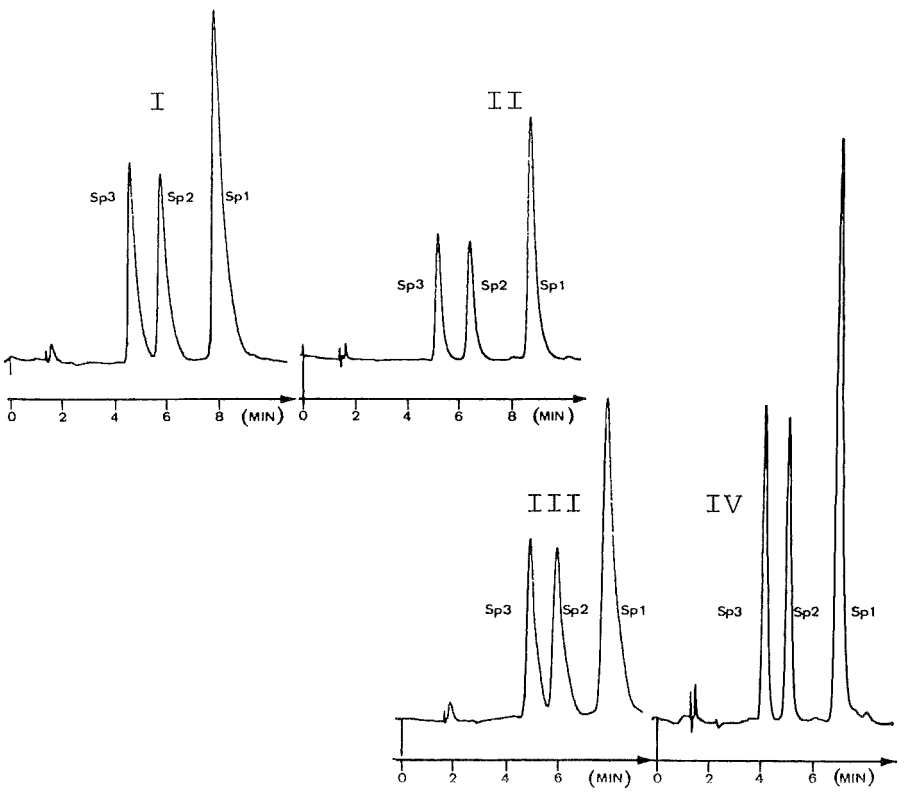


FIGURE VIII

Separation of Spiramycins on four types of silica gel columns.
Eluent : 70A-30B with 0.05 % diethylamine (B with 4.55 % H_2O)
Flow : 1.0 $ml.min^{-1}$
Detection : 232 nm
Temperature : 45°C
Columns : 150 mm x 4.6 mm I.D.

I : Lichrosorb Si60
II : Spherosil XOA 600
III : Lichrospher Si100
IV : Spherisorb S-5-W

TABLE IV

Column-efficiencies (N) and Resolutions (R_S) for Spiramycins and Turimycins as a Function of the Column Temperature

Column temperature	N Sp 1	R_S Sp 2 - Sp 3	N H_3	R_S H_4-H_5
25°C	3127	2.39	3761	1.07
35°C	3930	2.49	4815	1.19
45°C	4900	2.85	4715	1.15
55°C	5841	2.88	4769	1.13
65°C	6776	2.88	4615	1.09

peratures, but the resolution was satisfactory even at lower temperatures.

Effect of the silica gel adsorbent

With an optimized eluent system, consisting of 70A - 30B (with 4.55 % water in mixture B and 0.05 % diethylamine in the total) and a column temperature of 45°C, four types of silica gel were compared to investigate the contribution of their specific surface area or their pore diameter on the separation of the group of Spiramycins. Excellent separations were obtained with the different types, but a relationship between the capacity factors and pore diameters or specific surfaces could not be fount (Figure VIII).

CONCLUSION

Using a modified isohydric eluent system, Turimycin and Spiramycin macrolide antibiotics are well separated and both adsorption- and partition effects are

observed. When plotting k'-values againtst the percentage of water in mixture B, one may notice that the curvature obtained has the same slope as the water adsorption isotherm of silica gel (11) (Figure III).

Two hypotheses can be put forward. The first one is that capacity factors (or relative retentions on the column) of Spiramycins and Turimycins are increasing with increasing water content of mixture B, due to a partitioning process. In the second hypothesis, one may believe that the increased water content of mixture B (and hence increased mass of water on the silica gel surface), decreases the mass of diethylamine adsorbed on the silica gel surface. As a result of this decreased diethylamine concentration, the retention times of Spiramycins and Turimycins increase as evidenced by the data obtained when the diethylamine concentration was varied. (see : Variation of the diethylamine concentration in the eluent).

The first hypothesis was stated by following experiments : as only Turimycins did elute from the column at 0 % diethylamine in the eluent (see : Variation of the diethylamine concentration in the eluent), Turimycins H_5 and H_3 were used to check their behaviour with different water concentrations in the eluent at 0 % diethylamine. As can be seen from table V, where capacity factors of Turimycins H_5 and H_3 are given as a funtion of the percentage of water in mixture B at 0 % diethylamine, the capacity factors increased with increasing water content of the eluent, indicating a partitioning process.

Nevertheless, this may not exclude the fact that diethylamine may be washed out at higher water levels in the eluent and produce higher retentions of the macrolides.

TABLE V

Capacity factors of Turimycins H_5 and H_3 as a Funtion of the Percentage of Water in Mixture B at 0 % Diethylamine in the Eluent

% Water in mixture B	k' H_5	k' H_3
0 %	1.39	2.28
0.65 %	2.02	3.53
1.30 %	2.15	3.97
2.60 %	2.49	4.67
3.90 %	2.97	5.93
4.55 %	4.06	8.33

A water layer is build up on the silica gel surface and instead of adsorption chromatography on a highly deactivated adsorbent of which the activity is kept constant, mixed mechanisms of adsortion and partition take place.

Temperature effects are of minor influence, when using very low amounts (0.05 %) of organic base in the eluent, but they may contribute to a great extent to the efficiency of the column when using up to 0.3 % of organic base in the eluent. This can be explained by a lower uptake of base by the silica gel at higher temperatures as low organic base concentrations give very high plate numbers for the column.

Although the isohydric solent theory was based on the use of one particular silica gel (Spherosil XOA 600) other silica gels may be used, resulting in simular separation possibilities.

For the moment, the same experiments are performed in our laboratory on a great number of basis drugs and

the use of these isohydric systems in high-performance thin-layer chromatography on silica gel is also under investigation. Results will be presented in a next paper.

REFERENCES

1. Thomas J.P., Brun A. and Bounine J.P., Isohydric solvents in liquid-solid chromatography, J. Chromatogr., 139, 21, 1977.

2. Thomas J.P., Caude M., Brun A. and Bounine J.P., Le rôle de l'eau en chromatographie d'adsorption, Analusis, 5, 205, 1977.

3. Thomas J.P., Brun A. and Bounine J.P., A rational method of mobile phase optimization based on the use of isohydric solvents, J. Chromatogr., 172, 107, 1979.

4. Thomas J.P., Brun A. and Bounine J.P., Séparation de produits pharmaceutiques par chromatographie en phase liquide, Analusis, 8, 265, 1980.

5. Ōmura S., Suzuki Y., Nakagawa A. and Hata T., Fast liquid chromatography of macrolide antibiotics, J. Antibiotics, 26, 794, 1973.

6. Mourot D., Delépine B., Boisseau J. and Gayot G., Reversed-phase high-pressure liquid chromatography of Spiramycin, J. Chromatogr., 161, 386, 1978.

7. Bens G.A., Van den Bossche W. and De Moerloose P., Separation and Determination of components of Spiramycin in bulk powders and pharmaceutical preparations by high performance liquid chromatography, Chromatographia, 12, 294, 1979.

8. Bens G.A., Van den Bossche W. and De Moerloose P., HPLC method for the separation of Turimycin-H-complex antibiotics, H.R.C. & C.C., 2, 261, 1979.

9. Fricke H., Mühlig P. and Kühne Chr., Quantitative Dünnschichtchromatographische Bestimmung des Makrolidantibiotikums Turimycin H und einiger Abbauprodukte durch Densitometrische "in-situ" Messung mit

dem Bildanalyse gerät "Quantimet 720", J. Chromatogr., 153, 495, 1978.

10. Bens G.A., Van den Bossche W. and De Moerloose P., High-Performance Thin-Layer Chromatography of Some Sixteen-Membered Ring Macrolide Antibiotics, H.R.C. & C.C., 3, 433, 1980.

11. Halpaap H., Erzielung von reproduzierbaren Trennungen durch Verwendung von standardisierten Sorbentien in definierten Systemen, J. Chromatogr., 78, 77, 1973.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(8), 1467-1480 (1982)

A KINETIC AND QUANTITATIVE APPROACH TO PEAK SPLITTING PHENOMENA WHEN USING 2-PROPANOL AS MODIFIER IN ADSORPTION HPLC

J. MERTENS
Dienst Algemene Chemie
Vrije Universiteit Brussel
Pleinlaan 2
1050 BRUSSEL

ABSTRACT

When using 2-propanol as a modifier in adsorption chromatography, a "satellite" peak is observed if a shight excess of a polar chlorinated hydrocarbon, such as CH_2Cl_2, is injected.
This satellite peak whose appearance is related to the presence of 2-propanol, has been identified as a part of the CH_2Cl_2 injected. The satellite peak behaves chromatographically mainly as a normal solute peak and its area is proportional to both the amount of CH_2Cl_2 injected and the concentration of 2-propanol in the mobile phase and on the other hand inversely related to the flow-rate and the concentration of CH_2Cl_2 in the mobile phase.
A physico-chemical model is proposed based on the perturbance of the dynamic equilibrium when the sample enters the column. The model is compatible with the experimental results and explains the origin of the satellite peak as well as its chromatographic behaviour. It also allows a better understanding of the role of a modifier related to its adsorption-desorption process.

INTRODUCTION

The addition of a modifier, such as 2-propanol, to the mobile phase up to a concentration of 1 or 2% v/v, is of common use in adsorption HPLC. As mentioned in recent papers (1,3) there is still need for experimental and theoretical evidence to get a good understanding of the modifiying mechanism. On the other hand a modifier often causes peakshape distortion (2,3).

This paper deals with the kinetic and quantitative approach of a phenomenon, which to our knowledge has not been reported, namely the splitting up into two peaks of a single product injection.
When injecting methylenechloride, chloroform or 1,2-dichloroethane as a solute and making use of 2-propanol/iso-octane (0.5/99.5 % v/v) as mobile phase on a Si-or CN-modified Si-column, a second "satellite" peak is observed in addition to the solute peak. This second peak, characterised by a convex front tailing and a compressed rear, has been identified as a part of the injected solute.
Although it is also connected with the use of a modifier, in this case 2-propanol, this phenomenon of peak splitting is quite different from the "vacancy" peaks or displaced solvent peaks described in literature (4,5,6,7,8,9). A complex adsorption-isotherm and zone compression or peak shapening effects (10,11,12) could explain the convex shape of the peak, but not the peak splitting phenomenon. The origin of the "satellite" peak can neither be explained by the conventional dynamic approach nor by the thermodynamic "near by" equilibrium, usually described in relation to chromatographic processes (11,12,13,14).

We approach this peak splitting phenomenon by a dynamic model that also takes account of the quantitative aspect of the formation of the "satellite" peak. This model can also contribute to a better understanding of the fundamental behaviour of a modifier as it shows the Snyder-Soczewinski displacement model (15,16) to be more appropriate than the the Scott-Kucera solution interaction model (17,18,19) when low concentrations of high solvents in the mobile phase are involved.

EXPERIMENTAL

The chromatographic set-up consists of a Waters Assoc. M-6000 pump, a Waters Assoc. U6K injector equipped with a 2 ml sol-

vent loop and a Waters Assoc. R401 differential refractometer with external thermostatisation. The temperature of the flow-through cells is kept at 2°C below ambient to prevent degasing of the solvent in the cells. The RI detector output is connected to a Hewlett Packard 3370B integrator and a BD9 Kipp & Zonen recorder.

Columns used are : Lichrosorb Merck Si 100 (5µ), 250 mm x 4 mm
Chrompack Sil-60D-10CN (10 µ), nitrile, 250 mm x 4.6 mm
Waters Assoc. µPorasil, 10µ , 350 mm x 3.9 mm
Waters Assoc. µCN, Nitrile on µPorasil 10µ , 350 mm x 3,9 mm.

Home distilled CH_2Cl_2 (Solvay) and i-octane (Phillips Petroleum) are used for the major part of the experiments. The average water content of the CH_2Cl_2, as determined by GC analysis, is about 0.009%.
The other solvents such as 2-propanol, $CHCl_3$, 1,2-dichloroethane, CCl_4, and the CH_2Cl_2 used as control standard are HPLC or analytical reagent grade.
The sample injection volume varied from 1-100 µl, but 15 µl is used as a standardized amount for undiluted solvent samples. The standard flow-rate is 2 ml/min.
The mobile phase and the eluted peaks are collected at the detector outlet.
The gas chromatographic analysis of these samples is carried out on a Varian 3760 gas chromatograph equipped with a 6" x 1/8" column of 0.2% Carbowax 1500 on 60/80 Carbopack C (Supelco, Inc.) coupled to a FID detector. The column oven is isothermally heated at 70° C.
After four chromatographic runs, the iso-octane present in the sample, is flushed off by heating the column at 125° C during 10 minutes.

RESULTS AND DISCUSSION

Experimental results

- When injecting 1 µl or more of CH_2Cl_2, 1,2-dichloroethane, $CHCl_3$ the peak splitting phenomenon characterized by the presence of a "satellite" peak is observed as long as 2-propanol is present in the mobile phase. A detailed chromatogram (fig.1) shows the expected solute peak "S" and the "satellite" peak "P", the latter preceeded by a negative peak, obtained when injecting 15 µl of CH_2Cl_2 into a

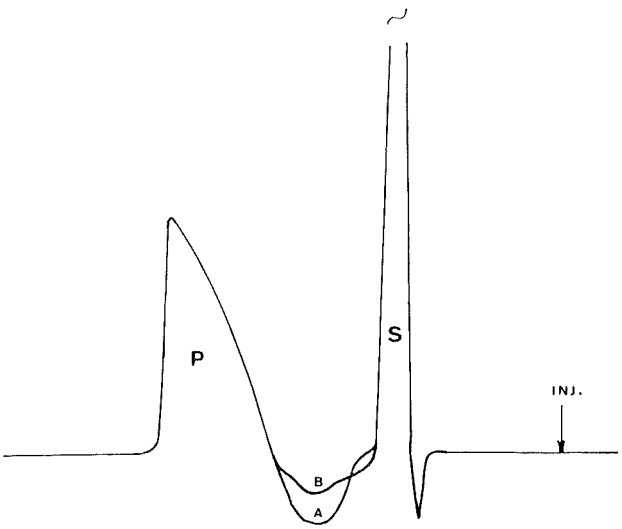


Fig. 1 : S : CH_2Cl_2 solvent peak; P : satellite peak; A : 15 µ CH_2Cl_2 injected; B : 150 µl of CH_2Cl_2 1/10 diluted in mobile phase.

mobile phase stream consisting of 2 propanol/iso-octane (0,5/99,5 % v/v) using a CN modified silica column. On the other hand no splitting effect is obtained when injecting CCl_4, 2-propanol or iso-octane.

The satellite peak has been identified by G.C. analysis (see experimental) as a portion of the injected chlorinated hydrocarbon, the remainder constituting the principal solute peak. The negative peak preceeding the satellite peak has been identified as a displaced 2-propanol peak.

When injecting a mixture of CH_2Cl_2 and $CHCl_3$ or 1,2-dichloroethane, both products are found in a common "satellite" peak.

If a more polar solvent, such as CH_2Cl_2, is present in the mobile phase the convex assymetry of the "satellite" peak decreases toghether with the backtailing of the negative 2-propanol peak.

One on the other hand when the same amount of the polar chlorinated hydrocarbon, diluted in the eluent is injected, a decrease of the negative peak is observed.

When plotting $1/t_{R'}$, $t_{R'}$ representing the corrected retention time of the "satellite" peak, as a function of the volumetric flow-rate $\bar{v}$, a linear relationship is obtained showing a slope equal to $1/V_{R'}$, $V_{R'}$ being the corrected retention volume.

As to the quantitative aspect of the satellite peak "P", it is experimentally proven that :

- The area P of the satellite peak increases linearly with the amount of solute injected up to 30 µl for a 4.6 mm x 250 mm column (fig.2) and can vary from 0-20% of the solute peak, depending on the experimental conditions described in the following section.

The area is directly proportional to the concentration of 2 propanol in the mobile phase Cp, (fig.3)

$$P = \alpha.Cp$$

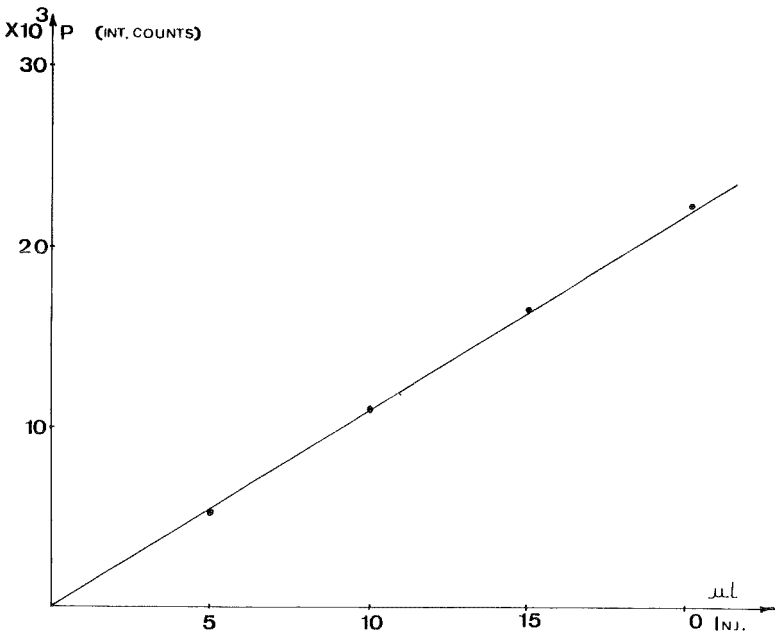


Fig. 2 : Area P of the satellite peak (integrator counts) as a function of the amount of CH_2Cl_2 injected; CN-column.

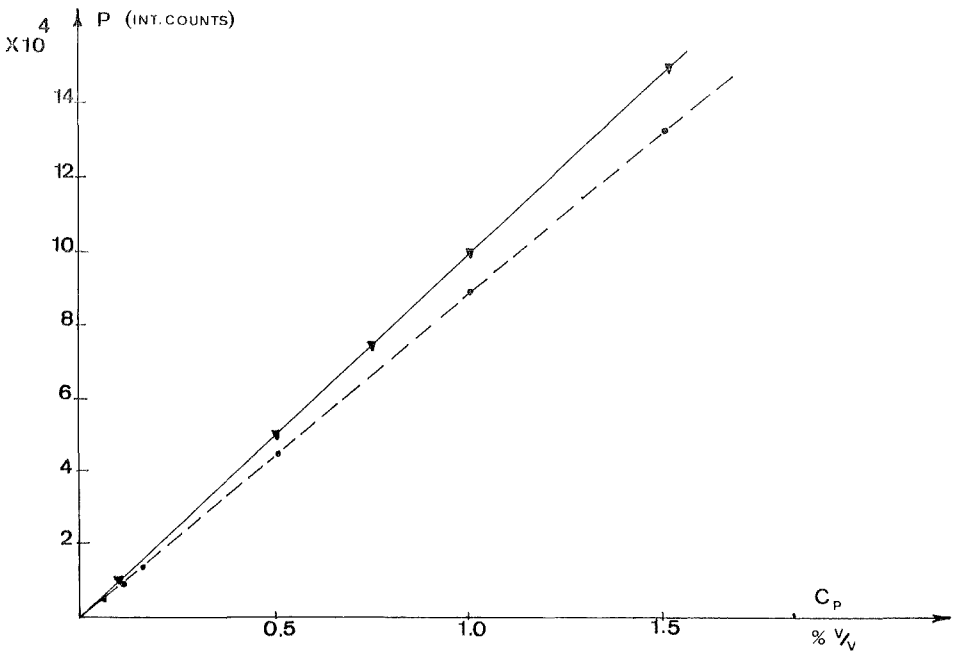


Fig. 3 : Area P (integrator counts) as a function of C_p; x C_p in % v/v in CH_2Cl_2/i-octane (30/70 v/v); --o-- CN column:—▼— Si column.

- When CH_2Cl_2 is already present in the mobile phase, the relation between P and the CH_2Cl_2 concentration in the eluent C_m, represented in fig. 4, can be written as :

$$1/P = a + bC_m$$

- P is inversely related to $\bar{v}$ as shown in fig. 5. Above a flow-rate of 4 ml/min the satellite peak begins to collapse with the solute peak and dissapears completely at higher flow-rates.

Discussion

Adsorption chromatography is governed by an adsorption-desorption process, which can generally be described by a "Langmuir-isotherm"-like expression (10, 12, 14, 22). Analogous to the Frenkel relation (23),

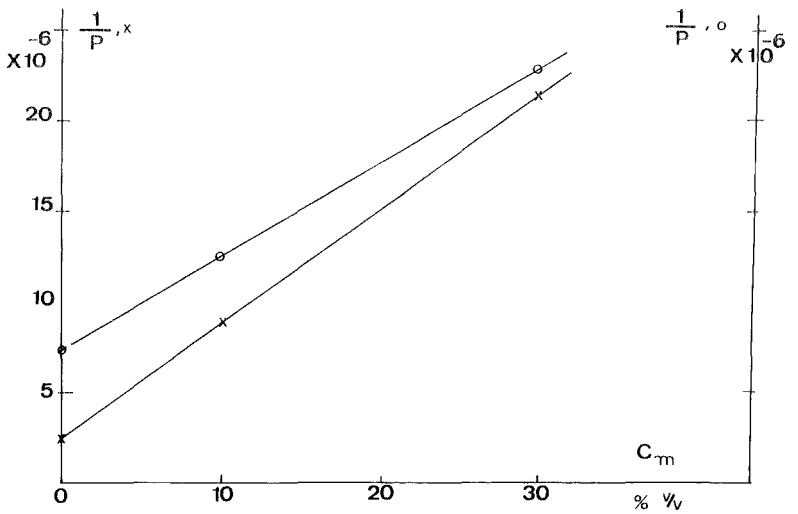


Fig. 4 : 1/P (integrator counts) as a function of C_m; CN-column.
Left ordinate : —X— 0,5 % 2-propanol in x CH_2Cl_2/100-x i-octane.
Right ordinate : --[●]-- 0,1 % 2-propanol in x CH_2Cl_2/100-x i-octane.

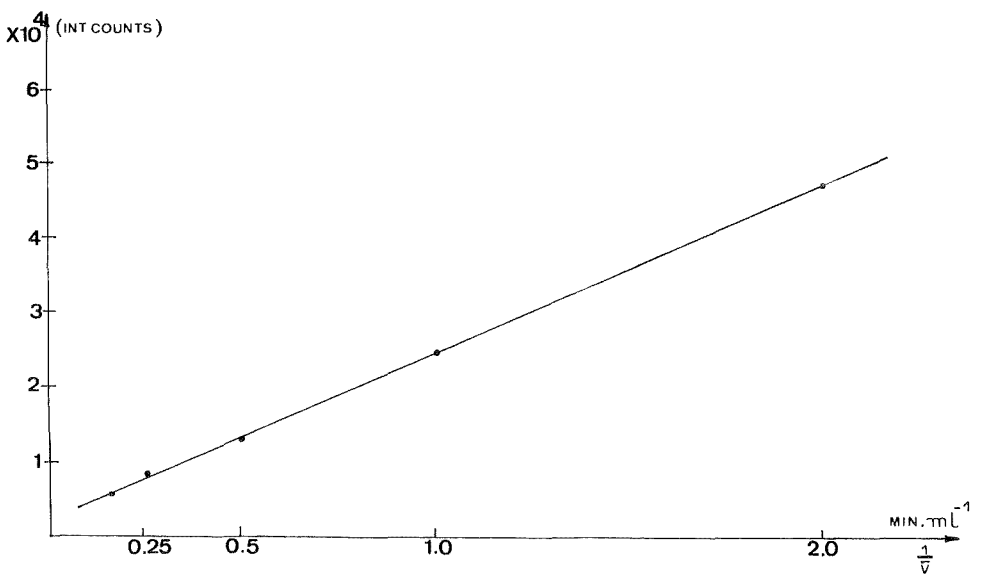


Fig. 5 : Area P (integrator counts) as a function of $1/\bar{v}$; 0.15 % 2-propanol in CH_2Cl_2/i-octane (30/70 v/v); CN column.

the residence or adsorption time τ of solute or solvent molecules on the active sites of the adsorbent can be defined as

$$\tau = \tau_o \, e^{\Delta G^t_{S,M} / RT} \qquad (3)$$

Here $\Delta G^t_{S,M}$ stands for the free energy of transfer, related to the difference of the interaction forces between solute -adsorbent and solute-mobile phase; τ_o is the proportionality constant (23).

It is assumed that the modifying interaction of 2-propanol, or any other modifier, can be described as a dynamic equilibrium and that the efficiency of occupying the more active sites is determined by its adsorption time τ on those sites and will depend on $\Delta G^t_{S,M}$, thus on the polarity of the mobile phase.

Polarity is used here in the general sense, namely the ability to engage in hydrogen bonding or dipole-dipole interactions.

