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SIMULTANEOUS CALIBRATION OF

MOLECULAR WEIGHT SEPARATION AND COLUMN

DISPERSION OF GPC BY COUPLING WITH LALLIS*

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ABSTRACT

It is shown theoretically and experimentally that both the calibration of the molecular weight separation and column dispersion can be evaluated simultaneously by coupling gel chromatograph with low angle light scattering photometer. The experimentally determined variation of the spreading factor with retention volume is quite similar to that obtained by Tung using reverse flow technique. A correction method is given for the lowering of inhomogeneity index printed by the data processor of the on-line GPC-LALLS.

INTRODUCTION

Gel Permeation Chromatography (GPC) is a powerful technique for polymer characterization, but the experimental chromatogram obtained is broadened by column dispersion (instrumental spreading). Strictly speaking, it is necessary to carry out a calibration for column dispersion in addition to the calibration for molecular weight separation. Calibration of column dispersion is a troublesome task since truly monodispersed polymer standards are unavailable. The experimental evaluation of the column dispersion was only possible by such sophisticated techniques as reverse-flow [1,2] or recycle [3,4] methods.

In the present work, it is shown theoretically and experimentally
that both the calibration of the molecular weight separation and
column dispersion can be evaluated simultaneously by coupling GPC with
a low angle laser scattering photometer (LALLS) using a number of poly-
dispersed samples with different average molecular weights while the
absolute values of them are unnecessarily known a priori. The exper-
imental determined variation of the spreading factor with retention
volume is quite similar to that obtained by Tung [1,2] using reverse
flow technique. The experimental number average molecular weight from
GPC-LALLS is higher than the actual one; and thus the inhomogeneity
index is lower [5,6]. In this work, a correction method for these
effects is also presented.

THEORETICAL

When LALLS is connected with GPC, the polymer sample in the
LALLS measuring cell is still polydispersed due to column disper-
sion, therefore the variation of the weight average molecular weight
of the polymer in the measuring cell with the elution volume V is
directly measured.

The relationship between the experimental chromatogram F(V) and
the true chromatogram $\overline{W}$ (V_A) can be expressed by Tung's integral
equation [7]

$$F(V) = \int_{V_R} \overline{W}(V_R)\ G(V,V_R)\,dV_R \tag{1}$$

where $G(V,V_R)$ is the instrumental spreading function, representing
experimental chromatogram of a truely monodispersed polymer with
V_R as its retention volume. Presume $G(V,V_R)$ is Gaussian and the
monodisperse calibration relation $M(V_R)$ of the gel chromatographic
column is

$$\text{Ln } M = A_M - B_M V_R \tag{2}$$

Yau et al. [8] had derived the relationship between weight and number
average molecular weight and elution volume as

$$M_W(V) = \frac{F(V - B_M \sigma_O^2)}{F(V)}\ \exp(\tfrac{1}{2}B_M^2\sigma_O^2)\exp(A_M - B_M V) \tag{3}$$

$$Mn(V) = \frac{F(V)}{F(V + B_M\sigma_0^2)} \exp(-\tfrac{1}{2}B_M^2\sigma_0^2)\exp(A_M - B_MV) \tag{4}$$

Where σ^2 is the spreading factor, i.e. the variance of the spreading function.

If F(V) is also Gaussian

$$F(V) = \frac{1}{\sigma_\tau\sqrt{2\pi}} \exp\{-\frac{1}{2\sigma_\tau^2}(V - \tilde{V})^2\} \tag{5}$$

in which $\tilde{V}$ and σ_τ^2 refer to the mean elution volume and the variance of the experimental chromatogram F(V) respectively. Substituting it into Eqs. 3 and 4 and introducing a parameter ξ defined as [9]

$$\xi^2 = (\sigma_\tau^2 - \sigma_0^2)/\sigma_\tau^2 \tag{6}$$

the variation of the experimentally determined average molecular weights of the eluted polymer with elution volume could be represented by [10]

$$Ln\ Mw(V) = [A_M - (1-\xi^2)B_M(\tilde{V}-\tfrac{1}{2}B_M\xi^2\sigma_\tau^2)] - \xi^2B_MV \tag{7}$$

$$Ln\ Mn(V) = [A_M - (1-\xi^2)B_M(\tilde{V}+\tfrac{1}{2}B_M\xi^2\sigma_\tau^2)] - \xi^2B_MV \tag{8}$$

Since the logarithm of the average molecular weight varies linearly with the elution volume, then Eqs. 7 and 8 can be written briefly as

$$Ln\ Mw(V) = Aw - BwV \tag{7a}$$
$$Ln\ Mn(V) = An - BnV \tag{8a}$$

where

$$Aw = A_M - (1 - \xi^2)B_M (\tilde{V} - \tfrac{1}{2}B_M\xi^2\sigma_\tau^2) \tag{9}$$

$$An = A_M - (1 - \xi^2)B_M (\tilde{V} - \tfrac{1}{2}B_M\xi^2\sigma_\tau^2) \tag{10}$$

$$Bw = Bn = \xi^2B_M \tag{11}$$

Determination of the Calibration Relation $M(V_R)$:

In coupling GPC with LALLS the function $M_W(V)$ is directly measured by experiments. If the distribution of the sample is nearly Gaussian Eq.7 should be complied. From the experimental data coefficients Aw and Bw of Eq. 7a can be obtained. Since the magnitude of the parameter depends both upon the spreading effect of the column and upon the distribution width of the sample, and on the other hand from Eq. 11 and the definition of ξ, it is obvious for a polydispersed sample in a real GPC column $o < \xi < 1$. Then the slope Bw of the experimental function

$M_w(V)$ must be smaller than the slope B_M of the monodisperse calibration relation $M(V_R)$. They must cross each other at a certain point. The coordinates of the cross point of $M_w(V)$ and $M(V_R)$ can be solved from Eqs. 2 and 7. The coordinate of the cross point volume V_w is

$$V_w = \tilde{V} - \tfrac{1}{2}B_w\xi^2\sigma_\tau^2 \tag{12}$$

applying Eq. 11 it can also be expressed as

$$V_w = \tilde{\tilde{V}} - \tfrac{1}{2}B_w\sigma_\tau^2 \tag{13}$$

Since the mean elution volume $\tilde{V}$ and the variance σ_τ^2 of a sample can be obtained from $F(V)$ and the coefficients A_w and B_w are available from GPC on-line with LALLS, the cross point volume V_w of that sample on $M(V_R)$ could be evaluated readily. Afterwards, by Eq. 7a, the molecular weight of this cross point is calculable from

$$M(V_w) = \exp\{A_w - B_wV_w\} \tag{14}$$

After a number of samples are measured, the line connecting all the cross points $(M(V_w), V_w)$ is just the calibration relation $M(V_R)$ of the GPC column studied.

Calibration of Spreading Factor $\sigma^2(V_R)$:

The instrumental spreading effect of a gel chromatographic column may be described by the magnitude of the spreading factor σ^2 which, in turn, is a function of the retention volume V_R. Coupling LALLS with GPC presents a simple and direct experimental method for measuring σ^2 (V_R). As mentioned above, the experimental function $M_w(V)$ and calibration relation $M(V_R)$ can be obtained from the experimental results of several samples with different molecular weight by LALLS-GPC. With the aid of the relationship between the coefficients of $M(V_R)$ and $M_w(V)$, the parameter ξ might be determined with Eq. 11

$$\xi^2 = B_w / B_m \tag{15}$$

is readily calculable from the slopes.

From Eg. 8

$$\xi^2 = 1 - \frac{A_M - A_w}{B_w(V - \tfrac{1}{2}B_M\xi^2\sigma_\tau^2)} \tag{16}$$

ξ^2 is also solvable by iteration from intercepts. After substituting $\bar{V}$ and σ_T^2 obtained from F(V) into Eq.16, since ξ^2 is evaluated, from its definition σ_0^2 is also calculable by

$$\sigma_0^2 = (1 - \xi^2)\sigma_T^2 \tag{17}$$

Correction of Inhomogeneity Index:

In coupling LALLS with GPC the number of average molecular weight of the whole sample printed by the data processor is always larger than the true value and, therefore, leads to a lower inhomogeneity index $\langle M \rangle_w / \langle M \rangle_n$, although the correct weight average molecular weight can be obtained. It is because the eluted polymer in the light scattering cell is still polydispersed, due to the presence of instrumental spreading effect, even though the cell volume is rather small. The true weight and number average molecular weight of the whole sample should be

$$\langle M \rangle_w, \text{ true} = \int_V F(v)M_w(V)\,dV \tag{18}$$

and

$$\langle M \rangle_n, \text{true} = 1 / \int_V \frac{F(V)}{M_n(V)}\,dV \tag{19}$$

respectively, while the printed number average molecular weight of the whole sample was calculated by the data processor according to

$$\langle M \rangle_n, \text{cal} = 1 / \int [F(V)/M_w(V)]\,dV \tag{20}$$

in which $M_w(V)$ was used instead of $M_n(V)$ for calculation. Thus, the inhomogeneity index printed out by the data processor is

$$(\langle M \rangle_w / \langle M \rangle_n)_{GPC-LALLS} = \langle M \rangle_w, \text{true} / \langle M \rangle_n, \text{cal} \tag{21}$$

in turn it may be written as

$$\left(\frac{\langle M \rangle_w}{\langle M \rangle_n}\right)_{true} = \frac{\langle M \rangle_n, \text{cal}}{\langle M \rangle_n, \text{true}} \cdot \left(\frac{\langle M \rangle_w}{\langle M \rangle_n}\right)_{GPC-LALLS} \tag{21a}$$

where $(\langle M \rangle_n, \text{cal} / \langle M \rangle_n, \text{true})$ is the correction factor for the inhomogeneity index. Substituting Eqs. 7 and 8 into Eqs. 19 and 20, and taking the quotient we get

$$\frac{\langle M \rangle_{n,cal}}{\langle M \rangle_{n,true}} = \exp\{ B_M^2 \xi^2 (1-\xi_\tau^2) \} = \exp\{B_M^2 \xi^2 \sigma_0^2\} \tag{22}$$

substituting Eq. 15 into the preceding two equations we have

$$\left(\frac{\langle M \rangle_w}{\langle M \rangle_n}\right)_{true} = \exp\{B_M^2 \xi^2 \sigma_0^2\} \left(\frac{\langle M \rangle_w}{\langle M \rangle_n}\right) \text{ GPC-LALLS}$$

$$= \exp\{B_w B_m (1 - \frac{B_w}{B_M})\sigma_\tau^2\} \left(\frac{\langle M \rangle_w}{\langle M \rangle_n}\right) \text{ GPC-LALLS} \tag{23}$$

It could be seen that the correction factor is a value larger than one. When the sample is monodispersed, $B_w=0$; and when the column is ideal, $B_w=B_M$. The correction factor is equal to one at these two extreme cases. This correction factor depends upon the parameter ξ^2 and σ_0^2; that is to say, it depends both upon the instrumental spreading and the molecular weight distribution of the sample. The correction factor increases with increasing breadth of the molecular weight distribution. In this respect it differs from the correction factor for instrumental spreading of the inhomogeneity index calculated from the experimental chromatogram F(V) and the calibration relation $M(V_R)$. For the latter [10]

$$\left(\frac{\langle M \rangle_w}{\langle M \rangle_n}\right)_{true} = \exp\{-B_M^2 \sigma_0^2\} \left(\frac{\langle M \rangle_w}{\langle M \rangle_n}\right)_{GPC} \tag{24}$$

the correction factor is independent of the molecular weight distribution of the sample.

EXPERIMENTAL

Two groups of polystyrene samples were used. Samples of group A are fractions prepared by fractional precipitation of a self-polymerized polystyrene sample. Group B contents ARL polystyrene standards with narrow molecular weight distributions.

The experimental instruments used include an ARL 950 gel permeation chromatograph and a KMX-6 low angle laser light scattering photometer. They are connected together according to the manual and references [10-13]. The sample cell of the light scattering photometer is set between the GPC column and concentration detectors.

The GPC columns were packed with two kinds of deactivated silica
beads, prepared in our own laboratory. The experiments were carried
out at 35°C, using tetrahydrofuran as eluent with a flow rate of
ca. 1 ml/min. The volume of sample solution injected was 2 ml. with
concentrations from 0.001 to 0.002 g/ml. The elution volume was
counted by a syphon tube with a volume of 1.98 ml. The concentration
of the eluted polymer was detected by an ultraviolet detector at
245 nm. Signals from GPC and LALLS were fed into the dual pen
recorder and the KMX-6/DP data processor simultaneously. Program
mode GP1 was adopted. The following information was printed out
in six reports at the end of run: table and chromatogram of original
data, calculated concentration and molecular weight, graphs of
differential and integral molecular weight distribution and
molecular weight averages of the whole sample, etc.

The start of the run of the data processor is controlled
by a switch. The length of a run is specified beforehand. It
is divided into 150 data points within entire length of a run.
For the further treatment of the experimental data, the data
point number m was transformed into elution volume count no. V
by following equation:

$$V = Vs + \frac{\text{Flow Rate (ml/mn) X Run Length (mn)}}{150 \text{ X } 1.98 \text{ (ml)}} \cdot m$$

where V_s represents the count no. at the beginning of a run.

"GPC Delay" is one of the parameters input into the data
processor. It denotes the time needed for the eluent flow from
light scattering sample cell to concentration detector. It
should be measured precisely. For this purpose we took the GPC
column out of the system and let the sample injection valve be
directly connected with the detectors. As a small amount of
sample solution was injected, "GPC Delay" could be obtained
from the distance between the two peaks of the detector responses.

RESULTS AND DISCUSSIONS

The experimental data for samples printed out by the data
processor have been further treated and analyzed as follows:

TABLE 1

The Experimental Data of Polystyrene Samples by GPC and GPC-LALLS

Polymer		$\overline{V}$	σ_T^2	A_W	B_W	V_W	$M(v_W) \times 10^{-4}$
A	1	108.4	37.0	21.78	0.060	107.3	448
A	2	108.2	27.4	19.45	0.039	107.6	429
A	3	109.1	33.2	20.18	0.046	108.3	410
A	4	109.1	32.1	19.78	0.043	108.4	381
A	5	116.3	59.8	22.65	0.069	114.2	246
B	1	130.2	19.0	13.61	0.006	130.2	38.1
B	2	140.8	19.6	14.42	0.019	140.6	12.0
B	3	152.9	18.1	13.63	0.024	152.7	2.21
B	4	158.9	18.2	14.57	0.033	158.6	1.10
A	6	166.5	17.4	15.44	0.040	166.1	0.64

The mean elution volume $\overline{V}$ and the variance σ_t^2 are calculated from the printed original data table (report 1) of the experimental chromatogram F(V) according to their definitions. The results obtained are listed in Table 1, in which the unit $\overline{V}$ and σ_t are both in elution volume counts. The plotted diagram of the logarithm of the weight average molecular weights from report 3 versus the elution volume possesses good linearity except at the two extreme ends of the chromatogram; an example is shown in Figure 1. The departure from linearity at the tail parts of the chromatogram is mainly caused by too low a concentration of the eluted polymer in that region. The coefficients A_W and B_W were calculated by a linear regression method according to Eq. 7a using the middle portion of the data points, the results obtained are also listed in Table 1. The experimental functions $M_W(V)$ for the samples studied and thus obtained were drawn in Figure 2, where the dotted line represents the tail regions where the data points deviate from linearity. In Figure 2, the data lines of some high molecular weight samples are omitted so as to avoid crowding.
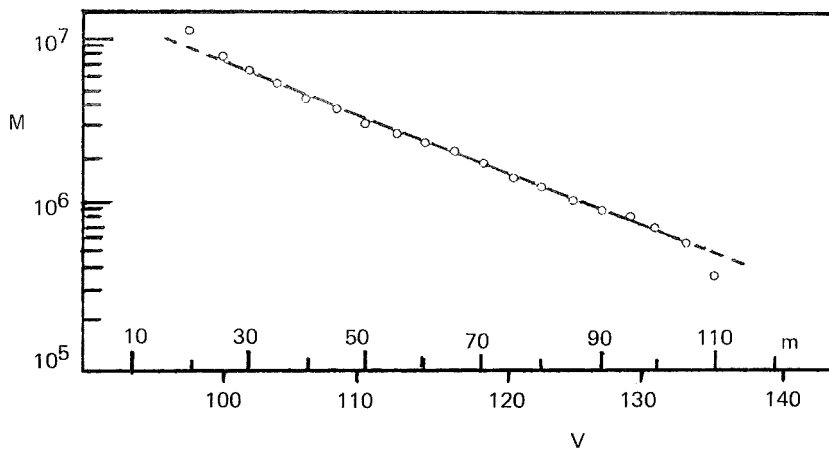
FIGURE 1.   Dependence of the experimentally determined
            molecular weight on the elution volume for
            sample A5.
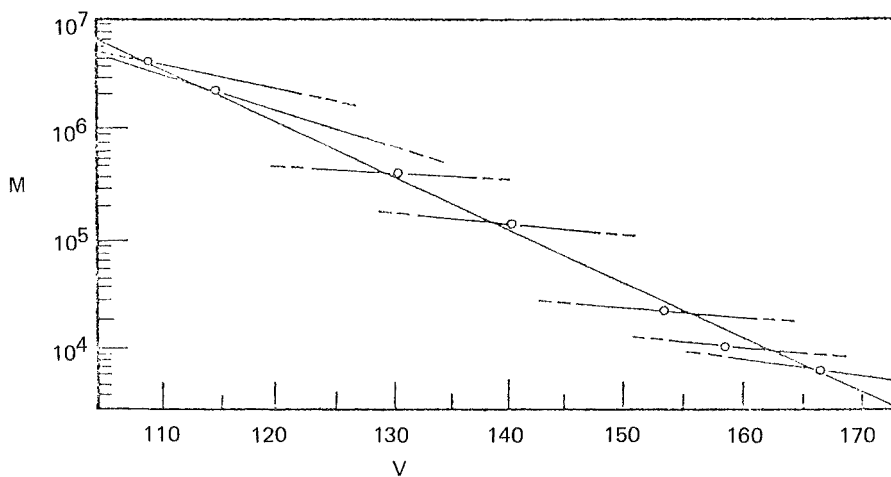
FIGURE 2.   The experimental relation $M_w(v)$ and the
            calibration relation $M(v_R)$ of the GPC column.

The volume V_w and molecular weight $M(V_w)$ at the cross point of
the experimental function $M_w(V)$ with the calibration relation $M(V_R)$
were calculated according to Eq. 13 and 14 from the mean elution
volume V, variance σ_τ^2 as well as the coefficients A_w and B_w of the
function listed in Table 1 and also drawn in Figure 2. By plotting
the logarithm of $M(V_w)$ against V_w, we get the monodisperse molecular
weight separation calibration relationship $M(V_R)$ for polystyrene of
the gel chromatographic column used.

$$\ln M = A_M - B_M V$$

with the coefficients

$$A = 27.42 \quad \text{and}$$
$$B = 0.115.$$

The parameter ξ^2 was then calculated from the slope or the intercept
of the experimental function $M_w(V)$ and the calibration relation
$M(V_R)$ according to Eqs. 15 or 16. Afterwards, the spreading factor
σ_0^2 was calculated from σ_τ^2 and ξ^2 according to Eq. 17. The calculated
results are all listed in Table 2. The parameters ξ^2 and the spreading

TABLE 2

The Spreading Factor for the Polystyrene Samples

Polymer	V_w	ξ^2		σ_0^2	
		From Slope	From Intercept	From Slope	From Intercept
A 1	107.3	0.536	0.533	17.2	17.3
A 2	107.6	0.345	0.346	17.9	17.9
A 3	108.3	0.407	0.407	19.7	19.7
A 4	108.4	0.379	0.375	19.9	20.1
A 5	114.2	0.617	0.633	22.9	22.0
B 1	130.2	0.051	0.057	18.0	17.9
B 2	140.6	0.172	0.177	16.2	16.1
B 3	152.7	0.211	0.196	14.3	14.6
B 4	158.6	0.295	0.282	12.8	13.1
A 6	166.1	0.358	0.375	11.2	10.9
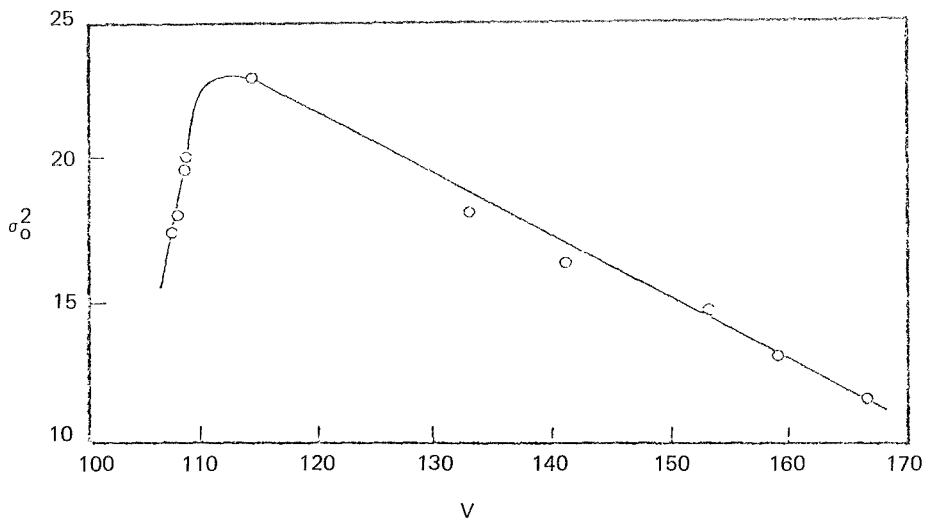
FIGURE 3.   Dependence of the spreading factor on the
            retention volume.

factors σ_O^2 obtained from the slope and from the intercept are practically
identical.   This fact indicates that the results of the mathematical
treatment given in the present article are valid.

For a polydispersed sample the spreading factor obtained by the
preceding method is an average value identified as

$$<\sigma_O^2> \; = \int_{V_R} \overline{W}(V_R)\,\sigma_O^2(V_R)\,dV_R$$

Since the molecular weight distributions of all the samples used are
rather narrow, as an approximation it may be looked upon as the
spreading factor of monodispersed polymer.   Taking V_w as the retention
volume of monodispersed polymer, the plot of σ_O^2 versus V_w is shown in
Figure 3.   It may be regarded as the   (V) function of the gel chroma-
tographic column used in the present work.   It can be seen from
Figure 3 that a maximum appears at the retention volume not far from
the interstitial volume of the column.   This phenomenon is identical
with that first observed by Tung [1,2] using a reverse flow technique.

TABLE 3

Experimental Data & Corrected Values of Inhomogeneity Indexes

Polymer		From Gel Chromatogram				From GPC-LALLS			
		$\langle M \rangle w$ x10^{-4}	$\langle M \rangle n$ x10^{-4}	$\langle M \rangle w / \langle M \rangle n$ Expt.	Cor.	$\langle M \rangle w$ x10^{-4}	$\langle M \rangle n$ x10^{-4}	$\langle M \rangle w / \langle M \rangle n$ Expt.	Cor.
A	1	509	322	1.58	1.27	461	404	1.14	1.28
A2	2	496	249	1.42	1.13	428	410	1.04	1.13
A	3	460	301	1.53	1.19	413	383	1.08	1.19
A	4	458	306	1.49	1.16	381	361	1.06	1.16
A	5	241	113	2.12	1.59	246	181	1.36	1.62
B	1	39.6	31.1	1.27	1.01	39.9	39.2	1.02	1.04
B	2	12.1	9.46	1.28	1.04	11.7	11.6	1.01	1.05
B	3	3.06	2.44	1.26	1.05	2.08	2.04	1.02	1.06
B	4	1.57	1.24	1.27	1.08	1.07	1.06	1.02	1.07
A	6	0.67	0.54	1.25	1.08	0.60	0.59	1.02	1.08

The weight and number average molecular weights, as well as the inhomogeneity index printed by the data processor, are listed in Table 3. The results calculated from the experimental chromatogram F(V) and the calibration relation $M(V_R)$ are also listed in the same table. It is obvious that the inhomogeneity index $(\langle M \rangle_w / \langle M \rangle_n)_{GPC-LALLS}$ from GPC with LALLS on line is lower, while that from the gel chromatogram and the calibration relationship $(\langle M \rangle_w / \langle M \rangle_n)_{GPC}$ is higher. Naturally, both of them are the consequences of the instrumental spreading. These two independent series of data were corrected by Eqs. 23 and 24 respectively. The corrected inhomogeneity indexes coincide with each other very well as shown by the data in Table 3. It indicates that the correction factor proposed in this article is also valid.

For the commercial polystyrene standards we used (group B samples), the manufacturer (ARL) only gave the weight-average molecular weights; no accurate inhomogeneity indexes had been given. The manual and the experimental values are listed together in Table 4; it indicates that

TABLE 4

Comparison Between Manual Values and Corrected Experimental
Values of Polystyrene Standards

| Polymer | $\langle M \rangle w \times 10^{-4}$ | | | $\langle M \rangle w / \langle M \rangle n$ | |
	Manual	LALLS	GPC-LALLS	Manual	GPC-LALLS cor.
B 1	39	38.5	39.9	<1.06	1.04
B 2	11	11.7	11.7	<1.10	1.05
B 3	2.04	2.02	2.08	<1.06	1.06
B 4	1.00	0.98	1.07	<1.10	1.07

an absolute characterization of polymer could be made by gel chromato-
graphy by coupling with a molecular weight detector such as LALLS.

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LONG-CHAIN ALKYLSILANE-MODIFIED POROUS SILICA[*]

BEADS AS GPC PACKINGS

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SUMMARY

A series of silica gels having various molecular weight exclusion limits has been modified by reaction with octadecyltrichlorosilane. Toluene being used as moving phase and hydroxyl-end blocked polydimethylsiloxane as solute, the chromatographic process on these modified silica packings is mainly controlled by size exclusion; an additional absorptive retention can still be observed with the low molecular species. Thus, calibration in this case should be performed using standard samples of the specific polymer to be determined.

INTRODUCTION

Porous silica gels, used as GPC packings, usually need to be modified with trimethylchlorosilane or hexamethyldisilazane[1-3] in order to suppress the adsorptive effect caused by surface silanol groups. It has been proven[4,5] that an appreciable number of unreacted silanol groups is still left on the surface of silica gels treated chemically with organochlorosilanes. The adsorptive

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effect due to remaining silanol groups can be very strong with
short-chain bonded phases[6], since solute molecules penetrated
through the phase to the surface easily. A variety of packing
materials for reversed phase liquid chromatography, prepared by
reaction of porous silica gels with long-chain alkylchlorosilanes,
has commercially been available for a long time, but reports on
the application of these modified silica gels for GPC have not
yet been found. In the case of long-chain bonded phases, as
noted by Kirkland[7], unreacted silanol groups are shielded by
an "umbrella" of tightly packed organic groups, and the adsorp-
tive effect may be reduced to a very low level. In this labor-
atory, a series of porous silica beads was modified with octa-
decyltrichlorosilane. Toluene being used as moving phase and
hydroxyl-end blocked polydimethylsiloxane as solute, the gel
chromatographic behaviour on the modified silica beads and the
residual adsorptive effects were investigated.

EXPERIMENTAL

Materials

    Octadecyltrichlorosilane was prepared by addition of silicon
tetrachloride to octadecyl Grignard reagent; the product was dis-
tilled and collected at 210-214°C/6mmHg. Other chemicals were
commercially available materials, AR and CP grade, and were not
treated before use.

    Cross-linked polystyrene gels, JD-type, were supplied by the
Chem Dept., Jilin University. Silica packings, NDG-L, which had
been treated by hexamethyldisilazane, were commercially available
(2nd Chem. Reagent Plant, Tianjin). Silica packings Nos.1-6 were
prepared by calcining spherical porous silica beads (Qingdao Sea
Chem. Plant). Two procedures were employed for the silica modi-
fication:

Method I: by reaction with hexamethyldisilazane, according to the
procedure described by Beijing Chem. Ind. Institute[8].

Method II: by reaction with octadecyltrichorosilane[4.7.9.]. A
common procedure is as follows: A given amount of silica beads,
whose surface had been fully hydrolyzed with HCl, was placed in
a round-bottom flask, heated at 150-160°C for 2-4 hours and followed
by cooling, in vacuum; then an excess of octadecyltrichlorosilane
dissolved at 10-20% in dry toluene was drawn in. It was assumed
that the amount of octadecyltrichlorosilane, Ws, required for
monomolecular layer bonding can be estimated by the following
relation:

$$W_s = \frac{Wg \times S \times 4 \times 10^{18} \times 388}{6.02 \times 10^{23}} \quad g$$

where Wg and S are the weight (g) and specific surface (M^2/g) of the
silica beads to be treated, respectively. The mixture was refluxed
with suitable stirring for 16-18 hours, while a small stream of dry
nitrogen was passed through the solution. After cooling, the silica
beads were separated from the solution by decantation, then washed
by column-elution with dry toluene, acetone, a mixture consisting of
10% H_2O and 90% acetone, methanol successively until the final eluate
was fully neutral. The silica particles were air dried and heated
at 120°C for 4 hours. For silica packing No. 1, after treating as
above, the reaction with octadecyltrichlorosilane was carried out
once more in much the same way, except using dry xylene instead of
toluene. In order to remove any residual silanol groups which could
have been formed by octadecyltrichlorosilane hydrolysis, usually,
the silica beads, treated as above, were further reacted with hexa-
methyldisilazane according to Method I.

Samples and Standards

    Anionically polymerized polystyrene standards were supplied by the
Chem. Dept. Jilin University.

    Polydimethylsiloxane samples (blocked by hydroxyl-end) were
obtained from this institute and fractionated at 15±0.1°C by addition
of methanol to their dilute solutions (1g/dl). Benzene[10] or ethyl

acetate[11] was chosen as the good solvent. Each fraction was re-
dissolved and reprecipated until a symmetrical peak of appropriate
width was obtained by GPC. The intrinsic viscosities of the fractions
were measured with a Ubbelodhe-type semi-microviscometer in toluene
at 25°C and the molecular weights were calculated by the relation[12]:

$$[\eta]^{25°C}_{toluene} = 0.828 \times 10^{-4} \ M^{0.72} \ dl/g \tag{2}$$

and the results are listed in Table II.

Gel Chromatography Operation

A gel chromatograph (Tianjin Anal. Instrum. Plant, Model SN-01A)
equipped with a differential refractometer was used. The elution
volume was measured by counting with a syphon of 2.5ml. Provided
that there are no special notes made in this paper, the chromatographic
separations were performed with a column of 1m length x 8 mm i.d.
at room temperature and flow rate range of 0.6-0.8ml/min. using toluene
as moving phase.

Solute recovery check was made as follows: At the same sample
size and instrumental sensitivity, a polydimethylsiloxane was
chromatographed with modified silica gel columns and a polystyrene
gel column respectively. Suppose the recovery of this polymer on
polystyrene gel column is 100%; then the recoveries on modified
silica gel columns may be expressed by S_1/S_2 ratio, where S_1 is
the peak area values obtained with modified silica gels and S_2,
with polystyrene gel.

RESULTS AND DISCUSSION

Chromatographic Properties of Modified Silica Gels

Molecular weight exclusion limit, ratio of pore volume to
interstitial volume, and packed-column efficiency were measured;
these results are listed in Table I. As shown in the last column
of Table I, the particle size exerts an obvious effect on column

TABLE I. Properties of silica packings

No. of Silica [a]	Modification method	Particle size (mesh)	App. density (g/ml)	Mol.wt. exclusion limit (PS)	Ratio of pore[b] volume to interstitial volume (V_i/V_o)	Column [c] efficiency (theor. plate per meter)
1	untreated	120-180	0.434	1.8×10^4	1.07	1570
1-I	I	120-180	0.468	1.5×10^4	0.99	–
1-II	II	120-180	0.537	1.2×10^4	0.82	1570
2	untreated	200-280	0.412	1.4×10^5	1.10	4500
2-I	I	200-280	0.428	1.4×10^5	1.10	5000
2-II	II	200-280	0.459	1.2×10^5	1.08	
3	untreated	160-200	0.448	4.4×10^5	1.07	2950
3-II	II	160-200	0.476	4.0×10^5	1.00	2580
4-II	II	160-200	0.447	8.0×10^5	1.03	2650
5-II	II	160-200	0.474	$> 4 \times 10^6$		2660
6-II	II	160-200	0.528	$> 4 \times 10^6$		

(a) The silica packings prepared by calcining in this laboratory were divided into six grades, denoted by Arabic numbers 1-6, according to their exclusion limit.
(b) Measured by GPC.
(c) Measured by using heptane as the solute and calculated:

$$N = 5.54 \times \left(V_R / 2\Delta V_{\frac{1}{2}} \right)^2$$

where $2\Delta V_{\frac{1}{2}}$ is the half-peak width.
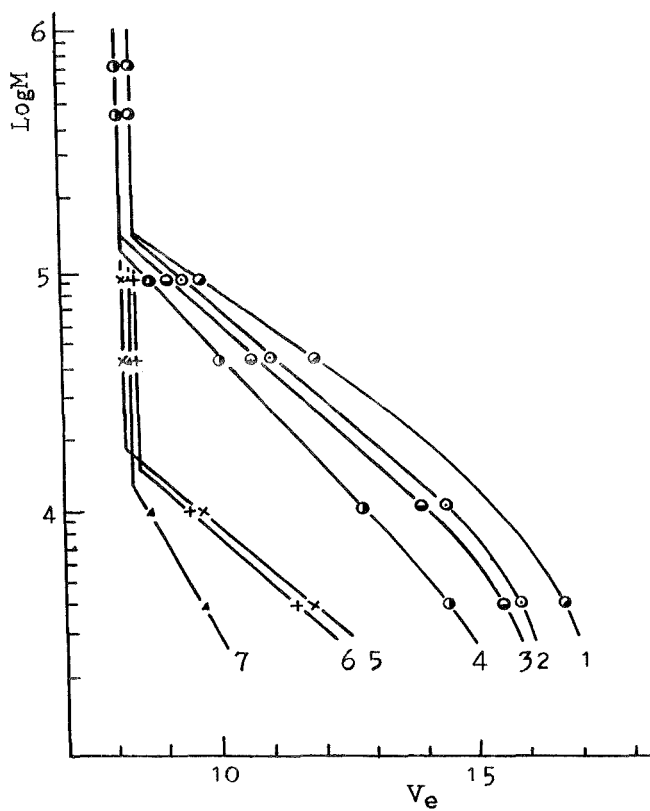
FIGURE 1.   PS calibration curves before and after packing modification.
Column of 1mm x 8mm i.d.; toluene as moving phase except
curves 2,5.