The residence time τ of 2-propanol on the sites of lower activity will be smaller, resulting in an increase of the accessibility of those sites to other molecules.

The latter type of sites can be called "available" sites.

The splitting of a solute peak, resulting in the formation of a "satellite" peak can neither be described by a "near equilibrium" model (12,13), nor by a non linear isotherm (6,10,11).

Our interpretation of the origin of peak splitting, due to the presence of a modifier such as 2-propanol in adsorption HPLC, is related to the disturbance of the dynamic equilibrium of the modifier-adsorbent interaction, starting as the injected solute enters the column. The appearance of peak splitting will also depend on the relative strength of the interaction forces the injected solute and the modifier present in the mobile phase with the adsorbent.

We present a first approach based on a simple dynamic model, omitting complex mathematical derivations and neglecting peak broadening diffusion phenomena.

For the sake of simplicity, but still obeying realistic conditions, the mobile phase is supposed only to consist of the modifier 2-propanol in iso-octane. As solute a small amount of CH_2Cl_2 is injected. We further assume that the iso-octane molecules cannot compete with the CH_2Cl_2 and 2-propanol molecules for adsorption sites and that

the sites occupied by 2-propanol are of higher activity, corresponding to its role of modifier.

Let'sconsider a small volume element V_i at the column inlet. When the amount of CH_2Cl_2 injected enters this element, the concentration in the mobile phase of CH_2Cl_2, $c_{M,m}$, is high while that of 2-propanol $c_{M,p}$, decreases considerabely.

At that moment $\frac{c_{S,p}}{c_{M,p}} \gg k_p$, kp being the partition coefficient at equilibrium. This causes the 2-propanol molecules to desorb from the more active sites at a rate given by

$$r_{d,p} = k_{d,p} \cdot c^{eq}_{S,p} \qquad (4)$$

$k_{d,p}$: the desorption rate constant of 2-propanol from the more active sites in contact with the CH_2Cl_2 plug.

$c^{eq}_{S,p}$: the concentration of 2-propanol in the absorbed state on the more active sites at equilibrium conditions before injection of the CH_2Cl_2 sample.

The injected CH_2Cl_2 entered in V_i is now involved into a twofold adsorption-desorption process.

1° The first being a fast dynamic interaction with the "available" sites of lower activity. This allows the mass balance of CH_2Cl_2 in V_i at that moment to be written as :

$$\frac{dc_m}{dt} = - \bar{\mu} \frac{dc_m}{dz} + k_{d,m} c_{S,m} - k_{a,m} c_m (c^{\circ}_{S,m} - c_{S,m}) \qquad (5)$$

$\bar{\mu}$: the mean linear flow rate; z : distance from the column inlet

$c_{S,m}$: the actual concentration of CH_2Cl_2 on the adsorbent

$c^{\circ}_{S,m}$: the concentration if all the "available" sites are occupied

c_m : the concentration of CH_2Cl_2 in the mobile phase

$k_{a,m}$ and $k_{d,m}$: the adsorption and desorption rate constants.

2° On the other hand the CH_2Cl_2 molecules also interact with the more active sites as they become available by the desorption of 2-propanol. As the amount of this kind of sites becoming available per unit of time, depends on the desorption rate $r_{d,p}$ of 2-propanol,

the adsorption rate $r'_{a,m}$ of CH_2Cl_2 will only depend on $r_{d,p}$ as long as its actual concentration, governed by eq.5, is sufficiently high. As the activity of these sites is higher than that of the "available" ones, the adsorption time τ of CH_2Cl_2 on these sites will also be larger (eq.3) than on the "available" sites. The desorbed 2-propanol, on its path through V_i, will be involved in an adsorption-desorption process different from the equilibrium state and will thus behave as a "displaced solvent" peak.

3° When the total amount of the CH_2Cl_2 injected has penetrated into the column, fresh eluent mixture enters V_i. Now $C_{M,p}$ increases rapidly in V_i and the 2-propanol molecules are involved in a fast adsorption process, taking the place of the CH_2Cl_2 molecules "adsorbed" on the sites of higher activity during 2°.
At the end of the column this results in a CH_2Cl_2 solute peak S (fast equilibrium, Cfr. 1°) and a retarded CH_2Cl_2 satellite peak "p" (slow exchange, cfr. 2° and 3°).

The area of the satellite peak P is directly related to the amount of CH_2Cl_2 involved in the adsorption process described in 2°. This amount is proportional to the adsorption rate $r'_{a,m}$ of CH_2Cl_2 on the sites of higher activity and the time Θ during which the adsorption occurs.
P can be estimated by

$$P \sim r'_{a,m} \cdot \Theta \qquad (6)$$

The desorption rate of 2-propanol is described by equotion 4 wherein $C^{eq}_{S,p}$ may be substituted by its value from the Langmuir-isotherm, as it corresponds to the adsorbed concentration of 2-propanol at equilibrium, before the injection of the CH_2Cl_2 sample :

$$C^{eq}_{S,p} = \frac{C_p \cdot C^\circ_{S,p} \cdot K^{eq}_p}{1 + K^{eq}_p \cdot C_p} \qquad (7)$$

K^{eq}_p is the adsorption equilibrium constant for 2-propanol
For Cp sufficiently small, this results in

$$r_{d,p} = k_{d,p} \cdot K^{eq}_p \cdot C^\circ_{S,p} \cdot Cp \qquad (8)$$

The assumption made about Cp in order to obtain equation (8) seems to be realistic as the maximal analytical concentration used is 1,5% v/v or 0.016 g/g.

The adsorption rate $r'_{a,m}$ of CH_2Cl_2 on the more active sites is mainly determined by the desorption rate of 2-propanol from those sites allowing equation (8) to be written as

$$r_{a,m} \sim r_{d,p} = k_{d,p} \cdot K_p^{eq} \cdot C_{S,p}^{\circ} \cdot C_p \qquad (9)$$

Substituting eq.9 in eq.6 gives

$$P \sim k_{d,p} \cdot K_p^{eq} \cdot C_{S,p}^{\circ} \cdot C_p \cdot \theta \sim \mathcal{K} \cdot \theta \cdot Cp \qquad (9')$$

We now consider θ' as the residence time of a small plug of CH_2Cl_2 passing through V_i .

As long as the residence time τ of 2-propanol on the more active sites is smaller than θ', the CH_2Cl_2 molecules can absorb on those sites and $\theta' = \theta$, θ is the time during which adsorption occurs and can be written as :

$$\theta = \theta' = \frac{V_i}{\bar{v}} \qquad (10)$$

$\bar{v}$: the volumetric flow rate

Substituting equation (10) in (9') gives :

$$P \sim \mathcal{K} \cdot V_i \cdot \frac{1}{\bar{v}} \cdot Cp \qquad (11)$$

Equation (11) clearly corresponds to the experimental results described above and shown in the Figures 3 and 5. On the other hand, if CH_2Cl_2 is present already in the mobile phase, before the injection of the sample, the adsorption time τ of 2-propanol decreases and the CH_2Cl_2 molecules now compete in the equilibrium adsorption-desorption process for their share of higher activity-sites.

According to Saunders (24) the resulting isotherm equation for 2-propanol, if Cp is small, can be written as :

$$C_{S,p}^{eq} = \frac{K_p^{eq} \cdot Cp}{1 + K_m^{eq} \cdot C_m^{eq}} \qquad (12)$$

with K_m^{eq} : the adsorption equilibrium constant for CH_2Cl_2 already present in the mobile phase

C_m^{eq} : the analytical concentration of CH_2Cl_2 in the mobile phase.

Substituting equation (12) in (4) and assuming that

$$r'_{a,m} \sim r_{d,p} \; ; \quad \text{gives}$$

$$r'_{a,m} = k_{d,p} \frac{K_p^{eq} \cdot Cp}{1 + K_m^{eq} \cdot C_m^{eq}} \qquad (13)$$

and

$$P \sim \frac{V_i}{\bar{v}} \cdot k_{d,p} \cdot \frac{K_p \cdot Cp}{1 + K_m^{eq} \; C_m^{eq}} \qquad (14)$$

with $\alpha = \frac{V_i \cdot k_{d,p}}{\bar{v}}$ and $b = \frac{\bar{v} \cdot K_m^{eq}}{V_i \cdot k_{d,p} \cdot C_p}$

equation (14) can be rearranged as

$$\frac{1}{P} = \frac{1}{\alpha Cp} + b \; C_m^{eq} \qquad (15)$$

It is shown that equation (15) is compatible with all experimental results. (see Figs. 3 and 5)

Our dynamic model accounts for both the interaction of the solute molecules with the adsorbent and the solvent. Our interpretation of the contribution of the adsorption time and $\Delta G^t_{S,M}$ (equation 3) to the fundamental role of the modifier can explain the Scott-Kucera interpretation of solvent interaction at higher concentrations of polar solvent in the eluent. At high "modifier" concentrations, in the range to 10-90%, as described in the experiments of Scott and Kucera (18), the polar solvent does not act as "modifier" anymore. In the model of these authors two mechanisms are mixed up, namely a selective occupation of sites of higher activity and the solute-modifier interaction at high modifier concentrations when all the sites of the adsorbent are saturated.

Our model is consistent with the remark of Snyder and Poppe (1) that solutes or solvents with lower polarity than the modifier can compete with the modifier molecules.

On the other hand our results prove that anommalous peak-shape effects and even peak splitting do occur when slow desorption kinetics of the modifier are involved and this in total agreement with the theory of the Snyder-Soczewinski displacement model (1,15,16) assuming a dynamic equilibrium for the modifier and a monolayer adsorption as proposed by Snyder, Poppe et al (1,15, 16,20,21,22).

CONCLUSION

It may be concluded that the experimental results do confirm the theoretical relations derived from the physico-chemical model.
The moderating role of 2-propanol, and of any modifier, is related to a dynamic adsorption-desorption process, whereby the adsorption time τ is a function of the interaction forces in both the mobile and solid phase.

When this equilibrium process is perturbated, due to the fact that the interaction forces and the related kinetic parameters of a solute are of the same order of magnitude as those of the modifier, a process resulting in peak splitting can occur. This will result in a more or less separated "satellite" peak, depending on the concentration and the interaction strength of the modifier and on the polarity of the mobile phase.

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REFERENCES

1. L.R. Snyder , H. Poppe, J. Chromatogr., 198, 375, 1980.
2. J.J. Kirkland, J. Chromatogr., 83, 149, 1973.
3. J. Punčochařová, J. Kříž, L. Vodička, D. Průšová, J. Chromatogr. 191, 81, 1980.

4. K. Slais, M. Krejci, J. Chromatogr., 91, 161, 1974.
5. L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, Second edition, John Wiley & Sons Inc., N.Y. 1979, chap. 19.2
6. R.M. Mc Cormick, B.L. Karger, Anal. Chem. 52, 2249, 1980.
7. R.M. Mc Cormick, B.L. Karger, J. Chromatogr., 199, 259, 1980.
8. J.F.K. Huber, R.R. Becker, J. Chromatogr., 142, 765, 1977.
9. R. Groh, J. Halász, J. Chromatogr., 199, 23, 1980.
10. F.Helfferich, H. Klein, Multicomponent Chromatography, Marcel Dekker, N.Y., 1970, chap. 3.
11. D.L. Peterson, F. Helfferich, J. Phys. Chem., 69, 1283, 1965.
12. J.C. Giddings, Dynamics of Chromatography, part 1, Marcel Dekker, N.Y. 1965.
13. J.C. Giddings, Anal. Chem., 35, 1338, 1963.
14. R.H. Perry, C.H. Chilton, Chemical Engineers' Handbook, 5th edition, Mc. Graw-Hill Kogakusha, Tokyo, 1973.
15. L.R. Snyder, J. Chromatogr., 92, 223, 1974.
16. E. Soczewinski, J. Chromatogr., 130, 23,1977.
17. R.P.W. Scott, P. Kucera, J. Chromatogr., 122, 35, 1976.
18. R.P.W. Scott, P. Kucera, ibid., 149, 93, 1978.
19. R.P.W.Scott, P. Kucera, ibid., 171 , 37, 1979.
20. J.E. Paanakker, J.C. Kraak, H. Poppe, J. Chromatogr., 149, 111, 1978.
21. E.H. Slaats, J.C. Kraak, W.J.T. Brugman, H. Poppe, J. Chromatogr., 149, 225, 1978.
22. E.H. Slaats, Ph. D. Thesis, Amsterdam 1980, chapter III.
23. A.W. Adamson, "The adsorption time" in Physical Chemistry of Surfaces, J. Wiley & Sons, N.Y. 1976.
24. D.L. Saunders, J. Chromatogr., 125 , 163, 1976.

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EFFECT OF SUBSTITUENTS ON THE RETENTION IN HPLC CHROMATOGRAPHY OF STRYCHNINE DERIVATIVES.

George M.Iskander, J.Strombom[+] and A.M.Satti.
Dept. of Chemistry, University of Khartoum,
P.O.Box 321, Khartoum, SUDAN.

ABSTRACT

The capacity factor k, relative retentions α_{SP} and $\log \alpha_{SP}$ values measured on μPorasil columns for 33 strychnine derivatives using $CHCl_3$:MeOH (containing ca 2% NH_4OH) (93:7) as eluent in normal-phase chromatography. The results allow for the estimation of the effect of various substituents on the retention of these alkaloids.

INTRODUCTION

The rapidly advancing technique of high pressure liquid chromatography made possible the microanalytical identification, as well as the preparative separation of synthetic and naturally occuring complex organic molecules which were, otherwise, insolable by the traditional t.l.c. and column chromatography techniques.

[+] Present address: Biomedicum center,U.of Uppsala,Sweden.

Many examples can be cited where both analysis and isolation of plant active substances which may have some physiological significance, was successfully achieved e.g. steroidal (1), tropane (2), morphine (3) and strychnos alkaloids (4).

The main aim of the present work is to give a qualitative correlation of the effects of substituents on the retention of compounds, which are strychnine alkaloid derivatives modified in the aromatic and non-aromatic part of the molecule. The results shown can lead to a "prediction"of the behavior of similar molecules when treated under the same conditions of HPLC.

EXPERIMENTAL

The present analyses were carried out on a high pressure liquid chromatograph consisting of the following parts: A Waters 6000 pump, a Waters U 6K Universal injector and a Varian Variscan 635 u.v. detector. Chromatograms were obtained at 254 nm wavelength. All measurments were performed on 8 μm Porasil column (300 x 4.5mm i.d).

The solvent system used was $CHCl_3$ and MeOH (containing 2% NH_4OH) (93:7). This was isocratically eluted at a flow rate of 3ml/min. at ca 2 500 psi, and at ambient temperature. Stock solutions of the solutes were made in the eluent and about 0.5-10 μg of each individual sample was injected. The elution time of an unsorbed solute, t_M, was measured as described earlier (5). The retention times of the solutes, t_R, was evaluated at the peak maxima of the symmetrical peaks. The capacity factors, $k=(t_R-t_M)/t_M$, and the relative retentions α_{SP} were also evaluated as described (5).

RESULTS AND DISCUSSION

The chromatograms in fig. 1 illustrate the speed and effeciency of HPLC for the analysis of the systems studied. The use of Porasil column at ambient temperature and the relatively high flow rates, together, afford ease of manipulation and efficiency.

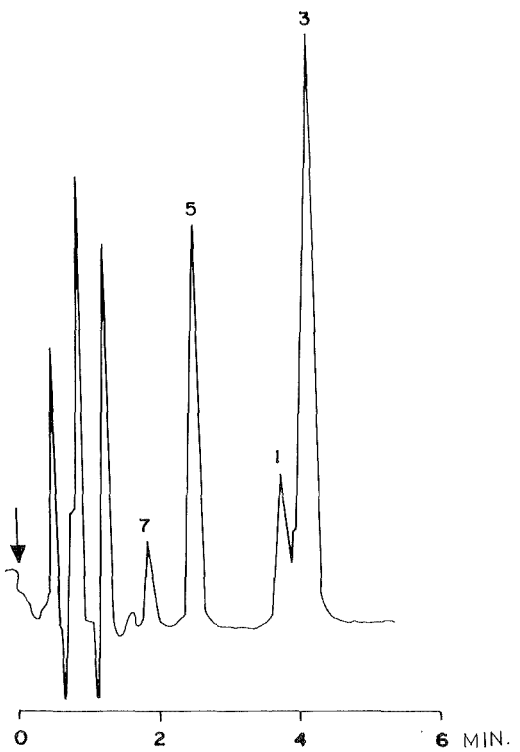
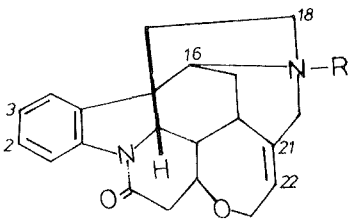


FIGURE 1

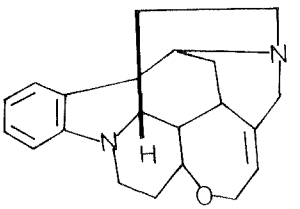
Chromatogram of some derivatives. The conditions and symbols are given in text.

In the present study, the use of the slightly basic solvent system allowed for the separation of the relatively polar substrates, which otherwise may need less strongly retentive columns.

The capacity factor k, the relative retention α_{SP} together with the corresponding $\log \alpha_{SP}$ values are given in tables 1,2 and 3 for 33 compounds of type I, II and III. The data in general offer an overview on the



I



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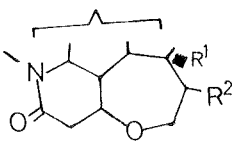

III

effect of the various substituents on the retention of such compounds. The results generally show the solute-solvent interaction wherein an increase in the energy of interaction between the solute and the solvent reduces binding. The interaction can be optimised to Van der Waals interaction, which is primarily dependent on the molecular size of the solute and the solvent, and the electrostatic interactions as is evident for the 2-carbamoyl strychnines (fig. 2). In this series, the increse in size of the "non-polar" alkyl fragment of the amide side chain, increases retention. In either case the plot

TABLE 1. Relative retentions and capacity factors of 2-carbamoylstrychnines.

Compound (I)	Name	α_{SP}	$\log\alpha_{SP}$	k
2	Amino	1.0	0.0	0.96
3	Acetamido	4.4	0.64	7.63
4	n-propion-amido	2.8	0.45	4.49
5	n-butyr-amido	2.0	0.30	3.12
6	iso-butyr-amido	2.0	0.30	2.96
7	n-valer-amido	1.7	0.23	2.33
8	iso-valer-amido	1.8	0.25	2.53
9	benzamido	1.4	0.15	1.74
10	pivalamido	1.4	0.15	1.74
1	strychnine	1.0	0.0	5.86

TABLE 2. Relative retentions and capacity factors of 2- and 3-substituted strychnines.

Compound (I)	Name	α_{SP}	$\log\alpha_{SP}$	k
3	2-acetamido	4.4	0.64	7.63
2	2-amino	1.0	0.0	0.96
11	2-hydroxy	0.69	-0.16	3.71
12	2-methane-sulfonamido	0.60	-0.22	3.12
9	2-benzamido	0.40	-0.40	1.74
13	2-p-toluene-sulfonamido	0.34	-0.47	1.35

(continued)

TABLE 2/ conti.

Compound (I)	Name	α_{SP}	$\log\alpha_{SP}$	k
14	2-methoxy	0.29	-0.53	0.96
15	2-bromo	0.20	-0.70	0.37
16	2-nitro	0.17	-0.77	0.18
17	3-acetamido	0.57	-0.24	2.92
18	3-amino	0.54	-0.27	2.73
19	3-hydroxy	0.54	-0.27	2.73
20	3-methane-sulfonamido	0.46	-0.34	2.14
21	3-methoxy	0.29	-0.53	0.96
22	3-nitro	0.26	-0.59	0.76
23	3-bromo	0.20	-0.70	0.37
24	2,3-dimethoxy	1.20	0.08	7.56

TABLE 3. Relative retentions of other strychnine and strychnidine derivatives.

Compound	Name	α_{SP}	$\log\alpha_{SP}$	k
(I) 25	16-hydroxy-strychnine	0.29	-0.53	0.96
(I) 26	16-methoxy-strychnine	0.17	-0.77	0.18
(I) 27	16-ethoxy-strychnine	0.17	-0.77	0.18
(I) 28	16-isopropoxy-strychnine	0.17	-0.77	0.18
(III; R^1=R^2=H)	21,22-dihydro-strychnine	0.28	-0.56	0.90
(III; R^1=R^2=OH)	21,22-dihydroxy-strychnine	0.62	-0.21	3.30

(continued)

TABLE 3/ conti.

Compound	Name	α_{SP}	$\log_{\alpha_{SP}}$	k
(I; R=O)	strychnine -N Oxide	1.06	0.025	6.25
(I; R=Me Cl^-)	N-methylstrychnine chloride	1.26	0.10	7.60
(II)	21,22-dihydrostrychnidine	0.74	-0.13	4.10

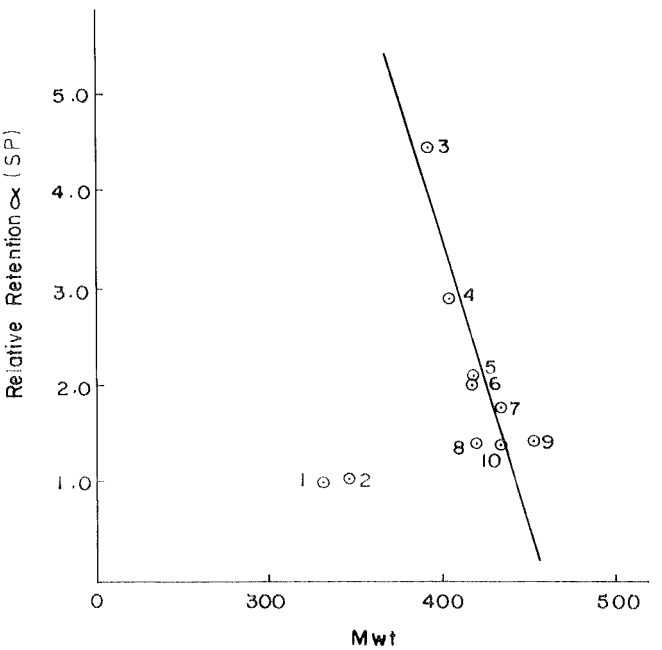

FIGURE 2

Relative retention vs. Molecular weight of 2-Carbamoylstrychnines.