1) Silica No.2
2) Silica No.2, in THF
3) Silica 2-I
4) Silica 2-II
5) Silica No.1 in THF
6) Silica 1-II
7) Silica 1-II

efficiency, but not much difference in efficiency can be found between the two packings, which were modified by Method I and Method II respectively (either Silica 1-I and Silica 1-II, or Silica 2-I and Silica 2-II).

It is important[13] that a monomolecular layer of organic groups should be chosen in silica modification, since the pore openings of gels, particularly those with small pore sizes, may be blocked by polymer layer, resulting in a drastic decrease in observed pore column. On this account, in our laboratory, the reaction of silica beads with octadecyltrichlorosilane containing three active functional groups have been well protected from moisture in the air. The value of V_i/V_o, as shown in the 6th column of Table I, have only a slight disparity from a comparison with Silica No. 2 and Silica 2-II, or Silica No. 3 and Silica 3-II. As regards Silica 1-II, however, the value of V_i/V_o is 22% less than before its modification; this might be attributed to the large specific surface of Silica No. 1, i.e. the great number of silanol groups available for reaction with the organosilane. Thus, this modified material has a high level of organic content which, of course, can occupy an appreciable amount of pore volume of the gels.

Effects of silica modification on the calibration curves are illustrated in Fig. 1.

Suppressing Adsorptive Effects

Adsorptive retention, which occurs simultaneously with the size exclusion process, is usually observed as excessive retention as well as tailing chromatographic peaks. When the adsorptive effect is strong, solute molecules may either be permanently retained in the column, or elute so slowly that the concentration of solute in eluate is below the minimum detectable limit. Accordingly, solute recoveries calculated from chromatograms are on the low side. In order to examine the residual adsorptive effect, hydroxyl-end-blocked polydimethylsiloxane (PDMS) samples of narrow or broad molecular weight distribution were chromatographed using various silica peaking which had been modified

by the two methods described above. For convenience, the same
code numbers are employed for silica packing and the corresponding
column packed with it.

The exclusion limit values are both approximately 1×10^5 for
Silica 2-I and Silica 2-II, but retention behaviour of PDMS fractions
on column 2-II is quite different from that on column 2-I. Fig. 2-b
shows a chromatogram of a mixed sample on column 2-II where narrow
distribution PDMS fraction V, PDMS fraction X and heptane are well
separated from each other; the values of V_e at peak position are
10.15, 14.8 and 17.4 respectively, in agreement with the order of
size separation. In the case of column 2-I, PDMS fraction X actually
elutes later than heptane and peak tailing is very pronounced (Fig.2-c).
A chromatogram of PDMS sample No. A (with broad distribution) lies
before the total permeation volume, 17.4 (Fig.2-a-1), and the polymer
sample recovery amounts to 100% on column 2-II, while a part of this
PDMS sample eluting is beyond the normal volume range for GPC
(Fig. 2-a-2) and the sample recovery detected is only about 80% on
column 2-I.
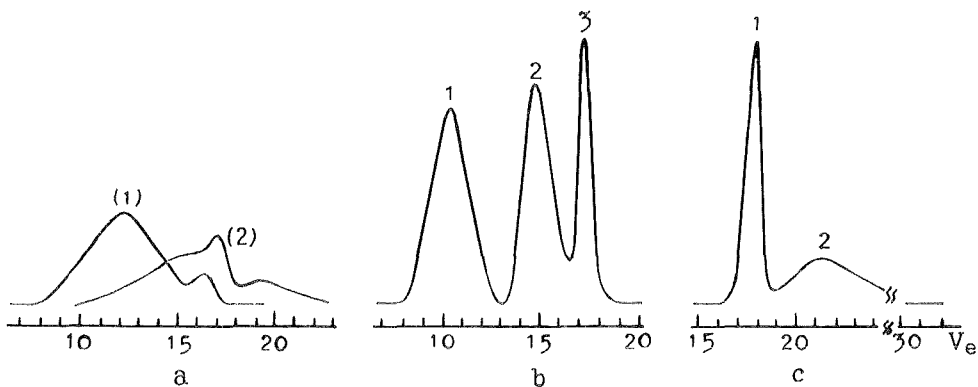
FIGURE 2.   Elution curves on column 2-I or column 2-II.
            a. PDMS sample No.A of broad distribution, $\bar{M}\eta$ 1.3×10^4
            (1) column 2-II; (2) column 2-I b1) PDMS fraction V, $\bar{M}\eta 6.07 \times 10^4$
            2) PDMS fraction X, $\bar{M}\eta 8.11 \times 10^3$, 3) heptane, column 2-II
            c.1) heptane, 2) PDMS fraction; column 2-I
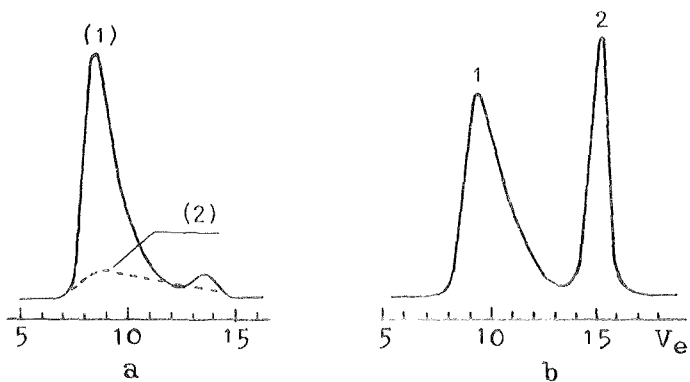
FIGURE 3.   Elution curves on column 1-I or column 1-II.

   a. PDMS sample No.A of broad distribution, $\bar{M}_\eta 1.3 \times 10^4$,

   (1) column 1-II; (2) column 1-I b.1) PDMS fraction X, $\bar{M}_\eta 8.11 \times 10$

   2) heptane; column 1-II

Silica I-II of exclusion limit 1.2×10^4 was treated twice with
octadecyltrichlorosilane for removing reactive silanol groups.
As usually expected in GPC, PDMS sample No. A or PDMS fraction
X can elute within a fixed volume, (Fig.3-a-1, Fig.3-b) and the
recovery also amounts to 100% on column 1-II.   On column 1-I,
however, the elution curve of PDMS sample No.A is scarcely to be
recognized because of the strong adsorption by the packing materials
(the dash line in Fig. 3-a).

   While the exclusion limit value is as high as 1×10^6 for Silica
NDG-4L, an obvious adsorptive retention can still occur on this
packing.   As shown in Fig. 4-b. a skewed and tailing peak is ob-
served for PDMS fraction VII on column NDG-4L and about a half
of the polymer sample elutes beyond the elution volume of heptane.
However, Fig.4-a shows a good separation based on molecular
sizes on column 4-II, the values of V_e being 11.3, 15.5 and 17.75
for PDMS fraction II, PDMS fraction VII and heptane, respectively.

   It is evident from the above that the adsorptive action by
remaining silanol groups, as might be expected, can be effectively
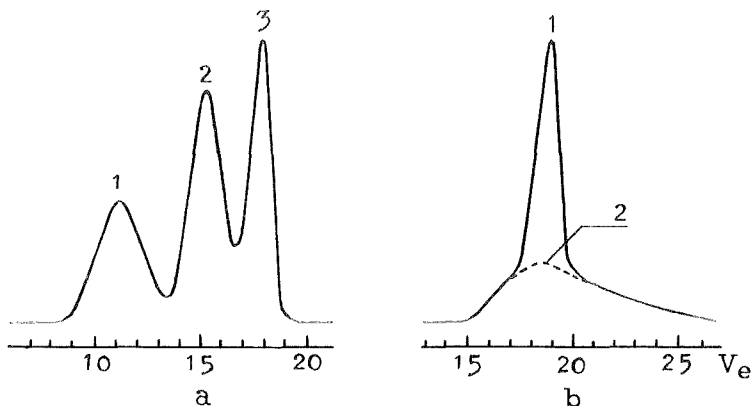
FIGURE 4.   Elution curves on column 4-II or column NDG-4L.
a. 1) PDMS fraction II, $\tilde{M}_\eta 2.87 \times 10^5$, 2) PDMS fraction VII, $\tilde{M}_\eta 3.26 \times 10^4$, 3) heptane; column 4-II. b.1) heptane 2) PDMS fraction VII; column NDG-4L.

suppressed by a long chain alkyl protective screen bonded onto the silica surface. In contrast to silica packings modified only with hexamethyldisilazane, on silica modified with octadecyltrichloro-silane the chromatographic process of hydroxyl-end-blocked poly-dimethylsiloxane in toluene is mainly controlled by size exclusion.

Examination of Universal Calibration Procedure

Gel chromatographic separation is based on molecular sizes. When there is no special interaction between solute and packing materials, hydrodynamic volume defined as $[\eta]M$ may be a universal parameter for calibration. It was proved with polystyrene gels by Dawkins[14] that polystyrene and polydimethylsiloxane followed the same M or $[\eta]M$ calibration in a good solvent such as chloroform. Andrianov et al.[15] also reported that the plots of $\log[\eta]M-V_e$ were in agreement with each other for polystyrene and polydimethylsiloxane blocked by trimethylchlorosilane in a GPC system using toluene and porous glasses. For hydroxyl-end-blocked polydimethylsiloxane, its

TABLE II. Intrinsic Viscosities, molecular weights and elution volume values of polystyrene standards and PDMS fractions.

No.	PDMS fractions				PS		
	$[\eta]$(dl/g)	M	$[\eta]$M	Ve(peak)	M(peak)	$[\eta]$M	Ve(peak)
I	0.870	3.84×10^5	3.33×10^5	23.5	2.6×10^6	1.22×10^7	20.1
II	0.705	2.87×10^5	2.02×10^5	23.85	7.2×10^5	1.32×10^6	22.35
III	0.420	1.40×10^5	5.88×10^5	24.95	3.7×10^5	4.20×10^5	23.25
IV	0.385	1.25×10^5	4.83×10^4	25.15	9.1×10^4	3.70×10^4	25.8
V	0.230	6.07×10^4	1.40×10^4	26.8	4.3×10^4	1.01×10^4	27.6
VI	0.164	3.79×10^4	6.22×10^3	28.3	1.2×10^4	1.11×10^3	30.2
VII	0.147	3.26×10^4	4.79×10^3	28.6	1.0×10^4	8.12×10^2	30.65
VIII	0.104	2.10×10^4	2.09×10^3	29.95	9.0×10^3	6.77×10^2	31.1
IX	0.083	1.47×10^4	1.22×10^3	31.0	5.0×10^3	2.45×10^2	32.0
X	0.054	8.11×10^3	4.38×10^2	32.2	3.7×10^3	1.46×10^2	32.4
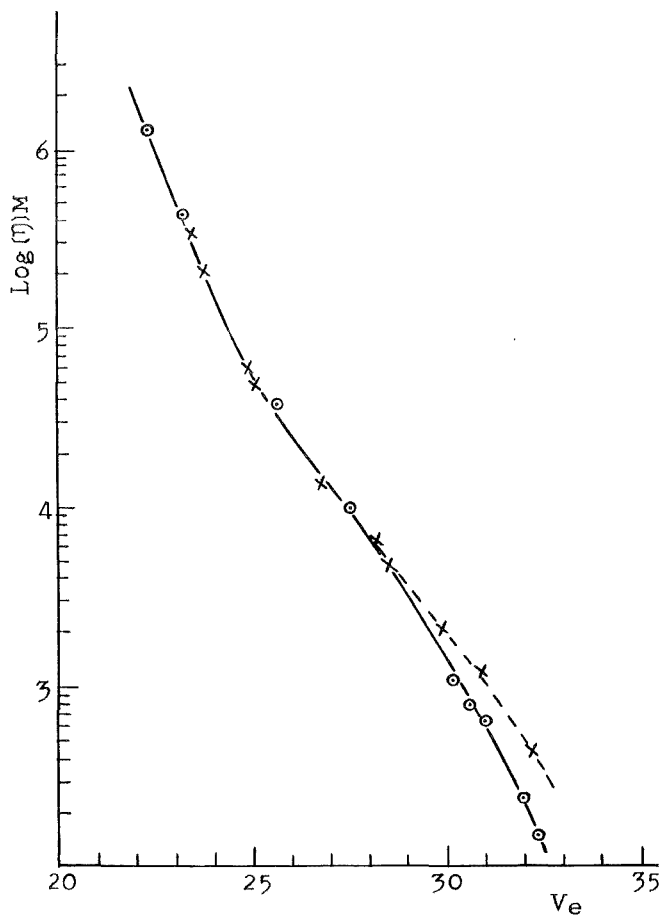
FIGURE 5.   M calibration curves
              two columns of 1m x 8mm i,d, in series, packed with Silica 5-II
              and Silica 2-II repsectively; toluene; 25°C.
              ⊙ PS, ˣ PDMS fractions.

chromatographic behaviour on modified silica gels would follow the
universal calibration prinicple if the adsorptive effect caused by
remaining silanol groups were suppressed to a level comparable
with experimental errors. Based on this thought, the values of
[η] M and elution volume from silica columns 2-II and 5-II in
series are listed in Table II and plotted in Figure 5 for polystyrehe
standards and PDMS fractions. The following relationship was em-
ployed to calculate the [η]M values for polystyrene[16]:

$$[\eta]_{toluene}^{25C} = 0.977 \times 10^{-4} \, M^{0.73} \quad dl/g \qquad (3)$$

Fig. 5 shows that the same   M calibration curves may be obtained
for polystyrene and PDMS fractions above a molecular weight of
approximately 4×10^{4}. On the low side of the curves, however,
PDMS fractions elute later than polystyrene of the same [η]M;
the lower the molecular weights of the polymers, the greater
is the difference in elution volume between them. In principle,
this may be interpreted as additional adsorptive retention; then
the calibration in such a case should be performed with standard
samples of the specific polymer to be determined in order to avoid
introducing an error into the molecular weight calculation.

Discussion Concerning "Adsorptive GPC"

The above residual adsorptive effect is further evidenced from
a comparison of molecular weight calibration on modified silica gel
columns and polystyrene gel columns. The log M-V_e plots are almost
in coincidence for polystyrene and PDMS fractions in the system
with toluene and polystyrene gel JD-103 of exclusion limit 1×10^{5}
(Fig. 6); however, a difference in molecular weight calibration
between the two polymers, especially those of molecular weights
below 4×10^{4}, can be found on silica column 2-II and column
1-II (Fig. 7). It is evident that the chromatographic behaviour
displays "adsorptive-GPC" characteristics for hydroxyl-end-
blocked polydimethylsiloxane on silica gels modified with octa-
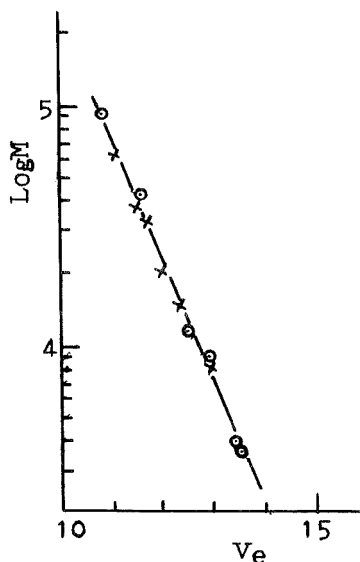
FIGURE 6.   Molecular weight calibration on polystyrene gel JD-103.
            ⊙ PS,   × PDMS fractions

decyltrichlorosilane.   In this case, the excessive retention is
due to the residual adsorption added to the size exclusion; the
adsorptive strength decreases with increasing molecular sizes,
since an increase in size exclusion is accompanied by a loss of
the surface available for adsorption[17].   Finally, when the
polymer molecular species are excluded from all of the pores,
the adsorptive effect also immediately disappears.
        As noticed by Gilpin[18] and Majors[4], if the porous
structure leads to molecular exclusion, the surface available
for chemical modification decreases.   It may be assumed that
there is actually no long-chain alkyl bonded phase on the
internal surface of some pore openings from which octadecyl-
trichlorosilane molecules are all excluded.   In general, the
polymer molecular sizes are large so that they are also excluded,
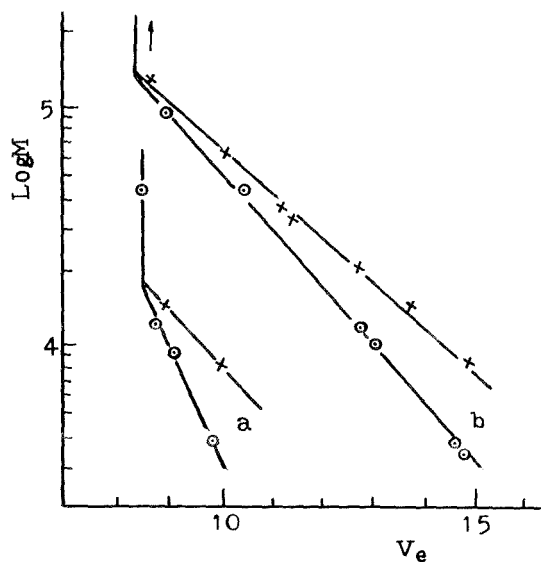
FIGURE 7.   Molecular weight calibration on modified silica gels.

(a) column 1-II, ⊙ PS, ˣ PDMS fractions

(b) column 2-II, ⊙ PS, ˣ PDMS fractions.
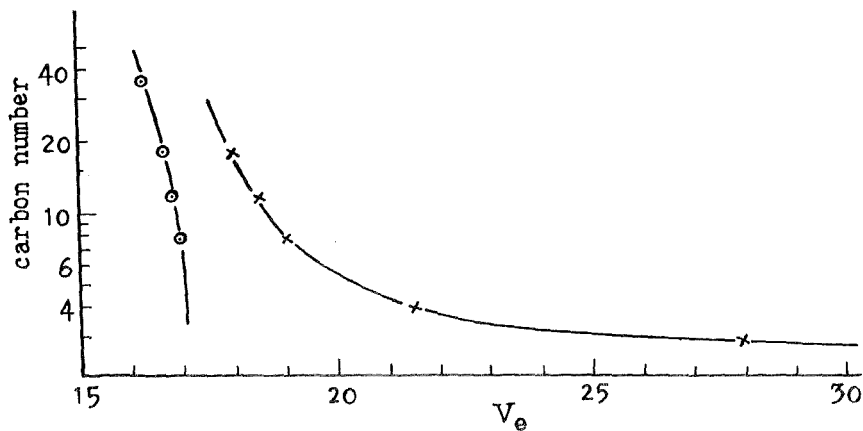
FIGURE 8.   Retention behaviour of n-alkanes and n-alcohols.

column 2-II; toluene. ⊙ n-alkanes, ˣ n-alcohols.

whereas the smaller molecules of polar solute may permeate
into such micropores, resulting in strong retention and in the
adsorptive effect becoming a controlling factor in chromato-
graphic process. For this, an experimental example is given in
Fig. 8, which qualitatively indicates that the differences
between V_e values for n-alkanes and n-alcohols of the same
carbon number increase rapidly as molecular weight decreases on
column 2-II.

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FLOW RATE DEPENDENCE OF SEPARATION AND
BROADENING EFFECTS IN GPC

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ABSTRACT [*]
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   The primary objective of this work is an investigation
of the separation and zone brodening effect in columns
packed with porous and nonporous materials, and estimation
of the accuracy of the broadening parameter, h, obtained by
a reverse flow method.   In order to study the separation and
dispersion phenomenon in the mobile phase, and that caused
by a mass-transfer process, columns packed with smooth glass
beads and porous silica columns were used.

INTRODUCTION
————————————

   In GPC, the relationship between the experimental
chromatogram F(v) and the chromatogram after the correction

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for zone broadening W(y) is given by the Tung's integral
equation (1)

$$F(v) = \int_{-\infty}^{\infty} W(y)\ G(v-y)\,dy \qquad\qquad (1)$$

where both v and y represent the elution volume, and the
function G(v-y) represents the overall zone broadening,
which is thought to have come from several sources: (a)
broadening caused by mixing outside the packed columns, (b)
broadening caused by mixing (diffusional and convective) in
the mobile phase within the packed columns, and (c)
broadening caused by the process of transfer of solute
between the mobile and stationary phases in the columns.
The effect of (a) has been shown to be small (2). The
effect of (b) and (c) have been shown to give Guassian
broadening for conventional chromatography,

$$G(v-y) = \frac{h}{\sqrt{\pi}}\ \exp[-h^2\ (v-y)^2] \qquad\qquad (2)$$

where h is a parameter describing the degree of the
broadening and is related to the standard deviation   of a
Gaussian distribution by

$$h = \frac{1}{\sigma\sqrt{2}} \qquad\qquad (3)$$

In order to use Tung's integral equation, the parameter h,
as a function of elution volume, must first be determined

experimentally for each calibration standard and column

combination.   There are two techniques that can be used to

determine the h value: the reverse flow technique (3) and

the recycle technique (4-5).   Using the reverse flow method,

Tung has found that the parameter h, as a function of

elution volume, passes through a minimum.   This is the

result of combined effects of the extent and rate of

permeation.   In the present work, the separation ability and

broadening effect in mobile phase, the flow rate dependence

of the elution volume on porous silica columns, and the flow

rate dependence of the function h(K), where K is the solute

distribution coefficient, were determined by means of a

reverse flow or recycle method.   The accuracy of the h value

obtained by the reverse flow method was also estimated.

EXPERIMENTAL
————————————

1) Equipment
   ——————————

    GPC Unit,Model SNJ-75-1, with a four-port valve (for

reverse flow technique) and two six port valves (one for

injection of the sample and the other for recycle technique)

was used in this study.   A differential refractometer and a

UV detector were used to monitor the solute concentration in

the eluent.

2) Columns
   ————————

    Five porous silica columns of 2000, 1200, 360, 160 and

100 angstrom mean pore sizes were used, each being 1m long

with 10 mm i.d.

TABLE 1
Standard Polystyrene Samples
Sample   Molec. Weight
———————   ——————————————————

PS-1   2350

PS-2   8500

PS-3   3.5X10^4

PS-4   1.1X10^5

PS-5   2.0X10^5

PS-6   4.7X10^5

PS-7   2.7X10^6

PS-8   3.7X10^6

Four columns, 1 m x 10mm i.d. were packed with nonporous glass beads of 44-53 micron particle size.

3) Samples
   ———————

The polystyrene standards were obtained from Waters Associates and are listed in Table I.   In addition to the above samples, a polystyrene NBS-706 sample, four PMMA samples, two PVC fractions, a SBS TR-1101 copolymer and benzene were used.

4) Experimental Conditions
   ——————————————————————

All measurements were performed at room temperature, THF was used as solvent, flow rate 0.5-5.0 ml/min.   The injection volume was 1.3 ml for porous silica columns and 0.5 ml for glass bead columns.

RESULTS AND DISCUSSION

1) Separation Ability and Zone Broadening Effect in the Mobile Phase.

It was considered that the mobile phase caused only the broadening of the solute zone but did not separate solutes with different molecular weights. Yau (6) used columns packed with nonporous glass beads and found no separation between a styrene monomer and a polystyrene sample, and, by means of this observation, indicated that separation by flow in the mobile phase was not an effective mechanism in GPC. Our experimental results for PS and PVC samples and benzene on nonporous glass bead columns are listed in Table II. This shows that the peak elution volume, Vp, depends upon the molecular weight of the samples. Vp decreases with increasing molecular weight, though the variation is small. In order to further confirm this effect, the recycle

TABLE 2
Elution Vol., Vp, & Broadening Parameters h and h'.

Sample	MW	Vp	h	h'
Benzene	78.1	38.09	2.46	2.41
PS-1	2350	38.07	2.17	2.04
PS-4	1.1×10^5	38.00	1.93	1.78
PS-6	4.7×10^5	37.87	1.89	1.79
PS-7	2.7×10^6	37.60	1.45	1.21
PVC-B	1.18×10^5	38.01	1.89	1.58
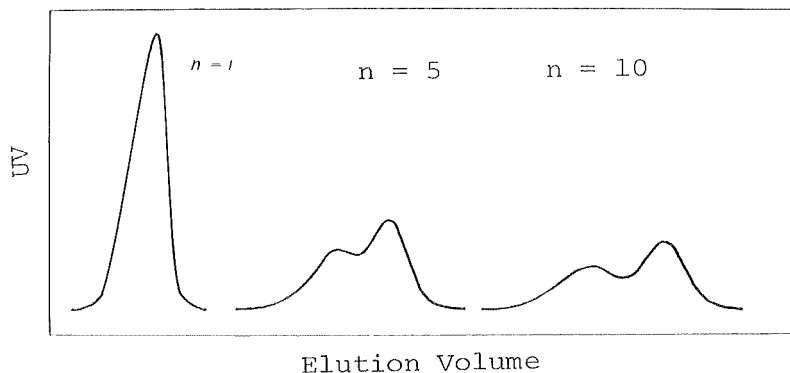
Figure 1.   Separation of mixture (PS-1 + PS-7) on nonporous
             glass bead columns

technique was used; this served the same purpose as the

lengthening of the column.   Figure 1 shows the recycle

chromatogram of a mixed polystyrene sample (PS-1 and PS-7),

n is the number of cycles.   At the first cycle there was

only a single peak of the mixed sample, but after several

cycles the peak was split into two.   The first two peaks

were further separated from each other with increasing cycle

number.   Thus, the separation ability of the columns was

well confirmed.   Flow rate dependence of V_p is shown in

Figure 2. V_p of both samples increase with increasing flow

rate, and the slopes of both curves are almost the same.

This result could not be explained in terms of separation by

flow and separation by steric exclusion because, in both

cases, the flow rate independence of V_p is usually expected

(7).   Explanation by restricted diffusion theory is not

possible either; it would predict that the elution volume

should decrease with increasing flow rate and that the
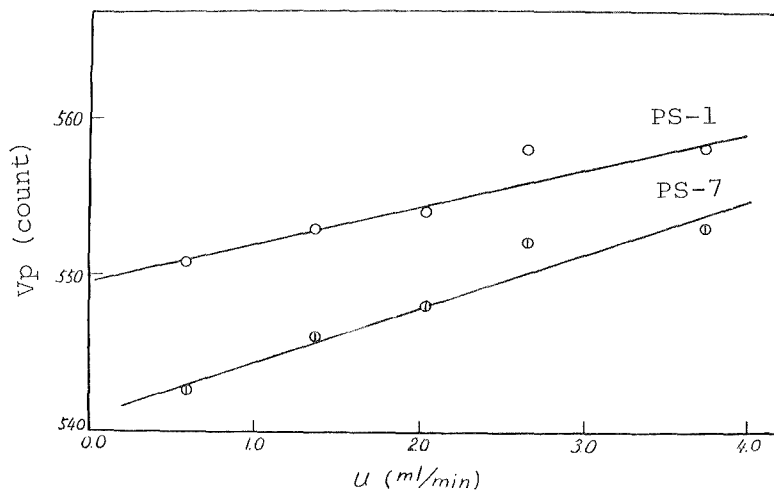
Figure 2. Variation of V_p with flow rate on nonporous
glass bead columns

elution volume of low molecular weight compounds should be
influenced by a variation of flow rate to a lesser extent
(8).

According to Kelley and Billmeyer(9) the plate height
equation describing dispersion in the mobile phase with a
velocity profile model is expressed by equation (4)

$$Hm = 2\phi Dm\ U^{-1} + 2\lambda dp + \frac{2kRc^2}{\phi DmU^{-1} + \lambda dp} \qquad (4)$$

where Hm is the mobile phase plate height, U is the
interstitial velocity, Dm is the diffusion coefficient for
the solute in the mobile phase, ϕ is the tortousity factor (ϕ
= 2/3), λ is an eddy diffusion proportionality factor (λ =
1/11), dp is the effective particle diameter, k is a
velocity profile constant, and Rc is the column radius.

Thus Hm is composed of molecular diffusion, eddy diffusion, and velocity profile terms. The molecular diffusion contribution to dispersion is ignored. This was supported by the experimental results of Copper (10). The eddy diffusion term is a function of particle size only, being independent of molecular weight of solute molecules. The third term, the velocity profile effect, is related to MW through Dm in the denominator.

In this work, the broadening parameter h (or h', the broadening parameter obtained from a straight-through flow chromatogram) is used to describe the dispersion effect. The relationship between Hm and h is

$$Hm = L \ (2Vp^2h^2)^{-1} \tag{5}$$

where L is the column length. The values of h and h' obtained from reverse flow and straight through flow chromatograms respectively for several PS, PVC-B and benzene are listed in Table 2. The fact that h' decreases with increasing MW is qualitatively in agreement with the prediction of the velocity profile model. But in equation (4) there was no factor related to the MWD of the solute. As mentioned above, the samples could be separated according to their MW's in the mobile phase, so we considered that a factor reflecting the solute MWD should be added to equation (4). This deduction was supported by the data of Table II. The narrow-distribution PS-4 and the broad-distribution PVC-B (Mw/Mn = 2.9) have almost the same values of Vp and h, but their h' values are obviously different from each other.

Here, similar h values mean that the broadening caused by
polydispersity has been almost cancelled by the flow
reversal, and the difference in h' simply reflects the
effect of polydispersity of the sample.

2) Effect of Reverse Flowing Process on Zone Broadening on
Nonporous Glass-Packing Columns
     As the low MW solute benzene is monodisperse in
molecular weight, the broadening parameters h and h' should
have the same value. The h and h' for benzene were
determined from reverse flow and straight-through flow
chromatograms. Figure 3 shows the flow rate dependence of Δ

$$\Delta = \frac{2(h-h')}{h + h'} \ 100 \ \%$$
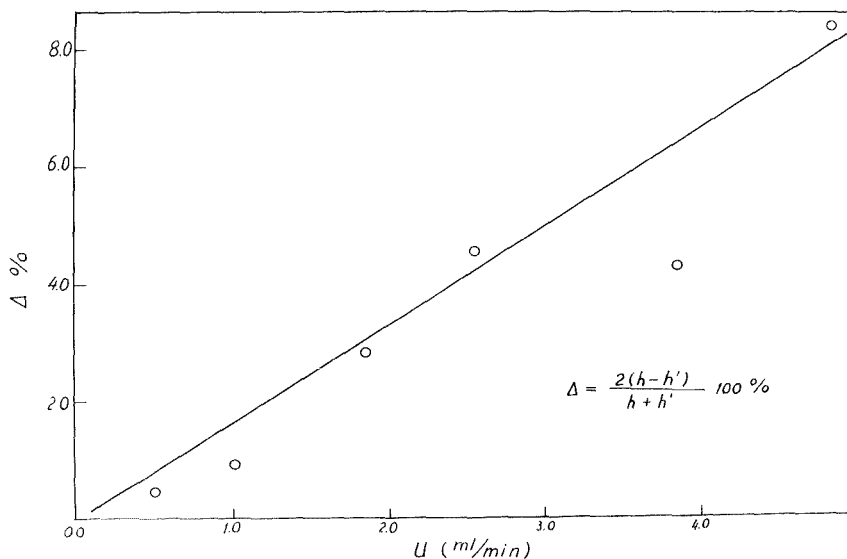
Figure 3.   Variation of Δ for benzene with flow rate on
            nonporous glass bead columns

($\Delta = 2(h-h')/h+h'$).   The fact that $h>h'$ could be explained
by the following assumption: According to Kelley and
Billmeyer's velocity profile model, while the fluid is
flowing in some fixed direction on the column, the velocity
profile is formed and the solute zone disperses to some
extent.   During the reverse flowing process, the fluid
changes its flow direction in a very short time; thus, the
velocity profile should be disturbed.   After this time, a
new velocity profile is established and the solute zone
further dispersed.   However, the extent of dispersion of
this reverse flowing experiment should be less than that of
the straight through flowing experiment, in which the
velocity profile is not disturbed.   Therefore, the value of
h becomes larger than that of h'.   It is expected that the
extent of disturbance is dependent upon the flow rate as
shown in Figure 3.

3) Flow Rate Dependence of Elution Volume on Porous Silica
Columns

      The dependence of peak elution volume on flow rate has
been reported by several authors giving conflicting results.
Yau (11) observed that the elution volume of PS high
molecular weight decreased with increasing flow rate in the
range from 0.1 to 10.0 ml/min.   Little (12), on the other
hand, found Vp was independent of flow rate in the same
range.   Spatorico (13) reported that a negligible dependence
of Vp on U was obtained in porous glass-bead packing
studies, whereas a small increase in Vp was observed with

increasing flow rate in the range of 0.2 to 1.0 ml/min on

polystyrene gel studies.   Recently Aubert and Tirrell (14)

and Gudzinowicz and Alden (15) came to the conclusion that

the elution volume increased with increasing flow rate in a

range of 1.0 to 3.0 ml/min on porous silica columns, and

offered an explanation based upon a flow-rate dependent

equilibrium distribution coefficient.   Moreover, Boni et al.