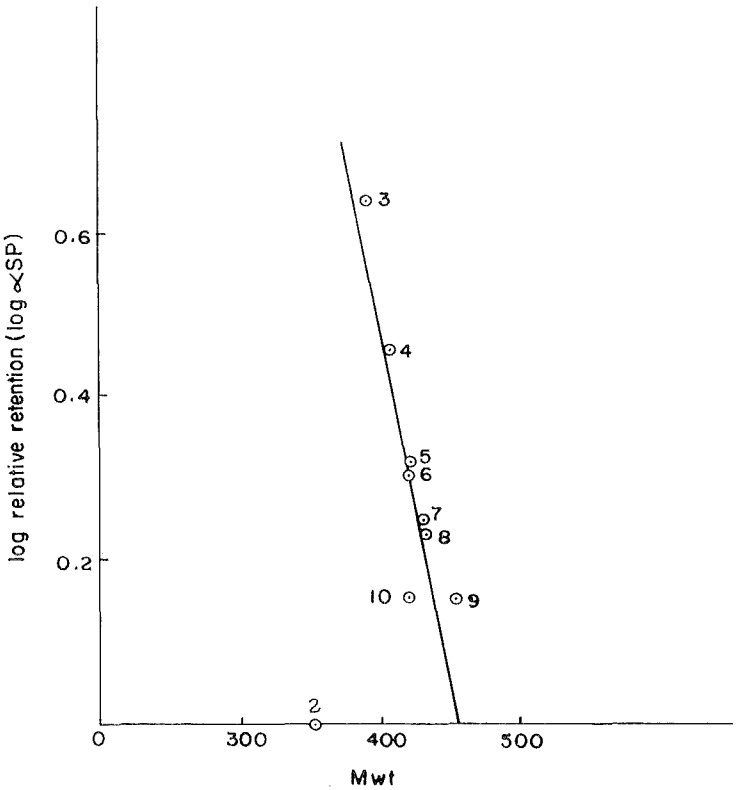

FIGURE 3
Relative retention vs. Molecular weight of 2- Carbamoylstrychnines.

of the relative retention α_{SP} or $\log \alpha_{SP}$ versus molecular weight evidently show a linear relationship (figs. 2 and 3). In other words, the effect of the methylene group increment on the aliphatic side chain is surprisingly consistent. This is, however, unmatched by the benzamido group even though the replacement of a methyl group by an aromatic ring significantly increases

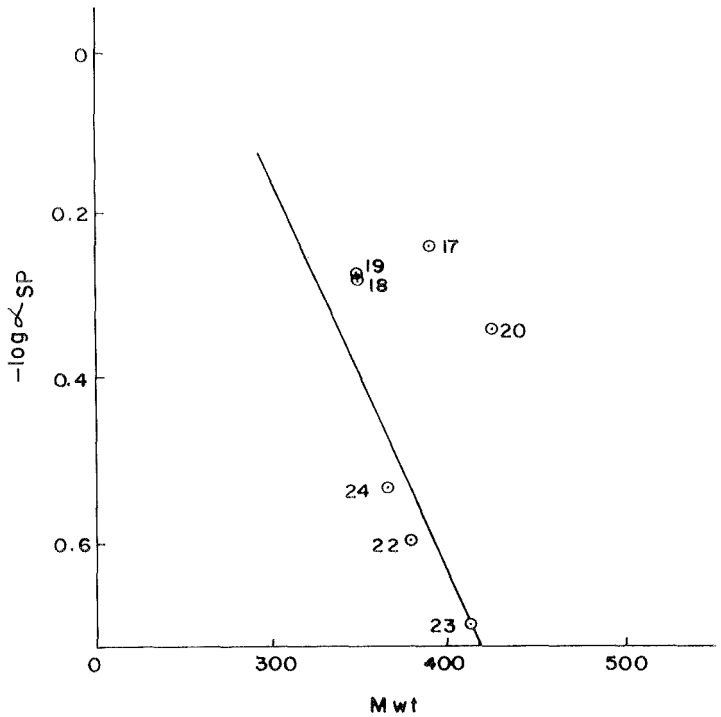

FIGURE 4
-Log Relative retention vs. Molecular weight of 3-Substituted strychnines.

the molecular size. On the other hand, with the solutes studied of approximately similar dimensions and with a fixed eluent, the magnitude of the relative retention α_{SP}, is primarily affected by the polarity of the respective substituents (tables 2,3) and (figs. 4,5). Compounds with a "zwitterionic moiety" as in strychnine N-oxide (I; R = O) and quaternary strychnine salts (I; R = Me Cl^-) show a markedly reduced biophobicity and hence maximum retention values. Similar findings were reported

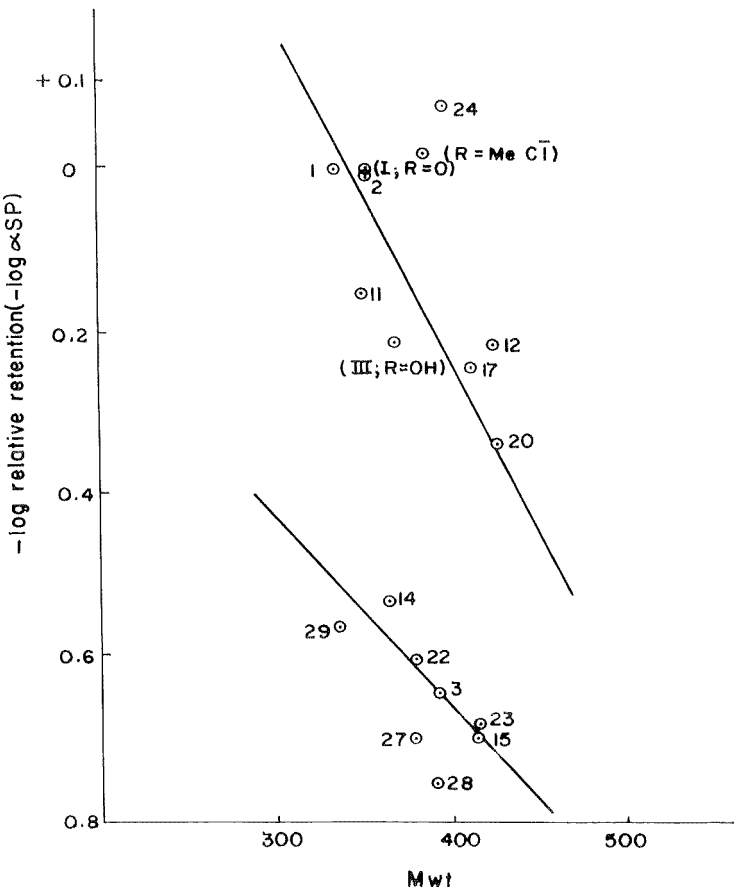

FIGURE 5

-Log Relative retention vs. Molecular weight of Strychnine and Strychnine derivatives.

for the effects of substituents on the retention values of catecholamines (6). This is expected in view of the behavior of monopoles, dipoles and zwitterions (7).

It is apparent that the substituents on C-2, C-3 and on the nitrogen atoms provide most drastic changes on retention (tables 2,3). The replacement of a C-2 and/or C-3 hydrogen atom(s) on the aromatic ring reduces retention even though the molecular properties, e.g. dipolemoments, are markedly changed (cf figs. 4,5). The 2-acetamido and 2,3-dimethoxystrychnines are surprisingly anamolous. These positions are, however, notably found to be phytochemically most effective (8). The sample population here is not large enough to establish a statistically valid quantitative structure-retention relation; nevertheless, the quantitative aspect of replacing a hydrogen at C-2, C-3 and C-16 on retention of these alkaloids is clearly illustrated.

To summarise, the lengthening of the hydrocarbon chain of a given substituent is associated with an equivalent increment on the relative retention and capacity factors in this chromatographic system. In contrast, polar functional substituents, cause a change in the net dipolemoment of the substrate molecule and thus rigorously change the αC_{SP} and k factors, which in many instances are unequalled by the relative increase in the molecular size.

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REFERENCES

(1) Hunter, I.R, Walden, M.K, Wagner, J.R. and Heftmann, E., J. Chromatogr., 119, 223, 1976.

(2) Verpoorte, R. and Svendsen, A.B., J. Chromatogr., 120, 203, 1976.

Stutz, M.H. and Sass, S., Anal Chem., 45 12, 2134, 1973.

(3) Knox, J.H. and Jurard, J., J.Chromatogr., 82, 398, 1973.

(4) Verpoorte, R., and Svendsen, A.B., J.Chromatogr., 109, 441,1975.

Verpoorte, R. and Svendsen, A.B., ibid., 100,227,1974.

(5) Horvath, C., Methods Biochem. Anal., 21, 79, 1973.

(6) Molnar, I. and Horvath, C., J.Chromatogr., 145, 371, 1978.

(7) Horvath, C., Melander, W. and Molnar, I., Anal. Chem., 49, 47, 1977.

(8) Iskander, G.M., Bohlin, L. and Ali, Y., Acta Pharm. Suec., 12, 461, 1975.

Iskander, G.M. and Bohlin, L., ibid., 157, 431, 1978.

FIRST SYMPOSIUM ON ADVANCES OF TLC AND HPLC

May 14-15, 1982
Biological Research Center
Hungarian Academy of Sciences
Szeged, Hungary

Edited by

S. PONGOR
Institute of Enzymology
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and

HALEEM J. ISSAQ
NCI – Frederick Cancer Research Facility
Frederick, Maryland

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PRE-COLUMN DERIVATIZATION IN HPLC OF AMINO ACIDS AND PEPTIDES

Gyula Szókán
Institute of Organic Chemistry, Eötvös University, H-1088
Budapest, Muzeum krt. 4/B, Hungary

The development of high preformance liquid chromatography with permanently bonded non-polar, so called reversed phase packings has been useful in the separation of a variety of peptides and amino acid derivatives. Limits to the detectability of underivatized substances are on the order of several nanomolar. Sensitivity enhancement became very important for the analysis and determination of peptide hormones, their metabolites and fragments, trace constituents and amino acids existing in very little quantity in biological and clinical samples. Therefore chemical derivatization techniques have been introduced into HPLC, too. By derivatization not only sensitivity enhancement , but selectivity can be achieved.

Pre- and post column derivatization have been applied in liquid chromatography. Post-column techniques are very well known by different types of amino acid analyzers and other instruments based on them.

Pre-column techniques offer some further advantages: simple procedure, economy, no restrictions by the solvent system and change of retention behaviour. The derivatization reaction must be rapid and quantitative, or at least reproducible. Formation of artifacts or of several derivatives of one compound can occasionally occur.

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For pre-column derivatization we have worked with dansyl-chloride, fluorescamine and o-phthaldialdehyde as reagents.

Chromatographic separations were performed on a laboratory assembled intrument of which the principal components were a reciprocating piston pump (Type 1515; Orlita, Giessen, G.F.R.) and a variable-wavelength photometer fitted to a 10 µl flow-cell (Model 212; Cecil, Cambridge, Great Britain). Column effluents were monitored at 215, 254 or 280 nm depending on the materials. For detection of fluorophores Waters M 420 fluorimeter was coupled to the instrument . Injection was made by Rheodyne injector. The efficiency of separations was increased by gradient elution. Peptides were synthetized by the Peptide Research Group of Hungarian Academy of Sciences.

Dansylchloride is well established as a reagent for the detection of amino acids and as an N-terminal reagent for peptides and proteins allowing detection at very high sensitivity. The conditions under which it reacts with amino and other functional groups in proteins and the stability of the resulting derivatives to acid hydrolysis have been extensively studied. Dansyl-amino acids are both fluorescent and ultraviolet absorbing. Some HPLC methods have also been developed for separation of dansyl-amino acids.

Wilkinson (1) applied reversed phase columns (µ Bondapak C_{18}, Spherisorb ODS) with linear gradient formed from acetonitrile and sodium phosphate buffers of approximately neutral pH. In order to avoid the problem of reproducibility, that arises from gradient the best possible resolution of the Dns-amino acids under isocratic conditions was sought. It was found, that the addition of glacial acetic acid to the solvent system used, resulted in faster elution of the Dns-amino--acids, but more importantly reduced tailing of the peaks (2). Using these experiences we have elaborated a method for separation of the three dansylated lysines: α, ε and bis-Dns-lysine. Resolution was achieved on PARTISIL-PAC 10 column isocratically by acetonitril-water eluent containing 1% acetic acid. Sharp peaks were obtained (see Fig.1.). The method was used in the analysis of branched polypeptides, for example Poly(Lys(Ala)m). The different bond types of lysines were determined.

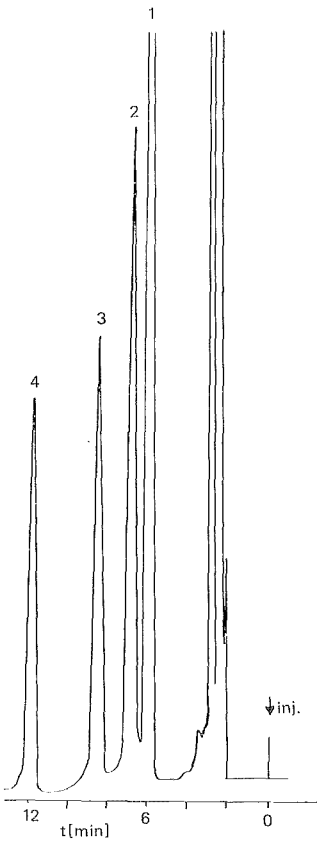

FIGURE 1. Chromatogram of dansylated lysines.
Column: Partisil PAC-10µm, 250 X 4.6mm
Flow: 1.0 ml/min, Pressure: 60 bar
Solvent: AcN-H2O-AcOH, 35:65:1
Detection: UV, 254 nm
Peaks: 1. ε-Dns-Lys k' 2.0
2. bis-Dns-Lys k' 2.4
3. α-Dns-Lys k'3.25
4. Dns-Ala k' 4.9

Recently fluorescamine (FLUORAM) and o-phthaldialdehyde (OPA) ha been used as very sensitive reagents to make fluorescent labelled amino acids and peptides.

Fluorescamine (4-phenylspiro-furan-2(3H)-1'phtalan)--3,3'-dione) reacts with primary amino groups to produce highly fluorescent derivatives. The half time of the reaction is 200-500 msec for most amino acids at pH 9. In post column techniques it is very well known. By pre-column derivatization unfortunately in the case of amino acids two fluorescent derivatives are formed because of second ring closure . Therefore it can be used well in the case of peptides with free amino groups. Live (4) reported results on the HPLC analysis of FLURAM derivatives of oxytocin, [Arg^8]-vasopressin and 16 analogs on Whatman-Partisil-10 ODS column. Wu and coworkers (5) demonstrated, that FLURAM-enkephalins are separated from each other using Tris-methanol solvent and RP-18 column. The nanogram level of sensitivity of this method compares favourably with other HPLC methods using UV, electrochemical or post-column fluorimetry detection.

o-Phthaldialdehyde reacts with amino groups in alkaline media in the presence of a reducing agent such as 2-mercaptoethanol to form a substituted isoindole. It is a highly fluorescent derivative which may be excited at 340 nm and emits at 455 nm. Lindroth (6) and Hodgin (7) elaborated HPLC-method for separation of OPA-amino acids using RP columns.

The potential of pre-column derivatization by FLUORAM and OPA has been demonstrated in our laboratory for monitoring fragments of α-MSH (α-melanocyta stimulating hormone).

The primary structure of α-MSH is

CH_3CO-Ser^{1}-Tyr-Ser-Met-Glu^{5}-His-Phe-Arg^{8}-Trp-Gly^{10}-Lys-Pro-Val^{13}-NH_2

The derivatization reagent was prepared by dissolving 27 mg of OPA in 0.5 ml of ethanol. Boric acid solution (0.4M) was adjusted to pH 9.5 with 1M NaOH. 20 microliters of 2-mercapto-ethanol and the OPA solution was added to 5 ml of borate buffer. The reagent mixture was allowed to stand for 24 hrs prior to use. 100 microliters of reagent was used for labelling of 20-30 μg peptide fragments. The structure of the derivatives were investigated, too.

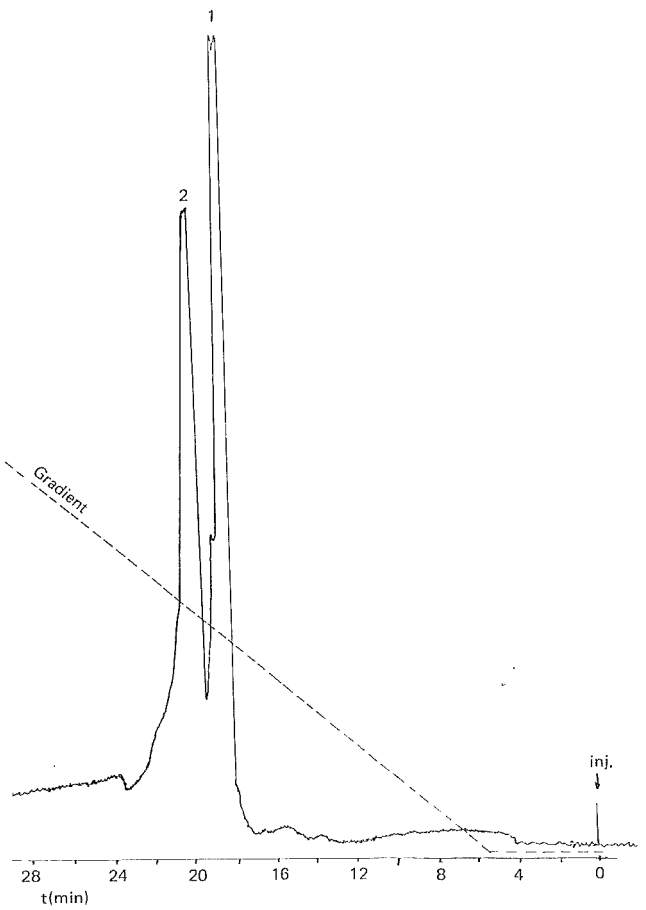

FIGURE 2. Chromatogram of o-phthalaldehyde derivatives of alpha-MSH fragments.
Column: ODS-Hypersil-5µm, 125 X 4 mm.
Flow: 1.4 ml/min, Pressure: 70 Bar
Solvent: AcN-H2O-TFA
A: 15:85:0.1 B: 90:10:0.1
Gradient: 2.2%B/min (-------UV profile)
Detection: Waters M-20 Fluorimeter
Peaks: 1. 0.3 microgram H-8-10-OH fragment, k' 8.5
2. 0.2 microgram H-11-13-NH2, k' 9.2

The efficiency of the separation was increased by gradient elution using acetonitrile-water eluent containing 0.1% trifluoroacetic acid (see Fig.2).

REFERENCES

1. J.M. Wilkinson: J. Chrom. Sci. 16, 547 (1978)
2. K.T. Hsu, B.L. Currie: J. Chromatogr. 166, 555 (1978)
3. W. McHugh et al.: J. Chromatogr. 124, 376 (1976)
4. D.H. Live: Peptides (edited by M.Goodman, J. Meienhofer) p. 41 (1977)
5. K.M. Wu, J.W. Sloan, W.R. Martin: J. Chromatogr. 202, 500 (1980)
6. P. Lindroth, K. Mopper: Anal. Chem. 51, (11) 1667 (1979)
7. J.C. Hodgin: J. Liquid Chrom. 2, 1047 (1979)

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IODINATION OF PEPTIDE HORMONES AND PURIFICATION OF IODINATED PEPTIDES BY HPLC

T. Janáky, G. Tóth[+], B. Penke[+], K. Kovács[+], F. A. László
Endocrine Unit, First Dept. of Medicine,
[+]Department of Medical Chemistry,
University Medical School, Szeged, Hungary

ABSTRACT

Hexagastrin, Arg^{8}-vasopressin, oxytocin, Tyr^{1}-somatostatin, and ACTH 1-39 were iodinated in order to yield precursors for tritium labelling or radioiodinated tracers for radioimmunoassay, respectively. The heterogeneous mixture of iodination products was purified via reversed-phase high-performance liquid chromatography. Iodination of peptides resulted in a marked increase in retention time on the reversed-phase adsorbent. A simple and quick method was applied for purification of radioiodinated peptides on a Sep-pak® C-18 cartridge for rapid sample preparation.

INTRODUCTION

The synthesis of iodinated peptide hormones has two aims:
1. Radioactive labelling for RIA and/or receptor binding studies.

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2. Synthesis of non-radioactive, halogenated precursor peptides for further work of trititiation /I → $^{3}_{1}H$ exchange by catalytic hydrogenolysis/.

1. Although numerous methods are known and have been applied for many years, there are some problems in the iodination of small peptides. One of the best-known methods is Hunter-Greenwood iodination /1/, using chloramin-T oxidizing agent for the iodid → iodine reaction in solution. During this procedure some amino acids /Met, Trp, His, Cys/ can also be oxidized partly or quantitatively by the excess of reagent in the solution. A short reaction time /5, 10 or 20 seconds/ is necessary to minimize side-reaction. Under the conditions applied /highly diluted reaction mixture, short reaction time/, only a part of the peptide reacts and it is necessary to separate the monoiodinated peptide from the oxidized by-products in order to increase the specific radioactivity. Numerous techniques have been employed for purification /high-voltage electrophoresis /2/, ion-exchange chromatography /3/, polyacrylamide gel electrophoresis /4/, thin-layer chromatography /5/ gel electrophoresis /6/ and isoelectric focusing /7//. Sediah /8/ used high-performance liquid chromatography for the purification of iodinated Leu-enkephalin, β-MSH, angiotensin, lysin-vasopressin and ACTH.

More recently, a new oxidizing reagent, "iodogene" /11/ has been introduced for the iodination of peptide hormones. It has some advantages over chloramin-T: the reagent has a very low solubility in water and present adhered as a thin film on the wall of the reaction vials, thus not being in a less direct contact with the peptide molecule in solution. The peptide is dissolved in water, radioactive iodide is added to the solution and the mixture is poured into the reaction

vial containing iodogeme on the wall. After some minutes the reaction mixture can be poured out of the vial and purified.
2. For introduction of non-radioactive iodine into the Tyr--residue of peptides, there are two possibilities:

a/ working with mono- or diiodotyrosine during peptide synthesis,

b/ iodination with unlabelled iodine by the methods mentioned for radiolabelling /1,11, 10/ or directly with elemental iodine /9/.

Our aim was to iodinate peptides /hexagastrin, Arg^8-vasopressin, oxytocin, somatostatin and ACTH/ in order to obtain radioactive-labelled hormones for RIA or to have iodinated peptide precursors for tritiation. HPLC proved to be the method of choice for the separation of mono- and diiodinated peptides from the starting material and the oxidized by-products.

MATERIALS

Protected hexagastrin /Boc-Tyr-Ala-Trp-Met-Asp-Phe-NH_2/, mono- and diiodo-hexagastrin, Arg^8-vasopressin and oxytocin were synthetized in our laboratory. Tyr^1-somatostatin was purchased from Beckman /Geneve, Italy/, ACTH 1-39 was a generous gift from NIAMDD /Bethesda, USA/. ^{125}Iodine /as $Na^{125}I$/ was purchased from the Central Isotope Institute, Budapest. Vycor glass powder 140 mesh was bought from Vycor Glass Works, Corning N.Y. USA.

The high-performance liquid chromatograph consisted of a Waters 6000 A pump and a universal liquid chromatog raph injector /Waters U6K/, coupled to an LKB Uvicord III fixed wavelength /20 nm/ UV monitor with an 8 µl. flow-through cell. Methanol and acetonitril were used as supplied by E. Merck /Darmstadt,

Germany/. Water was glass-distilled and deionized. The freshly prepared buffers were passed through a 0.45 um Sartorins membrane filter. Elution was effected isocratically at room temperature.

METHODS AND RESULTS

1. Hexagastrin

In order to obtain precursor substances for tritiation and standard substances for HPLC purification, we synthesized hexagastrin, monoiodo- and diiodo-hexagastrin in a stepwise manner, using Boc-monoiodo tyrosine and BOC-diiodotyrosine for the syntheses /the details of the synthesis will be published elsewhere/. The three compounds were separated on a Partisil-10 ODS column /25 cm x 4.6 mm/, applying the following elution solvent: 0.1 M triethylammonium phosphate /pH = 3.20/- acetonitrile 55:45 /v/v/ /flow rate 2 ml./min/

Compound	Retention time
BOC-hexagastrin	4.7 min
BOC-monoiodo hexagastrin	7.3 min
BOC-diiodo hexagastrin	12.7 min

Iodination of the hormone resulted in a marked increase in retention time on the reversed phase adsorbent, owing to the hydrophobicity of the iodo group.

BOC-diiodohexagastrin proved to be suitable as a precursor for tritiation. The radioiodination of hexagastrin was a difficult task: we found that a small amount of iodogene too dissolves in water and can oxidize the methionine residue. The iodination reaction is very slow /as a result of working in highly diluted solutions/, but a longer reaction time leads to oxidation

of the peptide. We made a compromise: if the reaction time was 30-60 seconds, the yield of radiolabelled monoiodinated hexagastrin was ~ 10 % after HPLC purification on a Partisil column.

2. Vasopressin

Arg^8-vasopressin was synthesized with the solid-phase method on Merrifield polymer. It was found that the iodogene method was not suitable for iodination of vasopressin on a preparative scale: a large amount of unidentified oxidized vasopressin derivative was formed during the reaction. Therefore, iodination was performed with the method of Flouret /9/, applying elemental iodine in alcoholic solution and a reaction time of ~ 10 minutes. HPLC purification of the reaction mixture on a Partisil-10 ODS-2 column /15 cm x 4.6 mm/ yielded diiodo-Arg^8-vasopressin as main product /~ 40 %/. Only a small amount of vasopressin and monoiodo-vasopressin remained in the reaction mixture. The solvent system: 0.05 M ammonium acetate /pH = 6.5/ - methanol 6:4 /v/v/; flow rate 1.0 ml/min.

Compound	Retention time
free iodine	1.2 min
Arg^8-vasopressin	5.3 min
Monoiodo-Arg^8-vasopressin	19 min
Diiodo-Arg^8-vasopressin	36 min

Radioiodination of Arg^8-vasopressin with the Hunter-Greenwood method gave similar results, but owing to the short reaction time /35 sec/ and highly diluted solution /5 µg AVP in 50 µl solution/, monoiodo-Arg^8-vasopressin was the main product. Yields after HPLC purification with the above method: monoiodo--AVP ~ 40 %, diiodo-AVP ~ 8-9 %. /After radioiodination with $^{125}I_2$, the peptides were adsorbed on Vycor glass beads before HPLC purification./

3. Oxytocin

Oxytocin was synthesized on solid phase and radioiodinated with $^{125}I_2$ /Hunter-Greenwood method, 5 ug oxytocin in 50 ul aqueous solution, reaction time: 35 sec/. The reaction mixture was separated on a Nucleosil 5 C-18 column /25 cm x 4.6 mm/. The solvent system: 0.01 M ammonium acetate /pH = 4.0/ - acetonitrile 4:1; solvent flow rate 2 ml/min.

Compound	Retention time
Oxytocin	4.8 min
Monoiodo-oxytocin	11.4 min
Diiodo-oxytocin	15.6 min

After purification the main product, monoiodooxytocin, had a very high specific radioactivity /1800 Ci/mmol/.

4. Somatostatin

Somatostatin does not contain any tyrosine-residue for iodination, and we therefore applied the Tyr^1-analogue of somatostatin. 2.5 ug peptide was radioiodinated with the Hunter-Greenwood method, the peptides were adsorbed on Vycor glass beads, and after desorption purified on a Partisil ODS-2 Column. The solvent system: 0.01 M ammonium acetate /pH = 4.00/ - acetonitrile 7:3, solvent flow rate 1.5 ml/min.

Compound	Retention time
Tyr^1-somatostatin	13.0 min
Monoiodo-Tyr^1-somatostatin	50.0 min
Diiodo-Tyr^2-somatostatin	91.0 min

The main product in this case too was the monoiodinated peptide, the amount of diiodinated compound being very small.

5. A simple separation of iodinated peptides on Sep-Pak cartridge

Since the iodinated peptides have much longer retention times on reversed phase adsorbents than those of the original peptides, a very simple technique proved suitable for the separation of these compounds from the reaction mixture after iodination. This technique is also known from the literature /6/: the separation is performed on a Sep-Pak® C 18 cartridge /Waters/ for rapid sample preparation. Solvent system: 1 % trifluoroacetic acid containing a methanol gradient from 5 to 90 %. The peptides containing tyrosine were iodinated as mentioned above /the reaction time for ACTH 1-39 was 20 sec/, pre-purified with adsorption on Vycor glass beads and purified on a Sep-Pak® C 18 cartridge equilibrated with 1 % trifluoroacetic acid. The results are as follows:

Compound	Methanol content of gradient
Monoiodo-Arg^{8}-vasopressin	45-50 %
Monoiodo-oxytocin	55 %
Monoiodo-Tyr^{1}-somatostatin	65 %
Monoiodo-ACTH 1-39	60-65 %

DISCUSSION

Four peptide hormones were iodinated and purified on reversed-phase high-performance liquid chromatography and on Sep-Pak® C-18 cartridge. Both methods are very simple, do not require any complicated instruments and give pure iodinated peptides suitable for RIA measurements.

REFERENCES

1. Hunter, W. M. and Greenwood, F. C., Preparation of iodine-131 labelled human growth hormone of high specific activity, Nature 194, 495-496 /1962/.
2. Haber, E., Koerner, T., Page, L. B., Kliman, B. and Purnode, A., Application of a radioimmunoassay for angiotensin I to the physiologic measurements of plasma renin activity in normal human subjects
J. Clin. Endocrinol. 29, 1349-1355 /1969/.
3. Düsterdieck, G. and McElwee, G. in "Radioimmunoassay Methods"/Eds. Kirkham, K.E. and Hunter, W.H./ Churchill, Livingstone, London /1971/ pp. 24-38.
4. Corvol, P., Rodbard, D., Drouet, J., Catt, K. and Menard, J.,
Monoiodinated angiotensins: preparation and characterisation by polyacrylamide gel electrophoresis and isoelectric focusing
Biochim. Biophys. Acta 322, 392-400 /1973/.
5. Gros, C., Pradelles, P., Rouget, C., Bepoldin, O., Dray, F., Fournier-Zaluski, M.C., Roques, B. A., Dollard, H., Llorens-Cortes, C. and Schwartz, J. C.,
J. Neurochem. 31, 29-39 /1978/.
6. Sheidah, N. G., Dennis, M., Corvol, P., Rochemont, J. and Chrétien, M.,
A rapid high-performance liquid chromatography purification method of iodinated polypeptide hormones
Anal. Biochemistry 109, 185-191 /1980/.
7. Moulin, M. A., Camsonne, R., Bigot, M. C. and Debruyne, D., A practical proposal for arginine-vasopressin radioimmunoassay, Clin. Chim. Acta 88, 363-374 /1978/.

8. Fyhrquist, F., Wallenius, M. and Hollemans, H.J.G., Radioimmunoassay of vasopressin in unextracted plasma Scand. J. clin. Lab. Invest. 36, 841-847 /1976/.

9. Flouret, G., Terada, S., Tang, F., Nakagawa, S. H., Nakahara, T. and Hetcher, O., Iodinated neurohypophyseal hormones as potential ligands for receptor binding and intermediates in synthesis of tritiated hormones Biochemistry, 16, 2119 /1977/.

10. Salacinski, P., Hope, J., McLean, C., Clement-Jones, V., Sykes, J., Price, J. and Lowry, P.J., A new simple method which allows theoretical incorporation of radio-iodine into proteins and peptides without damage. J. Endocr., 81, P 130 /1979/.

11. Fraker, P. J. and Speck, J.C.Jr., Protein and cell membrane iodinations with a sparingly soluble chloroamide-1,3,4,6-tetrachlore-3a,6a-diphenylglycoluril Biochem. Biophys. Res. Comm. 80, 849 /1978/.

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ANALYSIS OF POLYDISPERSITY OF OLIGOMERS AND POLYMERS BY ADSORPTION THIN-LAYER CHROMATOGRAPHY

E.S. Gankina and B.G. Belenkii

Institute of Macromolecular Compounds of the Academy of Sciences of the USSR, Leningrad, /USSR/

Thin-layer chromatography /TLC/ has been widely used for the analysis of virtually all classes of low molecular weight organic substances.

In 1968 Inagaki /Japan/ /1/ and Belenkii and Gankina /2/ demonstrated that TLC could be successfully applied to the separation of high molecular weight compounds, such as synthetic polymers. This method has recently become a powerful analytical tool for the investigation of various types of polydispersity of polymers and oligomers. The principal mechanisms of the separation of polymers in TLC are the adsorption and

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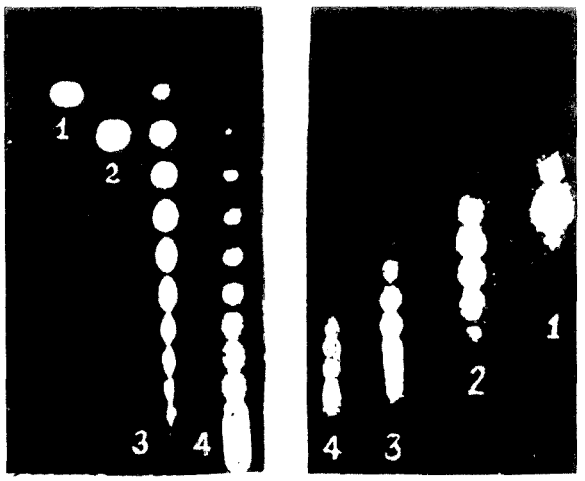
the precipitation mechanisms. Precipitation TLS has been widely used by Inagaki for the separation of homopolymers and copolymers according to their molecular weight !MW/ /3,4/. The present authors dealt mainly with the development of the adsorption TLC /ATLC/ of polymers /5, 6/. The investigation of experimental possibilities of using ATLC of high polymers and oligomers was developed simultaneously with the theory of this method. This theory showed that the adsorption and the exclusion /gel-permeation/ chromatography of polymers and oligomers exhibit the same mechanism and also elucidated the mechanism of polymer distribution according to MW. composition and functionality /7, 8/. This theory made it possible to separate polymers and oligomers by ATLC according to one of these types of polydispersity.

Now one should consider some peculiarities of the TLC of macromolecular compounds and demonstrate wide possibilities of using it for the investigation of various kinds of polydispersity of oligomers and high polymers.

ATLC allows the separation of homopolymers over a wide range of MW from polymer homologues in oligomers /MW 300/ up to polymers with MW of $2 \cdot 10^6$. Fig. 1 shows the ATLC of oligomers. One can clearly see the high resolution of TLC permitting the separation of polystyrene /PS/ with MW of 600 into individual polymer



a b

Fig.1.

ATLC of (a) polystyrenes with Mn: (1) 314, (2) 418, (3) 600, (4) 900 in cyclohexane - benzene (14:3) and (b) poly(- methylstyrene) fractions (1, tetramer, 2, hexamer, 3, octamer, 4, decamer) in carbon tetrachloride - heptane (2:1) on KSK silica gel.

homologues from 3 to 13-mers /9/. Fig. 1 also shoes the ATLC of oligo / -methylstyrene/. It can be seen that the 4-mer contains a small admixture of the 3-mer but 5-mer, 6-mer and 7-mer are a mixture of several polymer homologues. For example, 5-mer is a mixture of homologues from 4-mer to 8-mer.

When polymers are separated according to their MW by ATLC, the porosity of the adsorbent plays an important

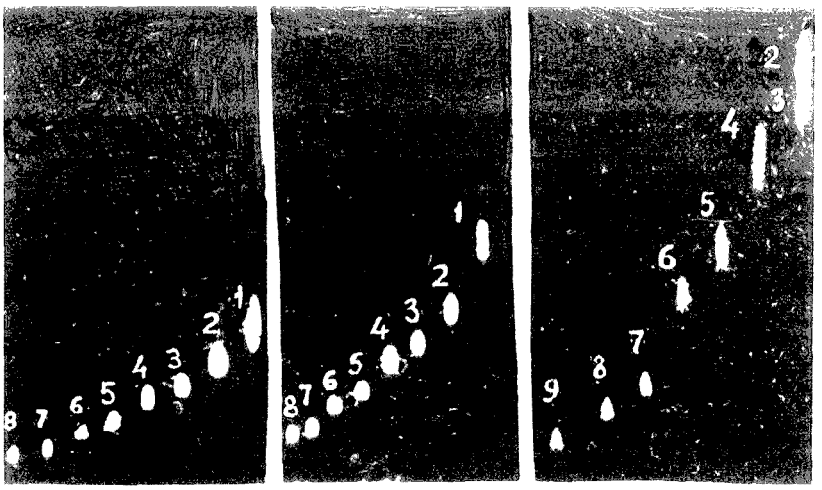


Fig.2.

Effect of pore size of silica gels / a) $\varnothing_p$ = 60 Å, b) $\varnothing_p$ = = 100 Å and c) $\varnothing_p$= 500 Å/ on the R_F of narrow-disperse PS (M_w /Mn 1.1) with M_n: (1) $9.6 \cdot 10^3$, (2) $20 \cdot 10^3$, (3) $36 \cdot 10^3$, (4) $49 \cdot 10^3$, (5) $111 \cdot 10^3$, (6) $193 \cdot 10^3$, (7) $404 \cdot 10^3$, (8) $773 \cdot 10^3$, (9) $1.99 \cdot 10^6$ in ArLC in cyclohexane - toluene - 2--butanone (17:2:1.6) (a,b) and (17:2:1.4) (c).

part /10/. The pore diameter of silica gels commonly used in TLC is 60 Å /"Merck"/ or 100 Å /"KSK", USSR/. Fig. 2 shows the separation of PS with MW ranging from 10.10^3 up to 10^6 on silica gels of different porosities. It is evident that a silica gel with the pore diameter of 60 Å permits the separation of PS only up to the MW

of 50.10^3; when the pore diameter is 100 Å, effective separation up to the MW of 400.10^3 takes places; for the separation of polymers up to the MW of 10^6 it is necessary to use silica gels with the pore diameter of 500 Å.

It should be noted that the exclusion limits of ATLC correspond to much higher MW than those of gel-permeation chromatography /silica gels with the pore diameter of 60, 100 and 250 Å correspond to MW of 10^4, 5.10^4 and 2.10^5/. This fact can be explained by tje theory if ATLC showing that a macromolecule adsorbed in pores undergoes great conformational changes. When the pore size decreases /up to exclusion limit/, the adsorption energy of macromolecules increases. Precipitation TLC does not allow the separation of oligomers but is very effective for the separation of high polymers with MW up to 24.10^6 /11/.

Otoca has demonstrated for precipitation chromatography, macromolecules never penetrate the pores but interact only with the outer surface of adsorbent particles. The nature of the adsorbent does not play an important part in this process /12/.

ATLC not only permits the separation of polymers over a wide range of MW but is also very sensitive to adsorption active end groups in homopolymers. It is known that one sugar residue per PS molecule with MW 100.10^3 greatly increases its adsorption activity as compared to

that ot common PS of the same MW /13/. On the basis of these data TLC allowed the estimation of the true percentage of grafting for PS grafted onto cellulose after the acid hydrolysis of the copolymer /11/. The possibility of the separation of PS of similar MW but with one or two carboxyl end groups or without any carboxyl groups was also shown my Min /14/.

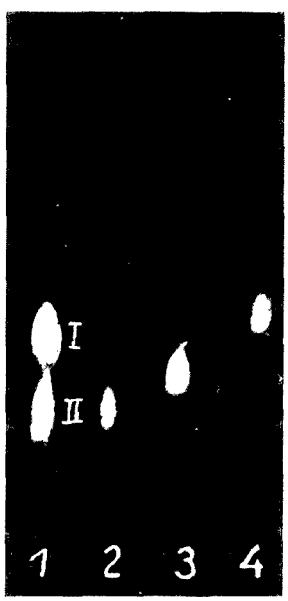


Fig.3.

ATLC of PS with M_n (1) $100 \cdot 10^3$ and narrow-disperse PS with M_n: (2) $404 \cdot 10^3$, (3) $193 \cdot 10^3$ and (4) $111 \cdot 10^3$ in cyclohexane - - benzene - acetone (12:4:0.7) on KSK silica gel.

ATLC was widely used to evaluate the homogeneity of homopolymers. Fig. 3 shows the ATLC of PS which according to the data of exclusion and precipitation chromatography, is narrow-disperse and unimodal. However, it can be seen that PS consists of two components one of which contains an adsorption active and group and is more strongly adsorbed /its R_F value is lower/.

The application of gradient ATLC to the separation of random polymers and block copolymers according to their composition reveals interesting possibilities. Fig. 4 illustrates the gradient ATLC of styrene-acrylonitrile /ST-AN/ random copolymers with an AN content ranging from 12 up to 44.7% /wt./ AN /15/ and that of vinylchloride - vinylacetate /VC-VA/ containing 6 to 28% /wt./ of VA /16/. It is clear that by using the chromatography of an unknown copolymer together with marked copolymers of known composition one can rapidly evaluate the compositional heterogeneity of random copolymers. Thus, the ST-AN copolymer investigated by us and located in the middle of the chromatogram contains about 25-31% of AN.

The most important factor here is that the adsorbability of copolymers with MW 50.10^3 is much more closely related to their heterogeneity of composition than to MW. This results from the theory and is proven experimentally. However, the more similar is the che-

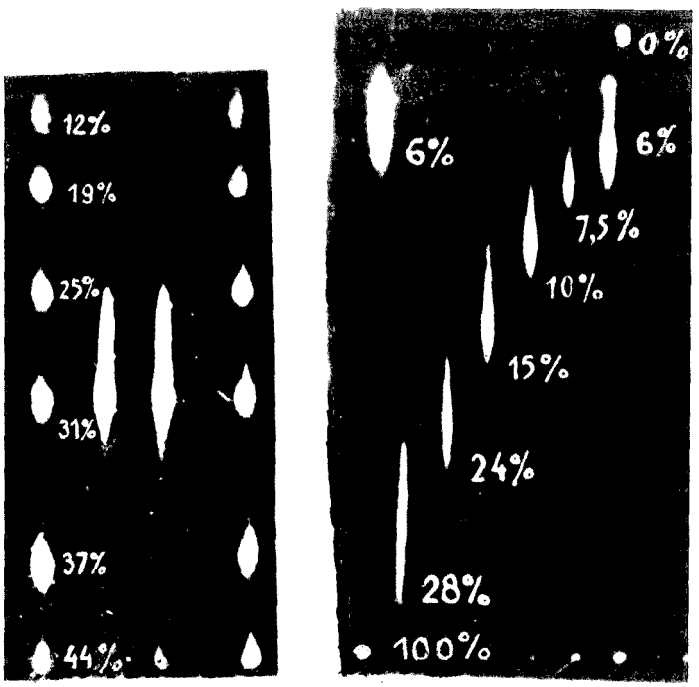
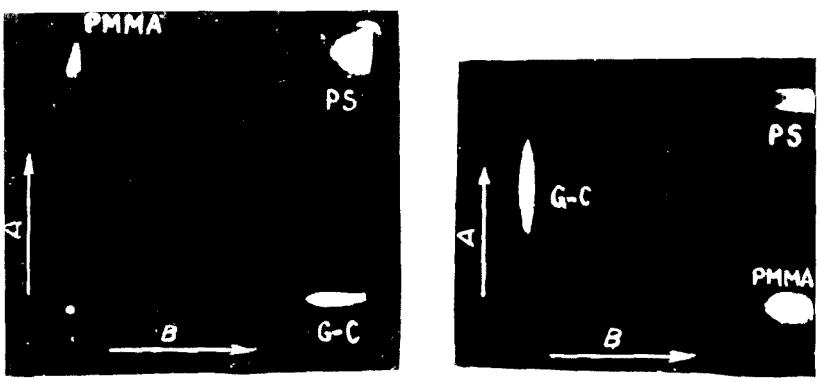


Fig. 4.

Gradient ATLC of (a) styrene-acrylonitrile (ST-AN) random copolymers (12-44% wt. AN) in carbon tetrachloride - methylene chloride (2:5) ——→ acetonitrile (3ml) (0.12 ml/min) and (b) vinyl chloride - vinyl acetate (VC-VA) random copolymers (6-28 % wt. VA) in 1.2 dichloroethane - carbon tetrachloride (1:1) (6 ml) ——→ 1.2 dichloroethane - 2-butanone (5:2) (6 ml) (0.24 ml/min) on KSK silica gel /15, 16/.

mical nature and, therefore, the adsorbability of copolymer units, the harder it is to carry out the fractionation of copolymers according to composition with the aid of ATLC regardless of their MW.

The important problem of the analytical chemistry of polymers has been the determination of the admixtures of homopolymers in block and graft copolymers. To solve this problem a number of TLC techniques was used. Fig.5



a b

Fig. 5.

Two-dimensional ATLC of polymethylmethacrylate-polystyrene (PMMA-PS) graft copolymer. (a) chromatogram in acetone - - acetic acid (12:2) in direction A and in chloroform - - 2-butanone (12:2) in direction B, (b) chromatogram in cyclohexane - benzene - acetone (12:4:0.7) in direction A and in acetone - acetic acid (12:2) in direction B on KSK silica gel.

shows the use of two dimensional TLC for the determination of homopolymers in the polymethylmethacrylate- - polystyrene /PMMA.PS/ graft copolymer /5/. It is evident that by varying the eluent one can either displace both homopolymers and the graft copolymer arranging them on the chromatogram in different corners of the plate or displace only homopolymers into different plate corners, the graft copolymer remaining in the centre of the plate.

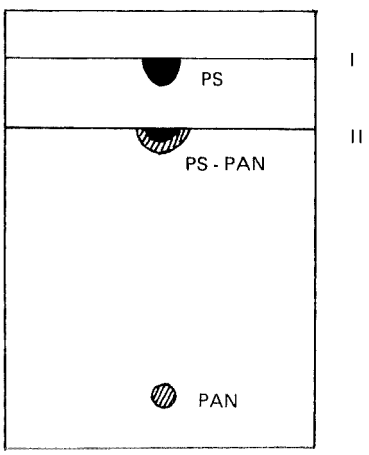


Fig. 6.

ATLC with multiple development of polystyrene-polyacrylonitrile (PS-PAN) block copolymer in toluene (I step) and toluene-dimethylformamide (1:1) (II step) on KSK silica gel. (● - black colour - 1.8 % $KMnO_4/H_2SO_4$, ⊛ - yellow colour - 10 % NaOH).

For this purpose multiple development TLC can also be used. Fig. 6 shows the use of this technique for the investigation of a polystyrene - polyacrylonitrile /PS.OAN/ block copolymer /17/. It can be seen that the PS homopolymer moves at the toluene front. At the following elution step in the toluene - dimethylformamide system the PS-PAN block copolymer moves together with the front of the eluent, the PAN homopolymer remaining at the start.

Additional identification of these compounds was based on the colour test. The chromatogram was treated with a 1.8% $KMnO_4/H_2SO_4$ solution and a 10% NaOH solution with subsequent heating for 10 minutes at 140-150°C. /$KMnO_4$ detects only PS with the appearance of black colour whereas NaOH detects only PAN with yellow colour. The spot of the block copolymer turns black in the centre and yellow at the periphery.

If gradient ATLC is used, one can separate homopolymers from the block copolymer and simultaneously determine its composition and compositional homog Fig. 7 shows the gradient ATLC[x] of the polystyren

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x/ Here and below TLC was carried out on plates 6 x 9 c with a silica gel layer with pore diameter of 10 ± 5 mm. The separated samples were detected my spraying the chromatograms with a 1.8% solution of $KMnO_4/H_2SO_4$ with subsequent heating at 140 to 150°C for 10 min. Polymer zones were developed as black spots on white background.

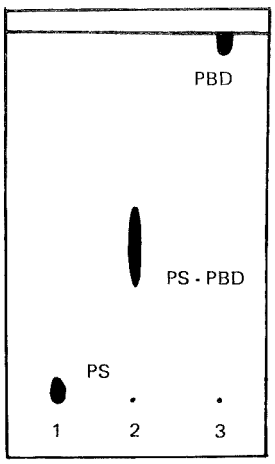

Fig. 7.

Gradient ATLC of (2)polystyrene-polybutadiene (PS-PBD) block copolymer and homopolymers: (1) PS and (3) PBD in cyclohexane - toluene (3.5:2.5) ——→ toluene (6 ml) (0.12 ml/min) on KSK silica gel.

- polybutadiene /PS-PBD/ block copolymer. It is clear that under these conditions the block copolymer moves in the centre of the plate whereas the less adsorbed homopolymer /PBD/ moves together with the front of the eluent and the more adsorbed homopolymer PS remains at the start.

ATLC provides great possibilities for the separation of oligomers according to their MW and functionality. It was shown that the ATLC of oligomers with active end groups makes it possible to observe three

types of R_F dependence on MW: when R_F increases with MW when it decreases and when R_F is independent of MW. The latter case provides the best conditions for the separation of oligomers according to their functionality /9/.

Fig. 8 illustrates the three above mentioned types of the R_F dependence upon MW for poly/ethylen oxides/ /PEO/. These dependences are related to different adsorbabilities of end and in-chain /or central/ units

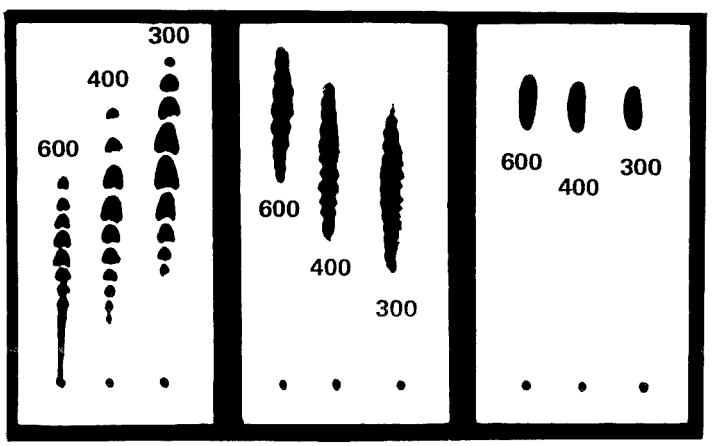

Fig.8'.

ATLC of poly(ethylene oxides) with M_n: 300, 400 and 600 on (a) KSK silica gel in pyridine - water (0.1:10), (b) Al_2O_3 in chloroform - ethanol (10:1) and (c) KSK silica gel in chloroform - pyridine (5:7).

of oligomers. When both types of units are adsorbed, adsorption increases with MW /negative R_F dependence/. When the adsorbability of the central units is zero /zero energy of adsorption/, the adsorbability of oligomers molecules does not depend on MW and they are separated only according to the number of adsorption-active end groups /according to functionality/.

We investigated non-functional, mono- and bifunctional oligoisoprenes /OI/ of different MW sent to us by Dr. Pokorny /Prague, CSSR Academy of Sciences/.

Fig. 9 shows the ATLC of these samples. It is clear that non-functional and monofunctional oligomers can be separated. Moreover, the mobility of non-functional oligomers decreases and that of bifunctional oligomers increases with increasing MW. Az eluent with a high displacing power is used to separate mono- and bifunctional oligomers. Non-functional OI move with the solvent front /i.e., according to the mechanism of exclusion chromatography/ and mono- and bifunctional OI are also separated according to MW. The R_F value increases with MW. It is evident that under these conditions the analysis of bifunctional and monofunctional oligomer admixtures can be carried out and the evaluation of the MW of functional OI can be made.

The possibility of the separation of 1.2-polybutadienes according to the type of functionality by

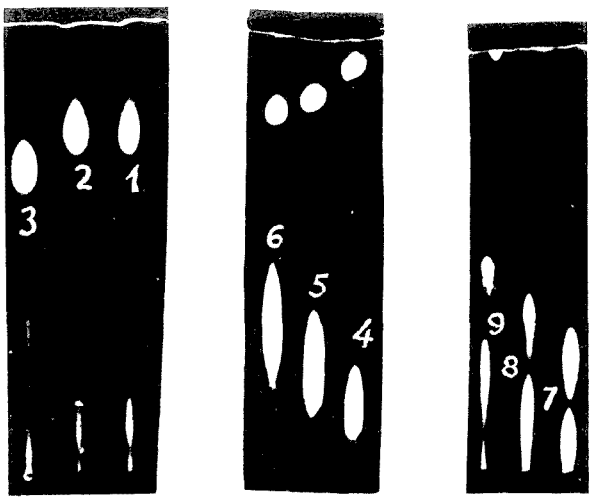

Fig. 9.

ATLC of oligoisoprenes (OI): (a) non-functional[x)] OI with M_n: (1) 1600, (2) 3200 and (3) 9300 and (b) monofunctional OI with M_n: (4) 2600, (5) 5700 and (6) 9600 in heptane - chloroform (10:6) and (c) bifunctional OI with M_n: (7) 4300, (8) 8600 and (9) 9200 in cyclohexane - toluene - 2-butanone (10:1:0.5) on KSK silica gel. /[x)] non-functional and monofunctional impurity components are located above samples 4-9/.

ATLC without their separation according to MW has been demonstrated /18/.

Using the effectiveness of ATLC for separating oligomers according to their functionality and MW we analyzed a complex oligomer compound. It was shown that the sample investigated was a mixture of two triols of poly /polypropylene oxide/ /PPO/ with different MW. One can see in Fig. 10 that in two-dimensional chromatography in water - pyridine the separation of PPO occurs

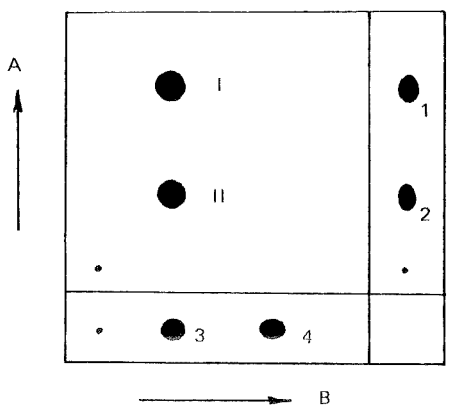

Fig. 10.

Two-dimensional TLC of an oligomer compound in water - pyridine (18:1.5) in direction A and in water-saturated ethylacetate in direction B on KSK silica gel. Poly(propylene oxides) (PPO) with M_n: (1) 425, (2) 1220, (3) 500 (PPO-triol) and (4) 425 (PPO-diol).

according to MW and the compound is separated into two components /I and II/ corresponding to PPO with MW 425 /1/ and 1220 /2/. When chromatography is carried out in water-saturated ethylacetate, PPO are separated according to their functionality /PPO - triol - 3 and PPO-diol - 4/, both components of compound moving on the triol level with MW of 500.