(16) reported that the observed Vp passed through a maximum

as flow rate was increased (0.2 - 2.0 ml/min).   Mori (17),

on the other hand, found an opposite result, i.e., that the

observed elution volume passed through a minimum as flow

rate was increased on Microstyragel columns in the range of

0.5 to 4.0 ml/min.   This problem was investigated in the

present work with porous silica columns.   A siphon was used

to collect the effluent liquid for the purpose of monitoring

the flow and the elution volume.   The volume of discharged

liquid was corrected as follows.   The weight per count of

collected liquid, A, at flow rate, U, was weighed.   The

relationship between A and U is given in Figure 4.   The

calibration curve may be expressed by a linear equation.

$$A = A_o + \alpha U \tag{6}$$

and the corrected elution volume Vp may be calculated using

equation (7)

$$Vp = (Vp)_{app} \left(1 + \frac{\alpha}{A_o} U\right) \tag{7}$$

where $(Vp)_{app}$ is the experimentally measured elution volume

at the flow rate U. A_o and α could be obtained from the
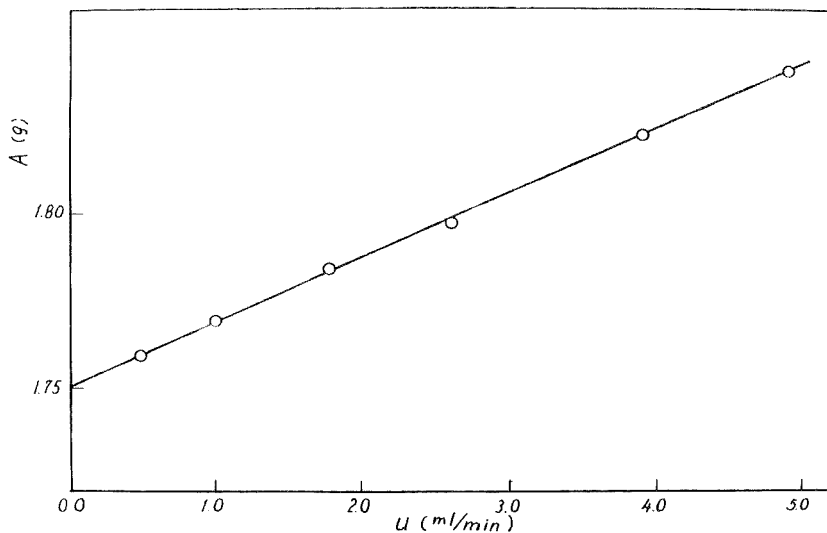
Figure 4.   Flow rate dependence of discharged efflux

calibration curve.   As the evaporation rate in the siphon
chamber of the solvent used (THF) was very small (less than
0.4 ml/24 hr), its effect on elution is ignored.   It was
found that the corrected elution volume is independent of
flow rate for all samples used, in the range of 0.5 to 5.0
ml/min on porous silica columns, as shown in Figure 5.

The GPC calibration curve is given in Figure 6, from
which the void volume is obtained (V_o = 68.5 count).   The
total permeation volume, V_o + V_i , is equal to the elution
volume of benzene (V_o +V_i = 150.1 count).   Thus, the pore
volume V_i is equal to 81.6.   Using these data, the,
distribution coefficient K for all standards were
calculated.
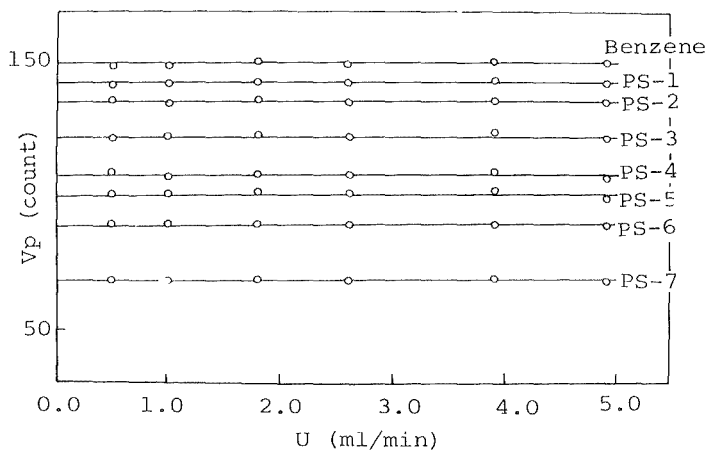
Figure 5.   Flow rate dependence V_p on porous silica columns
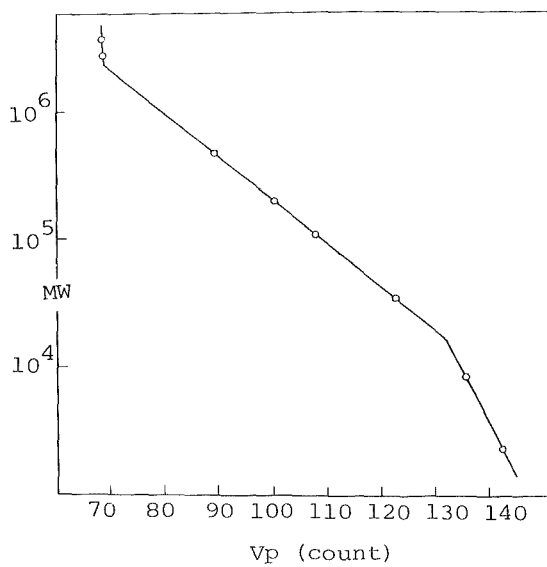
Figure 6.   GPC calibration curve of porous silica columns
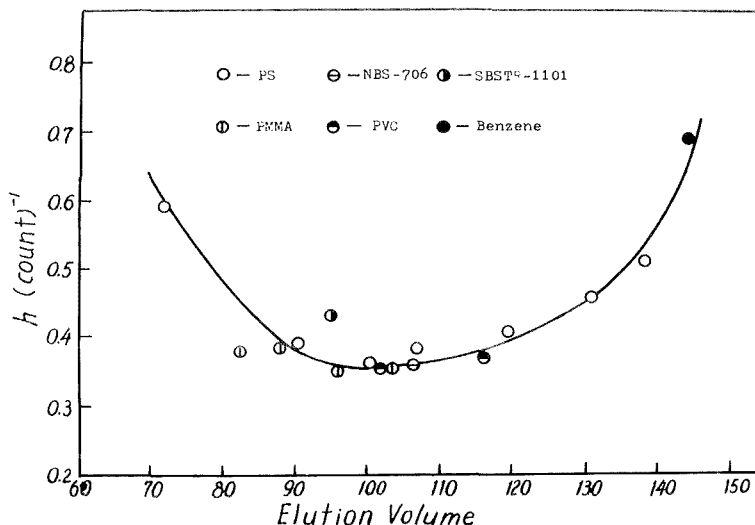
Figure 7.   Variation of parameter h with elution volume

4) Zone Broadening Effect on Porous Silica Columns

     The broadening parameter h of seven PS standards, four
PMMA samples, two PVC fractions, a broad MWD sample PS
NBS-706, a SBS TR-1101 copolymer and benzene were determined
by using reverse flow method.   Figure 7 shows the observed h
parameter as a function of elution volume.   All points fall
on a single curve.   This is in agreement with Tung's
experimental result (3) that the relationship between h and
V_p is universal, independent of chemical structure and MWD
of samples.

     The relationship between parameter h and distribution
coefficient K was determined at different flow rates as
shown in Figure 8.   It shows that the value of h is strongly
dependent upon flow rate.   However, these curves are very

similar in shape for different flow rates except for
smallest two. This means that $h(V_p)$ (or $h(K)$) functions
are defined primarily by pore structure of the packing
materials. Recently Wenner and Halasz reported the result
of a study of the relationship between the zone broadening
effect and pore size distribution of stationary phase.
These authors found that the plots of plate height vs
relative molecular weight, and of pore volume freqency vs
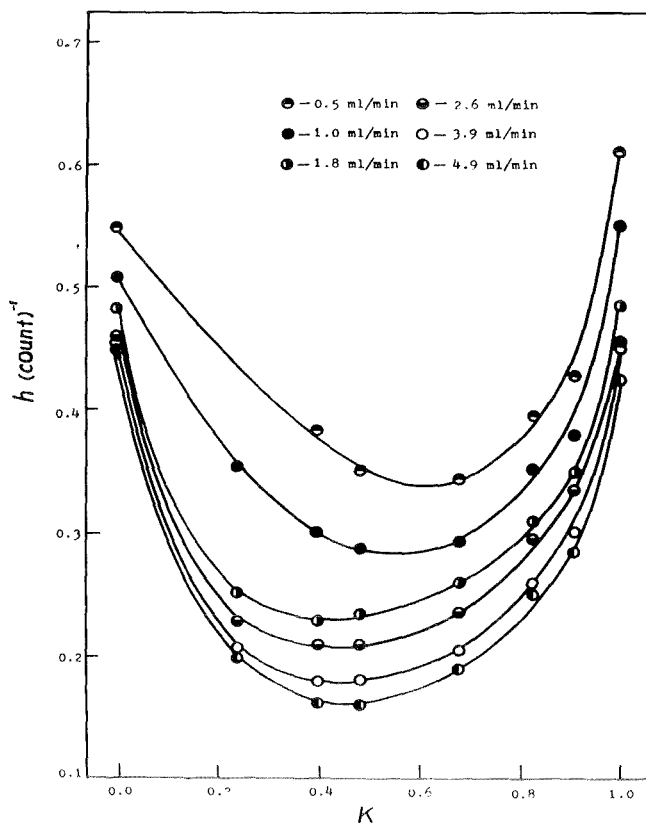
Figure 8. Flow rate dependence of $h(K)$

pore diameter showed very good agreement. The results of
that paper and of the present work complement each other.

5) Effect of Reverse Flowing Process on Zone Broadeing with
Porous Silica Columns

       As described above, for the nonporous glass bead
columns, the parameters h and h' for benzene on porous
silica columns were determined from reverse flow and
straight through flow chromatograms obtained at different
flow rates. For all flow rates h)h' are obtained, and Δ as
a function of flow rate U is given in Figure 9. It reveals
that the difference between h and h' decreases with

$$\Delta = \frac{2(h-h')}{h+h'} \cdot 100\%$$
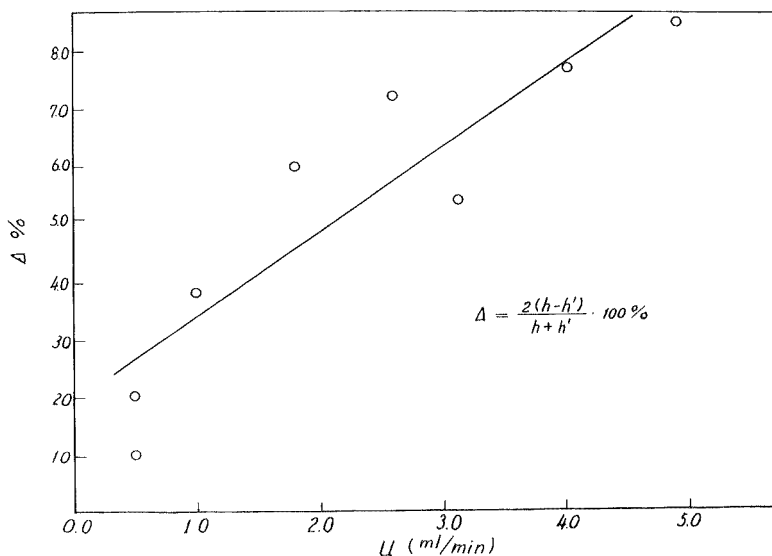
Figure 9.   Variation of Δ for benzene with flow rate on
            porous silica columns

decreasing flow rate in agreement with the result obtained
for nonporous glass bead columns.   Therefore the explanation
based upon the assumption of velocity profile disturbance
during the reverse flowing process is valid in this case.
The inequality of h and h' obtained for benzene ought to be
expected for polymer samples.   The fact that h>h' indicates
that the use of the h value obtained from the reverse flow
method to correct the zone broadening effect may lead to
slight underestimation.   However, this effect may be ignored
when experiments are run at low flow rate.

CONCLUSION

     The experimental results show that polymer samples may
separate in the mobile phase of GPC columns according their
molecular weights; thus, a factor reflecting a sample's
polydispersity ought to be added to the plate height
equation describing mobile phase dispersion.   After
correcting for the syphon volume, the elution volume is
independent of flow rate in the range of 0.5 to 5.0 ml/min
on porous silica columns.   The relationship between h and V_p
(or K) is universal, independent of chemical structure and
MWD of polymers.   The curves of h (K) are similar in shape
for different flow rates.   The broadening parameter,
obtained from a reverse-low chromatogram, h, is larger than
that obtained from a straight-through-flow chromatogram h'
for benzene on both nonporous and porous packing columns.
This fact indicates the use of the h value obtained from the
reverse-flow method may lead to slight underestimation of

the zone broadening effect in GPC. But this influence may

be ignored when the GPC experiments are run at low flow

rates.

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A STUDY OF MECHANICAL DEGRADATION OF *

POLYMER IN HIGH PERFORMANCE GPC

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ABSTRACT

Four narrow distribution polystyrene samples (M = 2.7 x 10^6 , 6 x 10^6 , 6.5 x 10^6 , 7 x 10^6) were dissolved in tetrahydrofuran and the solutions were passed through a Shodex A-80M column at a concentration of approximately 1 x 10^{-3} g/ml, injection volume of 500 microliters, and a flow rate of 2 ml/min (i.e., maximun flow rate allowable for this column). Molecular weights of eluants were then determined by viscosity and laser light scattering methods; concentrations were determined by ultra- violet spectrophotometry. From the results of analysis of the eluate, it was shown that no significant degradation was detectable for all four samples in this colulmn which was packed with a cross-linked polystyrene gel. When a silica gel (irregular shaped) column was used, under same operating conditions, only sample PS-4, with a molecular weight of M = 7 x 10^6 underwent degradation up to 15%. High pressure exerted on the column is believed to be the main cause of the degradation.

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INTRODUCTION

When a polymer solution passes through a GPC column at high pressure, the high molecular weight portion of the sample may be degraded by shearing stress. The degradation of polystyrene ($M = 10^7$) and polyisobutylene ($M = 10^6$) in conventional GPC has already been reported [1,2]. The degradation is expected to be more serious in high performance and high temperature GPC. Degradation was observed for polystyrene ($M = 4 \times 10^6$) in 1,2,4-trichlorobenzene at 135 °C and for PE ($M = 7.5 \times 10^5$) in the same solvent and temperature, even at low flow rate [3]. As a result of degradation in the high molecular weight portion of the sample, errors are introduced both to the calibration curve and to the calculated average molecular weight for high molecular weight samples. Therefore it is worth while to look deeper into the problem of degradation of high-molecular weight polymer after passing through a GPC column. GPC chromatographers are very much concerned about the question of what is the upper limit of molecular weight of polymer that will not undergo degradation in high performance GPC at room temperature. Since polystyrene

is widely used as standard sample to calibrate GPC columns, our study initially involved this polymer.

EXPERIMENTAL

1. Instruments:

Two sets of columns were used in a Waters Associates Model ALC/GPC 244 instrument. The first set was a Shodex A-80M column (inner diameter = 0.8 cm, theoretical plates = 18000 plates / 50 cm). The second set was a silica gel (irregular shape) column (inner diameter = 0.8 cm, 50 cm in length theoretical plates = 16000 plates / 50 cm). This prepared porous silica gel was supplied by Jilin Institute of Chemical Industry.

2. Samples

PS-1   M = 2.7 x 10^6 (Waters standard sample)

PS-2   (Polysciences, Inc.)

$\overline{M_w}$ = 6 x 10^6 (measured by LALLS in our laboratory)

PS-3   (Jilin Institute of Chemical Industry)

PS-4   (PS polymerized at room temperature and fractionated twice)

3.   Eluent: Tetrahydrofuran (THF)

4.   Detection method for molecular weight and
     concentration:

     The experimental procedure was carried out as
follows: A sample was collected as it eluted from the
chromatographic column.   Its MW was determined and
compared with the value obtained for a "blank" that was
not passed through the GPC column.   The concentration
of eluate was determined with a UV Spectrophotometer
(Specord UV-VIS):   Molecular weights of the samples
were determined with a laser low angle light scattering
photometer (Chromatix KMX-6).   Due to the extremely low
concentration of the eluate, the experimenatal error in
the light scattering measurement was rather large.
Intrinsic viscosities were also measured to supplement
the light scattering data.   Any changes in intrinsic
viscosities were taken as a measure of the degradation.
The low concentration (ca 3 - 8 x 10^{-5} g/ml) of sample
in the eluate from GPC column necessitated a proper
choice of viscometer.   Two viscosimeters with quite
long efflux times (to = 27'18"8 for THF at 25°C) were
selected and an effluent time difference, Δt , between

polymer solution and pure solvent of more than 10 seconds could be obtained.   In such a way, the intrinsic viscosities $\{\eta\}$ of dilute solutions were determined with good reproducibilities.

GPC Experiment:

Since degradation occurs most readily at high concentration and high flow rate, a high concentration of $1 - 2 \times 10^{-3}$ g/ml and a high flow rate of 2 ml/min were used for both the Shodex A-80M and silica gel columns.

RESULTS AND DISCUSSION

(1)   Four polystyrene samples ($M = 2.7 \times 10^6 - 7 \times 10^6$) were injected into a Shodex A-80M column at a concentration of approximately 1×10^{-3} g/ml, injection volume of 500 microliters and a flow rate of 2 ml/min, (pressure gauge indicating 300 psi).   All of the fractions were collected in one bottle and analyzed by the viscosity method.   The determinations were repeated several times.   The results are listed in Table 1. Under identical operating conditions, solutions of two

TABLE 1. [η] of polystyrene samples prior to and after passage through a Shodex A-80M column

sample No.	sample conditions		$C \times 10^5$ g/ml	η_r	$[\eta]$	$[\eta]$	$[\eta]-[\eta]^1$	$\dfrac{[\eta]=[\eta]^1}{[\eta]}$ %
1	PS-1 $M=2.7\times10^6$	prior	4.9239	1.024	487.4			
	2ml/min 300psi	after	5.25	1.025		476.2	11.2	2.3
2	PS-2 $Mw=6\times10^6$	prior	7.3903	1.065	852.5			
		after	7.03	1.063		867.7	-15.2	-1.8
	2ml/min 300psi	after	6.82	1.061		865.1	-12.6	-1.5
3	PS-3	prior	5.2181	1.048	919.9			
		after	4.22	1.039		925.1	- 5.2	-0.6
	2ml/min 300psi	after	5.65	1.054		955	-35.1	-3.8
4	PS-4	prior	7.818	1.079	984.9			
		after	3.20	1.032		1000	-15.1	-1.5
	2ml/min 300psi	after	7.95	1.081		994	- 9.1	-0.9

TABLE 2. [η] of polystyrene samples prior to and after passage through a silica gel column

sample No.	sample condition		$CX10^5$ g/ml	η_r	$[\eta]$	$[\eta]$	$[\eta]-[\eta]^1$	$\dfrac{[\eta]-[\eta]^1}{[\eta]^1}$ %
1	PS-2 Mw=6x10⁶	prior	5.4964	1.047	855.1			
		after	5.71	1.048		840.6	14.5	1.7
	2' ml/min	after	5.26	1.045		855.5	-0.4	-0.05
	1500 psi	after	5.72	1.049		856.6	-1.5	-0.18
2	PS-4	prior	5.0667	1.050	986.8			
		after	5.26	1.044		836	150.8	15.3
	2' ml/min	after	5.23	1.045		837.5	149.3	15.1
	1500 psi	after	5.21	1.044		844.5	142.3	14.4
	1 ml/min	after	5.25	1.047		897	89.8	9.1
	800 psi	after	5.24	1.047		896	90.8	9.2

polystyrene samples (M = 6 x 10^6 , 7 x 10^6) were passed through the silica gel column (pressure gauge indicating 1500 psi). The viscosities of the fractions were determined similarly. Results are given in Table 2.

(2)   It is obvious from Table 1 that all four samples passing through the Shodex A-80M column were not degradated at all. On the silica gel column, the situation was quite different. Sample PS 4 underwent degradation to a extent of approximately 15%, (see Table 2). Flow rate and injection concentration on the two columns were the same. The only difference was pressure on the two columns. The pressure on the silica column was much higher than that of Shodex A-80M column. We believe that column pressure is vital to the occurrence of shear degradation.

(3)   The data of Table 1 and Table 2 show that the degradation of PS-THF system is not so serious in the high performance GPC column at room temperature as we first thought. Our data pointed to the fact that mechanical degradation is negligible when PS samples with molecular weights up to 6 x 10^6 are used as standard samples for column calibration.

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DETERMINATION OF POLYOLEFIN ADDITIVES
BY NORMAL-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
FOLLOWING SOXHLET EXTRACTION

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ABSTRACT

Methods were developed for the determination of three classes of polyolefin additives. These classes are: mono and diglycerides, tertiary C_{12}-C_{16} alkyldiethanolamines, and alkyldithiopropionates. Soxhlet extractions were performed on 50 g ground samples with chloroform for 2 hours. The extracts were concentrated and the additives were determined by high-performance liquid chromatography on a μ-Porasil stationary phase. The methods were studied with both polyethylene and polypropylene.

INTRODUCTION

Recently a series of methods were published from this laboratory for the determination of additives in polypropylene and polyethylene by normal phase high performance liquid chromatography (HPLC) following hot decalin extraction (1-3). Decalin extraction can be performed in about an hour and can be used for many types of additives as long as the additives possess good chromophores (3). The present work describes methods based on Soxhlet extractions with chloroform which can be used for determining several additives which are not amenable to the decalin extraction/HPLC method.

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MATERIALS AND METHODS

Instrumentation The liquid chromatograph used in this study was a
Waters Model 204 liquid chromatograph equipped with a model 6000A
pump. The injectors were a Valco 6000 psi injector or a Micromer-
itics Model 725 Autoinjector, both equipped with 50µL sample
loops. Elution was monitored with a Waters Model 450 variable
wavelength ultraviolet absorption detector or a Waters Model 401
Differential Refractometer detector, and a 10 mV strip chart
recorder. The column used was a 3.9 mm i.d. x 30 cm µ-Porasil
column packed with 10 micron porous silica obtained from Waters
Associates, Milford, Mass.

The grinder was a Wiley mill with 10 mesh screen, cooled with
liquid nitrogen. The Soxhlet extractor used had a 500 mL capacity
solvent flask. Ground sample was extracted from 45 mm x 123 mm
cellulose thimbles obtained from Fisher Scientific.

Reagents Reagent grade chloroform, 1,2-dichloroethane, and abso-
lute ethanol for use as HPLC solvents were filtered through Milli-
pore type FH 0.5 micron filters prior to use. Ammonium hydroxide
(27% aq) was obtained from Mallinkrodt and used as received. The
extraction solvent was reagent grade chloroform and was used with-
out pretreatment. Armostat 310 and Armostat 410 were obtained
from Armak, Chicago, IL.; Atmul 84 was from ICI America, Inc.,
Wilmington, Del.; DLTDP and DSTDP were obtained from American
Cyanamid Co., Bound Brook, N. J.

Procedure Portions of 50 g samples of polymer were weighed accur-
ately after grinding to 10 mesh, and placed in Soxhlet thimbles.
To prevent the samples from floating in the extraction solvent,
the cutout tip of an extraction thimble and a 1 cm layer of 3 mm
glass beads were placed above each sample. A 250 mL portion of
extraction solvent was poured into each of the boiling flasks.
After 2 hours extraction time, the extract solutions were trans-
ferred to 400 mL beakers and evaporated to about 10 mL on a
steam bath. The solutions were transferred quantitatively to 25
mL volumetric flasks and diluted to volume with chloroform. The

TABLE I

Chromatographic Conditions Used for the Separation of Additives on μ-Porasil.

ADDITIVE	MOBILE PHASE	RETENTION VOLUME, mL	DETECTOR
Armostat 310	Chloroform:Ethanol: Ammonia (80:20:0.1)	5.6	Refractive Index
Armostat 410	Chloroform:Ethanol: Ammonia (80:20:0.1)	5.6	Refractive Index
Atmul 84	Chloroform:Ethanol: Ammonia (95:5:0.05)	6.3	Refractive Index
DLTDP	1,2-Dichloroethane	7.9	UV, 230 nm
DSTDP	1,2-Dichloroethane	6.3	UV, 230 nm

solutions were allowed to sit for several minutes until any small polymer particles present floated to the top, leaving a clear solution near the bottom of the flask. Using a Pasteur pipet, 1-5 mL portions of the clear bottom solutions were transferred to 20 mL scintillation vials, from which the HPLC injections were made.

The HPLC conditions used for the determination of the additive are listed in Table I. Duplicate injections of each standard and sample solutions were made. Peak heights were measured to the nearest 0.5 mm, and in cases where the peak widths for sample extracts were not uniform, area measurements were made by measuring also the peak widths at half the peak heights to the nearest 0.1 mm using a peak magnifier. The amount of each additive was determined from each sample injection by comparing peak heights or areas for sample and standard.

RESULTS AND DISCUSSION

A requirement for the determination of additives by the decalin extraction/HPLC method (1-3) is that the additives possess a strong chromophore above 230 nm, in the region where a mobile phase solvent such as dichloromethane does not absorb ultraviolet

radiation strongly. Typically levels of such additives in decalin
following extraction range from about 0.02-0.2 mg/mL. This is
about two orders of magnitude too dilute for HPLC determination
following decalin extraction of the five common additives which
are the subject of this work. These are Atmul 84 (mono-and
diglycerides), DLTDP (dilaurylthiodipropionate), DSTDP (distearyl-
thiodipropionate), Armostat 310 (ethoxylated tallow amine), and
Armostat 410 (ethoxylated coconut oil amine). Concentrations of
these additives should be 1-10 mg/mL or greater. To obtain
extracts with these concentration levels, an extraction procedure
with a volatile solvent is required. Several such procedures have
been described (4-6). Reported extraction times were as long as
48 hours (4).

In the present work, to determine the minimum amount of time
required to extract the additives of interest from ground polyole-
fin matrices, Soxhlet extractions were performed with chloroform
for time intervals ranging from 1-8 hours. The levels of addi-
tives were determined by HPLC following a 1:10 concentration of
the extract solution. The amounts of additives extracted are
listed in Table II. It is apparent from Table II that the extrac-
tions are almost complete following 1 hour and no significant
additional amount of additives are extracted after 2 hours extrac-
tion time. All subsequent analyses were performed using 2 hour
extraction times.

Response Linearity of response with peak height was established
for injected amounts for up to 465 µg Armostat 310, 510 µg
Armostat 410, 500 µg Atmul 84, 241 µg DLTDP, and 465 µg DSTDP.
Some nonlinear response with peak height and area was observed
between 241 µg and 482 µg DLTDP injected. This serves to illus-
trate that the region of linear of response should be established
for the particular liquid chromatograph and detector used for this
type of analysis.

Accuracy To ensure that the additives were being extracted in a
quantitative manner studies were performed with polymers prepared

TABLE II
Weight Percent Additives Determined in 50 g Polymer Samples at Various Extraction Times

ADDITIVE	POLYMER	TIME OF EXTRACTION, HOURS	WT %
Armostat 310	PE	1	0.12
		2	0.14
		4	0.15
		8	0.16
Armostat 410	PP	1	0.19
		2	0.18
		4	0.19
		8	0.18
Atmul 84	PP	1	0.28
		2	0.30
		4	0.28
		8	0.31
DLTDP	PE	1	0.025
		2	0.029
		4	0.020
		8	0.030
DSTDP	PP	1	0.26
		2	0.26
		4	0.24
		8	0.24

with known amounts of additives. The results of this study are listed in Table III. The data in Table III indicate good recoveries for the additives.

Precision The pooled standard deviations for the additive determination were calculated and are listed in Table IV. These data indicate good precision for the Soxhlet extraction/HPLC method.

Limits of Detection Limits of detection for the method as written were calculated assuming 2 mm peak heights for both the RI detector set at 16X sensitivity and the UV detector set at 0.2 Absorbance. This represents a S/N level of about two. The limits of detection, expressed as wt. % in original samples were 0.002% Armostat 310, 0.004% Armostat 410, 0.02% Atmul 84, 0.003% DLTDP, and 0.007% DSTDP.

TABLE III

Results of Studies on Polymer Samples with Known Amounts of
Additives.

SAMPLE	ADDITIVE	SPECIFIED WT. %	DETERMINED WT. %
PE	ARMOSTAT 310	0.10	0.068
		0.10	0.072
		0.20	0.14
		0.20	0.15
		0.40	0.31
		0.40	0.32
		0.60	0.48
		0.60	0.47
PP	ARMOSTAT 410	0.05	0.034
		0.05	0.034
		0.10	0.083
		0.10	0.085
		0.20	0.18
		0.20	0.18
		0.40	0.39
		0.40	0.38
PP	ATMUL 84	0.10	0.068
		0.10	0.068
		0.20	0.18
		0.20	0.17
		0.40	0.43
		0.40	0.42
		0.80	0.76
		0.80	0.75
PE	DLTDP	0.01	0.018
		0.01	0.014
		0.03	0.029
		0.03	0.026
		0.10	0.052
		0.10	0.046
		0.20	0.12
		0.20	0.11
PP	DSTDP	0.05	0.070
		0.05	0.052
		0.10	0.092
		0.10	0.088
		0.20	0.26
		0.20	0.24
		0.40	0.42
		0.40	0.45

TABLE IV

Precision Data for the Additives

ADDITIVE	POOLED STANDARD DEVIATION[a]	RANGE OF VALUES, WT. %
ARMOSTAT 310	0.0089	0.068-0.48
ARMOSTAT 410	0.0050	0.034-0.39
ATMUL 84	0.0055	0.068-0.76
DLTDP	0.0063	0.014-0.12
DSTDP	0.020	0.052-0.45

a. Calculated from the data in Table III.

Interferences Usually more than one additive is used in a poly-
olefin formulation. Thus, in determining an additive, it is
important to check possible interferences with other additives
which could be present in the polymer. Studies indicated that BHT
and Irganox 1010 do not interfere with the determination of Armo-
stats 310 and 410. However, a peak due to a minor component in
the additive Weston 618 might be an interference if this additive
is present. A small component in Weston 618 also might interfere
in the determination of Atmul 84. However, the additives DSTDP,
DLTDP, BHT, and Ethyl 330 do not interfere in the determination of
Atmul 84. In the determination of DLTDP and DSTDP, some potential
interference was encountered with Topanol CA and Irganox 1010,
however, the interference from the latter additive was slight.
The additives CGL-144, Kemamide E, Irganox 1076, TNPP, and BHT do
not interfere with the determination of DLTDP or DSTDP. In gen-
eral, interference studies should be done with any other known
additives present.

CONCLUSION

The procedure described in this work should be amenable to
several types of additives which do not possess good chromophores
for sensitive detection in dilute solution. The 2 hour Soxhlet

extraction conceivably could be coupled with other specifically designed normal-phase HPLC systems, or nonaqueous reversed-phase systems as well.

ACKNOWLEDGEMENT

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CHARACTERIZATION OF NONIONIC POLYACRYLAMIDES
BY AQUEOUS SIZE EXCLUSION CHROMATOGRAPHY
USING A DRI/LALLSP DETECTOR SYSTEM

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ABSTRACT

Herein is reported an experimental investigation of the molecular weight characterization of nonionic polyacrylamides by aqueous SEC with a DRI/LALLSP detector system. Methodology for the use of the DRI/LALLSP detector responses to determine the molecular weight calibration curve and the peak broadening parameter, σ^2 (variance of a Gaussian instrumental spreading function) over a wide molecular weight range has been developed. The method is based on the use of a broad MWD standard made by blending Polysciences broad MWD standards and a generalized analytical solution of Tung's integral equation for the detector response corrected for peak broadening. Molecular weight averages measured by SEC/DRI/LALLSP are in excellent agreement with those measured offline by LALLSP.

INTRODUCTION

The use of SEC with a LALLSP-based detector system for molecular weight characterization of organic and water-soluble polymers is a recent event.[1,2,3] In fact, methodology for the

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interpretation of the detector responses is still in the
development stage and to date, quantitative methods for the
estimation of the molecular weight calibration curve and σ^2 across
the chromatogram have been published for one polymer, dextran.[3] A
strong interest in the molecular weight characterization of
polyacrylamides has recently developed, largely due to the great
potential shown by these polymers for enhanced oil recovery via
polymer flooding. In fact, the molecular weight distribution of
the polymer must be tailored for each reservoir. This, in part,
has motivated the present investigation. Polyacrylamides are a
good choice for other reasons, the most important of which is the
lack of well-characterized standards, whether of narrow or broad
MWD. In addition, a suitable packing/mobile phase system has been
developed for nonionic polyacrylamides.[4]

THEORY

Tung's integral equation is the starting point for all
rigorous methods of correction for peak broadening. For the case
of a Gaussian instrumental spreading function, the integral
equation takes the form

$$F(v) = \frac{1}{\sqrt{2\pi\,\sigma^2(v)}} \int_0^\infty W(y)\,\exp(-(v-y)^2/2\sigma^2(v))\,dy \qquad (1)$$

The use of $\sigma(v)$ rather than $\sigma(y)$ is an approximation which should be valid when the variation of σ with retention volume or molecular size in solutions is not excessive. Molecular weight averages in the detector cell are given by

$$\frac{\overline{M}_K(v,uc)}{M(v)} = \frac{F(v-(K-1)\,D_2(v)\,\sigma^2(v))}{F(v-(K-2)\,D_2(v)\,\sigma^2(v))} \exp[(2K-3)(D_2(v)\,\sigma(v))^2/2] \quad (2)$$

where

$\overline{M}_K(v,uc)$ is the Kth molecular weight average of the contents of the detector cell at retention volume v.