In the foregoing discussion various examples are given of the separation of oligomers with functional groups exhibiting a highes adsorption activity than the central units. It is interesting to study the behaviour of oligomers with end groups of lower adsorption activity than the central units.

Fig. 11 shows the MW dependence of R_F for oligostyrenes with the secondary butil end group and non-alkyl oligostyrene. It is evident that when ATLC is carried out on silica gel, the secondary butil group has a lower adsorption activity than styrene units and therefore oligomers of this type have a greater R_F value than their non-alkyl analogues /19/.

Summarizing one should consider the main advantages introduced by the ATLC of polymers into the field of chromatography and analytical chemistry of polymers.

In the field of chromatography the method of the ATLC of polymers made it possible to increase the limits of using TLC for the analysis of macromolecular compounds

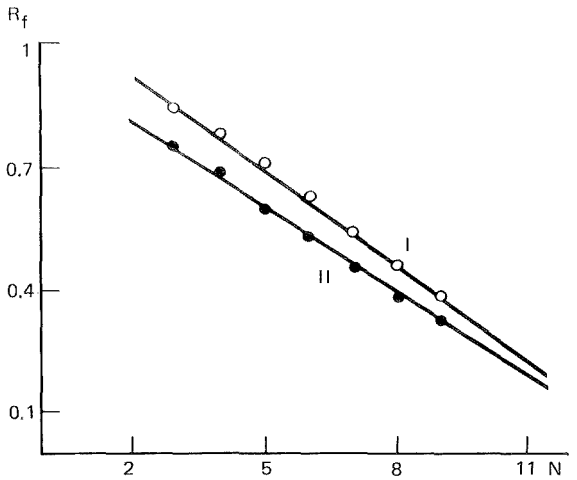

Fig. 11.

Dependence of R_F of oligostyrenes (I) with secondary butil group at the chain end and (II) non-alkyl species on MW (or degree of polymerization, N). ATLC in cyclohexane - benzene (20:0.5), twice on KSK silica gel /19/.

/with MW 10^6/. Moreover, taking as an example the analysis of functional oligomers it was possible to demonstrate the role of negative adsorption in adsorption chromatography. It manifests itself, for instance, in the positive MW-dependence of R_F for mono- and bifunctional OI.

Just as the highly effective GPC, ATLC demonstrated that polymers are a continuous series of polymer homologues with monotonously rising MW.

In the field of the analytical chemistry of macromolecular compounds the development of the ATLC of polymers provided a solution to a number of new problems that other physical and physico-chemical methods were unable to solve, such as to determine the functionally of oligomers apart from their polydispersity according to MW, separate macromolecules according to their geometry, stereoisomery, functionality and compositional heterogeneity regardless of their MW.

As to the fractionation of polymers and oligomers according to their MW, the advantage of GPC, this quantitative method of analysis of the molecular weight distribution of homopolymers, oligomers and block copolymers, are quite evident. However, one can successfully use ATLC for the express determination of the MW of homopolymers and oligomers. This method also permit the simultaneous determination of the homogeneity of polymers.

Hence, TLC is a highly effective and at the same time a simple, cheap and rapid method of investigation of various types of polydispersity of synthetic polymers and oligomers.

REFERENCES

1. H. Inagaki, H. Matsuda and F. Kamiyama, Macromolecules, 1 /1968/ 52o.

2. B.G. Belenky, E.S. Gankina and L.D. Turkova, Lektsii 2nd Shkoly po Metodam Ochistki i Otsenki Chistoty Monomerov i Polymerov. Chernogolovka, 1968, p. 216

3. H. Inagaki: In Fractionation of Synthetic Polymers. L.H. Tung /ed/. New Xork, Marcel Dekker, 1977, chap. 7.

4. H. Inagaki; In Adv. Polymer Sci. H.J. Cantow /ed./, Berlin Heidelberg, Springer Verlag, 24 /1977/ 190-

5. B.H. Belenky, E.S. Gankina, J. Chromatogr., 53 /1970/ 3.

6. B.G. Belenky, E.S. Gankina, J. Chromatogr., 141 /1977/ 13.

7. A.M. Skvortsov, B.G. Belenky, E.S. Gankina, M.B. Tennikov, Visokomol. Soed., A20 /1978/ 678.

8. B.G. Belenkii, E.S. Gankina, M.B. Tennikov, L.Z. Vilenchik, J. Chromatogr., 147 /1978/ 99.

9. B.G. Belenky, M.D. Valchikhina, I.A. Vakhtina, E.S. Gankina, O.G. Tarakanov, J. Chromatogr., 129 /1976/ 115.

10. H. Halpaap, K. Klatyk, J. Chromatogr., 33 /1968/ 80.

11. H. Inagaki: In Chromatography of Polymers and Polymers in Chromatography. 6-th Diskussion Conference, Prague, 1978, CL 11/1.

12. E.P. Otocka, M.Y. Hellman, P.M. Muglia, Macromolecules, 5 /1972/ 227.

13. T. Taga, H. Inagaki, Angew. Makromol. Chem., 33 /1973/ 129.

14. T.-I. Min, T. Miyamoto, H. Inagaki, Bull. Inst. Chem. Res. Kyoto Univ., 53 /1975/ 381.

15. E.S.Gankina, S.I. Ganicheva, V.M. Beliaev, J.J. Kever, B.G. Belenky, J. Chromatogr., 208 /1981/ 381.

16. J. Janca, J. Kroupova, S. Pokorny, J. Stehlicek, J. Kalal, E.S. Gankina, J.J. Kever, B.G. Belenky, J. Chromatogr., 171 /1979/ 81.

17. I.G. Krasnoselskaya, E.S. Gankina, B.G. Belenky, B.L. Erasalimsky, Visokomol. Soed., A19 /1977/ 999.

18. T.-I. Min, T. Miyamoto, H. Inagaki, Rubber Chemistry and Technology, 50 /1977/ 63.

19. B.G. Belenky, E.S. Gankina, B.N. Zgonnik, T.P. Krasnova, E.Yu. Melenevskaya, P.P. Nefedov, Visokomol. Soed. B20 /1978/ 574.

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MONITORING OF D-PENICILLAMINE IN CLINICAL PRACTICE BY IONEXCHANGE TLC

Péter Kiss

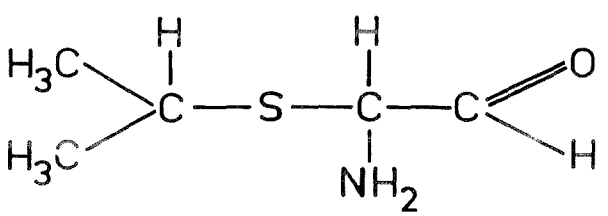
Apáthy Children's Hospital, Budapest

and

Judit Kovács

Section of Pediatrics, Péterfy Hospital, Budapest

D-Penicillinamine /Fig. 1/, dimethylcysteine, a metabolite of penicilline is a comparatively stable thiol compound, which has been first identified as a component of the penicilline molecule.



D – PENICILLAMIN

* Presented at the First Symposium on Advances of TLC and HPLC, May 14-15, 1982, Szeged, Hungary.

The biological action of D-Pa is attributed to its amino-thiol properties, such as chelation of metals, reaction with carbonyl groups etc. Its therapeutic application dates back to the late fifties when it was used with success in the treatment of Wilson disease, a copper storage disorder. Since then the D-PA proved to be a very efficient drug in various diseases.

In clinical practice, D-PA is used in the following cases:

Newborn period: hyperbilirubinemia, prevention of retrolental fibroplasia.
Metabolic diseases:Wilson disease, cystinuria.
Chronic diseases: rheumatoid arthritis, scleroderma, chronic hepatitis.
Metal poisoning: Hg, Pb.

Pharmacology - toxicity

1. Only the D form is effective and it is less toxic.

2. The toxicity is both dosage and time dependent.

3. A number of side effects may occur during long-term treatment, such as: bone marrow damage /anaemia/, renal failure /proteinuria/, loose of taste and smell sensing, gastro-intestinal symptoms, allergic reactions. All this unfavourable symptoms disappear rapidly after cessation of the therapy.

Mode of treatment

There are two ways of D-Pa administration:

1. in acute diseases, e.g. in hyperbilirubinemia of newborns intravenous mode of administration is preferred.

2. in chronic illnesses for long-term treatment oral application is employed.

The wide range of possibilities of clinical applications as well as the control of long-term treatment require a careful and reliable drug monitoring. Since the drug causes sometimes severe side effects and the dosage schedule is based upon only empirical data, a sensitive and selective method is needed for the determination of D-Pa in body fluids.

The aim of the present work was to develop a procedure suitable for the determination of D-Pa in small quantities in blood among others such as filter paper eluates.

The D-Pa which is actually an amino acid containing a thiol group could be well separated on Fixion sheets and could be visualized by means of the usual ninhydrin reagent.

Method

Blood samples in 50-100 µl quantities are drawn by fingertip or heel puncture and are collected either in heparinized tubes or dried on filter paper.

For deproteinization as well as for elution from the filter paper a 10% aqueous trifluoroacetic acid solution is used.

After deproenization the excess TFA is removed, since it may interfere with the chromatographic procedure.

The chromatography is carried out on Fixion 50X8 chromato-sheets, using citrate buffer pH 4.4 /Na^+ - 0.3M/:

citric acid.$2H_2O$	14.1 g
NaOH	8.0 g
NaCl	5.85 g
HCl /37%/	5.0 ml
deionized water ad	1000.0 ml

The 200 x 200 mm chromatosheets were developed in standard TLC tanks at +4°C in a refrigerator. Drying and staining with ninhydrin was carried out as described previously.

D-Pa appears as dark pinkviolet spot between leucine and valine /Fig. 2 and 4/.

Quantitative evaluation was performed by a Telechrom video-densitometer type OE-976.

Results

D-Pa /100 mg/kg bodyweight/ was administered intravenously to hyperbilirubinemic newborn infants.

At first the drug was administrated as a single intravenous injection. Fig 2 shows the developed chromatogram and it could be established that after two hours the drug completely disappears from the blood stream. The densito-

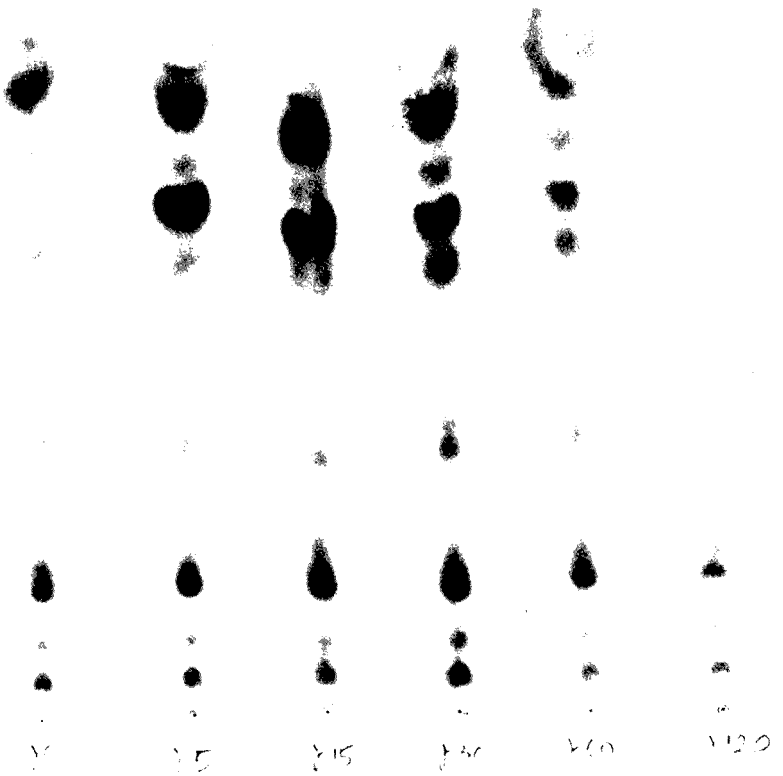


Fig. 2

Distribution of D-Pa in blood serum after administration of 100 mg/kg i.v.

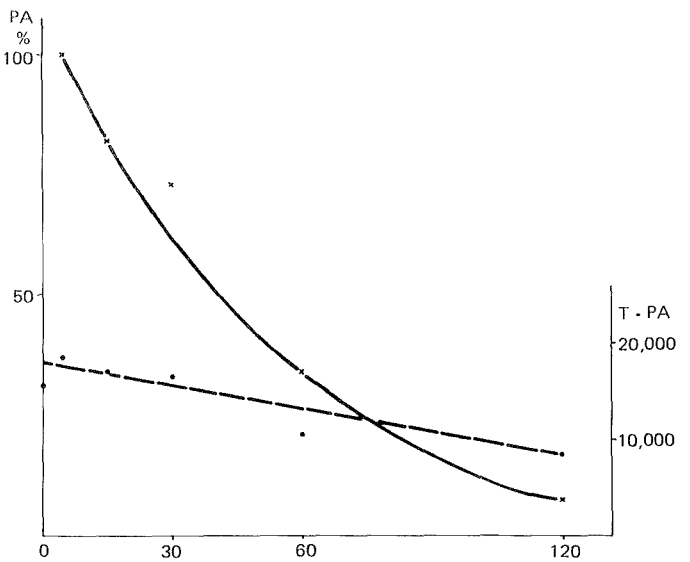


Fig. 3

Videodensitometric readings of the chromatogram shown on Fig. 2

metric evaluation confirms quantitatively these observations /Fig. 3/.

Secondly, the administration was followed by continuous drop infusion. This mode of employment ensures a steady drug level throughout the time of infusion, usually 5 hours long /Fig. 4 and 5/.

These findings correspond with our clinical observations, that especially in the treatment of hyperbili-

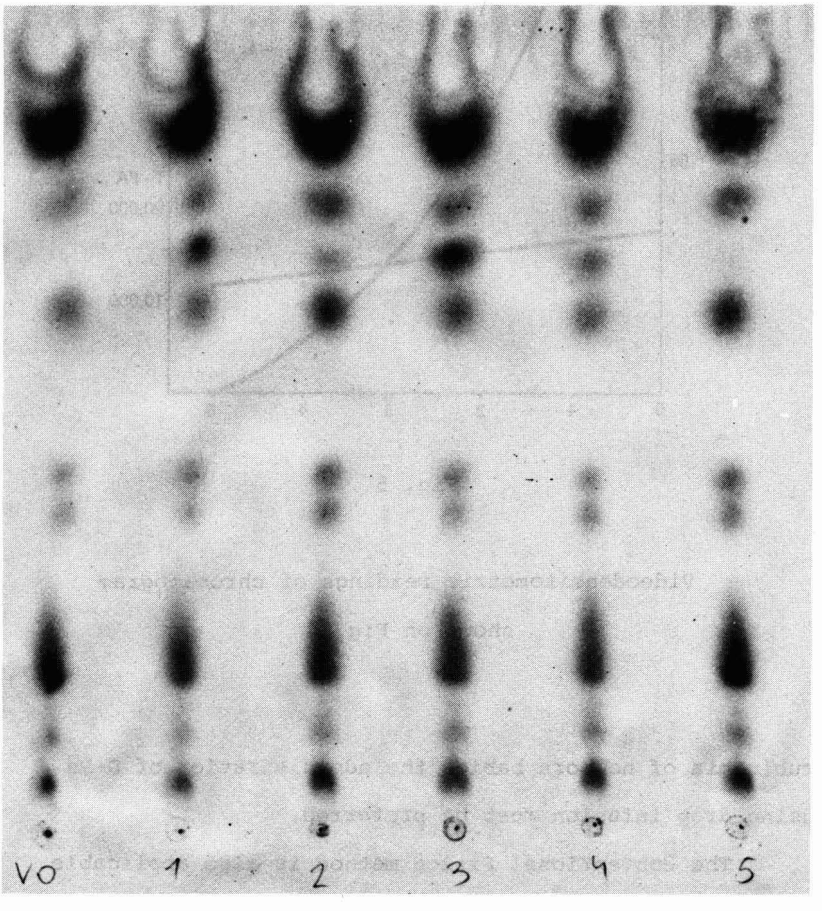


Fig. 4

Distritubiton of D-Pa in blood serum after administration of 100 mg/kg in drop infusion

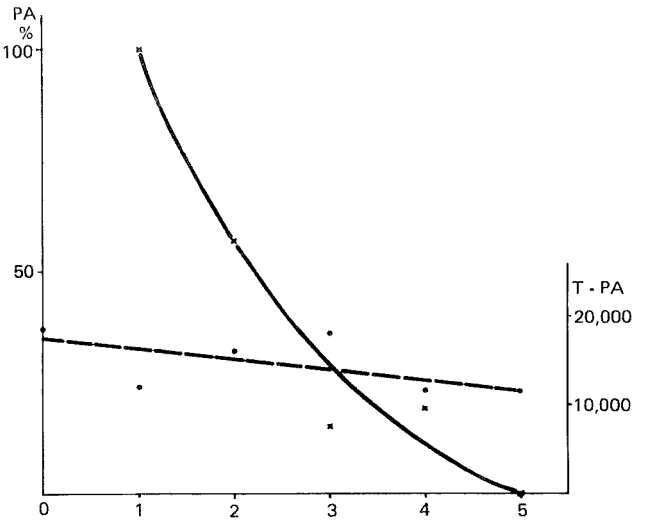


Fig. 5

Videodensitometric readings of chromatogram shown on Fig. 4

rubinemia of newborn babies the administration of D-Pa using drop infusion must be preferred.

The conventional Fixion method is also applicable detecting D-Pa in urine. It is especially indicated in the therapy control of cystinuric patients. D-Pa is excreted unaltered in the urine. The urine sample may be applied onto the chromatosheet without previous deproteinization or desalting. D-Pa be well distinguished even in the presence of cystine /Fig. 6 /.

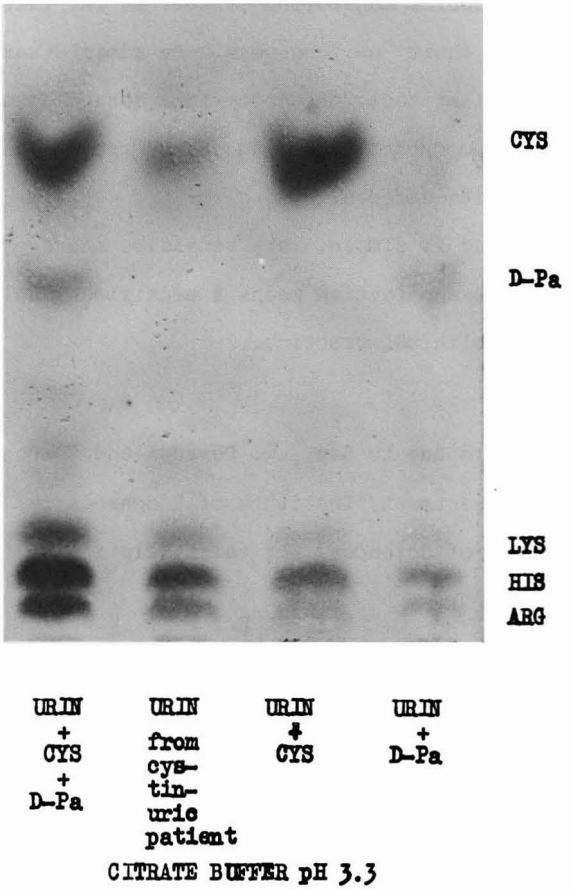


Fig. 6

Detection of D-Pa in urine

Summarizing:

The ion exchange TLC method developed by us is suitable for regular monitoring as well as for therapy control during short or long-term D-Pa administration. It is a convenient tool for appropriate dosage schedule, which is very important regarding the prevent ion of the possible side effects.

The method is simple, very sensitive and reproducible and its application means a significant help in the every day clinical practice.

Thanks are due to Drs. T. Dévényi and S. Pongor /Enzymology Department, Institute of Biochemistry, Hungarian Academy of Sciences/ for their help and assistance.

REFERENCE

S. Pongor, Judit Kovács, P. Kiss and T. Dévényi /1978/ Acta Biochim. Biophys. ASH 13, pp. 123-126

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OVERPRESSURED THIN-LAYER CHROMATOGRAPHY AND ITS APPLICATIONS

Z. Lengyel[1], E. Tyihák[2] and E. Mincsovics[2]

[1]LABOR Instrumental Works, Esztergom-Budapest, HUNGARY

[2]Research Institute for Medicinal Plants, Budakalász, HUNGARY

In recent years, a rapid progress can be observed both in column and planar liquid chromatographic techniques. In the field of liquid column chromatography the most spectacular achievement was the development of high-performance liquid chromatographic /HPLC/ systems by means of several special instruments and sorbents /1, 2/. As regards planar techniques, the most significant break-through is the development of highperformance thin-layer chromatography /HPTLC/ /3/ based on the application of fine-particle sorbents. Both techniques proved to be very useful in many fields of chemical analysis, although the use of the latter is more restricted, mainly to micro chromatographic studies.

It appeared, thus, logical to develop such a planar liquid chromatographic technique that would approach HPLC as regards stability and standardization of chromato-

* Presented at the First Symposium on Advances of TLC and HPLC, May 14-15, 1982, Szeged, Hungary.

graphic conditions but still would conserve the well known advantages of TLC and HPTLC such as visual evaluation, the use of selective and specific reagents, simultaneous study of a large number of samples and topographic identification of the separated substances.

The pressurized ultramicro chamber

Earlier, we developed a closed TLC chamber in order to study the relationship of conventional TLC and column chromatography /CC/ /4/. In this simple chamber /termed as ultramicro /UM/ chamber/, the sorbent layer is covered by a glass plate so that the end of the cover plate is not immersed into the solvent, in order to eliminate interfering capillary effects. The elimination of solvent vapour was first realized by this chamber. However, the advantages of a totally closed chamber were realized only later, when we developed the pressurized UM chamber /PUM chamber/ /5-8/.

The essential feature of PUM chamber of circular and linear types is that the sorbent layer is completely covered with a flexible membrane under an external pressure so that, in the closed chamber, a layer of water /a water-cushion/ forms between the Plexiglass cover-plate and the flexible and fixed membrane, and the vapour space above the layer is virtually eliminated. Solvent admission under overpressure has been solved by means of a pump system /Fig. 1/.

By adjusting the solvent, by means of a pump system in PUM chamber, it is possible to separate substances with

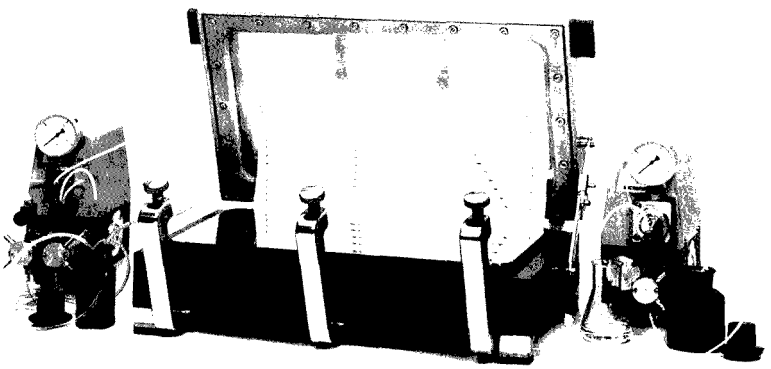

Fig.1

Top views of the CHROMPRES 10 chamber /LABOR Instrumental Works, Esztergom-Budapest, Hungary/ in open form after a two-directional separation of dyes.

optional development distances. This active form of the planar liquid chromatography is the so-called overpressured thin-layer chromatography /OPTLC/ which integrates the advantages of the classical TLC /9-10/, modern HPTLC /11-12/ and HPLC /1-2, 13/.

In the PUM, the external pressure on the flexible cover-membrane must always be higher than the input pressure of the solvent. The input pressure of the solvent increases linearly with increasing solvent migration distance. An in-

crease in the solvent flow velocity always results in higher input pressures, which must be taken into account by choosing an appropriate external pressure on the membrane /14/.

The PUM chamber constitutes one system with the water - dosing pump of larger delivery and the eluent dosing pump of smaller delivery.

In the PUM chamber of linear type, linear migration of the solvent front is achieved by impregnating the edges of the layer and either placing a narrow plastic sheet on it or by making a narrow channel in the layer before attaching the solvent inlet. Paraffin or various plastic dispersions can be used for impregnation. In one-directional OPTLC, three edges of the plate are impregnated. Two-directional separation can, however, be carried out by impregnating only two edges of the chromatoplate and attaching the solvent inlet to the middle of the chromatoplate /Fig. 2/. This developing system is especially suitable for the efficient separation of a large number of samples.

Triangular /15/, anticircular /16/ and circular /3/ separations can be achieved, simply, by special impregnation of the respective shape.

By changing the inlet openings of the eluent, possibility is equally provided for one- or two-directional linear, circular, triangular /anticircular/ running.