$M(v)$ is the molecular weight calibration curve and $D_2(v)$ is the slope of the molecular weight calibration curve which is given by

$$M(y) = D_1(v)\,\exp(-D_2(v)y) \tag{3}$$

If it is further assumed that the size distribution of polymer solute in the detector cell is Gaussian, it follows that

$$W(v,y) = \frac{F(v)}{\sqrt{2\pi\,\overline{\sigma}^2(v)}}\,\exp(-(y-\overline{y}(v))^2/2\overline{\sigma}^2(v)) \tag{4}$$

The detector response corrected for peak broadening is then given by[6]

$$W(v) = F(v)(\sigma(v)/\overline{\sigma}(v))\,\exp(-(v-\overline{y}(v))^2/2\overline{\sigma}^2(v)) \tag{5}$$

$$\overline{y}(v) = v + \frac{1}{D_2(v)} \ell n\left(\frac{F(v+D_2(v)\ \sigma^2(v))}{\sqrt{\ F(v-D_2(v)\ \sigma^2(v))\cdot F(v+D_2(v)\ \sigma^2(v))}}\right) \qquad (6)$$

$$\overline{\sigma}^2(v) = \sigma^2(v) + \frac{1}{D_2^2(v)}\ \ell n\left(\frac{F(v-D_2(v)\ \sigma^2(v))\cdot F(v+D_2(v)\ \sigma^2(v))}{F^2(v)}\right) \qquad (7)$$

Equation (5) may be applied to either the DRI or the LALLSP detector response. Whole polymer molecular weight averages corrected for peak broadening are given by

$$\overline{M}_K(c) = \int_0^\infty W(v)\ \overline{M}^{K-1}(v)dv\Big/ \int_0^\infty W(v)\ \overline{M}^{K-2}(v)dv \qquad (8a)$$

$$\overline{M}_K(c) = \int_0^\infty F(v)\ \overline{M}_K^{K-1}(v,uc)dv\Big/ \int_0^\infty F(v)\ \overline{M}_K^{K-2}(v,uc)dv \qquad (8b)$$

The above equations are employed to develop quantitative methods for determining the molecular weight calibration curve and $\sigma(v)$ across the chromatogram.

EXPERIMENTAL

Instrumentation and operational details for aqueous SEC employed follow.

Columns: 3/8 inch ID X 4-6.5 ft. dry packed with CPG-10 glass
           packing having pore sizes of 700, 1000 and 3000 Å and
           particle size, 200/400 mesh.

Mobile phase: 0.20 M Na_2SO_4 with 1 g/25 l Tergitol NPX (Union

              Carbide Corp.) in deionized distilled water.

Mobile phase flowrate: 1 ml/min.

Detectors: Waters R-401 DRI and Chromatix KMX-6 with angle 6-7°

            and field stop 0.15.

Inline filter: 0.45 micron Millipore.

Sample loop: 2.0 ml with 0.01-0.1 wt.% polymer solute.

Temperature: ambient.

Polymer standards: nonionic   polyacrylamide   standards   from

                  Polysciences (Warrington, PA) and blends of

                  these standards.

dn/dc: was measured with a Chromatix KMX-16 laser differential

        refractometer at λ = 632.8 nm and 23°C for nonionic

        polyacrylamide standards in 0.02 M Na_2SO_4 with 1 g/25 l

        Tergitol NPX. Values measured were 0.176 (PAM500), 0.168

        (PAM1000) and 0.174 (PAM2000).

A_2:   the second virial coefficient, A_2, was not used to determine

        $\overline{M}_W$(v,uc) as the error involved was neglible at the polymer

        solute concentrations employed.[3]

RESULTS AND DISCUSSION

    Molecular weight calibration curves for single columns

containing one pore size are shown in Fig. 1. These calibration
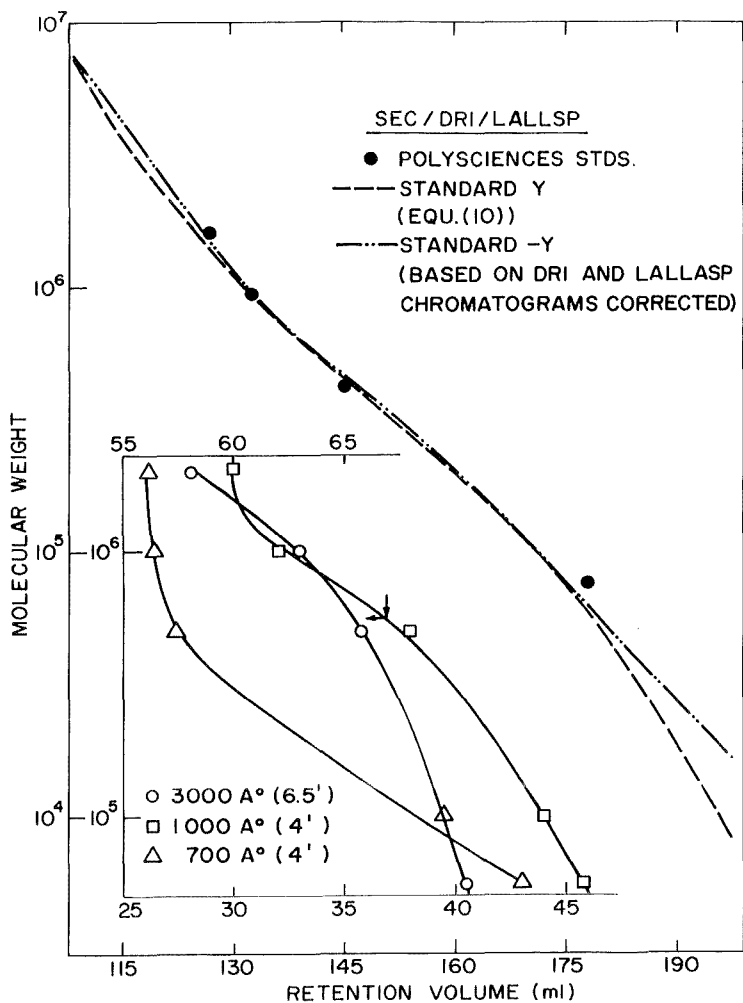
FIGURE 1:   Molecular weight calibration curves for single columns
            and a column combination obtained by different
            procedures.

curves are approximate as they were obtained using Polysciences broad MWD nonionic polyacrylamide standards and plotting log $\bar{M}_W$ versus peak retention volume. $\bar{M}_N$ values for these standards are not available from the Supplier. These approximate calibration curves are useful in establishing an effective column combination which gives good peak separation over a wide range of retention volumes. It will be later shown that a column set containing 1-4 ft. column with 700 Å pores, 1-4 ft. column with 1000 Å pores and 1-6.5 ft. column with 3000 Å pores gives a molecular weight calibration curve with excellent peak separation and which is almost linear over a wide molecular weight range.

Typical DRI/LALLSP detector responses for PAM2000 (a 2×10^6 $\bar{M}_W$ Polysciences standard) are shown in Fig. 2. The LALLSP response is very noisy and this is a direct result of the use of a 0.45 μm filter inline. In a previous study with dextrans, a 0.22 μm filter was used and a far less noisy LALLSP response was obtained.[3] To prevent the capture of large polyacrylamide molecules on the inline filter, larger pores are required. The noise level of the LALLSP response was reduced by continually flowing mobile phase at a flowrate of 0.1 ml/min through the columns for long periods of several days to a week before injecting polymer solute. The flowrate is increased to 1.0 ml/min and run for 4 to 6 hours before injection. An acceptable noise level for the LALLSP detector could not be obtained with a 0.65 μm inline filter.

DRI

LALLSP

RETENTION VOLUME (ml)
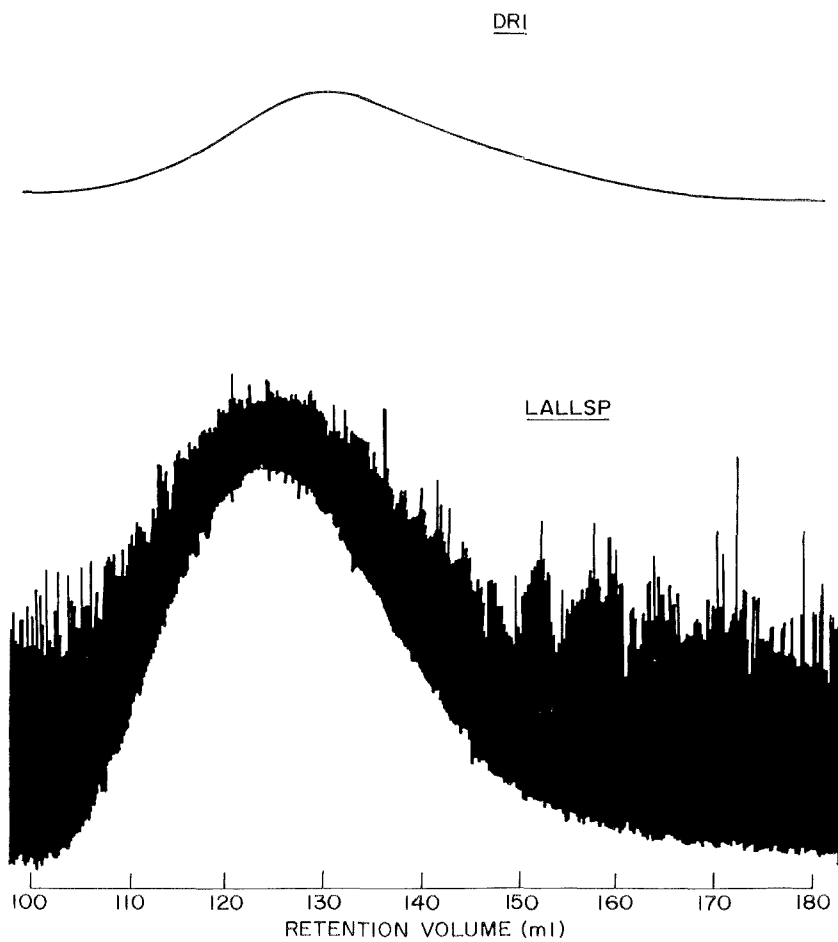
FIGURE 2:   DRI/LALLSP detector responses for PAM2000 (a
            Polysciences nonionic polyacrylamide standard with
            nominal $\overline{M}_W = 2 \times 10^6$).

A calculational procedure for finding M(v) and σ(v) over a wide range of molecular weights and retention volumes has been reported.[3] This procedure was used here with a blend of Polysciences standards obtained by mixing PAM2000 (42 wt.%), and PAM500 (58 wt.%). The DRI/LALLSP detector responses are shown in Fig. 3. The molecular weight calibration curve, M(v) was obtained by setting $\bar{M}_W(v,uc) = M(v)$ near the peak position of the individual Polysciences standards and this calibration curve is shown in Fig. 1. This calibration curve and the DRI/LALLSP detector responses in Fig. 3 were employed with eqn. (2) to
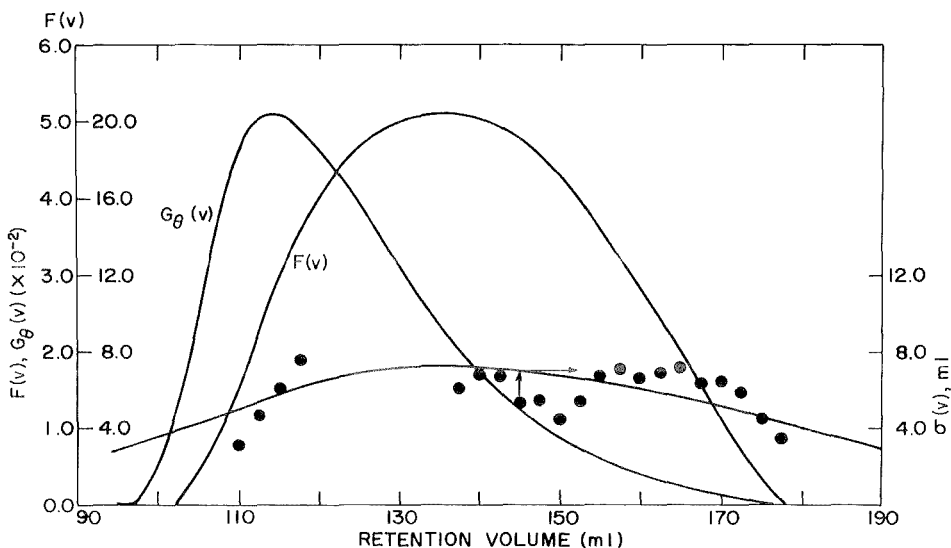
FIGURE 3:   DRI/LALLSP detector responses and peak broadening parameter (σ(v)) for a blend of Polysciences standards (PAM2000 (42 wt.%) and PAM500 (58 wt.%)) designed for σ(v) determination.

estimate σ(v) over the retention volume range of the blend.   A

single-variable search was used to find σ(v) and these results are

also shown in Fig. 3.   There is considerable scatter in the σ(v)

values due to the noisy LALLSP signal; however, the variation of

σ(v) with retention volume seems to follow the usual broad bell

shape with a relatively small variation with retention volume.   A

very broad standard, STANDARD-Y, was made by blending PAM200 (13
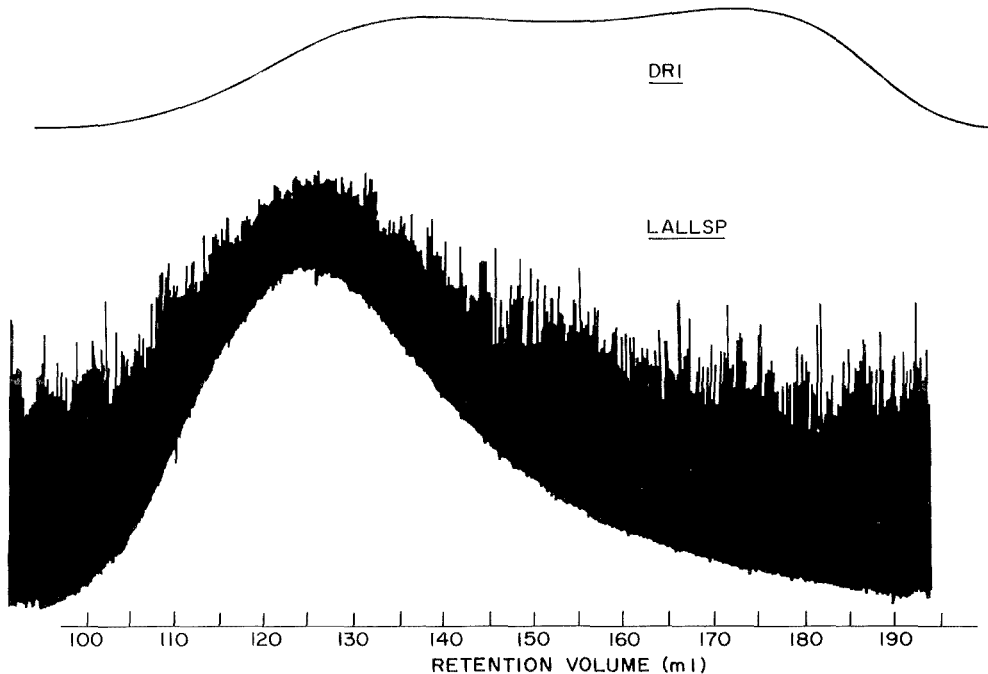
FIGURE 4:   DRI/LALLSP detector responses for STANDARD-Y, a blend
            of Polysciences standards (PAM2000 (13 wt.%), PAM500
            (65 wt.%), PAM74 (22 wt.%)) designed for molecular
            weight calibration curve determination.

wt.%), PAM500 (65 wt.%) and PAM74 (22 wt.%) and was designed to permit the determination of M(v) over a wide range of molecular weights with the DRI/LALLSP detector system. With this standard, it is possible to set $M(v) = \bar{M}_W(v,uc)$ over a wide range of molecular weights and thus find M(v) without the need to correct for peak broadening. The DRI/LALLSP detector response for STANDARD-Y are shown in Fig. 4. To investigate the error involved in M(v) when employing STANDARD-Y and neglecting the peak broadening corrections, a procedure with corrections for peak broadening was used. In particular, the sensitivity of the calculated M(v) to $\sigma(v)$ was investigated. Details of this calculational procedure follow.

It is assumed that in a narrow range of retention volumes $(V_i - V_{i+1})$, the calibration curve is linear. Eqn. (2) may then be written as

$$\ell n\left(\frac{\bar{M}_W(v_i, uc)}{\bar{M}_W(v_{i+1}, uc)}\right) = D_2(v_{i+1} - v_i) + \frac{D_2^2}{2}(\sigma^2(v_i) - \sigma^2(v_{i+1}))$$

$$+ \ell n\left(\frac{F(v_i - D_2 \sigma^2(v_i)) \cdot F(v_{i+1})}{F(v_{i+1} - D_2 \sigma^2(v_{i+1})) \cdot F(v_i)}\right) \qquad (9)$$

Given $\sigma(v)$, eqn. (9) is used to solve for $D_2(v)$ and then eqn. (2) is used to find M(v). The results of this computational procedure

TABLE 1

Determination of the Molecular Weight Calibration Curve
M(v) Using STANDARD-Y — Sensitivity of M(v) to σ(v)

Retention volume (ml)	$\overline{M}_W$(v,uc)/M(v)				
	σ(v) actual	0.5 σ(v)	0.8 σ(v) (ml)	1.2 σ(v)*	1.5 σ(v)*
118.75	0.734	0.955	0.974	--	--
123.75	0.876	0.798	0.933	--	--
128.75	1.006	1.003	1.004	--	--
133.75	1.065	1.014	1.036	1.118	--
138.75	1.078	1.018	1.048	1.125	--
143.75	1.069	1.017	1.044	1.100	--
148.75	1.065	1.016	1.041	1.095	1.153
153.75	1.052	1.014	1.033	1.073	1.118
158.75	1.046	1.012	1.030	1.065	1.096
163.75	1.056	1.014	1.036	1.078	1.118
168.75	1.052	1.013	1.034	1.074	1.115
173.75	1.078	1.019	1.050	1.115	1.183
178.75	1.100	1.023	1.062	1.141	1.23
183.75	1.113	1.027	1.070	1.166	1.26

* Calculation at low retention volumes were not possible because of excessively large σ(v).

using $\sigma(v)$ values shown in Fig. 3 are summarized as the molecular weight calibration given by eqn. (10).

$$\ln M(v) = 57.52 - 0.789v + 4.93 \times 10^{-3} v^2 - 1.102 \times 10^{-5} v^3 \qquad (10)$$

This calibration curve is also shown in Fig. 1. The calculational procedure was repeated using $\sigma(v)$ values smaller and larger than those shown in Fig. 3 to illustrate the sensitivity of the calculated $M(v)$ to $\sigma(v)$. These results are tabulated in Table 1. It is clear that STANDARD-Y can be used to determine $M(v)$ with small error when peak broadening corrections are neglected. This is true for the retention volume range; 128-183 or molecular weight range, $5 \times 10^4 - 2 \times 10^6$. Similarly, very broad MWD standards covering higher molecular weights could be made by blending in higher molecular weight polyacrylamides when available.

DRI detector response (raw and corrected for peak broadening) for Polysciences standards PAM2000, PAM1000 and PAM500 are shown in Fig. 5. Corrections are largest at the high molecular weight end of the chromatograms as expected. Corrections for peak broadening were made using eqns. (5,6,7) and the $\sigma(v)$ values in Fig. 3. Detector responses (raw and corrected for peak broadening) for STANDARD-Y obtained using the DRI/LALLSP detector system are shown in Fig. 6. Again, eqns. (5,6,7) and $\sigma(v)$ values shown in Fig. 3 were used to determine $W(v)$. The corrected responses ($W(v)$ chromatograms) were then used to calculate directly the molecular weight calibration curve. It is now
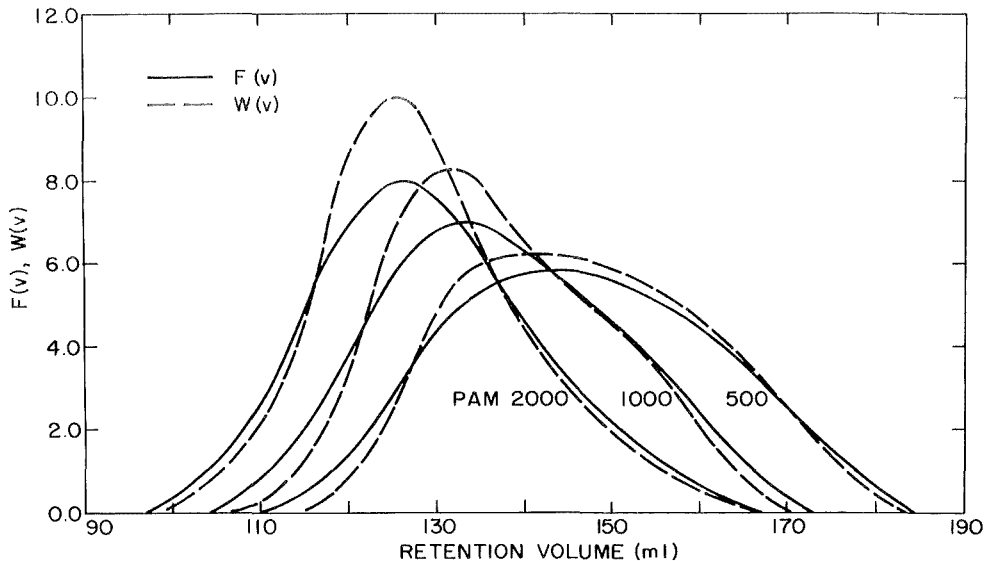
FIGURE 5:   DRI detector responses for Polysciences standards (raw
            (F(v)) and corrected for peak broadening (W(v)) using
            an analytical solution of Tung's integral equation).

correct to set $M(v) = \bar{M}_W(v)$ at all retention volumes across the

detector responses.   The $M(v)$ thus obtained is also shown in Fig.

1.   The agreement among the molecular weight calibration curves

obtained by three different methods is good.   In Fig. 7 are shown

the $\bar{y}(v)$ and $\bar{\sigma}^2(v)$ values obtained for the STANDARD-Y from the DRI

response and also from the LALLSP response.   The agreement among

these values obtained from responses of very different shape is

gratifying and suggests that both responses be used to obtain

reliable estimates of $\bar{y}(v)$ and $\bar{\sigma}^2(v)$ over the full molecular

weight range of the sample.
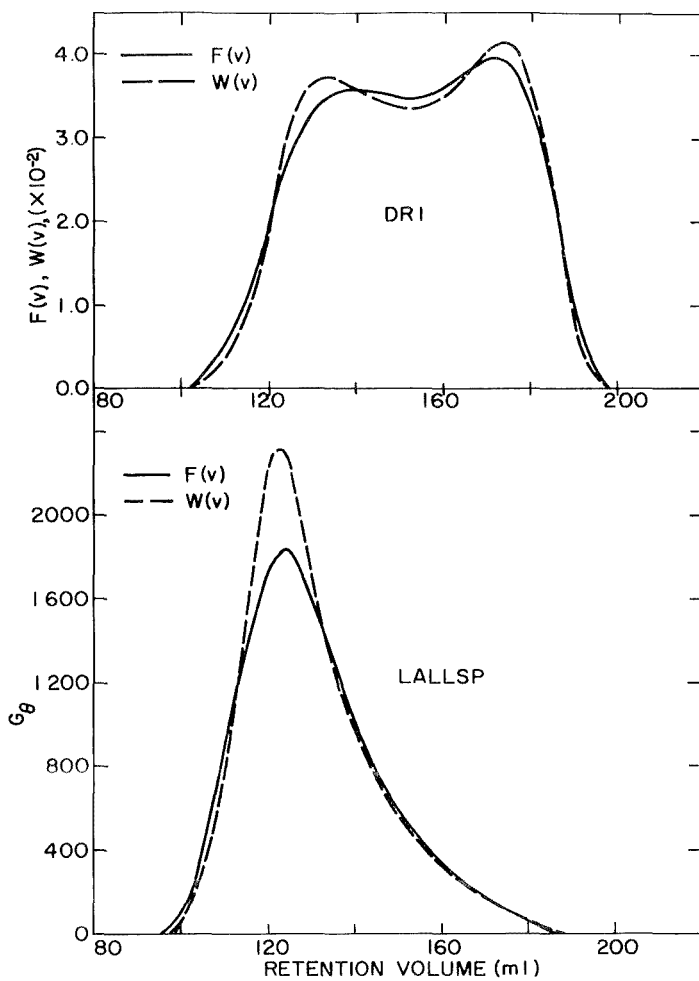
FIGURE 6: DRI/LALLSP detector responses for STANDARD-Y (raw and
           corrected for peak broadening).
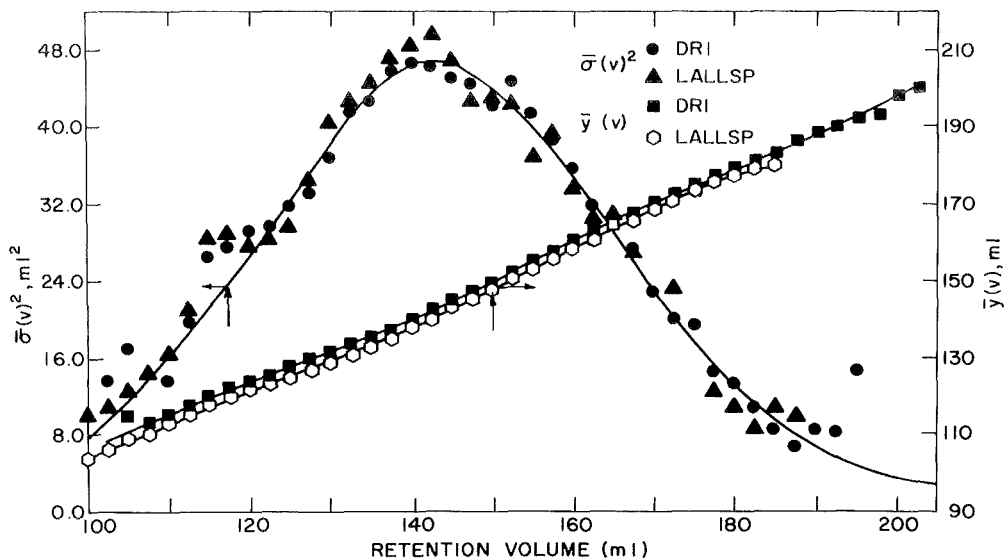
FIGURE 7:   Peak broadening parameters for detector cell contents
            $(\overline{\sigma}^2(v)$ and $\overline{y}(v))$ obtained for both DRI and LALLSP
            detector responses.

TABLE 2

Whole Polymer $\overline{M}_N(c)$ and $\overline{M}_W(c)$ Values by SEC and $\overline{M}_W$ by LALLSP
Offline

Sample	$\overline{M}_W$ (LALLSP) $\times 10^{-3}$	$\overline{M}_N(c)$ (SEC/DRI/LALLSP)* $\times 10^{-3}$	$\overline{M}_W(c)$	$\overline{M}_N(c)$ (SEC/DRI)** $\times 10^{-3}$	$\overline{M}_W(c)$
PAM2000	2,180	1,198	2200	959	1,880
PAM1000	1,000	608	964	551	988
PAM500	532	283	521	288	571

*   Calculated using eqn. (8b).

** Calculated using eqn. (8a).

Finally, whole polymer molecular weight averages $\bar{M}_N(v)$ and $\bar{M}_W(c)$ (averages corrected for peak broadening) measured by SEC using different calculation paths are tabulated in Table 2. The agreement among $\bar{M}_W$ values is within experimental error.

SUMMARY AND RECOMMENDATIONS

Methodology for the interpretation of DRI/LALLSP responses to provide the molecular weight calibration curve and peak broadening parameters (σ^2) over a wide range of molecular weights has been developed and applied successfully to the molecular weight characterization of nonionic polyacrylamides.

The recommended procedure for the molecular weight characterization of nonionic polyacrylamides by aqueous SEC is to use a LALLSP based detector system to determine the molecular weight calibration curve and peak broadening parameters with a specially blended very broad MWD standard, such as STANDARD-Y. The MWD and molecular weight averages for other polyacrylamide samples should then be measured using a mass concentration detector, such as DRI, with application of the analytical solution of Tung's integral equation to provide W(v). In other words, the LALLSP based detector system need only be used for calibration purposes.

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RETENTION OF IONIZED SOLUTES IN
REVERSED-PHASE HIGH PERFORMANCE
LIQUID CHROMATOGRAPHY

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ABSTRACT

The mechanism of retention in reversed-phase high performance
liquid chromatography is affected by both solute-eluent interac-
tions and the nature of the stationary phase. The hydrophobic ex-
pulsion of ionized solutes plays a major role in affecting solute
behavior in the water-rich range of hydroorganic eluents. In the
water-lean range of eluent composition, there is little hydropho-
bic expulsion, and specific interactions between the solute and
surface can be observed. The nature of the surface affects the
retention of a variety of ionized species, both large cations and
anions. Octadecylsilane (ODS) bonded phases can exhibit two dif-
ferent binding sites: one exhibiting a weak interaction and the
second a strong specific interaction with a solute. Styrenedi-
vinylbenzene polymeric surfaces exhibit the potential for weak
dispersion interactions, and in addition pi-bonding interactions
with a solute. A variety of solutes have been injected in a water:
methanol eluent system in order to assess solute-surface effects
on reversed-phase supports.

INTRODUCTION

The mechanism of retention in reversed-phase high performance

liquid chromatography has been the subject of controversy. It has

been variously proposed that partitioning of sample molecules oc-

curs into a "liquid" phase defined by the surface and associated

molecules, or that sample molecules adsorb onto the surface (1).

Whatever the nature of the solute-stationary phase phenomena, the

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mobile phase affects the extent of interaction of solute with the
stationary phase, either by modifying the surface (2-7) or by in-
teracting directly with the solute in the mobile phase.

Solute-mobile phase interactions can be formulated in terms
of the total energy of interaction of a solute molecule with the
molecules of solvent in the mobile phase. The total energy re-
flects the sum of all possible interactions which include disper-
sion interactions and specific interactions (electrostatic dipole
interactions, electron donor-acceptor interactions and bond for-
mation)(8). These solute-solvent interactions promote the affinity
of the solute for the mobile phase. Countervailing the attractive
interactions is the solvophobic affect (9,10) or hydrophobic ef-
fect when water is the solvent. Water exhibits the strongest
manifestation of this solvophobic effect, and so hydrophobic ef-
fects predominate in the binary eluent systems commonly employed
in reversed-phase HPLC; namely hydroorganic mixtures of water and
methanol, or water and acetonitrile. The hydrophobic expulsion is
generally attenuated by increasing the concentration of organic
modifier. Eventually at high levels of added modifier, e.g. (80:
20) methanol:H_2O for the alkylsulfonates (11), a water-lean eluent
composition is attained and hydrophobic effects are attenuated.
The capacity factor, k is then independent of the number of methy-
lenes in the alkyl chain (11). The eluent composition at which
solvophobic effects are attenuated is dependent on the nature of
the solute, but in general solvophobic effects are negligible at
90% methanol in the mobile phase.

In addition to solute-mobile phase interactions, solute-
stationary phase interactions can play a major role in determining
the extent of retention of a solute in reversed-phase chromato-
graphy. Solvent-surface interactions have been observed on the
surface of C_{18} columns (2-7). Solute-surface interactions are
similar. The interactions may be due to the solute adhering to
the C_{18} group bonded to the silica support (10). In this case,
one can envision a competitive effect in which organic solvent

molecules solvating the n-alkyl bonded chains in the stationary
phase are displaced by solutes of greater hydrophobicity (6).
Alternatively, the solute can interact with adsorbed organic mod-
ifier (3,6). Finally, the nonaqueous modifier in the mobile phase
may be extracted into the bonded phase, and solutes can then par-
tition between the mobile phase and the modified bonded phase (2).
Apart from the solute-hydrocarbonaceous phase interactions, silan-
ophilic interactions (12-14) can be operative causing tailing of
chromatographic peaks and the enhancement of retention of "non-
retained" species at extremely low levels of water. Obviously the
nature of solute-stationary phase phenomena in reversed-phase
chromatography is complex and subject to controversy.

In order to diminish the hydrophobic effects of the mobile
phase in reversed-phase chromatography, experiments were performed
using a water-lean eluent system, (90:10) methanol:H_2O. Solvo-
phobic expulsion of most species is negligible at this eluent com-
position, so that the driving force for retention will not be the
effect of solvent in forcing the solute to the hydrocarbonaceous
surface, but rather potential favorable interactions of the solute
with the stationary phase. In this way solute-stationary phase
interactions can be evaluated without having hydrophobic inter-
actions override the other operative interactions.

In order to assess the role of the stationary phase, two
distinct stationary phases were evaluated: (1) an octadecylsilane
bonded support and (2) a styrenedivinylbenzene co-polymer adsor-
bent. The phases differ significantly in their surface character-
istics.

Finally, quaternary ammonium salts and organic acids were
used as solute ions. These species are most affected by the na-
ture of the support since they can adsorb onto a surface (15). It
is assumed that sample molecules adsorb onto the surface rather
than partition into a liquid phase. An electrical double-layer
model can then be used to describe the retention behavior of ion-
ized solutes in reversed-phase HPLC. Ions which have no primary

solvation layer or are able to lose it on the side facing the ad-
sorbent surface can displace some of the adsorbed solvent molecules
and contact the surface. This contact adsorption is largely gov-
erned by the properties of the given ion. An array of contact ad-
sorbed solvent molecules and solute ions is known as an inner
layer, and the loci of the centers of the contact-adsorbed ions
defines the inner Helmholtz plane (IHP). Solvated ions which can-
not contact adsorb approach the surface up to a distance known as
the outer Helmholtz plane (OHP). These ions are non-specifically
adsorbed since the electrostatic interactions are essentially in-
dependent of the chemical properties of the ions. Counter ions
associated with the contact-adsorbed ions are found in the diffuse
layer outside the OHP. This model is derived from the Stern-Gouy-
Chapman theory of the electrical double layer, and has been used
for describing the adsorption of aromatic ammonium compounds on
Amberlite XAD-2 (16). A similar model was invoked by Bidlingmeyer
et.al. (10) and R. S. Deelder and J. H. M. Van Der Berg (17).