It follows from this brief account that the successful application of OPTLC requires a special instrument and precoated /impregnated/ chromatoplates from various inorganic and organic sorbents. Manufacturing of both products

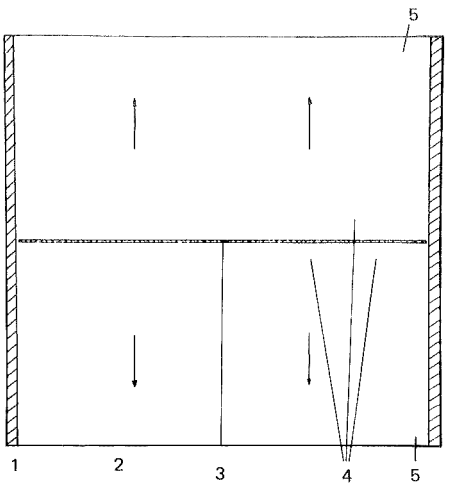

Fig. 2

Special pre-coated plate /e.g. SILPRES N/ to OPTLC for two--directional development. Schematic drawing 1 impregnated edges; 2 channel freed from sorbent; 3 solvent inlet; 4 place of samples; 5 front of solvent

is in progress, former at LABOR Instrumental Works /Esztergom-Budapest, Hungary/, latter at REANAL Fine Chemicals /Budapest, Hungary/ in cooperation with LABOR Instrumental Works.

Theoretical aspects

In classical TLC the distance between the solvent front and the solvent source /z_f/ is related to separation time /t/ by a quadratic equation /$z_f^2 = k.t$/ /17/ where the constant

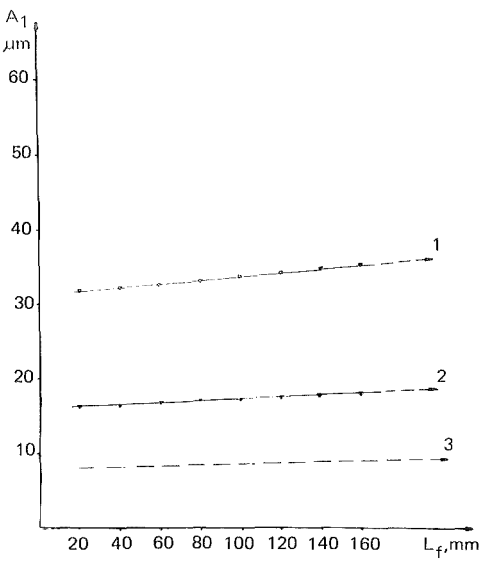

Fig. 3

Variation of the average plate height /H/ versus development length
Mobile phase, methylene chloride; investigated substance, Butter Yellow; external pressure on the membrane, 1.0 MPa; flow-rate of solvent, 20 cm^3/h; calculation of average plate height values according to Guiochon and Siouffi /18/
1 CHROMPRES 10 chamber, SILPRES N-1 experimental chromatoplate /d_p= 10-11 /um/; 2 CHROMPRES 10 chamber, SULPRES N-2 /d_p= 5-6 /um/; 3 CHROMPRES 10 chamber, SILPRES N-3 /d_p = 2-3 /um/

/k/ depends on the nature of stationary and mobile phases and also on sorbent particle size. The same quadratic is valid also in HPTLC /3/.

On the contrary, the law of linear OPTLC development can be described by a simple equation:

$$z_f = k^{OPTLC} \cdot t$$

The above relationship is valid not only in liquid-solid but also in liquid-liquid systems as well as in the "reversed phase" systems. In the circular OPTLC, however, the area to be wetted increases quadratically with the linear movement of the phase front. It is obvious from this fact that in circular OPTLC the classical law of TLC development is valid.

Optimum performance in linear OPTLC can be obtained with sorbent layers of very fine-particle and narrow size distribution as in HPLC. It is fact that the average plate height values in linear OPTLC diminish in direct proportion to decreasing of the particle size /Fig. 3/.

Due to the constant flow rate of the eluent in the linear OPTLC the theoretical plate number /N/ increases in a direct proportion to the distance /x/ while in the classical, normal saturated /N_s/ and unsaturated /N_{us}/, as well as in the ultramicro /UM/ chambers, an oppositional tendency dominates /Fig. 4/.

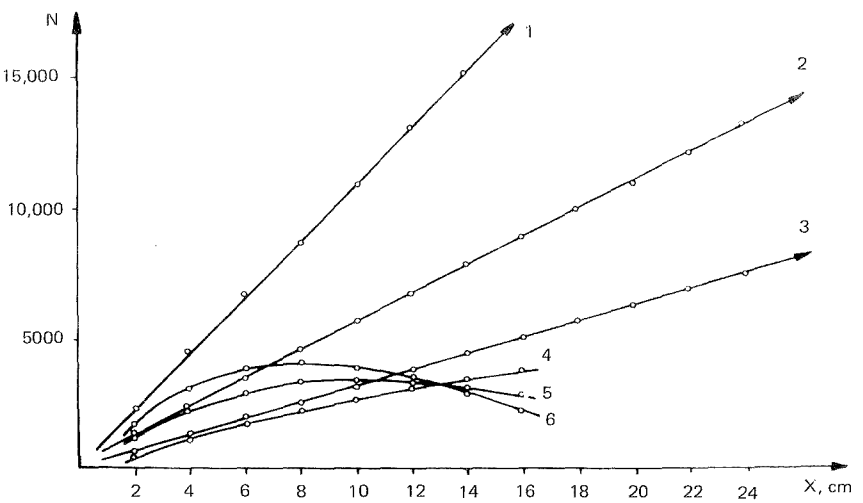

Fig. 4

Variation of the theoretical plate number /N/ values with the running distance /x/ in various chamber systems using various silica gel particle size Eluent, methylene chloride; materual, Butter Yellow 1 CHROMPRES 10, d_p = 2-3 /um; 2 CHROMPRES 10, d_p = 5 /um; 3 CHROMPRES 10, d_p = 11 /um; 4 N_s, d_p = 5 /um; 5 UM, d_p = 5 /um; 6 N_{us}, d_p = 5 /um

Application

A close fundamental relationship exists between the two alternatives of planar liquid chromatography: traditional TLC /and the modern HPTLC/ and OPTLC. There is, however, an essential difference between the two main techniques: in TLC /and in HPTLC/ a vapour phase is established above the sorbent layer in the normal /N/ chamber. In the sandwich /S/ chamber and also in so-called U-chamber /3/. The

characteristics of the vapour phase essentially depend on the composition of the solvent mixture and on the chamber system.

Starting from the fact that there is no vapour phase above the sorbent layer in OPTLC, it follows that one-component solvents can be conveniently used for separation. In the case of solvent mixtures consisting of two or more components, there are two possibilities: one is a normal separation using one-component solvents. The other possibility consists of demixing of the solvent mixture on the sorbent layer; in this case fronts, zones will build up. In OPTLC, therefore, in some cases we have to choose a new eluent system from the solvent mixtures, which is suitable for the efficient separation of the given substance group without solvent demixing.

According to our preliminary results, linear and circular OPTLC methods are suitable for the separation of various groups for organic substances, similarly to TLC and HPTLC, but with reasonably shorter separation times. Owing to the short separation times and the stable flow velocity, resolution values, especially those obtained with fine-particle plates, are excellent even in the case of longer solvent migration distances /e.g. 200-350 mm/.

Fig. 5 illustrates the efficiency of the separation in PUM chamber of linear type. It can be seen that the diameter of the spots is small, owing to the low degree of diffusion. It is obvious that the decreased time of separation yields better resolution.

Fig. 5

One-directional separation of dyes onf fine-particle silica gel layer /d_p = 5 /um/
Eluent, methylene chloride; external pressure on the membrane, 1.2 MPa; flow-rate, 20 cm^3/h

It is known that reversed-phase chromatography, using chemically bonded phases, is one of the most frequently chosen separation modes in HPLC. The usefulness of the technique arises from the development of suitable packings and the wide variety of eluting solvents. Because of the complexity of the retention mechanism, it is possible to separate, simultaneously, compounds of a wide polarity range with excellent resolution and short retention times. The outstanding pro-

perties of chemically bonded phases on various TLC plates were used in OPTLC for the separation of dimedone adducts of aliphatic aldehydes. Owing to the substantially shorter separation time and stable flow rate in the RP-OPTLC, the resolution values obtained on reversed-phase plate were also good in the case of longer solvent migration distances

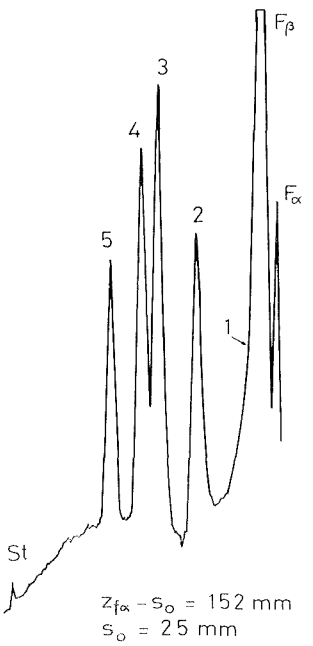

Fig. 6

Separation of dimedone adducts of formaldehyde and other aliphatic aldehydes on SILREVPRES N experimental chromatoplate Eluent, acetonitrile - 0.005 M KH_2PO_4 /4:6,V/V/; s_o=start distance, 25 mm; F_α = α -front; F_β = β -front; Z_f = front distance
OPTON-ZEISS PM Q III chromatogram spectrophotometer; , 264 nm; slit width, 7.0x0.26 mm; chart speed, 10 cm/min; 500-500 ng/substance 1 dimedone; 2 acetoldomedone; 3 formaldomedone; 4 propionaldomedone; 5 butiraldomedone

so that the spots of the substances separated were suitable for quantitative evaluation /Fig. 6/. It follows from these results that RP-OPTLC together with quantitative evaluation may be a useful tool in routine analysis.

REFERENCES

1. L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley-Interscience, New York, 1974.

2. Cs. Horváth and V. Melander, J. Chromatogr. Sci. 15 393 1977.

3. A. Zlatkis and R.E. Kaiser /Editors/, High-Performance Thin-Layer Chromatography, Elsevier, Amsterdam, 1977.

4. E. Tyihák and G. Held, in A. Niederwieser and G. Pataki /Editors/, Progress in TLC and Related Methods, Vol. II. Ann Arbor Sci. Publ., Ann Arbor, Mich., 1971.

5. E. Tyihák, H. Kalász, L. Kulacsy, J. Nagy, F. Körmendi, J. Knoll, E. Mincsovics and A. Gulyás, Process of overpressured thin-layer chromatography. Hung. Pat. 173.749, 1975-1979.

6. E. Tyihák,H. Kalász, E. Mincsovics and J. Nagy, Proc. 17th Hung. Annu. Meet. Biochem., Kecskemét, 1977.

7. E. Tyihák, E. Mincsovics and H. Kalász, J. Chromatogr. 174, 75 1979 .

8. H. Kalász, J. Nagy, E. Mincsovics and E. Tyihák, J. Liquid Chrom. 3, 845 1980.

9. E. Stahl /Editor/: Thin-Layer Chromatogrpahy, Springer-Verlag, New York, 2nd ed., 1969.

10. J.G. Kirchner, Thin-Layser Chromatography, Wiley, New York, 2nd ed., 1978.

11. R.E. Kaiser /Editor/, Einführung in die Hochleistungs-Dünnschicht-Chromatographie, Institut für Chromatographie, Bad Dürkheim, 1976.

12. W. Bertsch, S. Hara, R.E. Kaiser and A. Zlatkis /Editors/, Instrumental HPTLC, Hüthig-Verlag, Heidelberg, Basel, New York, 1980.

13. H. Engelhardt, Hochdruck-Flüssigkeits-Chromatographie, Springer, Berlin, Heidelberg, New York, 1975.

14. E. Tyihák, E. Mincsovics, H. Kalász and J. Nagy, J. Chromatogr. 211, 45 1981.

15. H. J.Issaq, J. Liquid Chrom. 3 753 1980.

16. R.E. Kaiser, HRC and CC 1 164 1978.

17. F. Geiss, Die Parameter der Dünnschicht-Chromatographie, Vieweg, Braunschweig, 1972.

18. G. Guiochon and A. Siouffi, J. Chromatogr. Sci. 16 470 1978.

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TWO PHASE, TWO-DIMENSIONAL TLC FOR FINGERPRINTING AND CONFIRMATION PROCEDURES

Thomas E. Beesley and Elaine Heilweil

Whatman Chemical Separation Inc., 9 Bridewell Place, Clifton, New Jersey 07014, USA

Two-dimensional /2D/ chromatography was first described by Kirchner in 1951. A sample applied to one corner of a 20 x 20cm plate approximately 1.5cm from each edge is developed in a mobile phase that resolves the polar constituents. It is then dried, rotated 90 degrees to the first direction and placed in a second mobile phase designed to resolve the more non-polar components. In this manner, a multitude of diverse compounds can be resolved.

The versatility of an open surface to separate a variety of complex samples gained momentum and in

*Presented at the First Symposium on Advances of TLC and HPLC, May 14-15, 1982, Szeged, Hungary.

1962 Stahl's Handbook on TLC referred to this method as the SRS technique and noted its ability to characterize changes in a given matrix after environmental exposure to Gamma rays, X rays, U.V. light, gases or heat. By 1967, Kirchner's Volume XII of Techniques of Organic Chemistry on TLC cited 77 references on two-dimensional TLC.

The technique further diversified to the use of dual media combinations. Strips of magnesium silicate with silicic acid were used on Bergamot oil, carbon and silica gel on ketones, and quite common was the use of parafin, silicone oil or undecane impregnated in the layer after the first development to create a reversed phase partition mode for the second direction. A wide variety of compounds were separated by these techniques from simple antioxidants in motor oil to serum proteins all dealing with complex matrices.

In studying these works and the comments of Stahl, it is apparent that not only can individual compounds be identified, but that the pattern of developed spots in a 2D development can become a fingerprint of the sources and further, that changes within the source can be identified as normal or abnormal, based on that pattern. This technique has been referred to as fingerprinting. Literature references on the identification of oil sources from ocean oil spills are now quite common.

Developing procedures for this method can prove quite harrowing, however, especially given the crude or complex nature of the starting sample. Samples often streak in the second dimension if composition differences are great. Reproducibility is poor under the best of conditions.

Recently it has been demonstrated that reliable procedures can be developed for fingerprinting emploxing a TLC plate which is composed of a three centimeter octadecyl bonded silica gel strip for reversed phase chromatography in one dimension and the remainder of the plate composed of a standard silica gel with high capacity for adsorption chromatography in the second dimension. Whatman refers to this combination as the Multi-K CS5 plate. The uniqueness of the combination resides in the ability to chromatograph without transfer, a complex mixture by both a non-polar mechanism and by the usual adsorption mechanism that deals with the compound's polarity differences.

The sample is applied to the reversed phase strip of the plate at a point 1.5cm from the edges and developed in the reversed phase partition mode, utilizing polar solvents. A major benefit at this point is that when working with crude samples, the usual interfering polar mass migrates to the solvent front, allowing a much cleaner analysis in the adsorption mode. Several operational points must be made at this stage.

1. The entire layer should be prewashed when dealing with these complex mixtures. This is accomplished by sandwiching several plates together with paper wicks at the end so all elutable material will migrate to the paper. Methanol/Acetone /50/50/ is best suited as a solvent for this task. Several plates may be washed simultaneously overnight and then activated at 110°C for one hour. Plates should then be stored in a dessicator so as not to readsorb environmental contaminants.

2. Chambers should be equilibrated for 10 minutes with paper pads for all developments.

3. To obtain an even solvent front in the first direction, scrape sufficient silica gel off the plate so that it is not immersed in the first mobile phase. An alternative to this is to spray the silica gel lightly with ethanol /masking the reversed phase strip/. In this method, the objective is to deactivate the silica gel sufficiently for the solvent front to migrate uniformly /Fig. 1 /.

4. Finally, the reversed phase strip, because of its binding requirements, must contain 0.5 M sodium chloride or equivalent buffer salt when mobile phases are used that contain in excess of 40% water. Ammonium acetate has also been used successfully, which of course, completely volatizes on drying the plate at 110°C for 10 minutes.

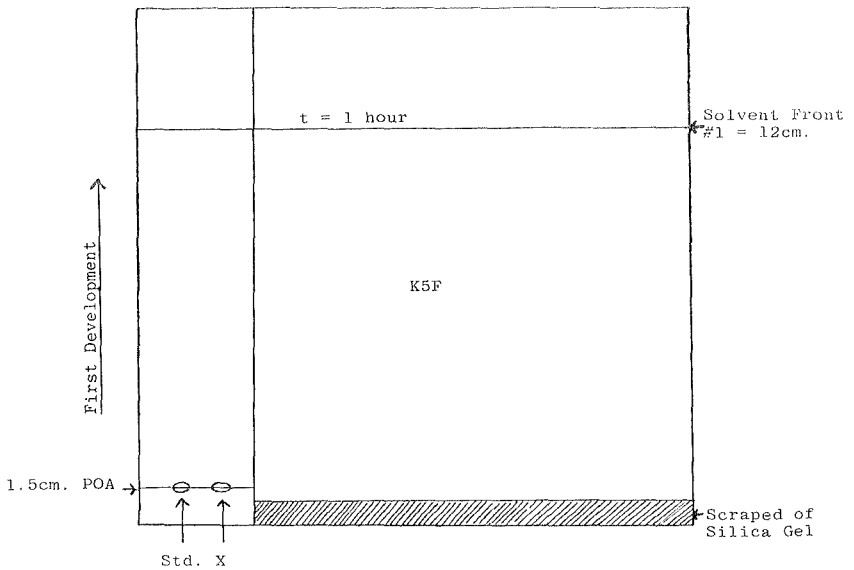

FIGURE 1

Separation in the second direction, i.e., the adsorption mode, on the CS5 plate, generally utilizes solvents of lower polarity to further resolve the components of the mixture. Typical K5F silica gel separations are obtained. A key feature at this point is the ability of these types of mobile phases to admix standards into the unknown while in the reversed phase strip to confirm identity /Fig. 2 /.

A known reference standard is, in effect, co-chromatographed in the second dimension. Admixing is commonly used in the adsorption mode by placing a known standard over the unknown mixture and co-chroma-

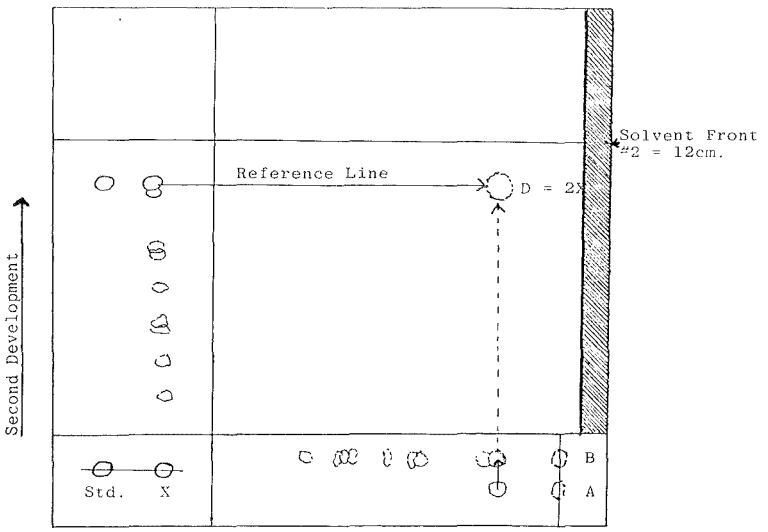

FIGURE 2
A moves to B and migrates as D with an increase in intensity = A + B

tographing. If the known and the unknown do not indicate separation after developing in the two directions, confirmation of the unknown's identity is assumed. Combining these two widely differing separation mechanisms, reversed phase partition chromatography in the first dimension, with adsorption chromatography in the second dimension, increases the reliability of this technique.

An example of this methodology could be the confirmed presence of morphine in a suspected urine specimen. A urine sample was extracted in the usual manner and 10 ul of extract was applied to the reversed phase strip with a known quantity of standard analyte /Morphine/ applied

next to it. It was developed up the reversed phase strip in Methanol /0.5 M Sodium Chloride solution /65/35/. After drying at 65°C for 20 minutes, the plate was again spotted with extract and standard at 90 degrees to the first direction and developed in the second mobile phase of Ethyl Acetate/Methanol/Water/28% Ammonium Hydroxide /85/13.5/1.0/0.5/ to 12 cm. After detection with methanolic Iodoplatinate reagent, the original suspected spot showed a greater intensity, indicating co-chromatography and confirmed identity.

Another example in a more difficult area for toxicology would be methadone confirmation.

The most intriguing application of this technique is in the area of fingerprinting. An example is lubricating oils taken from three different sources. The crude samples were extracted by shaking with 0.5% Acetic Acid in Methanol in equal volumes for 5 minutes and centrifuging to obtain a clear supernatant. Ten microliters of this supernatant were applied to the reversed phase and developed in 18 minutes to the 12 cm level in Acetonitrile/Water/Acetic Acid /90/10/1/, dried for 20 minutes at 65°C and then developed in the second direction to 12 cm /22 minutes/ in Hexanes/Chloroform /80/20/. After drying the plate, it was reacted by dipping in a 5% solution of Phosphomolybdic Acid and heated for 5 minutes at 120°C. Characteristic and reproducible spot patterns

/fingerprints/ were obtained which may be used in screening to denote changes in composition and/or to identify additives and therefore, possible sources.

Of particular note is the ability to develop mirror images by spotting the reversed phase strip at either end and developing toward the center line in the first mobile phase. Comparisons are made more easy /Fig. 3 /.

Secondly, known standards may be spotted at the outside edges in the second mobile phase development to establish identity. It is then possible to go back to scheme one to establish confirmation.

Coefficients of Variation for these oil patterns ran from a low of 4% for one spot to a high of 12%. It appears that some compounds are more variable and under some unknown influence contributing to this variation more than others.

A more recent example of the use of this technique is the identification and quantitation of several targeted sulfonamides in the muscle and liver tissue of turkeys and chickens. There are thirteen known sulfonamides currently in use. Of these, five are suspected carcinogens and levels have been set by the United States Department of Agriculture for their presence in meat products. They are often used in combination and therefore assays had to be developed to quantitate each of the targeted sulfonamides.

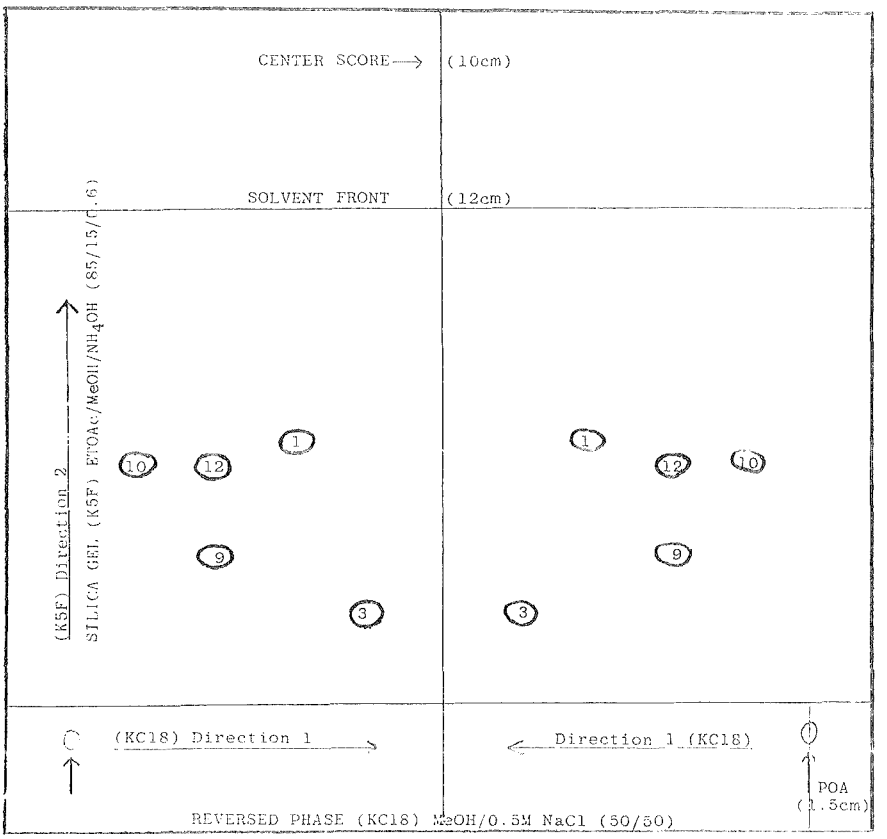

FIGURE 3
SEPARATION OF FIVE TARGET SULFONAMIDS

1. Sulfamethazine
3. Sulfathiazole
9. Sulfaquinoxaline
10. Sulfabromomethazine
12. Sulfadimethoxine

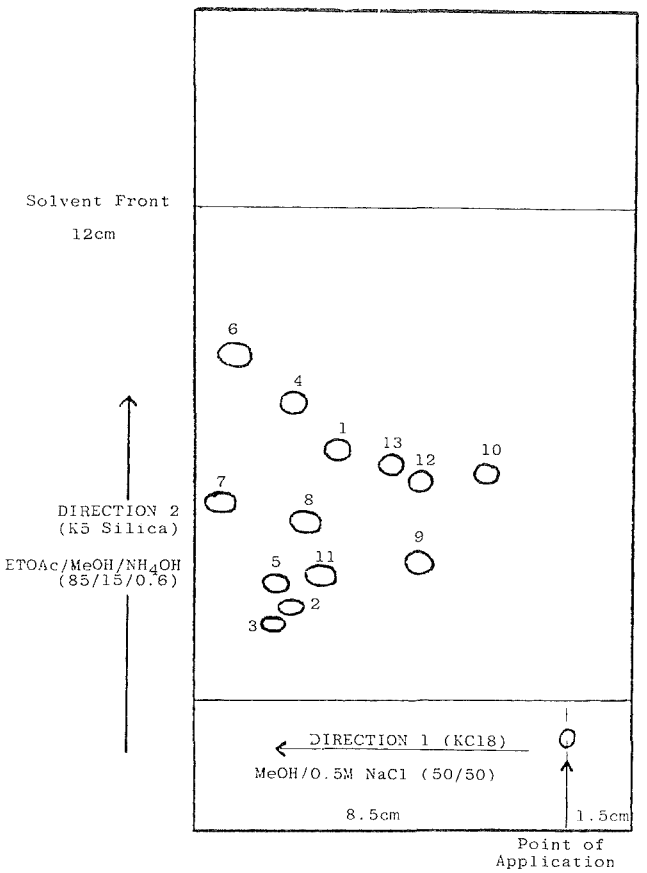

FIGURE 4.