EXPERIMENTAL

The chromatographic system employed in the retention studies
of the anions on the octadecylsilane bonded phase consisted of a
Waters system including an M6000 pump, a U6K injector, a Model 450
variable wavelength detector, a 720 system controller, and a 730
data module.

The chromatographic system used in all the remaining studies
was a Tracor, Model 985 solvent delivery system (Tracor Instru-
ments, Austin, TX, U.S.A.) with a model 7125 automatic syringe
loading sample injector (Rheodyne, Berkeley, CA, U.S.A.) with a
20 µL sampling loop, a model 970A variable wavelength detector
(Tracor), a model 951 HPLC pump (Tracor), and a model 3390A re-
porting integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The
octadecylsilica bonded column was a Partisil 5-ODS (Whatman, Inc.,
Clifton, NJ, U.S.A.), while the styrenedivinylbenzene copolymer
column was a PRP-1 (Hamilton Company, Reno, NV, U.S.A.).

Methanol (MCB Manufacturing, Cincinnati, OH, U.S.A.) was dis-
tilled, then aspirated through 0.50 µM Millipore filters. Filters
were purchased from Rainin, Woburn, MA. Water was distilled and
then aspirated through 0.50 µM cellulose acetate filters. The
mobile phases were degassed using helium prior to use. The mobile
phase flow rate was maintained at 1.00 mL/min.

Tetraalkylammonium salts, including tetramethylammonium
bromide, TMAB, tetraethylammonium bromide, TEAB, tetrabutylammonium
bromide, TBAB, tetraheptylammonium bromide, THAB, and hexadecyl-
trimethylammonium bromide (cetrimide), CTAB, were all purchased
from Eastman, Rochester, NY.

Acids and salts used in this study include: 2-naphthalene-
sulfonic acid, sodium salt (NAS) and picric acid (PA) purchased
from Eastman; toluenesulfonic acid (TS), sodium nitrate ($NaNO_3$)
and potassium nitrate (KNO_3) purchased from Fisher, Fair Lawn, NJ;
dichloroacetic acid (DCA) purchased from Aldrich.

All solutes were prepared as concentrated solutions and then
diluted to the desired mobile phase composition. All samples were
dissolved in the mobile phase. The retention volume of 10^{-4}M
$NaNO_3$ or 10^{-4}M toluenesulfonic acid was used as a measure of the
dead volume in all systems.

RESULTS AND DISCUSSION

Retention of Anions on ODS

A variety of completely dissociated aromatic acids and sodium
salts have been injected into a (25:75) methanol:H_2O eluent system.
In unbuffered systems, as the nanomoles of solute increase, there
is an increase in the capacity factor k_e of the solute. See
Figure 1. The capacity factor k_e is equal to $V_R - V_0 / V_0$ where V_R
is the eluting volume and V_0 is the void volume in the presence of
pore exclusion phenomena (18). The terminology is analogous to
that used in exclusion chromatography. V_M, the void volume in
general liquid chromatography is the sum of two factors, $V_M = V_e +
\phi V_i$ where V_e is the interparticle void volume and ϕV_i is the intra-

particle fluid volume explored by the solute. In the extreme case when $\phi = 0$, the solute does not penetrate the pores and the void volume, V_M is equal to the interparticle void volume, V_e. When $\phi = 1$, the solute penetrates all the pores and the void volume equals the sum of the interparticle and intraparticle void volumes. The value of ϕ should be constant in order to evaluate the capacity factor k which equals $(V_R - V_M) / V_M$. Using ionized solutes in un-buffered systems, the pore-penetrating ability of the solute varies with the ionic strength or amount of solute. The value of ϕ changes, thus changing the value of V_M. Since V_M is not constant the capacity factor k is difficult to evaluate. It is inappropri-ate therefore to correlate an increase in k with an increase in the chromatographic retention of ionized solutes in unbuffered sys-tems. For this reason, we have defined the expression, $(V_R - V_0) / V_0$ as the capacity factor k_e. Berendsen et.a. have considered the value of V_0 to equal V_R (19); however, it would be difficult to prove that at low concentrations of electrolyte ($<10^{-3}$M) there is no pore penetration at all ($\phi = 0$).

The effects in Figure 1 have been observed with ionized solutes on C_{18} columns (19,20), and have been explained in terms of a Donnan exclusion phenomena. On the basis of studies with reversed-phase supports, a salt is excluded from the pores of a packing at low electrolyte concentrations, presumably because of electrical charges on the phase support (19). Silica gel is known to have a negatively-charged surface at pH values above 2 because of the ionization of silanols (21). Alternatively, the adsorption of the first few molecules on the surface may impede any addition-al adsorption, and the existence of a pre-charged surface is not necessary in order for a Donnan expulsion phenomenon to be ob-served. This Donnan exclusion effect can be eliminated by adding a supporting electrolyte.

Counteracting the surface charge expulsion, the hydrophobic effect forces the solute to the surface. The expulsion of the anions from the eluent is based on molecular size such that the
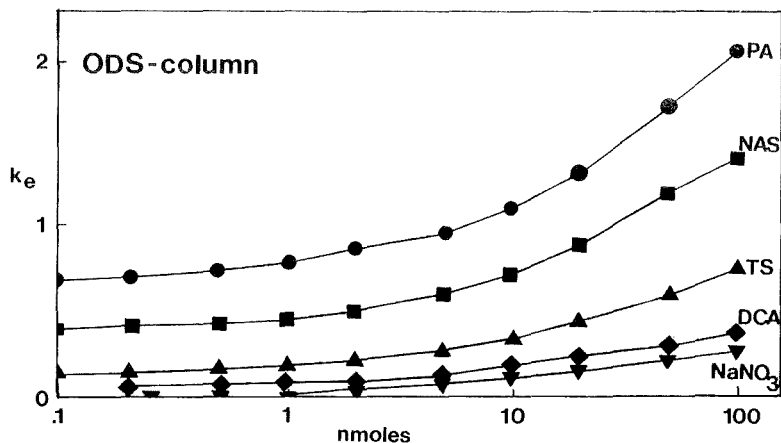
Figure 1.   Retention versus Amount Injected for a Series of Anions.
Mobile phase (25:75) methanol:water. Column: Whatman ODS-5.
Samples: PA = picric acid; NAS = naphthalene sulfonic acid; TS =
toluenesulfonic acid; DCA = dichloroacetic acid; $NaNO_3$. Detection
wavelengths: PA = 350 nm, NAS = 228 nm, TS = 203 nm, DCA = 203 nm,
NO_3 = 203 nm.

retention volume increases with number of carbon atoms (9). For
the following ionized species: DCA, TS, NAS and PA, the capacity
factor k_e increases with the increasing size of the anion. See
Figure 1.

     As the organic modifier concentration increases, k_e diminishes
and approaches that of the "unretained" $NaNO_3$ at a given concentra-
tion of solute. Eventually any hydrophobic effects are attenuated
and the molecular size does not affect the retention. There is no
hydrophobic expulsion which differentiates the anions from each
other, so that the concentration effects are identical. Figure 2
shows the similarity between the elution behavior of picric acid
and potassium nitrate. This behavior is observed for all the com-
pletely dissociated aromatic acids and salts investigated at (90:
10) methanol:H_2O.

     In order to substantiate that the variation in sorption of
solute is indeed due to an electrostatic expulsion of the eluite
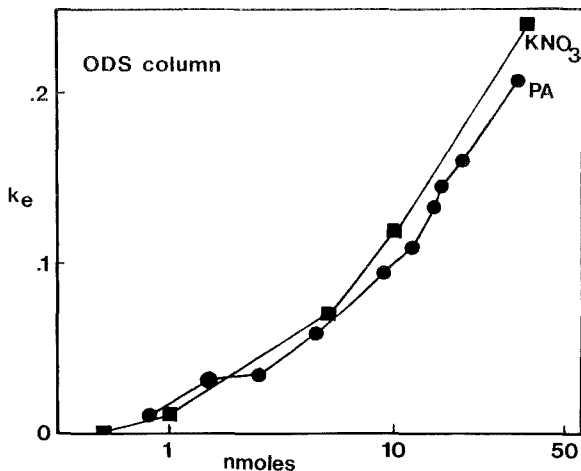
Figure 2. Retention versus Amount Injected. Mobile phase:
(90:10) methanol:water. Column: Whatman ODS-5. Samples: PA =
picric acid; KNO_3. Detection wavelengths same as Figure 1.

from the surface, $NaNO_3$ was added to the mobile phase. With in-
creased electrolyte concentration in the mobile phase, the ion ex-
clusion effect should be diminished, and the pores should then be-
come accessible to the solute (19). Maintaining the solute con-
centration constant, as the concentration of electrolyte in the
mobile phase increases, the retention of picric acid also in-
creases. The enhancement of retention on addition of electrolyte
to the mobile phase mimicks that of the behavior observed on
varying the amount of solute injected in the absence of electro-
lyte. "It is immaterial whether the enhanced ionic strength of
the mobile phase results from the injected salt itself or from
an electrolyte added to the mobile phase" (19). This would seem
to further support Donnan exclusion as the basis for the minimal
retention of solutes at low ionic strength. A simple "salting out"
effect cannot be invoked to explain the enhancement of retention
of solutes at high ionic strength because this effect would not be
paramount at ionic strengths below 0.1 M. (9).

Retention of Cations on ODS

Octadecylsilica bonded phases exhibit ion exchange properties due to the acidity of the silanols on the surface (21). Cations can exchange with the protons bound to residual silanol groups (21) and thus the nature of the support can be modified by adding electrolyte. Presumably, the surface will have a heterogeneous mixture of SiO^-H^+ and SiO^-M^+ (M^+ is the cation), with the exact surface concentrations determined by the concentration of electrolyte in the eluent. This cation exchange behavior has implications for the mechanism of retention of quaternary ammonium salts. These species have the possibility for interacting with the surface at two distinct binding sites. The lipophilic quaternary ammonium ion can be adsorbed onto the nonpolar surface (22). Alternatively, the quaternary ammonium ions might exchange against protons and any cations contributed by added electrolyte. These two distinct binding mechanisms were postulated by van der Houwen et.al. (23). Furthermore, the work of Melin et.al. (24) and Eksborg and Ekquist (25) supports the idea of two binding sites in alkyl-silica bonded phases.

In order to assess the mechanism of retention of cations on octadecylsilica bonded phases, a series of quaternary ammonium ions were injected into a water-lean eluent system (90:10) methanol:H_2O. For the small quaternary ammonium salts, TMAB and TEAB, there is a small increase in the retention with amount injected. See Figure 3. This can be explained in terms of Donnan expulsion. The two species are not differentiated on the basis of molecular size. Apparently, the difference in hydrophobicity of the two species is insufficient to produce any distinction in their chromatographic properties. As the size of the quaternary ammonium ion increases, the capacity factor k_e increases. Solvophobic effects appear to differentiate the large quaternary ammonium ions on the basis of size (k_e of THAB > TBAB > TEAB). This is not entirely unexpected since large quaternary ammonium ions are extremely solvophobic, and they might be expelled even in pure methanol as the eluent. It is assumed with the quaternary ammonium salts that the prominant basis of

Figure 3. Retention versus Amount Injected for a Series of
Quaternary Ammonium Salts. Mobile phase: (90:10) methanol:water.
Column: Whatman ODS-5. Samples: CTAB = hexadecyltrimethyl-
ammonium bromide, THAB = tetraheptylammonium bromide, TBAB =
tetrabutylammonium bromide, TEAB = tetraethylammonium bromide,
TMAB = tetramethylammonium bromide. Detection wavelength = 203 nm.

differential retention on the surface is due to the hydrophobic

expulsion of the salt, and that the secondary interactions between

solute and surface are not the primary basis for retention. Both

binding sites on ODS-bonded phases are presumably involved in the

retention of quaternary ammonium salts (23).

    Cetrimide, CTAB, exhibits anomolous retention behavior. See

Figure 3. Whereas all the spherically-symmetric surfactants show

a regular increase in the k_e as the nanomoles of solute injected

increase, CTAB exhibits a levelling effect at high solute concen-

trations. Presumably, this can be ascribed to the fact that CTAB

can more easily coat the surface by aligning its extended tail a-

long the C_{18} hydrocarbon moiety on the surface. This would then

cause a surface saturation to occur at relatively low concentra-

tions of solute. THAB on the other hand is a large spherical

hydrophobic species which has a positive charge located 7 carbon

atoms away from the end of the heptyl moiety. It cannot contact

adsorb as well on the surface because of the limited number of

carbon atoms which can align with the surface. It would therefore
not saturate the surface as completely as a linear long-chain
quaternary ammonium salt at a similar concentration.

Cations on PRP-1

Figure 4 depicts the retention behavior of quaternary ammonium
salts on a PRP-1 column. The PRP-1 column packing consists of
rigid spherical 10 μm particles which are a copolymer of styrene
and divinylbenzene. The packing material is an adsorbent which
can be used in the reversed-phase or normal phase modes. The sur-
face has no Bronsted acid character, and therefore there is no
possibility for bonding quaternary ammonium ions to cation-exchange
sites. The quaternary ammonium ions must therefore be adsorbed on
the surface. Recently, Cantwell and Puon (16) used a model derived
from the Stern-Gouy-Chapman double-layer theory for describing the
adsorption of aromatic ammonium compounds on Amberlite XAD-2. The
surface of PRP-1 is chemically similar to that of Amberlite XAD-2.
Therefore, the quaternary ammonium ions would form a primary layer
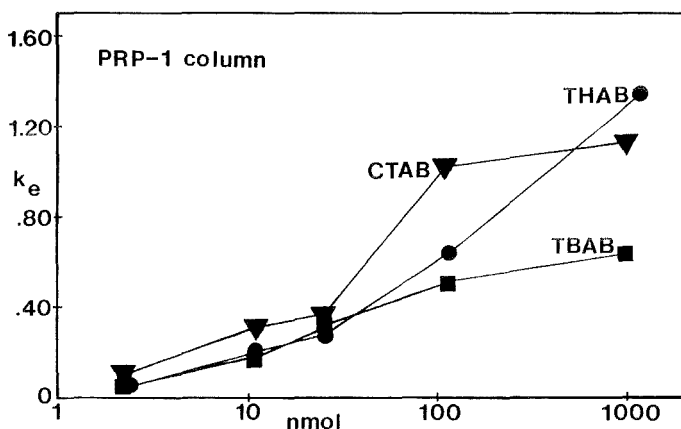
Figure 4. Retention versus Amount Injected for a Series of
Quaternary Ammonium Salts. Mobile phase: (90:10) methanol:water.
Column: PRP-1. Samples and detection wavelengths same as Figure 3.

adsorbed to the surface while the bromide counterions would then
be located in the diffuse layer.

In order to explain the chromatographic retention of these
ionized solutes, a Donnan exclusion effect can be invoked. As the
concentration of quaternary ammonium salt increases in the solute
plug, the local ionic strength increases, and the Donnan exclusion
is diminished. This explanation would correlate with that given
for the ODS bonded phases. Alternatively, a non-specific salting
out effect has also been postulated (26,27).

As far as the differing retention behavior, See Figure 4,
this can be explained in terms of hydrophobic and steric effects.
THAB is expelled more than TBAB by the eluent and therefore the
retention of THAB is greater than that of TBAB. CTAB again exhib-
its distinctive behavior differing from that of the spherically-
symmetric surfactants because of its ability to maximize dispersion
interactions with the hydrophobic surface. CTAB can align its
hexadecyl moiety with the surface and position its positive charge
away from the surface. THAB has its positive charge located 7 car-
bon atoms from the end of the heptyl moiety, and it is therefore
more difficult to isolate the charge from the surface. Contact
adsorption is easier for CTAB, and therefore surface saturation
would occur at lower concentrations of added solute.

The most important fact to be gleaned from the experimental
data is that the nature of the surface (for hydrophobic surfaces)
does not appear to have a dominant effect on the retention of large
cations. Even though ODS bonded-phases exhibit a potential ion-
exchange interaction, it appears that hydrophobic effects dominate
the retention behavior of quaternary ammonium salts on ODS and poly-
styrenedivinylbenzene copolymer surfaces.

Anions on PRP-1

Figure 5 shows the chromatographic behavior of a series of
ionized salts and completely dissociated acids on PRP-1. The
anions can contact adsorb on to the surface. Pietrzyk and Chu
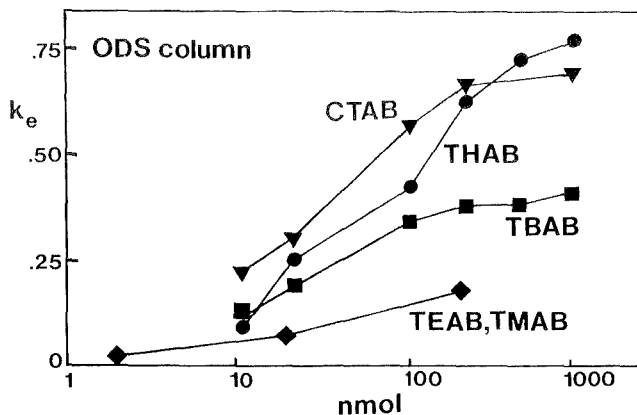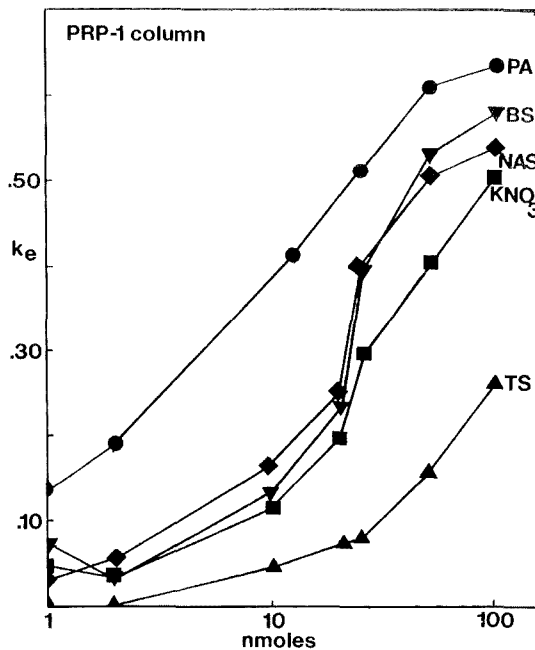
Figure 5. Retention versus Amount Injected for a Series of Anions.
Mobile phase (90:10) methanol:water. Column: PRP-1. Samples and
detection wavelengths same as Figure 1.

(27) envision the sorption of neutral aromatic acids as close to
a flat-wise orientation. If the organic acid is in its salt
form, the charged group disrupts the binding, and the orientation
of the solute is probably more end-on with the charged site away
from the XAD surface (27). Whatever the sorption orientation on
the surface, a charged layer is formed and Donnan expulsion can
occur in unbuffered eluents. This explains the increase in k_e
with amount of injected solute.

     Figure 5 demonstrates a variation in the relative retenion
of anions on the PRP-1 surface. This contrasts sharply with the
behavior observed on the ODS surface. At (90:10) methanol:water,
none of the anions injected are differentiated on the ODS surface.

All of the anions exhibit identical k_e values at all concentra-
tions. Hydrophobic effects are not operative for these homologues
at an eluent composition of (90:10) methanol:water. However, on
the PRP-1 surface, the nature of the anion affects the k_e at a
given concentration of solute. Since the effects cannot be at-
tributed to the eluent, solute-surface interactions on polystyrene-
divinylbenzene are implicated. The relative retention order for
the species is TS < KNO_3 < BS, NAS < PA. This is not the expected
order on the basis of size, but then again since solvophobic ef-
fects are not operative, this is not surprising. The explanation
for this ordering lies in the nature of the surface. Styrenedi-
vinylbenzene polymeric surfaces exhibit the potential for weak
dispersion interactions and in addition pi-bonding interactions
with the solute. Benzene sulfonic acid (BS) and the sodium salt
of naphthalene sulfonate (NAS) can align themselves with benzyl
groups on the surface and maximize the pi-bonding interactions.
These two aromatic sulfonates can orient themselves in such a way
as to position the negative charge away from the backbone of the
copolymer. The nitrate anion can undergo charge transfer complex-
ation with the aromatic moieties of the copolymer and exhibit re-
tention behavior analogous to that of the aromatic sulfonates.
Toluene sulfonic acid (TS) has lower k_e values at all concentra-
tions of solute. See Figure 5. This is attributed to the methyl
substituent on the benzene sulfonic acid. The methyl group pre-
sumably limits the distance of closest approach between the sur-
face and the aromatic ring. Finally, picric acid (PA) exhibits
the highest k_e values at all solute concentrations. Picric acid
is a relatively strong Lewis acid and the charge density of the
aromatic ring is low. Lewis acid-base behavior is expected then
between the benzyl groups of the copolymer and the aromatic tri-
nitrophenol. Picric acid is known to form adducts with aromatic
species, and therefore high retentivity is expected.

CONCLUSIONS

In water-lean eluents, where hydrophobic effects are diminished, surface effects can be observed. Anions interact with polystyrenedivinylbenzene surfaces on the basis of specific interactions. Anions do not exhibit any specific interactions with octadecylsilane bonded phases, unless of course the amount of water in the eluent is so low that silanols are not completely solvated. Large quaternary ammonium ions interact with both ODS and polystyrenedivinylbenzene surfaces in a similar manner. Solvophobic effects appear to dominate the retention behavior. Perhaps if an eluent system did not exhibit solvophobic expulsion of large quaternary ammonium ions, specific interactions between the ammonium ion and the surface could be observed; however, this has not been confirmed experimentally. Finally, both quaternary ammonium ions and aromatic acids and salts demonstrate an enhancement of retention as the amount of solute increases. This can be ascribed to Donnan expulsion of the solute at low ionic strength. The first molecules which adsorb on the surface, prevent the additional sorption of other molecules. If the solute concentration is then increased, the increased ionic strength of the solute plus swamps the Donnan expulsion effect and more solute molecules can then sorb to the surface.

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HIGH–PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE
METABOLITES OF THE POTATO GLYCOALKALOIDS, α–CHACONINE AND
α–SOLANINE, IN POTATO TUBERS AND POTATO PRODUCTS

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ABSTRACT

A high–performance liquid chromatographic method has been developed to separate and quantify the metabolites, γ–chaconine, β_1– and β_2–chaconine, γ–solanine and β_2–solanine, of the potato glycoalkaloids α–chaconine and α–solanine in potatoes and potato products. A carbohydrate analysis column and a solvent system of tetrahydrofuran–water–acetonitrile (55:8:37) were employed for the separation. Flow rate was 1.1 ml/min and the compounds were monitored at 215 nm. β_2–chaconine (0.63 mg to 29.75 mg/100 g dried weight) was present in all samples whereas the other glycosides of α–chaconine were only detectable in the animal feed products. It appears that some of the animal feeds may contain trace amounts of γ–solanine and an unknown which maybe β_1–solanine. Limit of detection for all glycosides was 0.05 µg/µl. Elution time for all the lower glycosides of α–chaconine was 8 min versus 16 min for the α–solanine group. These metabolic compounds were confirmed using thin–layer chromatography.

INTRODUCTION

The majority of glycoalkaloids found in commercial potato

varieties or tubers in breeding programs are of the solanidine

series which are comprised of α–chaconine and α–solanine and their

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A

B
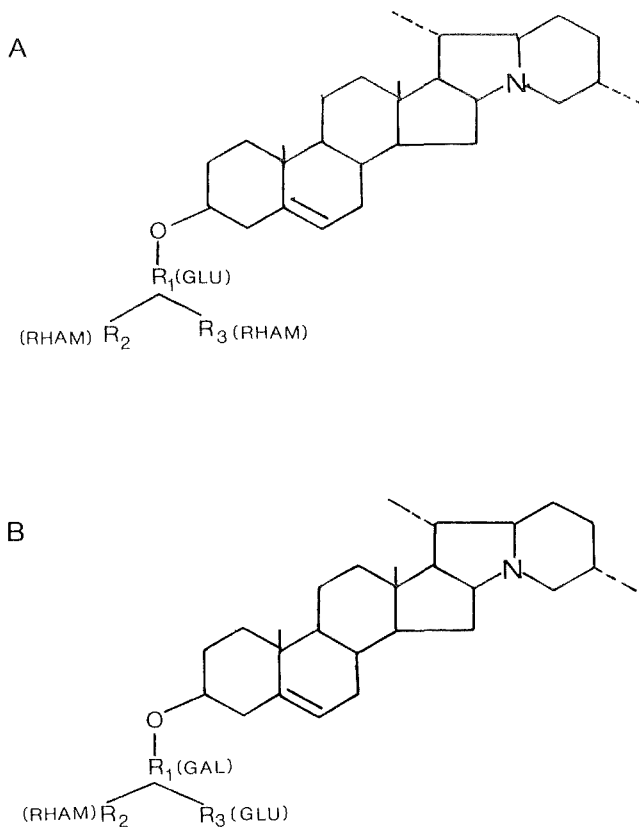
Figure 1. Metabolites of α-Chaconine (A) and α-Solanine (B),
γ-Chaconine (R_1), $β_1$-Chaconine ($R_1 + R_2$), $β_2$-Chaconine ($R_1 + R_3$),
γ-Solanine (R_1), $β_1$-Solanine ($R_1 + R_2$) and $β_2$-Solanine ($R_1 + R_3$).

metabolites (Figure 1). Because of their known acute toxicity

(1-3), their possible chronic toxicity (4-6), and their charac-

teristic bitter flavor (7,8), glycoalkaloids must be analyzed in

all new potato varieties before they can be released commercially.

     Of all the possible methods-colorimetric (9,10), titrimetric

(11), thin-layer chromatographic (12,13), gas chromatographic

(14,15) and high-performance liquid chromatographic (15-20)-

available to quantify total or individual glycoalkaloids, HPLC is

the most applicable to quantify these metabolites because it is

fast, accurate, reproducible and can be used to determine both in-

dividual and total glycoalkaloids. Analysis of the individual

glycoalkaloids is most important since they may vary in their de-

gree of bitterness and toxicity (7,8).

    Although there have been several HPLC methods developed for

determining glycoalkaloids (15-20), this is the first method that

can separate all the lower glycosides of α-chaconine and α-sola-,

nine in the same sample.

EXPERIMENTAL

Materials
    Solvents used for the extraction and for TLC of the glyco-

alkaloids were ACS grade (Fisher Scientific Co. Fair Lawn, NJ)

while those employed in the HPLC separations and to dissolve the

glycoalkaloids were HPLC grade (Fisher Scientific Co.).

    The glycoalkaloids, α-chaconine and α-solanine, were ob-

tained from the procedure of Bushway and Storch (21) while β_2-

chaconine was a gift from Eugene A. Talley, Eastern Regional Re-

search Center, USDA, Philadelphia, PA. The other glycosides were

obtained from acid hydrolysis of α-chaconine and α-solanine em-

ploying the procedure of Filadelfi (8).

    TLC plates were HP-KF high-performance silica gel plates 10 x

10 cm; 200 μ thickness (Whatman Inc. Clifton, NJ).

Apparatus

The HPLC system consisted of a Waters Assoc. (Milford, MA),

6000 A pump, a U6K injector, a Schoeffel variable-wavelength UV

detector (Westwood, NJ) and a Houston Instruments dual pen record-

er (Austin, TX).

Methods

Extraction: The procedure used to extract the tubers and po-

tato products was that of Bushway et al. (17) with one modifica-

tion. The extracting solution was methanol-water-acetic acid

(92:5:3).

HPLC separations: In order to establish the conditions needed

to separate the metabolic degradation products, α-chaconine

(9 mg/25 ml) and α-solanine (25 mg/25 ml) were acid hydrolyzed (8)

which yielded γ-chaconine and γ-solanine, β_1-chaconine, β_2-chaco-

nine, β_2-solanine and possibly a minute amount of β_1-solanine.

Operating conditions were: column, Carbohydrate analysis column

(Waters Assoc.); mobile phase, tetrahydrofuran-water-acetonitrile

(55:8:37); flow rate, 1.1 ml/min; column temperature, ambient;

wavelength, 215 nm; attenuation, 0.04 a.u.f.s.; and chart speed,

0.4 in/min. Before injecting 5 μl of each sample, they were fil-

tered through a 0.45 μm Millipore organic filter (Waters Assoc.).

TLC separation: The thin-layer chromatographic method em-

ployed was that of McCollum and Sinden (22).

RESULTS AND DISCUSSION

A chromatogram of the separation of the metabolites of

α-chaconine and α-solanine is shown in Figure 2. Elution time of
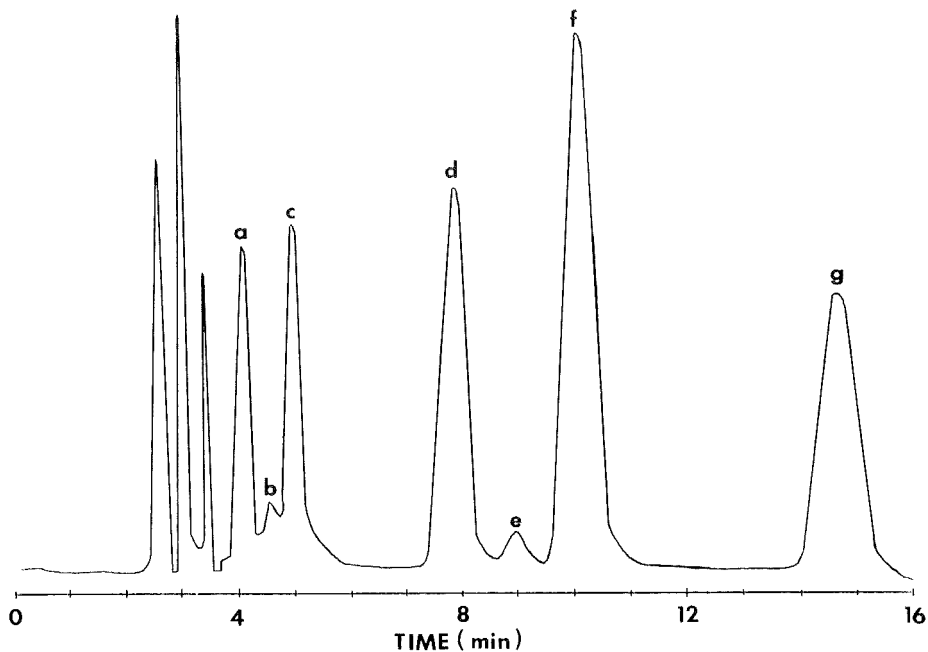
Figure 2. Chromatogram of the Metabolites of α-Chaconine and
α-Solanine. Peaks: a, γ-Chaconine; b, γ-Solanine; c, β_2-Chaconine;
d, β_1-Chaconine; e, unknown; f, α-Chaconine; g, β_2-Solanine.

the lower glycosides of α-chaconine was 8 min except in the pre-

sence of α-chaconine, in which case the elution time was 11 min

(Figure 2). The elution time for degradation products of α-sola-

nine was 16 min, not including α-solanine (Figure 2). α-Solanine

can best be analyzed using a previously developed method (17)

since it elutes very slowly in this solvent system. The reten-

tion time for the glycosides of α-solanine can be decreased by

increasing the water content of the mobile phase if the metabo-

lites of α-chaconine are not present. As can be seen in Figure 2,

TABLE 1

Level of Metabolic Degradation Products of α-Chaconine and α-Solanine in Potatoes and Potato Products.

Sample	γ-Chaconine	β₂-Chaconine	β₁-Chaconine	γ-Solanine	β₂-Solanine
	-----------------------------mg/100 g dried weight-----------------------------				
Potato Waste Animal Feed #1	1.76	15.25	1.30	ND*	ND*
Potato Waste Animal Feed #2	8.00	29.75	1.49	ND	ND
Potato Waste Animal Feed #3	2.60	22.00	1.75	ND	ND
Potato Waste Animal Feed #4	0.49	4.75	0.82	ND	ND
Potato Waste Animal Feed #5	0.70	13.00	1.30	ND	ND
Potato Peel Waste	ND	10.18	ND	ND	ND
Russet Burbank Potatoes	ND	0.63	ND	ND	ND
Norchip Potatoes	ND	1.00	ND	ND	ND
Kennebec Potatoes	ND	0.73	ND	ND	ND

ND=none detected at a detection limit of 0.05 µg/µl.

this time is needed for complete separation of all the metabolites.

When samples containing α-solanine are analyzed using this pro-

cedure, the injections can be staggered so that α-solanine does

not interfere with the other glycoalkaloids.

Several potato varieties and potato products were analyzed

for their content of metabolic glycosides and the results are pre-

sented in Table 1. Some of the feed products appear to have a

trace of γ-solanine and an unknown compound (Figure 3) that maybe

β_1-solanine (based on acid hydrolysis of α-solanine standard).