1. Sulfamethazine
2. Sulfisoxazole
3. Sulfathiazole
4. Sulfapyridine
5. Sulfadiazine
6. Sulfanilamide
7. Sulfaguanidine
8. Sulfamerazine.
9. Sulfaquinoxaline
10. Sulfabromomethazine
11. Sulfachlorpyridazine
12. Sulfadimethoxine
13. Sulfaethoxypyridazine

Standards of the thirteen sulfonamides separated from the matrix indicated a very clean and clear picture. A transparency can be prepared therefore, that when overlayed on a developed and visualized chromatogram, unknowns may be quickly identified. Both liver and muscle tissue responded identically /Fig. 4/.

An internal standard curve is recommended for quantitating. This can be accomplished by spotting two

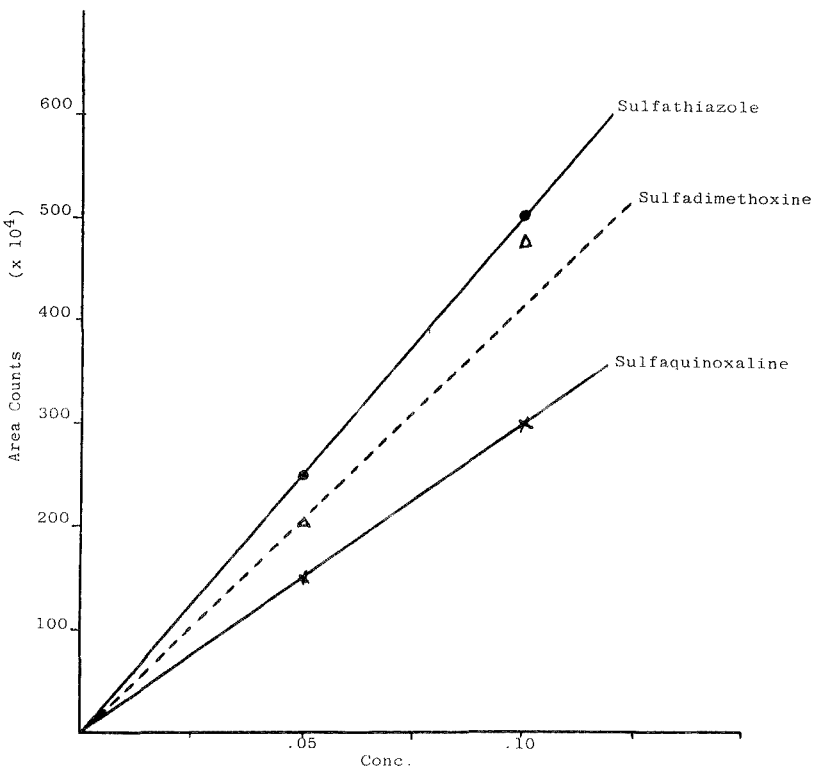

FIGURE 5

dilutions of chosen standards at the outside edges in the second development. From a scan of these two samples, a deviation from the standard curve can be established and a correction computed /Fig. 5/.

For the sulfonamide assay, standard deviations of 0.005 to 0.29 were computed, giving a coefficient of variation of 1-8%.

The two dimensional, two phase separation technique can be a reliable method for fingerprinting complex mixtures in crude extracts and perhaps,more importantly, as an identification method for solutes by comparison to the migration of authentic reference compounds in this two stage chromatography method.

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A NEW DETECTOR SYSTEM FOR CONTINUOUS-FLOW TLC

A. S. Bochkov
Institute of Organo-Element Compounds
Academy of Sciences
Moscow 117813, U.S.S.R.

SUMMARY

A new detector system for continuous-flow TLC is described that provides possibilities both for optical and for electrochemical measurements with low detection limits.

1. Introduction

The advantages of the continuous-flow thin-layer chromatography /CFTLC/ over the conventional TLC methods are higher speed and separation power. The benefits of CFTLC first came to the attention of a wide audience in 1955 when M. Mottier and M. Potterat /1, 2/ published their well known work. The onset of the modern CFTLC equipment goes back to the mid-1960s when E. Stahl /3/ introduced a convenient TLC chamber. This was followed by important works of M. Brenner and A. Niederwieser /4,5/, S. Hara /6/, F. Geiss and F. Schlitt /7/, E.V. Truter /8/, L.M. Libbey and E.A. Day /9/ and J.H. VanDijk /10/. The possibilities of CFTLC are now routinely available with

* Presented at the First Symposium on Advances of TLC and HPLC, May 14-15, 1982, Szeged, Hungary.

commercial equipment of the firms Camag /11/, Desaga /12/ and The Dime's Group, Inc /13/.

Since the selection of a mobile phase of adequate strength and selectivity is a major task is designing LSC separations, anything that simplifies this task will prove helpful. The right solvent can be chosen using, directly, the LC unit, but this is often a tedious, time consuming procedure. A much easier and faster one is to use TLC in the initial scouting for an appropriate mobile phase /14/. Under similar conditions separation by LSC and TLC proceed by the same retention mechanism, and R_f values in TLC can be used to predict k' values in LSC /same adsorbent and mobile phase/ by relationship $k' = /1-R_f//R_f$. However, there are several reports of the poor correlation between the TLC and LSC data. This can be accounted for by solvent demixing, variations in the adsorbent and adsorbent water content and by solvent concentration gradient in TLC. These difficulties are minimized in CFTLC as it is shown by H. Schmid, J. Cramer and H. Arm /15/. Their experimental installation is like a column chromatograph equipped with a thermostat , solvent system, sample injector for "wet dosing", chamber for evaporation of eluate, thin layer plate with glass support subdivided into two symmetrical parts of adsorbent - sample and reference. The detection takes place directly on the thin layer foil during the separation of sample components. The optical detector has a maximum of sensitivity at 550 nm /transmission/. The detection limit is about 10^{-9} g.

2. Experimental

Merck TLC Aluminium Sheets, Plastic Sheets, HPTLC pre-coated glass plates and home made plates /Silica gel 60, Merk/ without any binder. The experimental installation is similar to /15/ and includes solvent pumping system with controlled composition of the mobile phase during the development process. The electrochemical detection system is analogous to that described in ref. /16/. On plates with the glass support a small region of adsorbent layer is removed preliminarily and the light from fibre optic detection system passes through such optical tranparent region /17/ /Fig. 1/.

In the support of Aluminium and plastic TLC sheets apertures were made. If the support has a smooth surface, it is possible to utilize the reflectance detection system, such as described in ref. /17/. The diameter of the region without adsorbent and the aperture does not exceed 1-3 mm.

3. Results and Discussion

Excellent systems for CFTLC with the transparent optical region are HPTLC pre-coated plates from Merk, which compare favourably with well filled HPLC columns fith respect to the quality of packing /N-3450 for migration distance Z_f =60 mm/. With these plates the transparent optical region of any shape and size can be easily obtained. The efficiency of the plates prepared in the laboratory /layer thickness 0.4mm/ reaches N=700 for Sudan /Z_f=100 mm, Fig.2/.

Direct comparison of plate performance may present difficulties because of the variations in the quality of

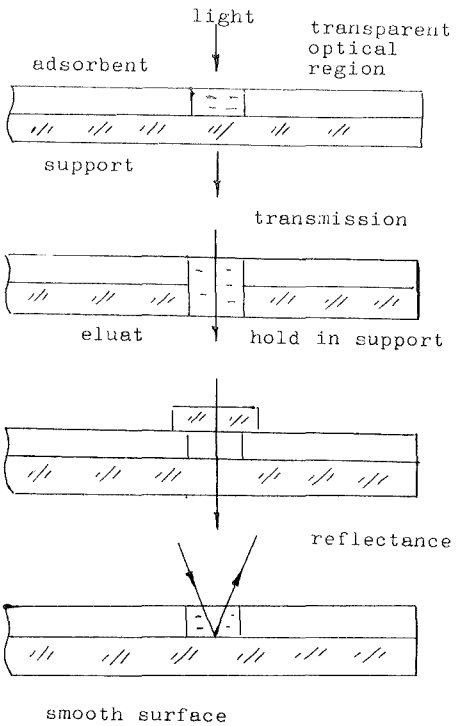

FIGURE 1

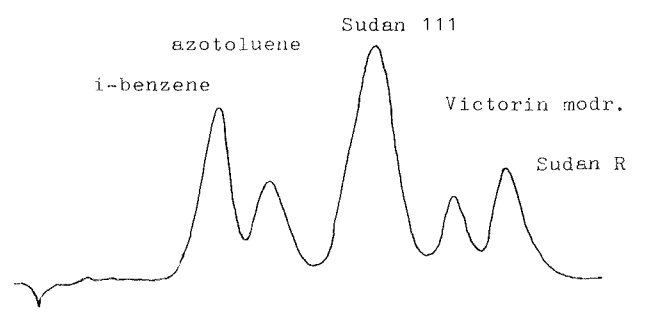

FIGURE 2

TLC plates and extra plate effects /e.g. sample injection, evaporation of the eluate, etc./.

In the same experimental conditions/transmission light/ the detection limit becomes /10 times/ lower for TLC plates in the laboratory than for precoated HPTLC plates /1,5--3 times/.

There are some problems in the reflectance detection due to specific properties of support, larger area of the transparent optical region, etc.

All the experiments were made on a linear chromatographic system, but the circular thin-layer and over-pressured thin-layer chromatography may be used as well /19-22/.

REFERENCES

1. Mottier M., Potterat M., Anal. Chim. Acta, 13, 46 /1955/.
2. Mottier M., Potterat M., Mitt. Lebensm. Hyg., 49, 454 /1958/.
3. Stahl E., Pat. N 1498678 /BRD/, 1962.
4. Brenner M., Niederwiesser A., Experientia, 17, 23 /1961/.
5. Brenner M., Niederwiesser A., Pat. N450005 /Switzerland/, 1963.
6. Shoji Hara, Pat. N. 1264234 /Great Britain/ , 1972.
7. Euroatom, Pat. N 442804 /Switzerland/, 1964.
8. Truter E., J. Chromatogr., 14, 57 /1964/.
9. Libbey L., Day E., J. Chromatogr., 14, 273 /1964/.
10. VanDijk J.H., Pat. N. 1262493 /Great Britain/, 1972.
11. Cataloge TL-8-E, Camag, Muttenz, Switzerland.
12. Desaga Catalogue, Desaga, Heidelberg, BRD.
13. The Dime's Group, Inc., Palo Alto, California, U.S.A.

14. Siouffi A.M., Guillemonat A., Guiochon G., J. Chromatogr., 137, 35 /1977/; "Regis Chromatography Catalog", Regis Chemical, II., U.S.A.

15. Schid H.U., Cramer I., Arm H., Chimia, 30, 62 /1972/.

16. DiStefano V., Marini P., Offenlegungsschrift BDR N2141936, 1971.

17. Berezkin V.G., Bochkov A.S., Inventor's Certificate N 752165, 1977; Bulletin "Otkrytiya, izobreteniya, promyshlennye obraztsy, tovarnye znaki", 28, 172 /1980/ /Russian/.

18. Kontes Densitometer- Prospect "Kontes", Viveland, N.J., U.S.A.

19. Berezkin V.G., Bochkov A.S., Quantitative Thin-Layer Chromatography, "Nauka", Moscow, 1980, p. 26 /Russian/.

20. Tyihak E., Mincsovics E., Kalasz H., J. Chromatogr., 174, 75 /1979/.

21. Gocan S., Studia Univ. Babes-Bolyai, Chemia, 1, 12 /1977/.

22. Warwzynowicz T., Soczewinski E., J. Chromatogr., 169, 191 /1978/.

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HIGH PERFORMANCE THIN-FILM CHROMATOGRAPHY IN BIOLOGICAL STUDIES

L.V. Andreev

Institute of Biochemistry and Physiology
of Microorganisms
Academy of Sciences
Pushchino, Moscow Region, 142292, U.S.S.R.

The conventional thin-layer chromatography /TLC/ method of Egon Stahl had not been significantly modified for a long time. But lately much attention was paid to the optimization of the rapidity and sensitivity of the method. The advances in column, gas and liquid chromatography very much contributed to it. In TLC, like in column chromatography, the use of very fine and homogeneous sorbent fractions was very promising. It appeared especially efficient in liquid chromatography where diffusion coefficients are low.

* Presented at the First Symposium on Advances of TLC and HPLC, May 14-15, 1982, Szeged, Hungary.

The use of fine sorbent fractions resulted in the drastic increase of the resolution power and decrease of the analysis duration as well as in 3-4 times shortening of the layer length. For the analytical resolution the layer about 100 /um in thickness /like in conventional TLC/ was used. That brought some researchers to the conclusion that the optimal diameter of sorbent particles was 5-7 /um and that the use of finer sorbent particles should decrease the resolution efficiency. We have shown /1-5/ that the use of still finer sorbent fractions coupled with the appropriate reduction of a layer thickness and the utilized length results in the constant increase of the resolution efficiency, rapidity and sensitivity. However, the sorbent layer area should be large enough not to require the use of complex microanalytical devices. Besides, for the full realization of the efficiency one needs further and further minimization of a starting spot size.

It has been shown that the optimal layer thickness is from 10 to 15 /um, its optimal length is 2-3 cm, and the optimal mean diameter of sorbent particles is 1-2 /um. Layers prepared on microscope glasses, 1x3 inches, are very handy. Using such a plate it is possible to obtain 2 two-dimensional and 2 or 3 unidimensional chromatograms simultaneously.

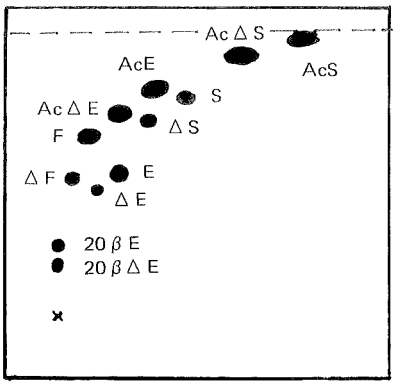

Fig.1. Two-dimensional chromatogram of steroids obtained on silica gel layer 1.5x 1.5 cm

The resolution power is perfectly illustrated by a two-dimensional chromatogram of steroids on a 1.5x1.5 cm silica gel layer /Fig. 1/.

The major problems in the work with ultra-fine sorbent fractions is the necessity of keeping the definite balance 1/ between the interaction of sorbent particles and a glass plate and 2/ between the particles themselves, because of the higher value of free surface energy. Therefore, a precise quantity of sorbent applied per a unit of a layer is a must. For different sorts of silica gel and its fractions the optimal quantity of sorbent varies within the range from

0.8 to 1.2 mg per cm^2 of support material which is by an order of magnitude less than for MERK HPTLC plates and by 1.5 order of magnitude less than for conventional TLC plates. Due to the higher surface energy of ultra-fine sorbent fractions the layers may be prepared without a binder or adding 1-2% of gypsum. The mechanical solidity of the layer is perfect. The disregard of the optimal conditions results in specific defects, e.g. regularly alternating sorbent "waves", "Liesegang ring"-like defects, rents, etc. Attention should be paid to the possible partial aggregation of particles which occurs upon the long-time storage of fractions, evet wet. Layers are formed mainly around such aggregations which makes the plates unfit for work /"speckled" plates/. To avoid this, a short-time ultrasound treatment of fractions prior to the preparation of layers is recommended /11/. The preparation of layers under optimal conditions is an extremely simple and highly efficient process. Coating of several hundreds of plates which will retain their resolution power for at least 8 years takes an assistant a day.

Based on the above-mentioned characteristics, such sorbent layers could rather be named "sorbent films". Therefore, the term "high performance thin-film chromatograohy" /HPTFC/ seems justified for the method of analysis on thus prepared sorbent.

It is convenient to prepare sorbents for HPTFC by method of sedimentation in appropriate solvents, e.g. in methanol - chloroform mixture for alumina /4/ and in water for silica gel /6/. For example Kieselgel 60 MERK is ground in a mill and sedimented in water in cylinders, 25 cm in hight. Fractions sedimented within the intervals of 3-6, 6-12, 12-24 and 24-48 hours are taken. The finer particles, usually making up 1-2% of the initial amount of Kieselgel, are removed. The coarser particles are ground again. Usually 4 final fractions make up 5%. For example, the grinding and fractionation of 940 g Kieselgel 60 MERK for preparative TLC yielded 42.4 g final fractions. The 1^{st} fraction /3-6 h/ was enough for preparing 335 plates for HPTFC using microscope glass as a support material, the 2^{nd} fraction, for 733 plates; the 3^{rd} for 830; and the 4^{th}, for 487 plates. Such a number of plates is adequate for obtaining more than 5000 two-dimensional chromatograms. With conventional TLC technique, 10000 times more sorbent would be spent. For the preparation of HPTFC plates carefully washed glasses are submerged into 1 ml suspension containing the necessary quantity of the sorbent and previously treated with ultrasound and then dried for a night.

HPTFC does not require special equipment. Plates are developed in glass chambers previously described /2/. Zones are detected by spraying using a usual spray.

Starting spots are applied by thin glass or metal capillaries. An analysis on 2 cm layer is usually done for the time from 2 to 10 min. In some cases HPTFC method yields chromatographic zones with diameters of several tenths of a millimeter. A layer thickness being 10-15 /um, the increase of sensitivity by 1-1.5 order of magnitude compared with conventional TLC is possible. For example, when analysing pyruvic acid in the form of quinoxalones we achieved the sensitivity of several 10^{-12} moles. After the resolution the plates were sprayed with 10% water solution of phosphoric acid, and zones of quinoxalones were visualized by intensive yellow-green luminescence. The technique may be used successfully for the group analysis of -ketoacids /7-10/- the major intermediates of the tricarboxylic acid cycle.

A high sensitivity of HPTFC allows the identification of components of complex mixtures chromatographed on conventional TLC layers. In a previous work /11/ a mixture of several dozens of substances - products of hydrocortisone microbial transformation was separated by a two-dimensional technique using "Silufol" plates. Some steroid zones were eluated from a layer and then identified by HPTFC method on alumina and silica gel layers in various solvent systems.

Mechanically solid films prepared of ultra-fine sorbent fractions may be successfully used for some

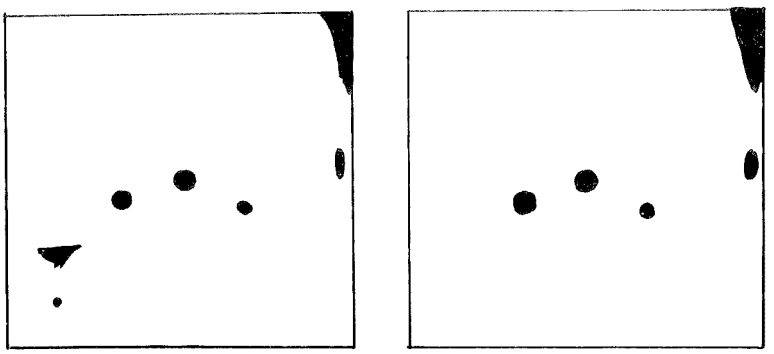

Fig.2. HPTFC of phospholipids of yeast mitochondria.
I - the extraction on a layer,
II- the analysis of previously extracted mitochondria.
Silica gel layer 2x2 cm.

analyses hardly done by conventional TLC method. Figure 2 presents chromatograms of phospholipids of mitochondria of the yeasts Endomyces magnusii, obtained by 2 different ways. In the 1st case water suspension of mitochondria was applied to a silica gel layer and then twice extracted on the layer by a two-fold development of the plate for a length of 3-4 mm with chloroform-methanol mixture /1:1/. In the 2nd case mitochondria were previously extracted according to Folch. Though both results were analogous, the 1st procedure is convenient for operating with microgram quantities of biological material.

A high analysis rate of HPTFC method makes it efficient for the regulation of high rate chemical pro-

cesses. It allows the control of the completeness of the reaction producing the derivatives for gas chromatography, e.g. fatty acid methylation or fatty hydroxyacid acetylation. In many cases the method, properly instrumentalized for the quantitative interpretation of thin-film chromatograms, is not inferior to gas chromatography in sensitivity and analysis rate.

Often the densitometry is not done immediately after obtaining a chromatogram and many substances are decomposed in air leading to misrepresentation of quantitative results. To do away with this inconveniency, the densitometry of negatives after photographing in visible or UV-light is recommended. This procedure was successfully used for the analysis of nucleosides and nitrous bases of nucleic acids /12-13/.

HPTFC method is an efficient analytical means, especially, for biochemical research. It solves the most of analytical problems in the studies of biological and environmental objects /5, 14, 15/.

REFERENCES

1. L.V. Andreev. Proceedings of the Symposium in memory of M.S. Tsvet, Abstracts /Russian/, Leningrad, 1972

2. L. Andreev, L.A. Golovleva, Z.I. Finkelstein, G.K. Skryabin. "Prikladnaya Biokhimiya i Mikrobiologiya" /Russian/, 1972, v. VIII, 1, 75-81.

3. L.V. Andreev, G.M. Daueva, L.A. Golovleva, N.V. Pechnikov. "Mikrobiologicheskaya Promyshlennost" /Russian/ 1971, 8, 20-23.

4. A. M. Bezborodov, L.V. Andreev, D.N. Chermenskii, T.A. Popova. "Prikladnaya Biokhimiya i Mikrobiologiya" /Russian/ 1971, v. VII, 5, 537-543.

5. L.V. Andreev, T.B. Tataryunas, V.N. Karnaukhov, In: "Biophysics of a Living Cell" /Russian/ Pushchino, 1971, 84-88.

6. L.V. Andreev, O.A. Evdokimova, USSR Inventor's Certificate N 607139, 20. IX. 1978 /Russian/

7. L.V. Andreev, G.P. Sapozhnikova, M.A. Rodionova "Prikladnaya Biokhimiya i Mikrobiologiya" /Russian/ 1974, v. 10, 6, 921-927.

8. L.V. Andreev, M.A. Rodionova, G.P. Sapozhnikova. In: "Mitochondria. Regulation of Oxidation and Conjugation" /Russian/ Moscow, "Nauka", 1974, 127-131.

9. N.M. Beletskaya, R.G. Rakhmankulova, L.V. Andreev. "Izvestiya Vyscykh Uchebnykh Zavedenij. Pishchevaja Technologiya" /Russian/ 1975, 2, 65-67.

10. N.M. Beletskaya, R.G. Rakhmankulova, L.V. Andreev. "Khlebopekarnaya i Konditerskaya Promyshlennost" /Russian/ 1976, 2, 15-17.

11. G.M. Mogilnitsky, K.A. Koshcheenko, L.V. Andreev "Mikrobiologiya", /Russian/ 1975, XLIV, 2, 351-356.

12. Ya. I. Buryanov, L.V. Andreev, N.V. Eroshina, O.F. Korsunsky, "Biokhimiya" /Russian/ 1974, v. 39, 1, 31-38.

13. A.M. Bezborodov, T.A. Popova, D.N. Chermenskii and L.V. Andreev "Folia Microbiol.", 1973, 18, 223-228.

14. L.V. Andreev, Z.I. Finkelstein, S.S. Belyaev, "Prikladnaya Biokhimiya i Mikrobiologiya" /Russian/ 1974, v. X, 2, 308-312.

15. L.V. Andreev, A.G. Kozlovsky, O.V. Kruglaya, A.M. Bezborodov "Mikrobiologiya" /Russian/ 1973, v. XLII, 3, 546-548.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(8), 1583-1595 (1982)

HIGH-SPEED VIDEO DENSITOMETRY: PRINCIPLE AND APPLICATIONS

S. Pongor

Institute of Enzymology, Hungarian Academy of Sciences
Budapest P.O.Box 7, Hungary
1507

In the past few years, TV-type multichannel detectors /Vidicon-, Plumbicon- and Orthicon tubes etc./ found application in several fields of analytical chemistry /1/. Recently, video-technique was introduced to the densitometric evaluation of thin-layer chromatograms as well /2/; the term 'video densitometry' is used in this context.

TV-type multichanel detectors reveal several properties unusual to commercially available densitometers. The main difference originates from the working principle: the scanning is carried out electronically rather than by mechanical movement of the specimen and/or light source. The scanning is very fast and its geometry is different from those used in traditional densitometers. These new

* Presented at the First Symposium on Advances of TLC and HPLC, May 14-15, 1982, Szeged, Hungary.

features called for novel technical solutions in instrumental design; on the other hand they allowed to adapt densitometric evaluation to new application fields. The present report deals with theoretical and practical aspects of using TV-type detectors in densitometry, with special regard to novel features and new possibilities of application.

General aspects

The working principle of TV-type detectors can be shortly described as follows: the image of the specimen /chromatographic spot/ is projected to a target-plate which is a two-dimensional array of unit detectors continuously scanned by an electron beam. The unit detectors behave like capacitors whose charge is proportional to incident light intensity and that are periodically discharged by the electron beam /Fig. 1/. Optical magnification /d/w using the terms of Fig. 1/ can be selected in a wide range according to the sizes of the chromatographic specimen. At 'usual' magnification /when a 200X200 mm chromatoplate fills the field of vision of the camera/, d/w is about 0.05 and several hundreds of unit detectors take part in the measurement of a single chromatographic spot or band.

For the purposes of mathematical treatment, the scanning scheme of video-densitometry can be considered as a stepwise two-dimensional scanning. The signal dis-

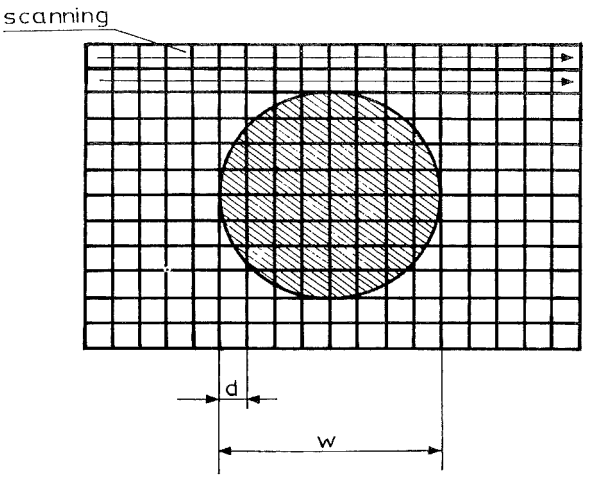

FIGURE 1

tortion of the spot density integral will thus contain two terms: the first one - common to all scanning geometries- originates from finite detector size /3/, the second one is a result of the stepwise nature of the scanning. According to our results /4/, in the case of an 'average chromatographic spot' /w = 5 mm, peak height = 0.5 absorbance/ the over-all signal distortion is below 1% of the spot density integral, and in the 'useful range' of quantitating a given component, it is nearly independent from spot-size data, indicating that the new scanning geometry is not a source of non-linearity.

Spectral sensitivity of Vidicon-type detectors allows quantitative determination in the visible range, extending the range to UV would need special detector de-

sign /quartz face plate and camera optics/. Detection limit for amino acids stained with ninhydrin lies in the nanomolar range /2-5 nanomoles depending on layer quality and spot shape/, as measured with a commercially available Vidicon tube /NOR-1" 2255 Heiman/. A further characteristic feature of Vidicone-type detectors as compared to conventional ones is the variance of detector response along the field of vision. This fenomenon originates partly from inhomogenities of the target plate and partly from that of illumination, and can be corrected for by shading correctors as used in video-techniques. Finally, it should be noted that the output signal of the TV-camera /the so called video-signal/ is too fast to be integrated by an ordinary integrator like those used in liquid and gas chromatography. On the other hand, combined with a high-speed integrator and data processing, TV-type detectors can drastically reduce measurement time as compared to conventional densitometers.

Application

Although the ide of building a Vidicon based densitometer dates back to the late sixties /5/, the first commercially available video densitometer /6/ /Telechrom OE 976, Chinoin-Budapest/ appeared only in 1976. The schematic diagram of this instrument is shown in Fig. 2. The instrument works according to a quasi double-beam

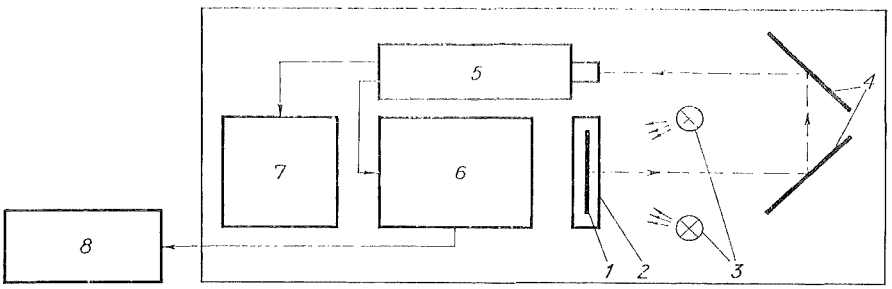

FIGURE 2

principle: the background intensity is sampled in each line of the scanning.

The new version of the instrument /Fig. 3/ can be used both in reflectance and in transmittance mode and

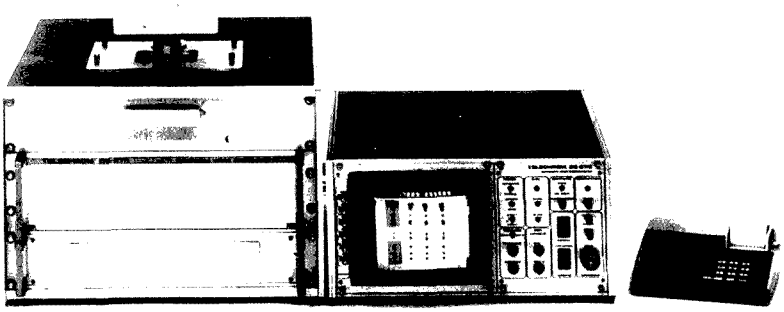

FIGURE 3

is equipped with UV light-source for fluorescence measurements. The control unit of the instrument fulfills two funktions. In contains the high speed integrator working according to a digital procedure /6/ that can be regarded as a three-dimensional extension of the area-measurement used in image analysis. The integrator is adapted to perform two integrations concomittantly, one of which is the component in question, the other one can be the total /coloured/ material of the sample or an internal standard. Another function of the control unit is the selection of the area of measurement. This is carried out by the operator with the aid of the monitor. Fig. 4 shows a schematic picture of the moni-

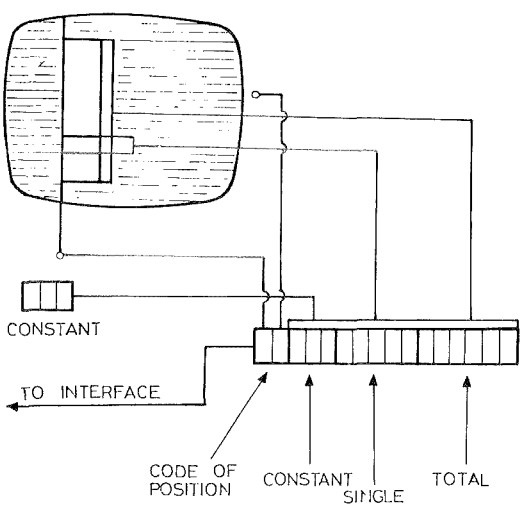

FIGURE 4

tor during measurement, indicating the flow-chart of the data-transfer.

The measurement is carried out either in manual or in automatic mode according to a pre-set program. Geometrically uniform chromatograms can be evaluated automatically, with small readjustments of the measurement program. The measured data are displayed on the monitor and also transferred to an on-line connected programmable calculator /Hewlett-Packard HP 97/. The calculator can be programmed to carry out the simpler calculations directly connected with the measurement, such as calibration curve fitting /reading from curve/, the use of internal and external standards, calculation and concentration per cents, etc. /7/. It is interesting to note that a densitogram is not required for the measurement. However, it can be displayed on the monitor and also on an optional recorder. As can be seen, the Telechrom video densitometer is adapted to large scale routine work and is especially suited to the direct determination of concentration per cents and component ratios. These properties made it possible to apply TLC methods in new fields of application as shortly summarized below. It should be mentioned finally, that, in addition to the hardware approach described above, the video signal may be processed also by software means to yield quantitative information. The fully computerized way is especially attractive since it may include all phases of signal processing /such as shading correction, spot selection and

integration, data processing etc./, with a substantial decrease in operator time.

Determination of essential amino acids in plant proteins

The concentration of the nutritionally limiting amino acid /usually lysine, tryptophan or methionine/ is a direct index of the nutritional quality and is used as a coarse ranking indicator in plant breeding programmes. Ion-exchange TLC on chromatosheets precoated with strong cation exchange resin /8/ makes it possible to separate each common /protein bound/ amino acid with a one-dimensional run. A few μl of plant hydrolysate is chromatographed according to this technique /using different sodium citrate buffers/, the chromatograms stained with ninhydrin are subsequently used for quantitation by videodensitometry. The concentration is expressed in per cent of the total amino acid content of the sample which is determined from the same chromatogram. Fig. 5 shows the determination of lysine in wheat. The spot density integral of lysine /L/ and that of the total amino acid content /T/ are linearily related to the sample size. However, their ration /the percentage of lysine in the total amino acid content/ is independent of the sample size within a broad range. Consequently, accurate weighing and sample application are not necessary provided that the amount of sample per spot is within this range. All this renders sample preparation simple and productive which is one of

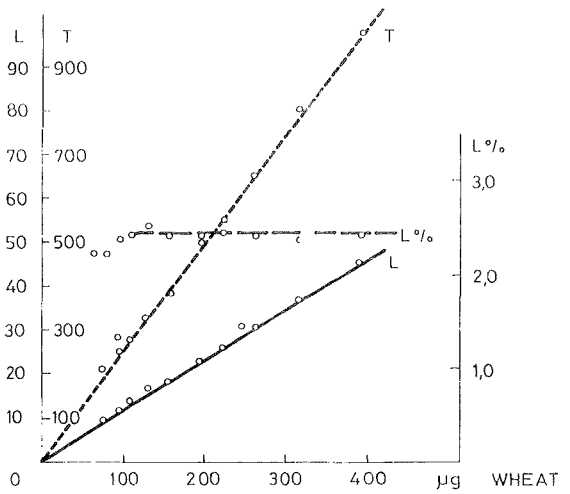

FIGURE 5

the ultimate reasons why the method can be applied economically in large scale screening programs. The video densitometric method showed good correlation with the amino acid analyzer, a comparison is shown in Fig. 6. The reproducibility of the method is characterized by a coefficient of variation of about 3-4%, including chromatography and hydrolysis /reproducibility of the densitometric measurement is below 1% c.v./.

The method has been applied in screening for lysine /2/ in cereals /barley /9/, wheat /10/, maize /13/, rye and sorgum /9/ / by different laboratories. The daily output was usually 300-800 analyses. Finally, a practical result should be mentioned: at the laboratory of the International Atomic Energy Agency /FAO/IAEA Seiberdorf,

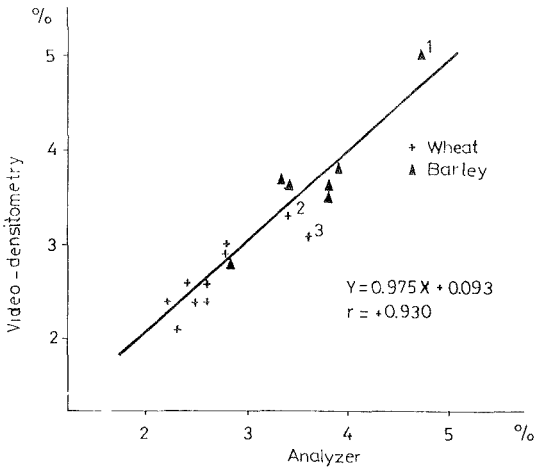

FIGURE 6

Austria/ about 16 000 wheat lines were analyzed in 1977 and a mutant was selected which contained about 30% more lysine than the control variety /9/.

The same system /ion-exchange TLC and video densitometry/ was used to determine biogenic amines in feed meals /16/ and diaminopimelic acid /17/.

Free amino acids in blood and in tissue samples

Amino acid metabolism disorders, such as phenylketonuria /phenylalaninaemia/, histidinaemia, lysinaemia, ornithinaemia, tyrosinaemia are manifested by an elevated level of one /or more/ of the above amino acids in the blood, curability of these diseases depends primarily on an early

diagnosis. Ion-exchange TLC in a pH = 4.2 ; Na^+ = 0.4 M sodium citrate buffer /14/ makes it possible to separate all of the above amino acids in one dimension, using 50-100 µl of blood dried on filter paper. Video-densitometry provides an adequately rapid quantitative answer and helps to identify even the mild forms of the above disorders, extending thereby significantly the scope of the screening.

A similar system was used to detect individual amino acid transforming enzymes in micro amounts of tissue samples /12/. Aliquots /a few µl/ were withdrawn from an enzyme reaction mixture and spotted directly to the chromatographic plate. The developped and stained chromatograms were analyzed by video-densitometry, the enzyme activity was calculated automatically from the slope of the progress curve. The sensitivity of this method offers new possibilities for clinical diagnostics.

Analysis of drugs

TLC technique is routinely used in many fields of the pharmaceutical industry. At the Chinoin Pharmaceutical Works, Budapest, TLC combined with video densitometry is used in the content uniformity test of multicomponent drugs /Pernovin, Amidazophen, Efedrin, ascorbic acid, Drotaverin etc./ /15/. Determination of D-Penicillamine in blood samples /11/ can be mentioned as an example of clinical application.

Although examples quoted in the present report - reflecting the author's scope of interest - con-

centrate mainly on amino acid analysis, we have to emphasize that video densitometry may be employed in most cases when general conditions of densitometric quantitation are fulfilled. TLC and other flat bed chromatographic techniques provided simple and efficient means for the separation of a wide range of substances and are ideally suited for large scale routine work. However, these techniques are now becoming neglected in many fields of chemical analysis for lack of an adequately productive quantitative method. We feel, that video densitometry may offer a viable alternative to solve a number of problems /such as the analysis of pesticides and food additives, screening for alkaloid producing plant lines etc./ where both the resolving power of a chromatographic technique and an adequately fast quantitative answer are equally important.

REFERENCES

1. Talmi, Y. /1975/: Analytical Chemistry, 47, 7. 658A

2. Dévényi, T. /1976/: Acta Biochim. Biophys. ASH 11, 1;

3. Goldman, J. and Goodall, R.R. /1968/: J. Chrom. 32 24;

4. Pongor, S., Candidat thesis, 1980

5. Hannig, K. and H. Wirth /1968/: Z. Anal. Chem. 243, 522;

6. Kerényi, Gy., T. Pataki and J. Dévényi /1976/: Hung. Patent No. 170-287

7. Pongor, S., A. Váradi, Gisela Hevesi and H. Glasschröder /1978/: Hung. Sci. Inst. 42, 1;

8. Dévényi, T., Hazai, I. Ferenczi, S. and J. Báti /1971/: Acta Biochim. Biophys. ASH 6, 385;

9. Sigurbjörnson, B., R.D. Brock and T. Hermelin /1978/: IAEA-SM 230 /Joint FAO/IAEA/GSP Program of Grain Protein Improvement/;

10. Belea, A. and F. Sági /1978/:Cereal. Res. Comm. 6, 15;

11. Pongor, S., J. Kovács, P. Kiss and T. Dévényi /1978/: Acta Biochim. Biophys. ASH 13, 123;

12. Elődi, P. and T. Karsai /1980/: J. Liquid Chrom. 3, 809-831

13. Szirtes, V., Pongor, S., Penczi. E. /1977/: Növénytermelés 26, 49;

14. Pongor, S., J. Kovács, P. Kiss and T. Dévényi /1978/: Acta Biochim. Biophys. ASH 13, 117;

15. Dávid, Á. /1979/ p.c.

16. Pongor, S., J. Krámer and E. Ungar /1980/: Chinoin, Budapest, J. HRC + CC 3, 93-94

17. Pongor, S. and K. Baintner /1980/: Acta Biochim. Biophys. ASH 15, 1-4.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(8), 1597-1600 (1982)

LC NEWS

AMPEROMETRIC CONTROLLER FOR ELECTROCHEMICAL DETECTION IN LC is a low cost and compact instrument suitable for your quality control, clinical, or routine-testing laboratory. This model features potential adjustment via a thumbwheel switch over a range of 0 +/- 1.99 V. Background currents may be offset over a range of 0 to 100 nA, making it suitable for most low potential oxidative and reductive applications. Bioanalytical Systems Inc., JLC/82/8, 111 Lorene Place, West Lafayette, IN, 47906 USA.

UNIVERSAL COMPUTING SAMPLE PROCESSING SYSTEM is designed to provide complete flexibility for handling liquid in a clinical environment. Up to 500 samples can be loaded into the analyzer tubes quickly and accurately with samples as small as 5 ul and reagent volumes up to 1 ml. Designed to be extremely flexible, this system offers an efficient way to handle liquid or transfer operations, including pipetting and diluting samples. Hamilton Company, JLC/82/8, Reno, NV, 89510 USA.

FAST PROTEIN LIQUID CHROMATOGRAPHY (FPLC) separates mixtures of proteins, peptides, and polynucleotides with excellent resolution, usually in 30 minutes or less. Fractionations of as much as 25 mg protein per run are achieved by ion exchange chromatography or chromatofocusing. A comprehensive methodology handbook provides a

practical guide to obtaining optimum results. Pharmacia Fine Chemicals, Inc., JLC/82/8, 800 Centennial Avenue, Piscataway, NJ, 08854, USA.

NEW COLUMNS FOR OLD column replacement service permits chromatographers to send in their old, obsolete columns to be emptied, cleaned, and repacked with the packing material of their choice. Columns are returned with a full evaluation report, as for new HPLC columns. HPLC Technology, JLC/82/8, P.O.Box 7000-196, Palos Verdes Peninsula, CA, 90274, USA.

EXPANDABLE COMMUNICATION SYSTEM for the chromatography lab uses a simple 3-wire connection to integrate chromatographs and data systems into an integrated network. This approach eliminates the need for a system controller or host computer to control communications. It can be interfaced with CRT terminals, printers, modems, computers via a communications interface board. Spectra-Physics, JLC/82/8, 3333 North First Street, San Jose, CA, 95134, USA.

SHORT HPLC COLUMNS perform many analyses faster and with reduced solvent consumption. Three-micron diameter packing materials yield efficiencies comparable to longer conventional columns. Advanced bonding and packing technology combine to assure long column lifetimes and consistent performance. Rainin Instrument Co., JLC/82/8, Mack Rd., Woburn, MA, 01801, USA.

ION CHROMATOGRAPHY USES STANDARD HPLC EQUIPMENT with RI detection. It uses an anion exchange process, with aromatic counter-ions to make inorganic anions detectable at the sub-ppm level with an ordinary RI detector. Chrompack, BV, JLC/82/8, P.O.Box 3, 4330 AA Middelburg, The Netherlands.

FIVE MICRON STEEL COLUMN guarantees high efficiency, speed, and column-to-column reproducibility. It combines spherical particles with the fast throughput of a 15 cm column. 35 chromatographic parameters and 56 physical and

chemical parameters are controlled during production to ensure optimum reliability and separations. Typically, 90,000 plates per meter are achieved with pressure less than 1000 psi at 0.7 ml/min (21 degrees C.). Waters Associates, Inc., JLC/82/8, 34 Maple Street, Milford, MA, 01757, USA.

PROTEIN SEQUENCING SOLVENTS eliminate solvent impurities that contribute to losses in PTH amino acid derivatives during protein sequencing even at the picomole level. Each solvent undergoes recovery testing using a representative mixture of 16 different PTH amino acid derivatives. This assures higher yields for Edman Degradation procedures. Burdick & Jackson Laboratories, JLC/82/8, 1953 S. Harvey St., Muskegon, MI, 49442, USA.

GUIDE TO MEMBRANE SEPARATION TECHNOLOGY is applications oriented. A unique "Applications Guide" leads one to the proper product for his needs, plus available literature. Millipore Corp., JLC/82/8, Bedford, MA, 01730, USA.

LC COLUMN SELECTOR switches five columns. It enables the user to exchange columns rapidly, without wrenches, and without subjecting fittings to repeated wear. A column that is switched off-line is sealed at both ends. One can be sure that no column is exposed to a solvent that is intended for another. Rheodyne, Inc., JLC/82/8, P. O. Box 996, Cotati, CA, 94928, USA.

PROGRAMMABLE WAVELENGTH DETECTOR is microprocessor controlled. It permits selection of any number of wavelengths in one-nanometer increments from 190 to 370 nm for optimal detection of all components. Utilizes keyboard entry that may be changed at any point and as often as desired. Varian Instrument Group, JLC/82/8, 2700 Mitchell Drive, Walnut Creek, CA, 94598, USA.

POST-COLUMN REACTION SYSTEM introduces reagents after the separation has taken place. Detection of derivatives is often more sensitive and

selective than the underivatized compounds, and the separation is, of course, unaffected. Kratos, Inc., JLC/82/8, 24 Booker Street, Booker, NJ, 07675, USA.

SEPARATE OPTICAL ISOMERS by HPLC with chiral columns. Preparative chiral sorbents are also available for scale-up of separations. J.T.Baker Research Products, JLC/82/8, 222 Red School Lane, Phillipsburgh, NJ, 08865, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(8), 1601-1604 (1982)

LC CALENDAR

1982

AUGUST 2 - 5: Int'l. Conf. on Ion Chrom., at the 24th Rocky Mountain Conf., Denver, CO, USA. Contact: Dionex Corp., 1228 Titan Way, Sunnyvale, CA, 94086, USA.

AUGUST 8 - 13: 34th Annual AACC Nat'l. Meeting, Anaheim, CA. Contact: M. Tuttle, Amer. Assoc. of Clinical Chemists, 1725 K Street, NW, Suite 903, Washington, DC, 20006, USA.

AUGUST 15 - 21: 12th Int'l. Congress of Biochem., Perth, Western Australia. Contact: Brian Thorpe, Dept. Biochem., Faculty of Science, Australian National University, Canberra A.C.T. 2600, Australia.

SEPTEMBER 12 - 17: National Amer. Chem. Soc. Meeting, Kansas City, MO, USA. Contact: A. T. Winstead, Amer. Chem. Soc., 1155 Sixteenth St. NW, Washington, DC, 20036, USA.

SEPTEMBER 13 - 17: 14th Int'l. Symposium on Chromatography, London. Contact: Mrs. Jennifer Chalis, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham NG1 4BU, U.K.

OCTOBER 10 - 13: 21st Annual Mtg. of ASTM Committee E-19 on the Practice of Chromatography, Marriott Hotel, New Orleans. Contact Mr K.

Riley, ASTM Headquarters, 1916 Race Street, Philadelphia, PA, 19103, USA.

OCTOBER 12 - 14: 3rd International Symposium On Chlorinated Dioxins and Related Compounds, International Congress Center, Salzburg, Austria. Contact: Prof. O. Hutzinger, University of Amsterdam, Nieuwe Achtergracht 166, 1018WV Amsterdam, The Netherlands.

OCTOBER 14: Ninth ANACHEM Symposium, sponsored by The Association of Analytical Chemists, Dearborn Inn, Dearborn, MI, USA. Contact: J. W. Auld, Detroit Edison Co., Detroit, MI, 48226, USA.

OCTOBER 14 - 15: "New Perspectives in Racemic Coumpound Separation" sponsored by CNR-PF Chimica Fine e Secondaria, Societa Chimica Italiana, and Universita degli Studi di Roma, in Rome, Italy. Contact: Prof. Domenico Misiti, Inst. di Chimica Organica, Via del Castro Laurenziano 9, 00161 Roma, Italy.

OCTOBER 19 - 20: short Course on LC/MS, Maison des Congres, Montreux, Switzerland. Sponsored by Int'l Assoc. of Environmental Anal. Chem. Contact: Dr. Alain Donzel, Workshop Office, Case Postal 5, CH-1052 Le Mont-sur Lausanne, Switzerland.

OCTOBER 21 - 22: 2nd Workshop On LC/MS and MS/MS, Maison des Congres, Montreux, Switzerland. Sponsored by Int'l. Assoc. of Environmental Anal. Chem. Contact: Dr. Alain Donzel, Workshop Office, Case Postale 5, CH-1052 Le Mont-sur Lausanne, Switzerland.

NOVEMBER 2 - 5: 1st Inter-American Congress in Forensic Medicine and Sciences, Pan-American Assoc. of Forensic Sci., Sacramento, CA. Contact: John D. DeHaan, Calif. Department of Justice Lab. Box 13337, Sacramento, CA 95813,USA.

NOVEMBER 11 - 14: Applied Seminar for the Association of Clincial Scientists, Chicago, IL. Contact: Dr. F. M. Sunderman, Jr., Dept. of Lab.

Medicine, Univ. of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT 06032.

NOVEMBER 16 - 18: Medical and Laboratory Instrumentation Soc. Annual Int'l. Congress and Exhibition, Sheraton-Washington Hotel, Washington, DC. Contact: John Wolf, MLIS, 11310 Palisades Court, Kensington, MD, 20895, USA.

NOVEMBER 17 - 19: Eastern Analytical Symposium, Statler-Hilton Hotel, New York. Contact: Dr. H. Issag, Frederick Cancer Res. Facility, P.O. Box B, Frederick, MD, 21701, USA, or Dr. D. Strumeyer, Rutgers University, Chem. Dept, New Brunswick, NJ 08903.

DECEMBER 6 - 8: 3rd Biennial TLC Symposium-Advances in TLC, Hilton Hotel, Parsippany, NJ. Contact: J.C. Touchstone, Hospital of the University of Pennsylvania, Philadelphia, PA, 19104, USA.

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MARCH 20 - 25: National Amer. Chem. Soc. Meeting, Seattle, WA, USA. Contact: A. T. Winstead, Amer. Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

MAY 2 - 6: VIIth International Symposium On Column Liquid Chromatography, Baden-Baden, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

MAY 30 - JUNE 3: International Conference on Chromatographic Detectors, Melbourne University. Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria, Australia 3052.

JULY: 3rd Int'l. Flavor Conf., Amer. Chem. Soc., The Corfu Hilton, Corfu, Greece. Contact: Dr. S. S. Kazeniac, Campbell Inst. for Food Research, Campbell Place, Camden, NJ, 08101, USA.

JULY 17 - 23: SAC 1983 International Conference and Exhibition on Analytical Chemistry, The University of Edinburgh, United Kingdom. Contact: The Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BV, United Kingdon.

1984

OCTOBER 1 - 5: 15th International Symposium on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

The Journal of Liquid Chromatography will publish announcements of LC meetings and symposia in each issue of The Journal. To be listed in the LC Calendar, we will need to know: Name of meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements for inclusion in the LC Calendar to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 1440-SMS, Fairfield, CT, 06430, USA.

INSTRUCTIONS FOR PREPARATION OF MANUSCRIPTS FOR DIRECT REPRODUCTION

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F. D. Pierce and H. R. Brown
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