We are presently trying to identify this substance. All samples
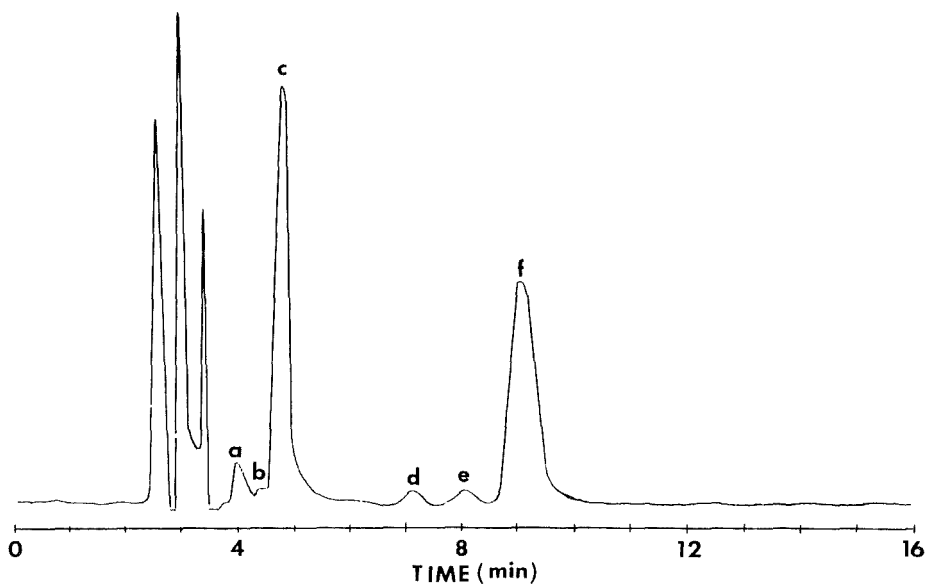
Figure 3. Chromatogram of a Potato Waste Animal Feed Product.
Peaks: a, γ-Chaconine; b, γ-Solanine; c, β_2-Chaconine; d, β_1-Chaconine;
e, unknown; f, α-Chaconine.

had at least one detectable degradation product of α-chaconine.
The potato waste animal feed samples contained all three metabo-
lites of α-chaconine while commercial tuber varieties had low
levels of β$_2$-chaconine. Large quantities of these degradation
glycoalkaloids were found in some of these animal feed products
(Figure 3 and Table 1). The presence of these high levels of in-
dividual glycoalkaloids could cause bitter flavor which could pre-
clude the use of the product as an animal feed. Also high a-
mounts of glycoalkaloids could have toxic effects on animals and/
or people consuming the animals. Further research is necessary to
determine if and to what extent the bitterness and toxicity pro-
blems exist.

The amount of β$_2$-chaconine in samples was calculated using a
standard curve. However, γ- and β$_1$-chaconine standards had not
been isolated at the time of this study so a response factor was
determined using the quantity of solanidine present in these two
lower glycosides and comparing this with β$_2$-chaconine. Such a
calculation can be performed since solanidine is the only portion
of the molecule that will absorb UV light at 215 nm. The identity
of these lower glycosides were confirmed using TLC.

The reproducibility of this method was tested on a potato an-
imal feed sample #5 (Table 1). Five subsamples of the product
which contained γ-, β$_1$- and β$_2$-chaconine were extracted and the
per cent coefficients of variation for three glycoalkaloids were
3.72, 3.28 and 7.59%. All coefficients of variation were below 10
per cent indicating that the method is reproducible.

This HPLC procedure offers a quick, accurate and reproducible method for plant breeders and toxicologists to determine the metabolites of α-chaconine and α-solanine in potatoes and potato products.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF THE NEW ANTINEOPLASTIC
9,10-ANTHRACENEDICARBOXALDEHYDE BIS[(4,5-DIHYDRO-1 H-IMIDAZOLE-2-YL)
HYDRAZONE]DIHYDROCHLORIDE (CL 216,942; BISANTRENE)

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INTRODUCTION

9,10-anthracenedicarboxaldehyde bis[(4,5-dihydro-1H-imidazole-2-yl) hydrazone] dihydrochloride, (Bisantrene, CL 216,942, or NSC-337766) (Fig. 1) is a new anthra-cenedione derivative which has significant antitumor activity in a number of animal tumor systems including L1210 leukemia, P388 leukemia, Liberman plasma cell tumor, B16 melanoma, Ridgeway osteogenic sarcoma and colon tumor 26 in mice (1). Although structurally, it bears some resemblance to doxorubicin, bisantrene differs in producing less myocardial toxicity at equitoxic doses. Therefore, Bisantrene may be a useful antitumor agent in doxorubicin sensitive tumors (2). In order to study the pharmacokinetics of the agent in conjunction with the phase I and II clinical trial in our institute, we developed an analytical method for Bisantrene. The sampling and analytical methods are rapid, reproducible, highly sensitive and applicable to the determination of the agent in plasma, urine and cerebrospinal fluid.

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Bisantrene .
NSC-337766
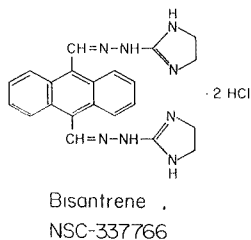
Figure 1. Structure of 9,10-anthracenedicarboxaldehyde bis[4,5-dihydro-
1H-imidazole-2-yl) hydrazone] dihydrochloride.

MATERIALS AND METHODS

Chemicals

Bisantrene (NSC-337766) was kindly supplied by Lederle Laboratories
Division, American Cyanamid Company, Pearl River, NY. Glass distilled
methanol was obtained from Burdick and Jackson labs (Saginaw, MI, USA).
All other chemicals were obtained from regular commercial suppliers.

Sample Preparation

Biological samples were obtained from patients receiving 40-250 mg/m^2
of Bisantrene intravenously. Blood was drawn into heparinized tubes and then
centrifuged at 12,000 X g for 10 min. in a Sorvall RC2-B centrifuge to
separate plasma from red blood cells. Urine was collected as voided.
A C_{18} Sep-Pak (Waters Associate, Milford, MA) was used as a minichroma-
tographic column to prepare the biological samples (3). The cartridge was
first eluted with 4 ml of methanol, followed by 4 ml of equal volumes of
methanol and water, 10 ml of 0.05 $\underline{M}$ sodium phosphate, 3 ml of biological
samples, and then 4 ml of 0.05 $\underline{M}$ sodium phosphate. The above eluates were
discarded. The column was washed with 6 ml of chloroform:methanol (2:1,v/v),
and the eluent was collected in a drying cup and evaporated to dryness
under a stream of nitrogen in a Brinkmann model SC/48 evaporator (Brinkmann

Instrument Co., Westbury, NY). It was then reconstituted with 50 μl of N-N-dimethyl acetamide and 250 μl of saline for the high performance liquid chromatography (HPLC) separation. Those urine samples that remained cloudy after reconstitution were recentrifuged at 12,000 X g for 15 min. and the supernatent collected again for injection into the HPLC.

HPLC Analysis

All analyses were performed on a Waters Associates liquid chromatograph (Milford, MA, USA) equipped with a Model M-6000A pump, U6K injector, and Schoeffel Model SF-970 fluorescent detector, (Kratos Analytical Company, Westwood, NJ) with the excitation wavelength set at 260 nm and emission set at 550 nm. Separation was achieved on an analytical reverse-phase μ Bonda-pak C_{18} column (Waters Associates, 4 mm x 30 cm, 10-mm particle size) using 0.02 $\underline{M}$ Ammonium acetate in 40% methanol, pH 4.0, as eluant at a flow rate of 1.5 ml/min. Retention times were determined by a Shimadzna chromatopec-EIA electronic integrator (Kyoto, Japan) and Brinkmann Instrument Model 2544 recorder.

Results and Discussion

Using the conditions described above, Bisantrene was eluted 8.5 min. after injection. In the urine samples, the separation of Bisantrene from other components may have been delayed by 0.2 to 0.5 min due to the presence of normal urinary constituents (Fig. 2). The preparation of the drug using C_{18} Sep-paks along with the procedures of evaporation, centrifugation and transfer of samples resulted in 75% recovery of the drug.

The standard curves for Bisantrene in plasma and in urine are shown in Fig. 3. The concentration is linear over a range of 125 to 2000 ng/ml. The lower limit of detection was approximately 2 ng/ml.

Figure 4 is a chromatograph of a patient's plasma after Bisantrene was administered at a dose of 250 mg/m^2. This HPLC technique is rapid, sensitive and reproducible. It provides easy detection of Bisantrene in both plasma and urine of patients receiving the drug. This assay is
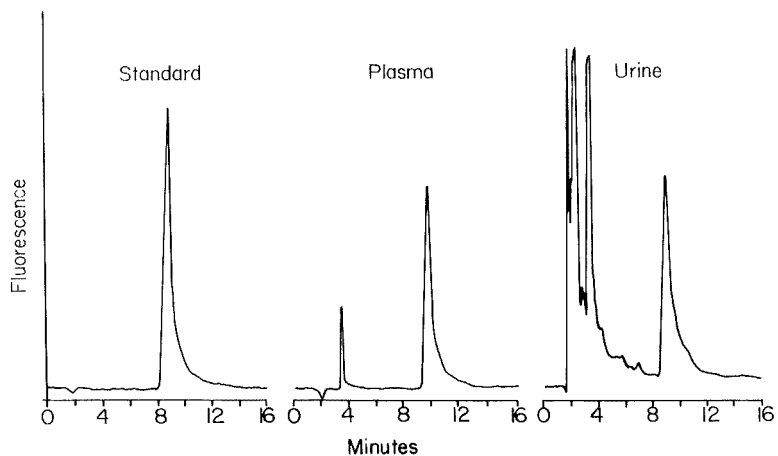
Figure 2. Elution profile of standard Bisantrene in aqueous solution (left), in human plasma (center) and in human urine (right) with fluorescence at 550 nm.
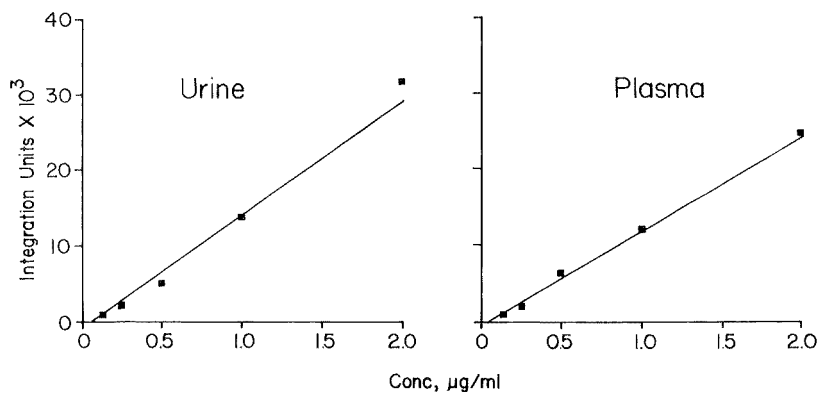
Figure 3. Standard curves of Bisantrene in plasma and urine.
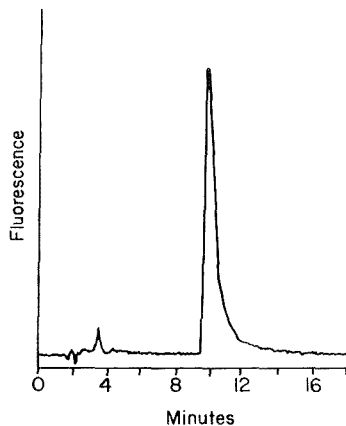
Figure 4. Chromatogram of a patient's plasma immediately after receiving

250 mg/m² of Bisantrene.

currently being used to study the clinical pharmacology of Bisantrene at

our institution.

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SEPARATION AND DETECTION OF MONOPYRROLE AND BIPYRROLE PRECURSORS

OF PRODIGIOSIN FROM SERRATIA MARCESCENS BY A COMBINED METHOD OF

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND SYNTROPHIC PIGMENT SYNTHESIS

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ABSTRACT

The monopyrrole (2-methyl-3-amylpyrrole) and bipyrrole (4-methoxy-2,2'-bipyrrole-5-carboxaldehyde) precursors of the tripyrrole pigment antibiotic prodigiosin, from Serratia marcescens were separated and detected by a combined method of high performance liquid chromatography (HPLC) and syntrophic pigment synthesis. The monopyrrole and bipyrrole precursors were extracted from mutants which were able to synthesize one but not both of the precursors. Mutant WF is incapable of synthesizing the bipyrrole while mutant 9-3-3 is unable to synthesize the monopyrrole precursor. By means of isocratic reversed-phase HPLC with a mobile phase of 70% methanol in water, the mono-pyrrole and bipyrrole precursors were well separated and resolved. The identity

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of the precursor peaks was establised by their ability to undergo syntrophic
pigment synthesis with the corresponding cells. The syntrophically synthesized
pigments shared similar spectroscopic as well as chromatographic characteristics
as the parent tripyrrole pigment, prodigiosin, extracted from wild type of
S. marcescens. The potential application of this method in the studies of
prodigiosin biosynthesis and in the quantitation of monopyrrole compounds
in clinical diseases is discussed.

　　Serratia marcescens produces a red tripyrrole pigment antibiotic,
prodigiosin. A Bifurcated pathway involving the enzymatic coupling of
the monopyrrole (2-methyl-3-amylpyrrole) (MAP) and the bipyrrole (4-methoxy-
2,2'bipyrrole-5-carboxaldehyde) (MBC) intermediate precursors to form pro-
digiosin has been proposed by various workers (1,2,3). Most of the evidence
for this pathway has been based on the syntrophic interactions of many
mutants (2,3,4). Syntrophic synthesis is the cross-feeding of precursors
for pigment formation in the cells. For example, mutant 9-3-3 which accumulates
the bipyrrole precursor (MBC) or mutant WF which produces the monopyrrole (MAP)
normally does not synthesize prodigiosin itself, but will produce prodigiosin
if the missing precursor is present.

　　Although thin-layer chromatography and gas liquid chromatography have been
used to separate and characterize MAP, high performance liquid chromatography
(HPLC) has not been applied in the study of either the monopyrrole or the bi-
pyrrole precursors of prodigiosin. The major advantages of HPLC over conven-
tional chromatography are better resolution, shorter analysis time and superior
precision, reproducibity and sensitivity. We have applied previously HPLC in
the analysis of prodigiosin extracted from a wild type S. marcescens 08 (6,7).
In this communication we report the results of HPLC separation of the mono-
pyrrole and bipyrrole precursors. Instead of using the tedious and expensive
chemical instrumentation for their identification, we used a combined method
of HPLC retention time, reaction of Erlich reagent (p-N,N-'diemthylamino-
benzaldehyde) and most importantly the ability to undergo syntrophic pigment
synthesis for their detection.

MATERIALS AND METHODS

Chemicals. Pyrrole, 2,4-dimethyl-3-ethyl-pyrrole (kryptopyrrole) and pyrrole
carboxaldehyde were purchased from Aldrich, Milwaukee, WI. All other chemicals
used were of analytical grade. Solvents used for HPLC analysis were HPLC grade
obtained from Fisher Scientific Co., Chicago, IL.

Growth of Bacteria

Serratia marcescens 08, a pigmented strain, was in stock in our laboratory.
Mutants WF and 9-3-3 were kindly supplied by Professor R. P. Williams, Baylor
College of Medicine, Houston, Texas. All cultures were grown in 2 l Erlenmeyer
flasks containing 1 l of liquid medium containing 0.5% Bactopeptone and 1%
glycerol on a rotary shaker at 27 C for 36-72 hr depending on the strain.
Bacteria were harvested by centrifugation at 10,000 x g, and washed with
distilled water. In the case of strain 08, harvested cells were submitted to
pigment extraction according to the procedure of Williams et al. (8) with
acetone followed by partition with petroleum ether. The supernatants of WF
and 9-3-3 were saved for monopyrrole and bipyrrole precursors extractions.

Extraction of Monopyrrole and Bipyrrole Precursors

The supernatant of mutant WF after 36 hr of growth and the supernatnat
of mutant 9-3-3 after 72 hr of growth were extracted with dichloromethane
according to the modified method of Wasserman et al (9). The dichloromethane
layers were collected after centrifugation at 10,000 xg for 25 min to break
the emulsions. In the case of the WF the organic phase was washed twice with
1/10 volume of 1 N NaOH followed by washing with 1/10 volume of water. A
liquid residue of monopyrroles (WF Extract) was obtained after the dried
organic phase (with anhydrous Na_2SO_4) was removed by fractional distillation.
The supernatant of mutant 9-3-3 was treated in a similar manner except that
the organic phase was first washed with 1 N HCl followed by 1 N NaOH and then
water. The dried organic phase was removed by distillation yielding the
crude crystals of bipyrrole precursors (9-3-3 Extract).

HPLC Analysis

A Beckman model 330 isocratic High Performance Liquid Chromatography with
a Pheodyne injector valve containing a 10 µl sample loop and a Perkin-Elmer

LC-55 variable wavelength UV-visible detector were used for separation and
identification of the monopyrrole and bipyrrole precursors. The column was
a 25 cm x 4.6 mm i.d stainless steel column and the precolumn was a 5 cm x 4.6
mm i.d. stainless column packed with Lichrosorb RP-18 of 10 μm particle dia-
meter. The following solvent systems were developed to analyse the pigment
and its monopyrrole and bipyrrole precursors from the various preparations:

    a) 25% dichloroethylene in methanol with 10 ppm of conc. HCl was used
       for the separation of any formed pigments (6).

    b) 70% methanol in water was used to separate both the monopyrrole and
       pyrrole precursors.

    c) 50% acetonitrile in water was found to be an additional suitable
       solvent system for the separation of the bipyrrole precursors.

The flow rate was 2 ml/min for all solvent systems.

Syntrophic Pigment Synthesis

    a) Syntrophic pigment formation in cells of 9-3-3 with monopyrrole
       precursor provided by cells of WF; Syntrophic pigment assays were
       carried out on Bactopeptone Glycerol-Agar agars. The upper half
       of each set of plates was heavily inoculated with 9-3-3 and the
       lower half was inoculated with WF. The plates were incubated for
       36 hr at room temperature (22-24 C) and observed for pigment formation.

    b) Syntrophic pigment formation in cells of 9-3-3 with monopyrrole
       precursor (MAP) or in cells of WF with bipyrrole precursor (MBC)
       separated and collected from HPLC analysis. WF Extract or 9-3-3
       Extract from 2 l of supernatants of cultures was dissolved in 3 ml of
       dichloromethane for HPLC separation. The combined eluents (8-24 ml) after
       5-6 injections of 10 μl each were extracted by adding 1 part of CH_2Cl_2 and 8
       parts of H_2O. A small amount of the eluent before extraction was allowed to
       react with 0.5% p-N,N'-dimethylaminobenzaldehyde in ethanol/conc. HCl (Ehrlich-
       Reagent). The organic layers was evaporated to dryness in N_2. Two ml of the
       corresponding cultures of 9-3-3 or WF were added to the residue and incubated
       for 3 hr to allow pigments to form. Culture of 9-3-3 was added to peaks
       collected from WF Extract whereas culture of WF was added to peaks collected
       from 9-3-3 Extract.

Any pigment synthesized syntrophically was extracted by acetone as described for the pigment from cells of strain 08.

RESULTS AND DISCUSSIONS

Recently we have applied HPLC in the analysis of the pigment components of S. marcescens, a gram(-) bacterium frequently indicted for causing many nosocomial diseases. We have studied the conditions under which the pigment components could be separated (6.7). By maintaining a small but constant amount of concentrated HCl in the mobile phase of 25% dichloroethylene in methanol on a reversed phase column, the pigment components could be separated and resolved in about 5 minutes. This method allowed preparative isolation of the individual components along with their hydrogen peroxide oxidized products. We suggested that components separated by this method and detected in the ultraviolet region may represent the precursors of the parent pigment, prodigiosin. Indeed, MAP has been shown to be a chemical degradation product of prodigiosin (9) as well as a precursor for its bio-synthesis (5). Systematic studies by HPLC of this and other degraded products may provide additional information in their possible role in the biosynthesis of prodigiosin.

Synthetic monopyrroles such as 2,4-dimethyl-3-ethylpyrrole (kryptopyrrole) having the structural similarity of MAP can also condense with MBC in a growing culture of 9-3-3 to produce the prodigiosin anologs. Other pyrrole derivatives such as pyrrole carboxaldehyde shares similar structural relationship with MBC. The UV-visible absorption spectra of these pyrrole derivatives provide us an important basis for the selection of the suitable wavelength for monitoring the analyses of the WF Extract and 9-3-3 Extract by HPLC (Figure 1). WF Extract had a maximum UV absorption at 210-235 nm which was shared by pyrrole and kryptopyrrole. 9-3-3 Extract had two maxima absorption (250 nm and 365 nm) in the UV spectra. The 250 nm absorption represents one of the two maxima of pyrrole carboxyaldehyde. An additional maximum absorption at 365 nm has also been reported for MBC.

Different results were obtained when the growth medium of mutant WF was extracted by various procedures under different conditions (Figure 2). Using
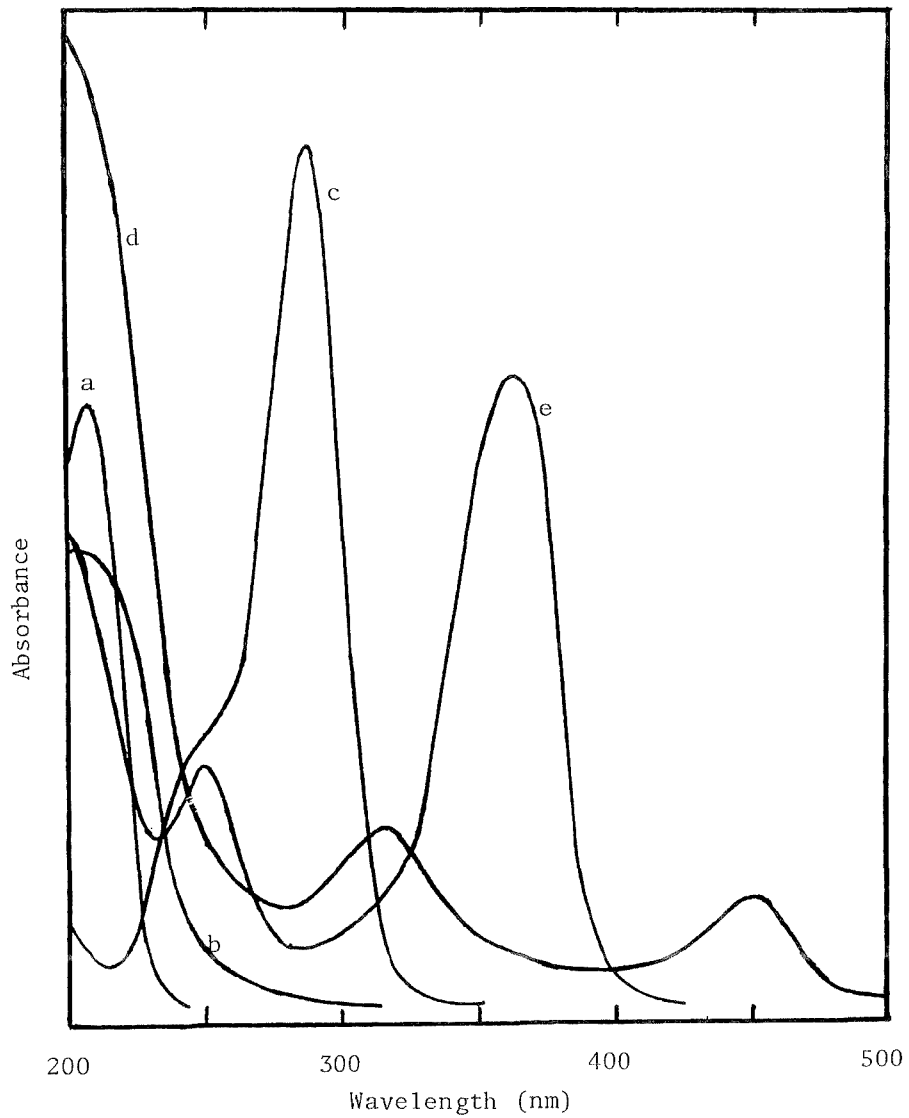
Figure 1. Ultraviolet-visible Spectra of pyrrole and pyrrole derivatives

    a)   Pyrrole

    b)   2,4-dimethyl-3-ethyl-pyrrole (kryptopyrrole)

    c)   Pyrrole carboxaldehyde

    d)   Extract from mutant WF

    e)   Extract from mutant 9-3-3
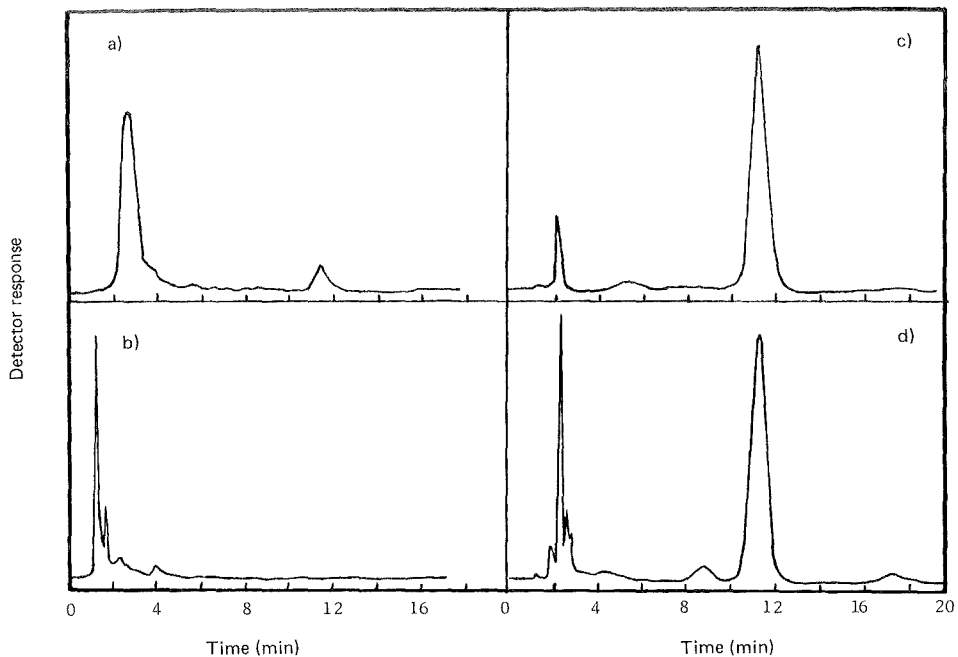
Figure 2. HPLC separation of monopyrrole precursors from S. marcescens

mutant WF.

Column packing: Lichrosorb RP-18, 10 um particle size

Solvent system: 70% methanol in water

Detection: 223 nm at 20 mV, 10 mm/min

Flow rate: 2 ml/min

a) Fresh extract from growth medium with diethyl ether

b) Extract from growth medium with diethyl ether after two months
    storage in the cold.

c) Fresh extract from growth medium with diethyl ether followed
    by washing with 1 N NaOH

d) Fresh extract from growth medium with dichloromethane followed
    by washing with 1 N NaOH

70% methanol in water as a mobile phase, the chromatogram of the freshly

prepared samples as extracted by diethylether according to the procedure of

Deol et al. (5) showed two main peaks, a strong peak and a small peak (Figure 2-a).

When this sample was stored in the cold for a period of two months, the first

peak was degraded into several small peaks and peak 2 in the original sample

almost disappeared (Figure 2-b). However, when either diethyl ether or dichloro-

methane was used as extracting solvent but washed with 1 N NaOH, four peaks with

retention times of 2.3 min, 8.9 min, 11.4 min and 17.3 min were obtained (Figure

2-c and 2-d). The first peak (R_t = 2.3 min) had similar retention time and UV-

visible absorption characteristics as pyrrole. Positive test with Ehrlich

reagent (p-N,N'-dimethylaminobenzaldehyde in acidic ethanol) showed it was

either pyrrole or pyrrole derivative. Peak 3 (R_t = 11.4 min) was the main

peak with positive Ehrlich Reagent test and with absorption characteristics

similar to those of kryptopyrrole (Figure 1). That this peak being a mono-

pyrrole precursor such as MAP was confirmed by the syntrophic assay technique

(Table 1). The remaining two peaks were not characterized, since neither one

was present in any signficiant amount.

The chromatograms of the extracts from the growth media of mutant 9-3-3

(9-3-3 Extract) were fairly simple (Figure 3). When 70% methanol in water

was used as a mobile phase, a single peak with retention time of 2.7 min was

obtained when monitored at either 250 nm (Figure 3-a) of 365 nm (Figure 3-b).

Again the synthrophic pigmentation test was positive when this peak was

collected and allowed to react with the monopyrrole precursor, MAP, in the cells

of mutant WF (Table 1). The UV-visible spectra were similar, if not identical,

to those reported for MBC (5). Therefore, we conclude that this peak is most

likely to be MBC. When 50% acetonitrile in water was used as the mobile phase,

the retention time of this peak shifted slightly giving a retention time of

3.1 min (Table 1).

The results obtained in this study clearly indicated that peaks separated

from either the extract of the supernatants of mutant WF or mutant 9-3-3

gave positive syntrophic pigmentation with cells of mutant 9-3-3 (test for MAP)

or cells of mutant WF (test for MBC). The retention time and the absorption

a)   b)

Detector response

0   2   4   0   2   4   6

Time (min)
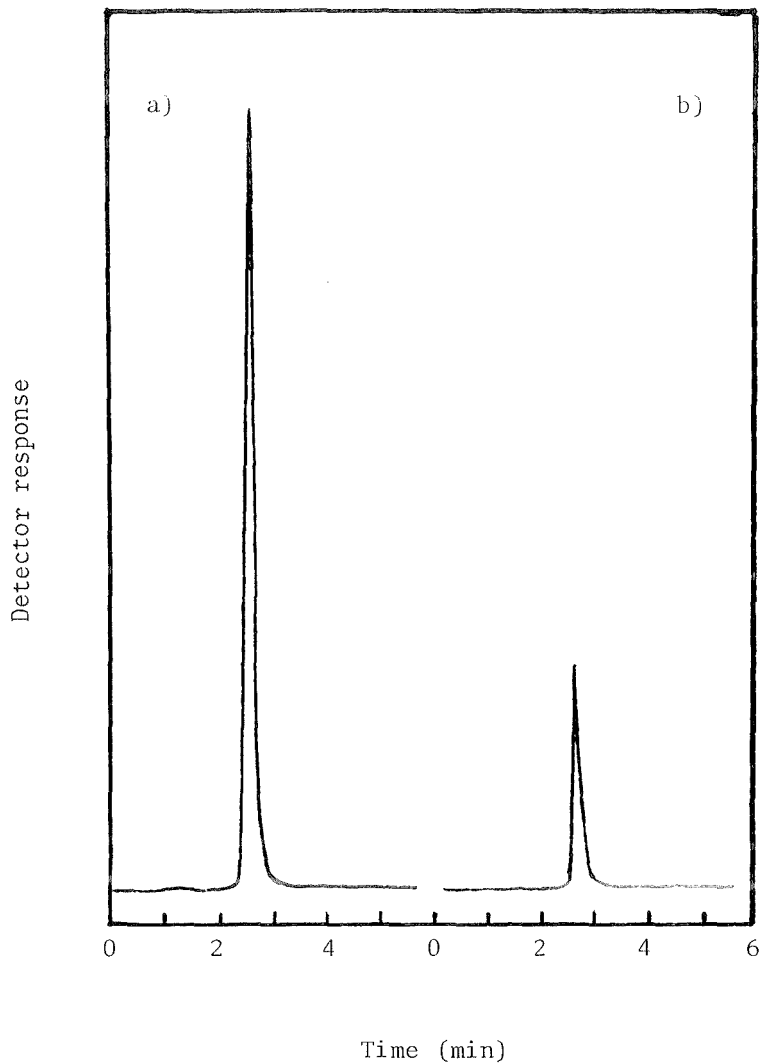
Figure 3. HPLC separation of bipyrrole precursor of S. marcescens mutant
           9-3-3
           Column packing and solvent system, see legend of Figure 2.
           Detection: a) 365 nm, b) 250 nm

Table 1. Summary of Retention Time of Pyrrole and Pyrrole Derivatives in Various HPLC Solvent Systems and their Results with Ehrlich Reagent and In Syntrophic Pigmentation Synthesis

Compounds or Extracts	Detection Wavelength in nm	Retention Time in Various Solvents (min)			Ehrlich Test	Syntrophic Pigmentation Assay
		25% dichloroethylene in methanol	70% methanol in water	50% acetonitrile in water		
Pyrrole and Derivatives						
Pyrrole	223	1.8	2.2	2.8	+	-
2,4-dimethyl-3-ethyl-pyrrole	223	2.0	4.8	8.8	+	+
WF Extract from growth medium of WF (source of MAP)	223		2.2 / 8.9 / 11.4 / 17.3		+ / - / + / -	- / - / + / -
Pyrrole Carboxaldehyde	250	1.9	2.1	2.2	-	ND*
9-3-3 Extract from growth medium of 9-3-3 (source of MBC)	250 / 365		2.7 / 2.7	3.1 / 3.1	- / -	+ / +
Pigment Extracts						
Extract from cells of 08	537	7.7			ND	ND
Extract from 9-3-3 cells after reaction with MAP from WF cells	537	7.7			ND	ND
Extract from 9-3-3 cells after reaction with peak (R_t = 11.4) from WF Extract	537	7.7			ND	ND
Extract from WF cells after reaction with peak (R_t = 2.7) of 9-3-3 Extract	537	7.7			ND	ND

*ND = not done

spectra of the resulting syntrophic pigments were similar to those of the

pigment of prodigiosin obtained from pigmented wild type strain 08 or the

pigments obtained after syntrophic synthesis between cells of mutant WF and

cells of mutant 9-3-3 (Table 1).   These results provide strong but simple

evidence for the presence of an active monopyrrole precursor in the growth

medium of mutant WF (MAP) and an active bipyrrole precursor (MBC) in the

growth medium of mutant 9-3-3.   In order to study the separation and resolution

of the monopyrrole and bipyrrole precursors in the sample, equal amount of WF

Extract and 9-3-3 Extract was mixed and the mixture was analysed under the

similar condition as described above (Figure 4-c).   The bipyrrole (MBC)

(R_t = 2.7 min) and the monopyrrole (MAP) (R_t = 11.4 min) were well separated

and resolved.   The reprodicibility of the separation provides for the first
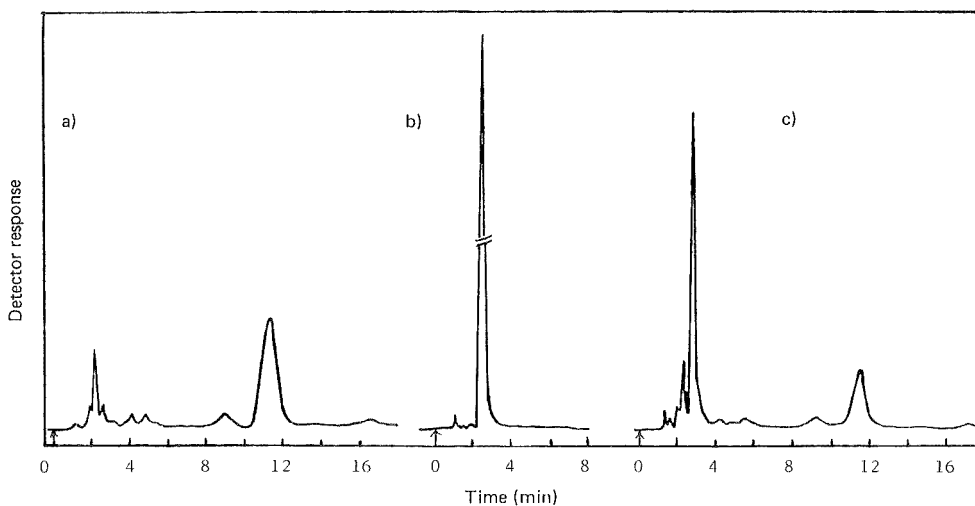
Figure 4.   HPLC profiles of the mixture of WF Extract and 9-3-3 Extract

   a)   WF Extract   b) 9-3-3 Extract   c)   mixture of WF Extract

   and 9-3-3 Extract (1:1, v/v)

   Solvent system: 70% methanol in water

   Detection: 235 nm

time a possible means to quantify the monopyrrole and bipyrrole precursors present in the same sample.

By combining the syntrophic pigmentation test and the powerful technique of separation and resolution of HPLC we believe we have established a simple and fairly rapid method for the detection of monopyrrole and bipyrrole precursors of prodigiosin. We are in the process of applying this method in the analysis of pigment and pigment precursors of various pigmented and non-pigmented clinical isolates of S. marcescens which might be responsible for many nosocomial diseases. Finally, it is worth mentioning that the method developed in this study could also be applied to the detection of other monopyrrole compounds in diseases, such as porphyria, psychosis and lead exposure (10).

ACKNOWLEDGEMENT

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DETERMINATION OF METHACHOLINE CHLORIDE BY ION-PAIR
HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method of analysis of methacholine chloride is presented,
which is adaptable to other choline esters. The method uses ion-
pair high-pressure liquid chromatography. Using 1-heptane
sulfonic acid optically transparent at low UV wavelengths as the
specific ion-pair, it was possible to assay for a specific
choline ester using ultraviolet detection at 210 nm without inter-
ference by hydrolytic by-products. The method can be used to
provide quality control and stability data on methacholine
chloride in 0.9% sodium chloride solutions.

KEY PHRASES

Choline   esters, ion-pair high-pressure liquid chromatography,

1-heptane sulfonic acid, ultraviolet detection at low wavelengths,

hydrolytic by-products, methacholine chloride stability and

quality control.

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INTRODUCTION

The choline esters acetylcholine, methacholine and
succinylcholine have been of considerable interest in pharma-
cology and therapeutics for many years. Solutions of these
compounds are considered relatively unstable, and must usually
be utilized shortly after preparation. Methacholine chloride
solution is currently receiving a great deal of attention as an
inhalation agent to assess the cholinergic hyper-reactivity of
asthmatics, when used in concentrations ranging from 0.075 to
25 mg/ml. The drug is not available commercially in solution,
and hospital pharmacies or chemical laboratories must prepare
solutions and obtain quality control data on each lot prepared.
Concerns about stability have greatly increased the cost of
providing test material.

Simple choline esters can be assayed by the method of Notari
and Munson (1), which is a general assay method that can be used
for a number of functional groups. The method depends on the
formation of a hydroxamic acid and subsequent colorimetric deter-
mination of the highly colored ferric hydroxamate. This
technique works fairly well for simple esters, but problems that
can occur with more complex esters include instability of the
color complex or failure of the hydroxamic acid to form. These
problems can be minimized by using an excess of ferric ion and
removal of water, but the exact conditions must be established
for each compound and/or system under study. Notari and
Baker (2) indicate another possible problem associated with this

assay method.   The procedure requires the addition of sodium
hydroxide during the conversion of the ester to the hydroxamic
acid.   There is a possibility of loss of yield of the hydroxamic
acid, due to hydrolysis of the ester or the hydroxamic acid to
the corresponding carboxylic acid.   Notari and Baker (2)
recommend using only the minimal amount of sodium hydroxide
necessary to give a convenient reaction time.   MacDonald et al.
(3, 4) used this method to study the stability of a 5 mg/ml
solution of methacholine chloride and have provided evidence of
reasonable stability under normal storage conditions.

It was felt that a high pressure chromatographic (HPLC)
assay that could be used for a wide range of related compounds
was desirable.   A reverse phase system was necessary as most
choline ester preparations are aqueous in nature and an extrac-
tion step would be undesirable.   Such a high pressure liquid
chromatographic system would eliminate any possibility of
hydrolysis during analysis, and would be more suitable than the
method of Notari and Munson (1) for determining more complex
esters.   An appropriate HPLC procedure would also be used to con-
firm the findings of MacDonald et al. (4) in relation to
methacholine.

Brown et al. (5) used ion-paired HPLC to develop an assay
for determining aprophen (2-diethylaminoethyl 2,2-diphenyl-
propionate), an anti-cholinergic which is chemically related to
the choline esters.   The choline esters absorb only in the short
wavelength ultraviolet (UV) region.   This precludes the use of

commercially available ion-paired reagents used by Brown et al.
(5) as these reagents have a UV cutoff at about 245 nm. The
current availability of ion-paired reagents with a UV cutoff at
205 nm has allowed the ion-paired technique to be applied to a
whole series of compounds, such as the choline esters, which were
previously very difficult or impossible to assay.

MATERIALS

Apparatus

The HPLC system consisted of a Waters Associates Model 6000A
solvent delivery system, U6K injector, Model 450 variable detec-
tor, and an Omni-Scribe 6000 dual pen recorder.

Reagents

HPLC grade methanol (Fisher Scientific Co., Milford, MA)
and Low-UV PIC-B7 (Waters Associates, Milford, MA) were used as
the mobile phase. Acetylcholine chloride (Calbiochem, La Jolla,
CA), methacholine chloride N.F. (J. T. Baker Chemical Co.,
Phillipsburg, NJ) and succinylcholine chloride (Burroughs
Wellcome Co.) were purchased.

The methacholine chloride obtained was stated to meet
national formulary specifications, i.e., it was not less than 98%
nor more than 102% of the labelled amount. It was stored in a
vacuum desiccator at -4° C in its original unopened container.
Because of the deliquescent nature of the drug all weighings were
made rapidly, and once a container of methacholine chloride was
opened any unused material was discarded.

TABLE 1

Optimal Mobile Phase Composition and Retention Times
for Choline Esters

Drug	% Methanol	Ion-Paired Reagent	Retention Time (Min)
Acetylcholine	10	90	12.6
Methacholine	25	75	9.6
Succinylcholine	40	60	7.8

METHODS

Procedure

A pre-packed 30 cm x 3.9 mm reverse phase octadecylsilyl column (μ-Bondapak C_{18}, Waters Associates, Milford, MA) was employed to chromatograph all compounds used in this study. The ion-paired reagent was prepared by dissolving the 20 ml vial of reagent into 480 ml of glass distilled water. The ratio of 0.01 M 1-heptane sulfonic acid and methanol used to prepare the mobile phase varied depending upon the compound to be studied (Table 1). The flow rate was 1 ml/min in all cases. All separations were performed at ambient temperature. Samples were introduced into the column through a continuous flow loop injector, and the resulting peak heights measured. Table 2 shows the conditions of analysis.

As methacholine chloride was of greatest interest, the detector wavelength of 210 nm was chosen. This is the UV maxima for methacholine chloride in this mobile phase.

TABLE 2

Optimum Conditions of Analysis

Mobile Phase	Table 1
Column	Reverse Phase C-18
Temperature	Ambient
Pressure	1500 psi
Absorbance Units Full Scale	0.02-0.1
Flow Rate	1.0 ml/min
Wavelength	210 nm

Method of Calculation

As no internal standard was used, an average peak height for each sample was obtained from three injections. The concentration of an unknown was determined from a standard curve prepared on the day of analysis. The values of the standards were subjected to linear regression analysis.

Precision

The precision of the method was assessed by the analysis on separate days of samples in 0.9% normal saline prepared from the same stock solution. At concentrations of 20 (n=5) and 5 mg/ml (n=8) the method yielded coefficients of variation of 0.67 and 2.74%, respectively.

Sensitivity

In the above analysis a signal-to-baseline noise ratio of 2 corresponds to a minimum detection limit of 0.08 mg/ml at 0.02 AUFS when 20 μl are injected.

Linearity

In the tested concentration range of 0 to 25 mg/ml, the
relation of concentration to peak height was linear. A typical
curve over these concentrations showed a slope of 2.679, a y-
intercept of -0.9460 and a correlation coefficient of 0.9998.

RESULTS AND DISCUSSION

The assay procedure proved quite reliable for methacholine
chloride and should be adaptable for a number of other related
compounds. The deliquescent nature of methacholine chloride must
be taken account of in preparing quality control samples and
samples for determining standard curves.

When the choline esters were subjected to basic hydrolysis
there was diminution or disappearance of the parent peak on the
chromatograph, with the hydrolysis by-products appearing at the
solvent front. When hydrolysis products were injected separately,
they showed no interference in the analysis of the three choline
esters.

The described HPLC procedure was also compared to the method
of MacDonald et al. (4). Table 3 represents the comparative data
obtained. The methods yielded similar results in this laboratory,
and indicate that for methacholine chloride the procedure used
by MacDonald et al. (4) is valid. MacDonald et al. reported
coefficients of variation between 1.37 and 2.47%, depending on
temperature. These precision data were better than this labora-
tory could obtain using the same method, as shown in Table 3.

TABLE 3

Comparison of Two Assay Methods for Methacholine

Method	Precision Data[a]			Calculated Value of Standard
	n	S.D.	C.V.	
Method of MacDonald et al.[b]				
5 mg/ml standard	5	0.393	7.08%	5.55 mg/ml
20 mg/ml standard	5	0.696	3.47%	20.03 mg/ml
HPLC Method				
5 mg/ml standard	8	0.152	2.74%	5.55 mg/ml
20 mg/ml standard	5	0.128	0.67%	19.27 mg/ml

Variation Between Methods
    for the 5.0 standard   S.D. = 0.0   C.V. = 0.0 %
    for the 20.0 standard   S.D. = 0.54   C.V. = 2.75%
[a]n = number of observations; S.D. = standard deviation; C.V. = coefficient of variation
[b]These values were obtained in this laboratory using the method of MacDonald et al.

There need be no concern about risk of hydrolysis if either

method is used.

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HIGH-PRESSURE LIQUID

CHROMATOGRAPHY ASSAY FOR DANE SALT

POTASSIUM (-)-N-(1-METHOXYCARBONYLPROPENE-2YL)-

p-HYDROXYPHENYLGLYCINE

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ABSTRACT

A rapid, ion-pair, high-pressure liquid chromatographic method for analysis of Dane Salt Potassium (-)-N-(1-Methoxycarbonylpropene-2-yl)-p-Hydroxy-phenylglycine was developed. Tetrabutylammonium hydroxide was used as a counter-ion in the mobile phase. A fixed wavelength detector (λ = 280 nm) and a μ-Bondapak C-18 column were employed. The percent relative range of the method (precision) was 0.6% (n = 3).

INTRODUCTION

Enamine protected amino acids are commonly used during the manufacture of penicillins and cephalosporins (1-9).

These compounds are referred to as Dane Salts. Potassium (-)-N-(1-Methoxycarbonylpropene-2-yl)-p-Hydroxyphenylglycine (I) is such a compound.

I

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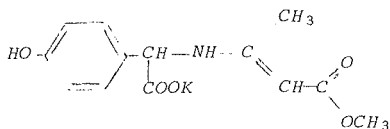
A method to determine the purity of this compound has not been reported

in the literature. Quality of this compound has a bearing on the

overall yield and quality of the penicillin or cephalosporin being

manufactured by it. Therefore, it was decided to develop a specific

HPLC assay for determination of purity of Dane Salt Potassium (-)-N--

(1-Methoxycarbonylpropene-2-yl)-p-Hydroxphenylglycine.

EXPERIMENTAL

Reagents:

Methanol, Burdick & Jackson, Muskegon, MI   49442.

Tetrabutylammonium Hydroxide, Eastman Chemical Company,
Rochester, New York   14650.

Potassium Hydroxide Solution (45%, w/v), Fisher Scientific Company,
Fair Lawn, New Jersey   07410.

Dane Salt Potassium (-)-N-(1-Methoxycarbonylpropene-2-yl)-p-Hydroxy-
phenylglycine (Reference Standard)
Bristol Laboratories, Syracuse, New York   13201.

Phosphoric Acid (85%, w/v), Fisher Scientific Company,
Fair Lawn, New Jersey   07410.

HPLC Conditions:

Column:	*μ-Bondapak C-18, 30 cm x 3.9 mm I.D., from Waters Associates, Milford, Mass., Catalog No. 27324.*
Pump:	*Milton Roy mini-pump, 5000 p.s.i. or equivalent.*
Mobile Phase:	*MeOH/Water (50/50) with 0.1N potassium hydroxide, 0.00693M tetrabutylammonium hydroxide adjusted to pH 7.0 with concentrated phosphoric acid.*
Flow Rate:	*1.5 ml/minute.*
Injector:	*Valco Loop Injector, 7000 p.s.i., 40-μl loop, Valco Instrument Co., Inc., Houston, Texas.*
Detector:	*Waters Model 440 or equivalent, 280 nm.*
Chart Speed:	*0.2 inch/minute.*

Suggested Attenuations & Retention Times:	*RT (min.)*	*Attenuations*
Dane Salt Potassium (-)-N-(1-Methoxy-carbonylpropene-2-yl)-p-Hydroxphenylglycine	*4.0 ± 0.5*	*0.05*
Benzocaine (Internal Standard)	*5.5 ± 0.5*	*0.05*

Mobile Phase Preparation:

Transfer 500 ml of methanol and 450 ml of distilled water to a 1-liter volumetric flask and mix the contents. Pipet 18.0 ml of tetrabutylammonium hydroxide titrant, and 8.5 ml of potassium hydroxide reagent to the flask and mix the contents. Adjust to pH 7.0 with phosphoric acid (approximately 6 ml). Dilute to volume with water. Filter the resulting solution through Whatman No. 5 filter paper (5.5 cm) placed in a Millipore filter holder.

Diluent Preparation:

Transfer 1.0 ml of Potassium Hydroxide Solution (2.25%, w/v) and 40.0 ml of methanol to a 100-ml volumetric flask. Dilute to volume with distilled water and mix well.

Internal Standard (Benzocaine Solution) Preparation:

Transfer 25 ± 1 mg of Benzocaine Solution to a 100-ml volumetric flask. Dissolve and dilute to volume with methanol and mix well. Transfer 10 ml of the resulting solution to a 100-ml volumetric flask, dilute to volume with methanol and mix well.

Master Standard or Sample Solution Preparation:

Transfer 30 ± 2 mg of Dane Salt Potassium (-)-N-(1-Methoxy-carbonylpropene-2-yl)-p-Hydroxyphenylglycine Reference Standard or sample, accurately weighed, into a 200-ml volumetric flask. Add 100 ml of methanol and swirl to dissolve (1) the contents. Dilute to volume with methanol and mix well.

Working Standard or Sample Solution Preparation:

Transfer 5.00 ml of Dane Salt Potassium (1)-N-(1-Methoxy-carbonylpropene-2-yl)-p-Hydroxyphenylglycine Reference Standard or sample solution and 10.00 ml of Benzocaine Solution into a 50-ml volumetric flask. Dilute to volume with methanol and mix well. Transfer 4.0 ml of the resulting solution to a 10-ml volumetric flask, dilute to volume with the diluent (2) and mix well.

NOTES

(1) If the standard or sample is not dissolved in methanol after swirling, sonicate for five minutes.

(2) Diluent should be added to the working solution immediately before injecting the final solution into the HPLC system.

Calculations:

1. Peak Height Ratio (R) $= \dfrac{\text{Peak Height of Analyte}}{\text{Peak height of Internal Standard}}$

   $R_{Std.}$ = Peak height ratio of standard solution.

   R_{Sple} = Peak height ratio of sample solution.

   Analyte = Dane Salt Potassium (-)-N-(1-Methoxycarbonylpropene-2-yl)-p-Hydroxyphenylglycine.

2. Standard Factor (F) $= \dfrac{\text{Std. wt. (mg) x Std. Purity (\%)}}{R_{Std.} \text{ x } 100}$

3. Assay Results:
   Percent Dane Salt Potassium (-)-N-(1-Methoxycarbonylpropene-2-yl)-p-Hydroxyphenylglycine

   $$= \frac{R_{Sple} \text{ x } F \text{ x } 100}{\text{Sample wt. (mg)}}$$

RESULTS & DISCUSSION

The purpose of this work was to develop a specific assay procedure for Dane Salt Potassium (-)-N-(1-Methoxycarbonylpropene-2-yl)-p-Hydroxy-

TABLE I

Spike Recoveries

Amount Added		Amount Found	
% Target	mg	mg	% Recovery
60	19.08	19.13	100.3
100	31.80	32.38	101.8

phenylglycine with a suitable level of precision and accuracy. Standard

linearity was checked by assaying standards ranging from 25 to 200% of

the target value with Benzocaine as internal standard.   Relative

standard deviation (2s%) for chromatographic variability was determined

by five injections of the standard solution and was found to be 0.6%.

Accuracy of the method was determined by spiking authentic Dane Salt

Potassium (-)-N-(1-Methoxycarbonylpropene-2-yl)-p-Hydroxyphenylglycine

at the 60 and 100% levels, respectively, to the actual sample solution

(containing 27.75 mg Dane Salt Potassium (-)-N-(1-Methoxy-carbonyl-

propene-2-yl)-p-Hydroxyphenylglycine.   The percent recoveres are

shown in Table I.

Percent relative range for precision of the method was 0.6% and was

determined by a single injection of triplicate sample preparations

for each of two samples.

Specificity of the assay was shown by injecting two known

degradation products, methylacetoacetate and p-hydroxyphenylglycine.

No interferences were noted. A search was conducted in order to

find a diluent in which Dane Salt Potassium (-)-N-(1-Methoxycarbonyl-

propene-2-yl)-p-Hydroxyphenylglycine could be dissolved without

significant degradation before injection. Dane Salt Potassium (-)-N-

(1-Methoxycarbonylpropene-2-yl)-p-Hydroxyphenylglycine was more stable
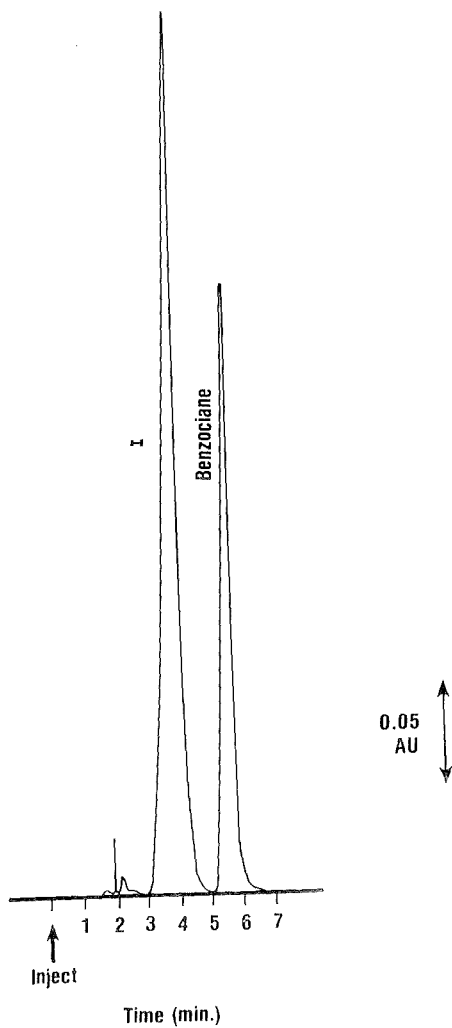
FIGURE 1   Sample Chromatogram

in methanol than in water or mobile phase alone over time.   When methanol

was used as a diluent, distorted peak shapes were produced during chroma-

tography.   When 40% methanol in H_2O, containing 0.023% potassium hydroxide

solution was used in the final dilution step, Gaussian peak shapes and

less than 1% loss after 10 minutes of Dane Salt Potassium (-)-N-(1-

Methoxycarbonylpropene-2-yl)-p-Hydroxphenylglycine was observed.   A

typical sample chromatogram is shown in Figure 1.

In summary, an ion-pair HPLC assay method has been validated.

The precision, accuracy and specificity of the method have been shown

to be good.

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HIGH PRESSURE LIQUID CHROMATOGRAPHIC DETERMINATION OF
PARABENS IN PHARMACEUTICAL PREPARATIONS
CONTAINING HYDROXYQUINOLINES

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ABSTRACT

A high pressure liquid chromatographic (HPLC) procedure for the
analysis of methyl paraben (MP) and propyl paraben (PP) in pharma-
ceutical preparations containing a halogenated hydroxyquinoline
(HHQ) is described. The method involves a separation of the
phenolic constituents, MP, PP and HHQ with a Bio-Rad AG®1-X8 anion
exchange resin, elution of the phenols with methanol after
acidification and a reverse phase HPLC separation of the parabens
using methanol - pH 6.5 buffer (60/40) mobile phase, a 30 cm x
3.9 mm (i.d.) column packed with Waters µBondapak C_{18} packing and
a guard column packed with Waters Bondapak C_{18}/Corasil packing.
Recovery, precision, specificity and interference data along with
the application of the proposed method for some commercial formu-
lations both with and without a hydroxyquinoline are described.

INTRODUCTION

Methyl and propyl parabens are used extensively as preserva-

tives in pharmaceutical, food and cosmetic preparations in order

to prevent the growth of microbials (1). Several analytical

methods have been reported in the literature for the analysis of

the parabens. These methods include colorimetry (2-3), gas

chromatography (4-5) ion-exchange (6-7) and reverse phase HPLC

(8-11), partition chromatography (12), adsorption chromatography

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(13-14), ultraviolet spectrophotometry (15), and thin-layer
chromatography (16-17).   However, when we employed the above
methods for the analysis of a pharmaceutical lotion preparation
which also contained the active ingredients iodochlorhydroxyquin
and hydrocortisone in addition to the parabens, none of the above
methods could be employed successfully due to one or more of the
following reasons: 1. high iodochlorhydroxyquin/parabens con-
centration ratio 2. phenolic properties of both parabens and
iodochlorhydroxyquin 3. high polarity of iodochlorhydroxyquin
(18) 4. weak basicity of iodochlorhydroxyquin 5. poor stability
of parabens in basic solutions 6. formation of emulsion during
clean-up procedure due to the excipients present in the sample
7. relative high solubilities of parabens in water and 8. pre-
sence of interfering components in some of the excipients such
as lanolin used in the lotion formulation (19).   In this publica-
tion we are reporting a procedure which is based on a preliminary
ion-exchange separation of the parabens and iodochlorhydroxyquin
from the formulation, elution of these compounds from the column,
precipitation and removal of a majority of iodochlorhydroxyquin at
a pH of 6.5 and a subsequent HPLC separation and quantitation of
the individual parabens.

MATERIALS AND METHODS

Apparatus

A modular HPLC instrument with a Waters Model 6000A solvent
delivery system, a Waters Model 440 absorbance detector (fixed
wavelength at 254 nm) and a Valco injection valve with 100 µL loop
was used. For HPLC separation, a Waters µBondapak C_{18} column,
30 cm x 3.9 mm (i.d.) and a guard column packed with Waters
Bondapak C_{18}/Corasil were used.

Mobile Phase

Mix thoroughly 600 mL of methanol and 400 mL of a pH 6.5
buffer solution. The buffer solution was prepared by adding the

appropriate amount of 0.1N sodium hydroxide to 250 mL of 0.1N monobasic sodium phosphate to obtain a pH value of 6.5 and then adjusting the volume to 1000 mL with water.

Chromatographic Conditions

A mobile phase flow rate of 1 mL/minute (isocratic) was used for the study. The column was maintained at room temperature and the detector sensitivity was maintained at 0.1 AUFS for MP and at 0.04 for PP.

Standard Preparation

Prepare a standard methanolic stock solution containing 2.25 µg of MP/mL and 1.25 µg of PP/mL. Dilute 25 mL of the stock solution to 50 mL with the pH 6.5 buffer solution. Filter and inject 100 µL into the column.

Procedure

Take 1.5 g of AG 1-X8 (chloride form) analytical grade anion exchange resin (Bio-Rad Laboratories) in a 25 cm x 10.5 mm (i.d.) glass chromatographic column equipped with a 200 mL reservoir at the top and a stopcock at the bottom and convert the resin into the hydroxide form with 1N sodium hydroxide. Wash the column with water and methanol. Extract an amount of lotion or cream sample equivalent to about 0.7 mg of total parabens three times with 15 mL of methanol, filter, if necessary, collect the clear liquid and dilute to 50 mL with methanol. Disperse the ointment sample with 25 mL of ether, add 15 mL of 0.1 N HCl, shake and collect the ether layer. Repeat the extraction twice with 25 mL of ether. Combine the ether layers and evaporate the ether. Dissolve the residue in 50 mL of methanol. Pass a 25 mL aliquot of the sample solution through the ion-exchange column and wash the column successively with 100 mL of methanol, 10 mL of 0.5N hydrochloric acid and 10 mL of water. Discard the eluants. Pass 100 mL of methanol through the column, collect the eluant and adjust the final volume to 100 mL. Dilute 25 mL of the sample solution to 50 mL with pH 6.5 buffer, centrifuge an aliquot and filter.

Inject 100 μL into the HPLC column. Compare the peak heights
(or the area) of the sample and the standard solutions and
calculate the amount of MP and PP in the sample.
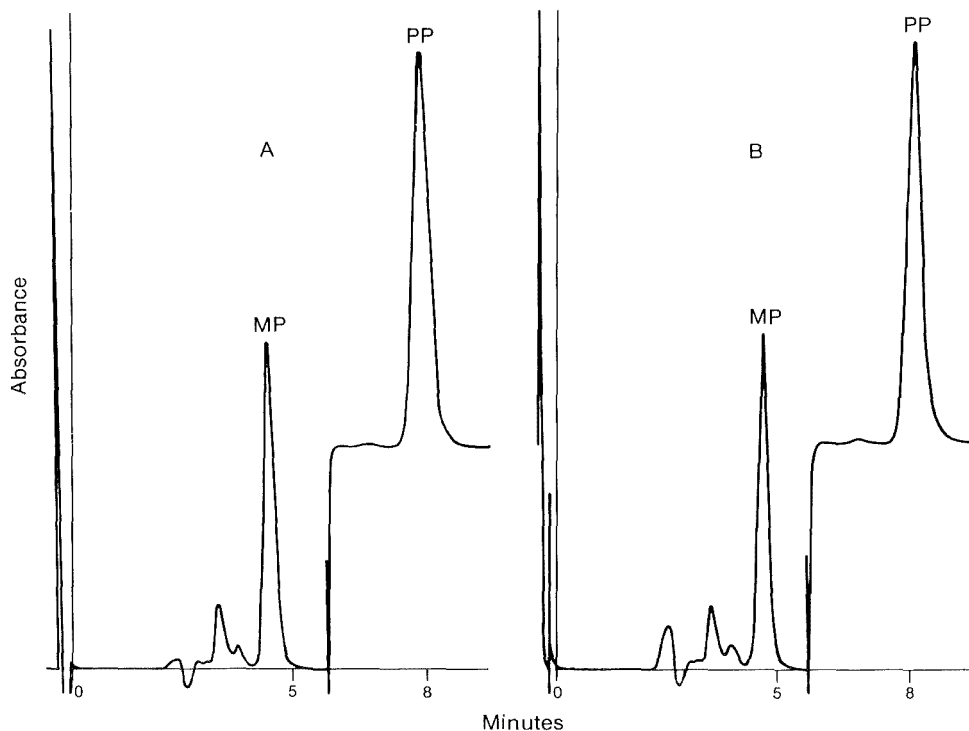
FIGURE 1

A. A typical chromatogram showing the separation of MP and PP
    from the analysis of a 1 g sample of a iodochlorhydroxyquin-
    hydrocortisone lotion formulation using the method described
    in the text. The detector sensitivity was changed from 0.1
    AUFS to 0.04 AUFS after the elution of MP.

B. Same as A except for the addition of 0.087 mg of parahydroxy
    benzoic acid to the sample before the analysis by the proposed
    method.

RESULTS AND DISCUSSIONS

A chromatogram obtained from a typical analysis of a lotion sample labelled to contain 0.07% of total parabens, 1% hydrocortisone and 3% iodochlorhydroxyquin is shown in Figure 1. Interference values of 2% and < 1% respectively were obtained for MP and PP when a paraben placebo lotion sample which contained hydrocortisone and iodochlorhydroxyquin was analyzed by the proposed method.   Samples of the placebo lotion samples were spiked with MP and PP at a level of approximately 80%, 100% and 120% of their amount present in a typical iodochlorhydroxyquin-hydrocortisone lotion sample and then analyzed by the proposed method.   The results, shown in Table 1, indicate that the recovery of MP is within 101-104% range and the recovery of PP within 97-99% range.

TABLE 1

Recovery and Linearity Data For
Iodochlorhydroxyquin-Hydrocortisone Lotion Formulation

Amount of Placebo g	Amount Added mg		Amount Found mg		Recovery %	
	MP	PP	MP	PP	MP	PP
1.004	0.331[1]	0.214[1]	0.346	0.212	104	99
0.952	0.414[2]	0.268[2]	0.419	0.260	101	97
1.014	0.497[3]	0.322[3]	0.515	0.318	104	99

MP = Methyl Paraben   PP = Propyl Paraben

[1]Approx. 80% of Label

[2]Approx. 100% of Label

[3]Approx. 120% of Label

TABLE 2

Parabens in a Iodochlorhydroxyquin-Hydrocortisone Lotion[5]

Sample	Current Method[4] % Total Parabens	Proposed HPLC Method		
		% MP	% PP	% Total Parabens
A[1]	0.07	0.044	0.024	0.068
A[2]	--	0.044	0.024	0.068
B[1]	0.066	0.044	0.022	0.066
B[2]	--	0.042	0.022	0.064
C[1]	0.070	0.046	0.025	0.071
D[3]	0.020[6]	0.044	0.023	0.067

[1] = Chemist #1 Lab 1
[2] = Chemist #2 Lab 1
[3] = Chemist #3 Lab 2
[4] = Recovery of Spiked Placebo was < 100% by this Alumina Column - UV method. However, the standard is treated in the same way as the sample in this method.
[5] = Label claim: total parabens = 0.07%.
[6] = Probably due to the poor reproducibility of the alumina employed

The results obtained from the analysis of four different batches of a commercial iodochlorhydroxyquin-hydrocortisone lotion are shown in Table 2. The results indicate that the proposed method is more accurate than a alumina adsorption column - UV combination method employed currently for this product. The results also show that the inter- and intra-laboratory reproducibilities of the proposed method are satisfactory.

In addition to the potential binding of parabens with the excipients present in some of the formulations (20-22), parabens are also known to hydrolyze to parahydroxybenzoic acid (PHBA) particularly in the basic solutions (23). In order to establish the specificity of the proposed method for MP and PP in presence of PHBA, a lotion sample containing MP, PP, iodochlorhydroxyquin

TABLE 3

Parabens in Some Typical Formulations

#	Sample	Claim, %	Found, %	
		Total Parabens	MP	PP
1	Crotamiton Cream	0.4	0.25	0.14
2	Flumethasone Pivalate Cream	0.04	0.021	0.014
3	HC Cream	NA*	0.19	0.09
4	HC Cream + 2% CQ added[1]	NA*	0.18	0.10
5	HC Lotion	NA	0.14	0.02
6	HC - ICHQ Cream	NA*	0.12	0.05
7	HC - ICHQ Ointment	NA	ND	ND
8	HC - DIHQ Cream	0	ND	ND
9	HC - DIHQ Cream	0.072[2]	0.044	0.027

[1] Sample #4 is same as sample #3 with 2% CQ added.
[2] Sample #9 is same as sample #8 with 0.044% MP and 0.028% PP added.

HC   - Hydrocortisone;   CQ   - Chlorquinaldol
ICHQ - Iodochlorhydroxyquin;   DIHQ - Diiodohydroxyquin

NA*   - Parabens present in formulations;
        amount not indicated.
NA   - Information not available.
ND   - No parabens detected by the proposed method.

and hydrocortisone was analyzed with and without added PHBA.
The results shown in Figure 1 indicate that PHBA does not
interfere with the analysis of MP and PP. It has to be pointed
out, however, that the proposed method as such cannot be employed
for the determination of PHBA due to the fact PHBA is retained on
the HPLC column under the conditions employed. If the quanti-
tation of PHBA is desired, one can accomplish this by substituting
water for pH 6.5 buffer in the mobile phase of HPLC and in the
final step of the sample preparation. This modification will
result in the elution of PHBA. However the reproducibility of
MP and PP quantitations are not satisfactory with the mobile
phase system that is not buffered.

        In addition to the analysis of iodochlorhydroxyquin lotion,
the proposed method was also applied to the analysis of parabens
in some other semi-solid formulations containing one or more of
the following: iodochlorhydroxyquin, diiodohydroxyquin, chlor-
quinaldol, crotamiton, flumethasone pivalate and hydrocortisone.
The results, included in Table 3, indicate that the proposed
method is applicable to these formulations as well.

        The proposed method has two minor disadvantages, first the
clean-up step is somewhat time consuming. However, the analysis
time can be shortened by carrying out the complete analysis in one
step using a short ion-exchange column in front of the HPLC column
and employing a series of valves in order to automate the clean-up
steps (24-26). The other minor disadvantage of the method is the
need for a periodic replacement of the guard column included in
the HPLC step. The guard column was included in order to elimi-
nate the potential drift in the baseline due to the elution of
iodochlorhydroxyquin particularly after repeated injection of
the sample solution.

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REVERSE-PHASE HPLC OF PROTEINS: EFFECTS OF
VARIOUS BONDED PHASES

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ABSTRACT

A variety of organic phases bonded to silica can be used effectively for the reverse-phase high performance liquid chromatography of large proteins. These include octyl, octadecyl, cyanopropyl and diphenyl bonded phases. The differences in retention characteristics and selectivity among these bonded phases is demonstrated with several standard proteins.

INTRODUCTION

The use of reverse-phase high performance liquid

chromatography (hplc) for the separation and purification of

peptides and small proteins has gained wide acceptance (1).

This same methodology for large proteins has been used in a few

instances (2,3) and shows promise for much wider use. The best

reverse-phase chromatography conditions for large proteins utilize

large pore silica supports (30-50nm pores) and slow flow rates

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(2,4). These are necessary due to the size of the larger proteins and their slow diffusion rates. Although a variety of organic bonded phases have been used for the isolation of peptides and small proteins (5,6,7), an examination of various organic bonded phases for the reverse-phase chromatography of large proteins has not been reported. In this paper we demonstrate that octyl, octadecyl, diphenyl, and cyanopropyl bonded phases can be used effectively in the reverse-phase hplc of large proteins. In addition, the differences in selectivity with these different bonded phases is described.

MATERIALS AND METHODS

Collagen (M.W. 100,000), β-lactoglobulin-B (M.W. 35,000), phosphorylase-B (M.W. 94,000), and human serum albumin (M.W. 68,000) were all purchased from Sigma. Pyridine, formic acid, propanol, and acetone (all from Baker Chemical Co.) were distilled over ninhydrin prior to use. The hplc system used was a Spectra-Physics 8700 equipped with a mixing chamber. The post-column fluorescamine detection system has been described previously (8). The chromatography buffers were 0.5M formic acid brought to pH 4.0 with pyridine and the same buffer containing 60% (v/v) 1-propanol. A flow rate of 0.75mL/min was used for all the chromatographies. The columns (4.6 X 250mm) were 'Bakerbond' Wide-Pore columns (Baker Chemical Co.) with 33nm pore size. Further details are provided in the figure legends.

RESULTS AND DISCUSSION

The advantage of using 33nm pore supports instead of 10nm pore supports is demonstrated in Fig. 1. The 33nm pore octyl column gives better separation of the standard proteins and
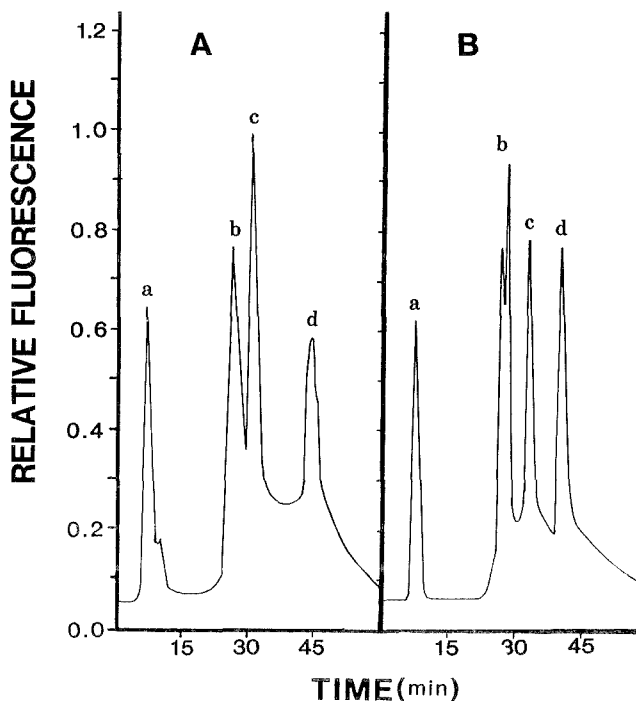

FIGURE 1. Comparison of 10nm and 33nm pore octyl columns. Phosphorylase B (10 μg), collagen (35 μg), human serum albumin (7 μg) and lactoglobulin B (10 μg) were dissolved in 1 mL of starting buffer for injection. This is the elution order of these proteins (identified a-d in the figure). The gradient was 1-propanol, 0-24% in 15 min, 24-48% in 55 min in 0.5M formic acid/ 0.4M pyridine pH 4.0 with a flow rate of 0.75 mL/min. Panel A is a Lichrosorb RP-8 column (10μ particles with 10nm pores). Panel B is a "Bakerbond" Wide Pore Octyl column (10 particles with 33nm pores).

partially separates the α_1 and α_2 chains of collagen which are unresolved on the 10nm pore octyl column. β-lactoglobulin-B elutes later from the small pore column and shows a small shoulder not seen on the large pore column. Thus, it appears that this protein is small enough to effectively penetrate the smaller pores. Although other factors may be influencing the differences between these columns none of the 10nm pore supports we have tested give resolution comparable to the 33nm pore supports.

In an effort to determine the effect that the various bonded phases would have on the elution of these proteins, identical amounts of each protein were chromatographed on each of the four types of columns using identical gradients (Fig. 2). From this figure it is clear that all four bonded phases produce excellent resolution. The gradient used was designed to separate these proteins on the octyl column. The protein elution times for each of the columns is shown in Table 1. Phosphorylase-B is only weakly retained (about 1 min beyond wash through) on all these columns. The columns all show good reproducibility and recoveries of the proteins ranged from 80-95%.

The octyl and octadecyl columns show very similar elution profiles and elution times (Fig. 2A and B, Table 1), although the collagen chains are separated to a slightly greater extent on the octadecyl support. This similarity is somewhat surprising in view of the large difference in hydrophobicity between the two bonded phases. This leads us to suggest that the octadecyl chain may be
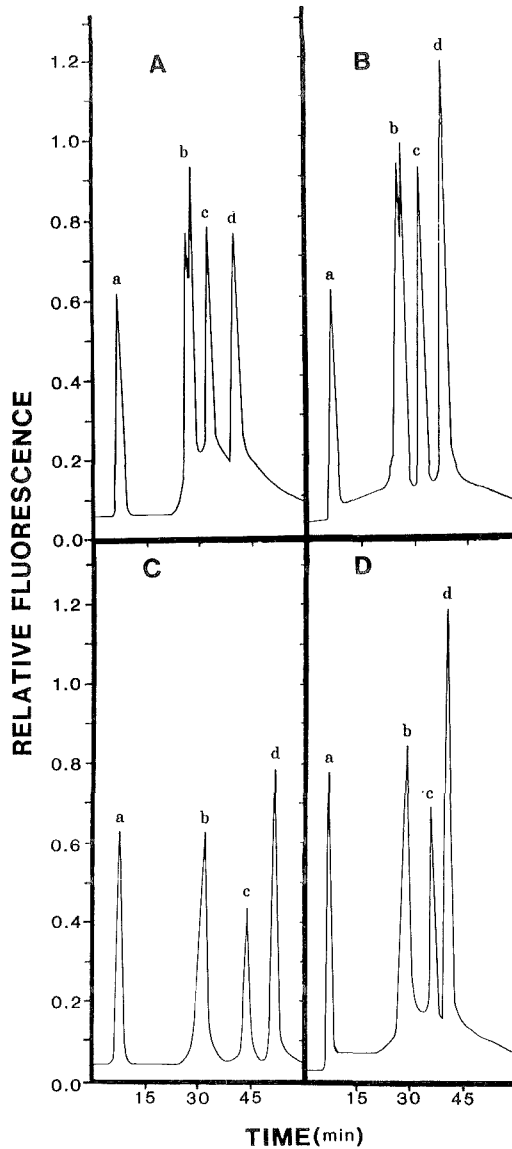
FIGURE 2. Elution from different bonded phases.
Proteins and gradient used were the same as in Fig. 1. The columns were all "Bakerbond" Wide Pore columns. a) Octyl, b) Octadecyl, c) Diphenyl, and d) Cyanopropyl. Protein elution order is the same as Fig. 1.

TABLE 1

COLUMN		ELUTION TIME (Min)		
	PB	CL	HSA	LGB
Octyl (10nm)[2]	7	26.5 (0.5)	31.5 (0.3)	45 (0.3)
Octyl[3]	7	27,28 (0.3)	34 (0.4)	41 (0.4)
Octadecyl[3]	7	27,28 (0.3)	33 (0.3)	38.5 (0.4)
Cyanopropyl[3]	7	29.5 (0.2)	36.5 (0.4)	41 (0.4)
Diphenyl[3]	7	32.5 (0.1)	45.3 (0.3)	53.5 (0.4)

[1]The times are averages of at least 3 chromatographies with the S.E.M. in parentheses.
[2]E.M. Merck Lichrosorb octyl column (10μ particles).
[3]"Bakerbond" Wide Pore columns (33nm pores and 10μ particles). All columns were 4.6 x 250nm.
[4]The peaks are phosphorylase B (PB), collagen (CL), human serum albumin (HSA), and β-lactoglobulin B (LGB).

folding back on itself under the aqueous conditions employed. Another possible explanation is that the proteins we used have no hydrophobic sites large enough to discriminate between the two different chain lengths. Lower ionic strengths or different buffer systems might be used to accentuate differences between these two bonded phases.

The cyano-propyl column shows a slightly different elution profile than the two aliphatic carbon chain bonded phases (Fig. 2D). There is no resolution of the collagen chains with this bonded phase. However, their elution times, as well as that of human serum albumin are later relative to the octyl column (1.5 and 2.5 min respectively). In contrast, β-lactoglobulin-B elutes at the same time from both bonded phases. This selectivity difference between the cyano-propyl column and the aliphatic chain columns, indicates these differences can be exploited for protein

purifications. The cyano-propyl bonded phase has been shown to be effective in separating small proteins that differ in glycosylation (9). These large pore cyano-propyl columns may also be useful in this type of application with large proteins.

Retention times of the standard proteins on the diphenyl bonded phase are significantly different than with the other three bonded phases (Fig. 2C). The three retained proteins, collagen, human serum albumin, and β-lactoglobulin-B, elute much later from this column, the latter two proteins by 11 and 13 min respectively. From this data and data obtained using small pore columns with peptides (6) it is clear that the diphenyl bonded phase is interacting with proteins in a different manner than the straight chain bonded phases. It is likely that this interaction involves a combination of hydrophobic and aromatic stacking effects. Since increased retention times are not seen on a phenyl column (unpublished results) it appears the diphenyl group is required to obtain stacking with aromatic residues on the proteins. The smaller effect seen with collagen may be due to the relatively low percentage of aromatic groups in that protein.

From the results obtained in these studies it is evident that larger proteins (M.W. 50,000) can be effectively separated by reverse-phase hplc. As has been demonstrated for peptides, the use of different organic bonded phases greatly facilitates purifications by taking advantage of the differences in selectivity among the bonded phases. The proper gradient

conditions are very similar for all these bonded phases as was demonstrated in Fig. 2 where the same gradient was used for all four bonded phases. Thus it is possible to take advantage of the differing elution characteristics and selectivity provided by the different bonded phases without a great deal of trial and error to determine the appropriate buffer and organic modifier conditions.

ACKNOWLEDGEMENTS

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RAPID ISOLATION OF NANOGRAM AMOUNTS OF

CRUSTACEAN ERYTHROPHORE CONCENTRATING HORMONE

FROM INVERTEBRATE NERVE TISSUE BY RP-HPLC *

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ABSTRACT

A reverse phase-high performance liquid chromatography (rp-hplc) method was developed for the rapid isolation of nanogram amounts of crustacean erythrophore concentrating hormone (CECH) from invertebrate nerve tissue. Tissue homogenates from the shrimp, Paleomenetes pugio, were subjected to a multistep work-up to remove proteins and lipids prior to analysis by rp-hplc. Samples were eluted with a concave gradient of 0.1% trifluoroacetic acid (TFA) verses acetonitrile. Detection at 210 and 254 nm combined with the use of highly efficient and end-capped columns permitted the determination of less than 5 ng of CECH. Pure CECH was isolated from the columns by fraction collection followed by lyophilization of the volatile TFA buffer.

INTRODUCTION

Reverse phase-high performance liquid chromatography (rp-hplc) is rapidly becoming the preferred method for the analysis and isolation of underivatized peptides from tissue. Recent improvements in pumps, detectors, and column technology have made it

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* Mention of a commercial product in this paper does not constitute an endorsement of this product by the USDA.

possible to detect or isolate nanogram amounts of peptide hormones from tissue samples (1-8).

Four invertebrate peptide hormones, proctolin (Arg-Tyr-Leu-Pro-Thr), locust adipokinetic hormone (LAKH, p-Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH$_2$), crustacean erythrophore concentrating hormone (CECH, p-Glu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH$_2$) and mulluscan cardioexcitatory neuropeptide (MCEN, Phe-Met-Arg-Phe-NH$_2$) have been isolated, identified (9-16) and have become available commercially. In all cases isolation of these peptides required the processing of large numbers of invertebrates followed by size exclusion chromato-graphy. For example, in the isolation of proctolin (9) 125 kg of whole cockroaches, Periplaneta americana, were required to yield 180 μg of pure peptide suitable for structural analysis.

Recently, Stone and Mordue (17) suggested the use of reverse phase-hplc to isolate small quantities of insect neuropeptides. In this paper we describe an hplc method for the rapid isolation of nanogram amounts of peptide hormone from invertebrate nerve tissue. The method is illustrated by a description of the techniques used for the isolation of crustacean erythrophore concentrating hormone (CECH) from 32 pairs of eyestalks removed from the shrimp, Paleomenetes pugio. The average weight of an eyestalk is 2 mg.

MATERIALS AND METHODS

Chemicals.

Triethylamine (Fisher, Hplc Grade)[1] was purified by fractional distillation through a short Vigreaux column. The middle fraction was further purified by passage through a C$_{18}$ Sep-Pak (Waters Associates). Phosphoric acid (Fisher, Hplc Grade) was used without further purification. Triethylammonium phosphate buffer (TEAP) at pH 2.20 was prepared by the method of Rivier (18). Trifluoroacetic acid (TFA, Baker, Analyzed Reagent Grade) was purified by fractional distillation through a short Vigreaux column and the middle fraction was taken. Hplc grade water was obtained from a Milli-Q System (Millipore). Acetonitrile (Burdick & Jackson or Fisher, Hplc Grade),

ethyl acetate (Fisher, Hplc Grade), methanol (Burdick & Jackson and
acetic acid (Baker, Ultrex Grade) were used without further purifi-
cation. Peptides (Peninsula or Sigma) were stored in the freezer.
Standard peptide solutions were made up at $0.50 \pm .04$ mg in 50 ml
0.1% TFA and stored in the refrigerator.

Crustaceans.

P. pugio (Gulf Specimen Co) were kept in salt water aquaria.
Live female lobsters, Homarus americanus, were purchased from a
local distributor and used immediately.

Hplc.

The hplc system consisted of a gradient liquid chromatograph
(Waters Associates) equipped with a Model 660 solvent programmer,
Model U6K injector, two Model 6000A pumps, Model 440 absorbance
detector and Model 450 variable wavelength detector. The latter
was connected to a Model MM 700 memory module (Schoeffel) to
provide baseline correction when necessary during the course of the
gradient at low wavelengths.

The columns, Supelcosil LC-18DB (Supelco) or Zorbax C-8
(Dupont) were protected by means of a short guard column of Pelli-
guard LC-18 (Supelco). Aqueous buffer was filtered and degassed
through a type HA filter (Millipore); acetonitrile solutions through
a type HF filter. The aqueous buffer was passed through a 61 x
0.78 cm column of Bondapak C_{18}/Porasil B (Waters Associates) in-
stalled in line between the high pressure output of the aqueous
pump and the mixer (Figure 1).

The columns were equilibrated by running several cycles of the
gradient over 1 min followed by isocratic elution at the starting
conditions until a stable baseline was achieved. Samples were ap-
plied to the equilibrated columns which were then eluted with a
concave gradient (Curve 5) of 90——> 40% aqueous buffer. Buffer
systems and optimal hplc conditions as determined experimentally
are described in Table 1. The elution was monitored simultaneously
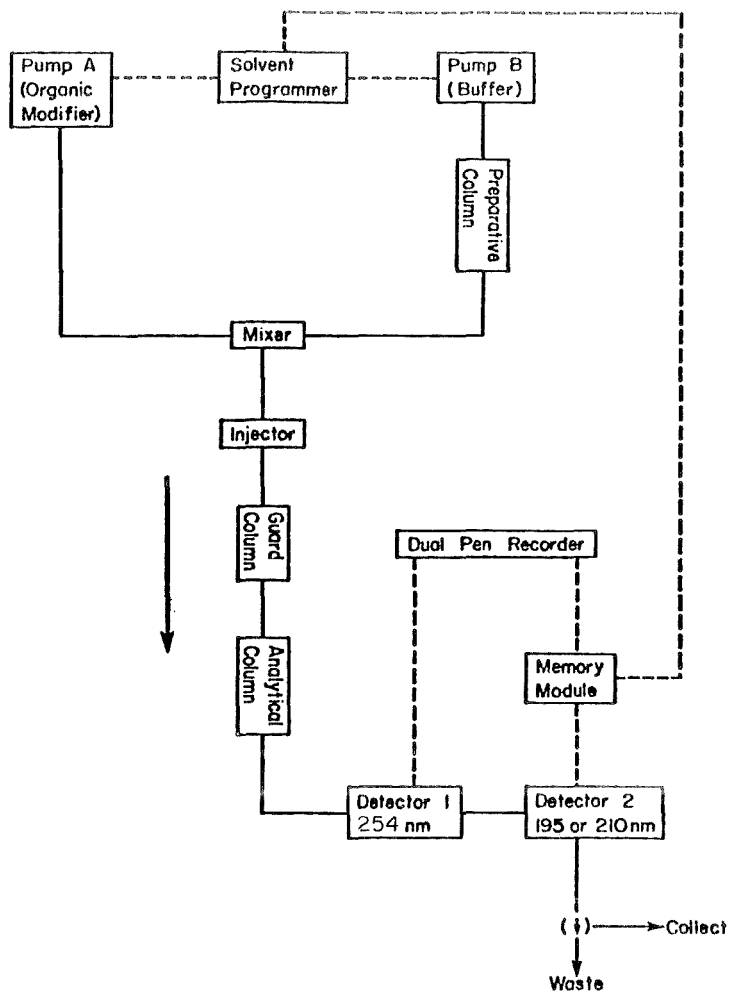at 254 nm (0.01 AUFS) and 210 or 195 nm (0.1 AUFS).

FIGURE 1. Schematic diagram of hplc system. Dashed lines are
electrical connections.

TABLE 1
Buffer Systems and Hplc Conditions Used for Analysis
and Isolation of Invertebrate Neuropeptides

Aqueous Phase B	Organic Modifier A	UV Detection (nm)	Hplc Conditions
0.25N TEAP pH 2.20	Acetonitrile	254,195	90——> 40% B/1.0 h/ 1.1 ml/min (curve 5)
0.1% TFA	0.1% TFA in Acetonitrile	254,210	90——> 40% B/1.0 h/ 1.0 ml/min (curve 5)

Sample preparation.

Pairs of eyestalks from as few as 10 shrimp, P. pugio, or
eyestalks and brain from the lobster, H. americanus, were removed
and immediately placed in 1 ml of chilled methanol-water-acetic acid
(90:9:1 by vol) in a 1.5 ml polyethylene centrifuge tube (Sarstedt).
The tissue was homogenized with a Polytron homogenizer (Brinkmann)
equipped with a PT 7 micro probe generator. The homogenate was
centrifuged in a Model RC-3B refrigerated centrifuge (Sorvall) at
4000 rpm at 5°C for 30 min. The supernatant was transferred into
a clean 1.5 ml polyethylene centrifuge tube and concentrated to
minimal volume under a stream of nitrogen. The resulting sample
was taken up in 1 ml of 0.1% TFA and extracted with ethyl acetate
(3x). The ethyl acetate layer was removed by decantation. Residual
ethyl acetate was removed under a stream of nitrogen. Final filtra-
tion was accomplished by use of a centrifugal filtration apparatus
(Rainin) equipped with a 0.45 Nylon-66 membrane filter centrifuged
at 1500 rpm for 10 min at 5°C.

Peptide isolation.

The entire filtered sample (<1 ml) was taken up in 1 cc
dispensable plastic syringe (Becton-Dickenson) equipped with a
N725 stainless steel needle (Hamilton) and injected into the hplc
apparatus. The gradient was begun immediately upon injection.
Fractions were collected in polyethylene vials and lyophilized in

a Model 25SRC freeze dryer (Virtis) equipped with a soda trap and
outside venting of the vacuum pump. The resulting freeze dried
samples were taken up in 100 or 200 μl of 2.7% NaCl for bioassay.

Bioassay.

Peptides and fractions were assayed for their erythrophore
concentrating activity as previously described (19) by injecting
them into the shrimp, P. pugio; induced erythrophore contractions
were then assigned values according to the scale of Hogben and Slome
(20). Effects of standard solutions of synthetic CECH in 2.7% NaCl
on the shrimp erythophores were measured at various times following
the injection. Injections (10 μl) were made into shrimp whose
eyestalks had been removed 24 hr earlier.

RESULTS AND DISCUSSION

Many buffer systems have been proposed for reverse phase-hplc
of peptides, but in our experience the most versatile for the
analysis of a wide range of peptides and small proteins is triethyl-
ammonium phosphate (TEAP) vs acetonitrile. This buffer system,
first described by Rivier (18), is characterized by its UV trans-
parency down to 190 nm and its excellent resolution. We have re-
cently reported (21) on the use of this buffer system at pH 2.20
in a concave gradient of 90——> 40% TEAP combined with a highly
efficient, end-capped column (Supelcosil LC-18DB) to analyze
small synthetic invertebrate peptides as well as larger peptides
such as insulin (Figure 2). Simultaneous detection at 254 and
195 nm permitted peak ratioing and detection of less than 5 ng of
peptide. The TEAP buffer system is also characterized by its
excellent recovery of peptides. Peptide peaks collected from the
columns could be reinjected after removal of acetonitrile with
little loss of peak height. (Table 2). However, despite the fact
that TEAP buffer has been reported (18) to be compatible with most
biological systems both in vitro and in vivo, we have found that
0.25N TEAP at pH 2.20 interfered with the bioassay of CECH and
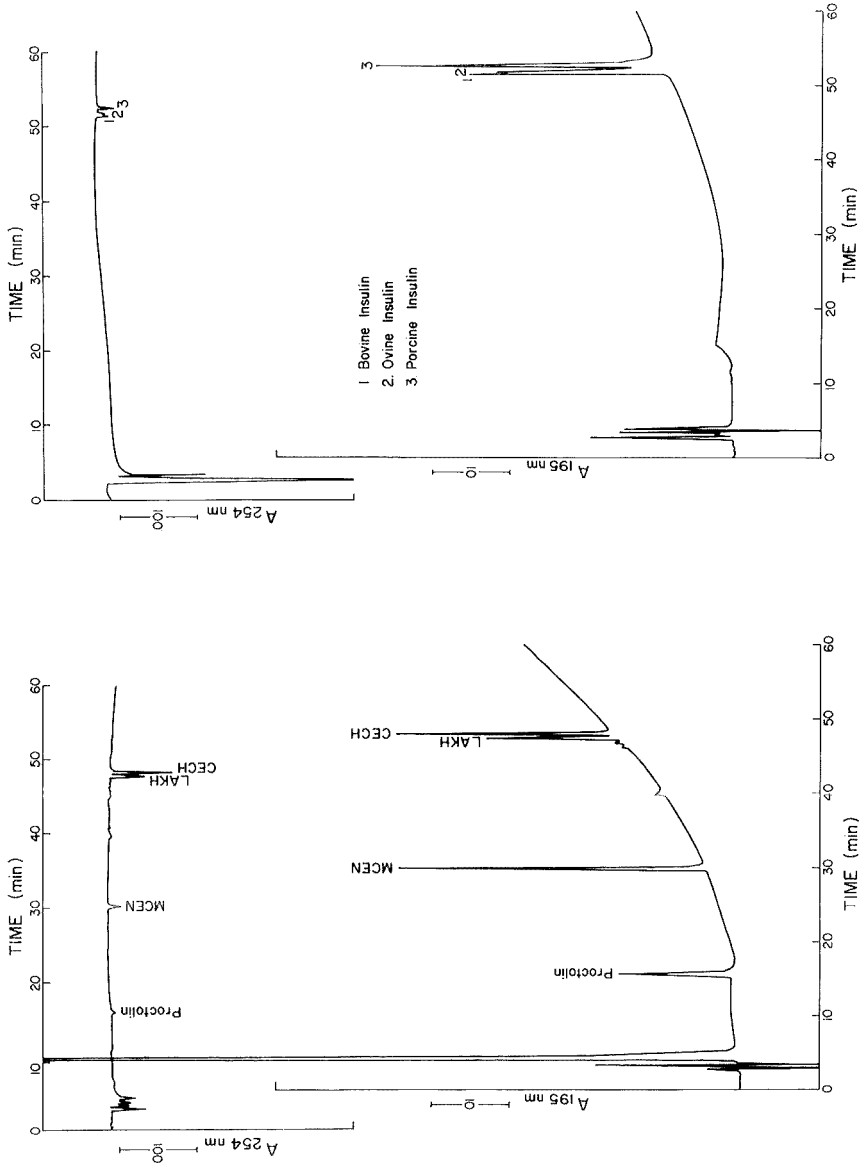LAKH (13). Since TEAP was non-volatile, high salt concentrations

FIGURE 2. Analysis of four invertebrate neuropeptides (left hand trace) and insulins (right hand trace) with TEAP buffer on the Supelcosil LC-18DB column. Buffer system and hplc conditions are described in Table 1.

TABLE 2
Recovery of Peptides from the Supelcosil LC-180B
Column Using TEAP and TFA Buffers[a]

Peptide	% Recovery	
	TEAP[b]	TFA[c]
Proctolin	71	90
leu-enkephalin	61	70
met-enkephalin	94	90
LAKH	–	81
CECH	–	66

a. Buffer and hplc conditions are described in Table 1.
b. Sample reinjected after removal of acetonitrile under
    nitrogen.
c. Sample reinjected in 0.1 ml 0.1% TFA after lyophilization.

would be expected when hplc peptide fractions were concentrated
prior to bioassay. TEAP-obtained fractions proved to be toxic to
bioassay animals and therefore undesirable for bioassay purposes.

A buffer system which eliminates these problems and allows us
to lyophilize fractions containing nanogram amounts of peptides
for later uptake in physiologically compatible solutions is 0.1%
TFA vs acetonitrile (2, 22, 23); it is volatile and yet, like
TEAP, is characterized by excellent resolution of small synthetic
invertebrate peptides as well as larger peptides such as insulin
(Figure 3). Recovery of lyophilized peptide fractions collected
from the columns is excellent (Table 2). A disadvantage of the
TFA buffer is its UV absorption at low wavelengths. Nevertheless,
0.1% TFA buffer can be run as low as 210 nm without excessive base-
line drift by addition of 0.1% TFA to the acetonitrile, so that the
TFA concentration does not change during the course of the gradient.
However, the absorbance of CECH in 0.1% TFA at 210 nm is about
50% less than in 0.25N TEAP (pH 2.20) at 195 nm (Figure 4).

We have utilized the 0.1% TFA buffer as a basis for develop-
ment of a method for the rapid isolation of nanogram amounts of
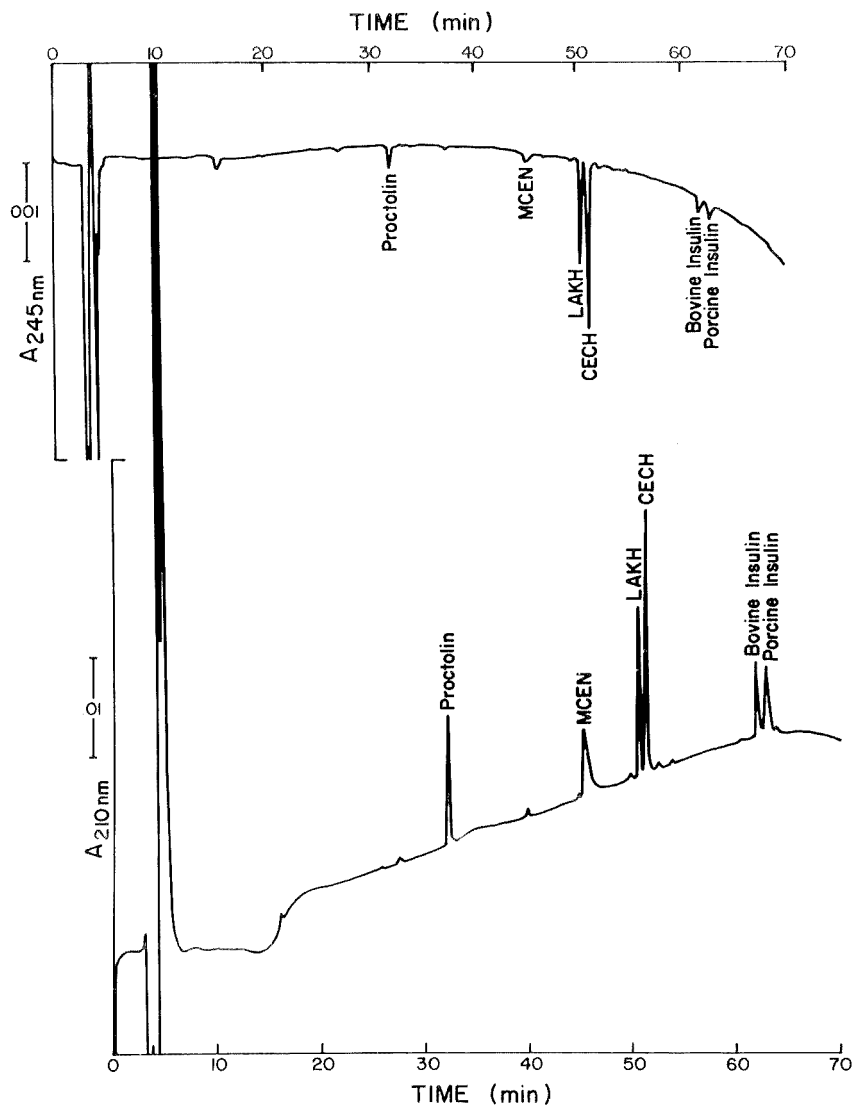neuropeptides from small amounts of invertebrate tissue. The

FIGURE 3.   Analysis of four invertebrate neuropeptides and insulins with TFA buffer on the Supelcosil LC-18DB column.   Buffer system and hplc conditions are described in Table 1.
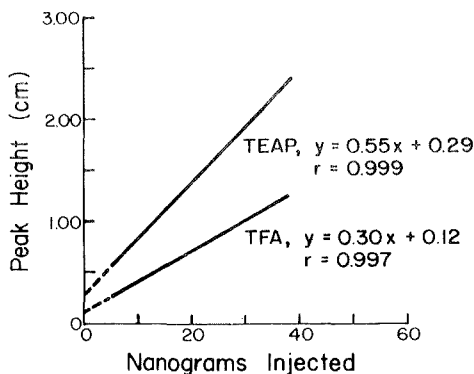
FIGURE 4. Regression analysis of data resulting from plotting peak height (cm) versus ng CECH injected over the range of 4.8-38.4 ng on the Supelcosil LC-18DB column with TEAP and TFA buffer systems. Buffer systems and hplc conditions are described in Table 1.

method, which involves several preliminary clean-up steps followed by reverse phase-hplc, is illustrated by the isolation of CECH from eyestalks of P. pugio. Eyestalks were homogenized in chilled acidic methanol to precipitate protein. The solvent system employed for this step, methanol-water-acetic acid (90:9:1 by vol) has previously been used by Holman and Cook (24) to homogenize insect tissue in the isolation of proctolin. Methanol was removed, lipids were extracted, and the sample was injected into the instrument with a plastic syringe after a final centrifugal filtration. Each step was designed to minimize losses. Therefore inert plastic was used throughout because CECH, like many other peptides, has been reported to adhere to glass (19). In addition, strongly acidic conditions were employed throughout to inhibit the action of any peptidases present.

Figure 5 is a representative elution profile for the isolation of CECH from the eyestalks of 32 P. pugio. The small sharp peak eluting at 51.5 min was identified as CECH by its retention time, absorbance peak ratio of 210 nm/254 nm, and bioassay of the collected peak. The CECH peak was readily observed with samples derived
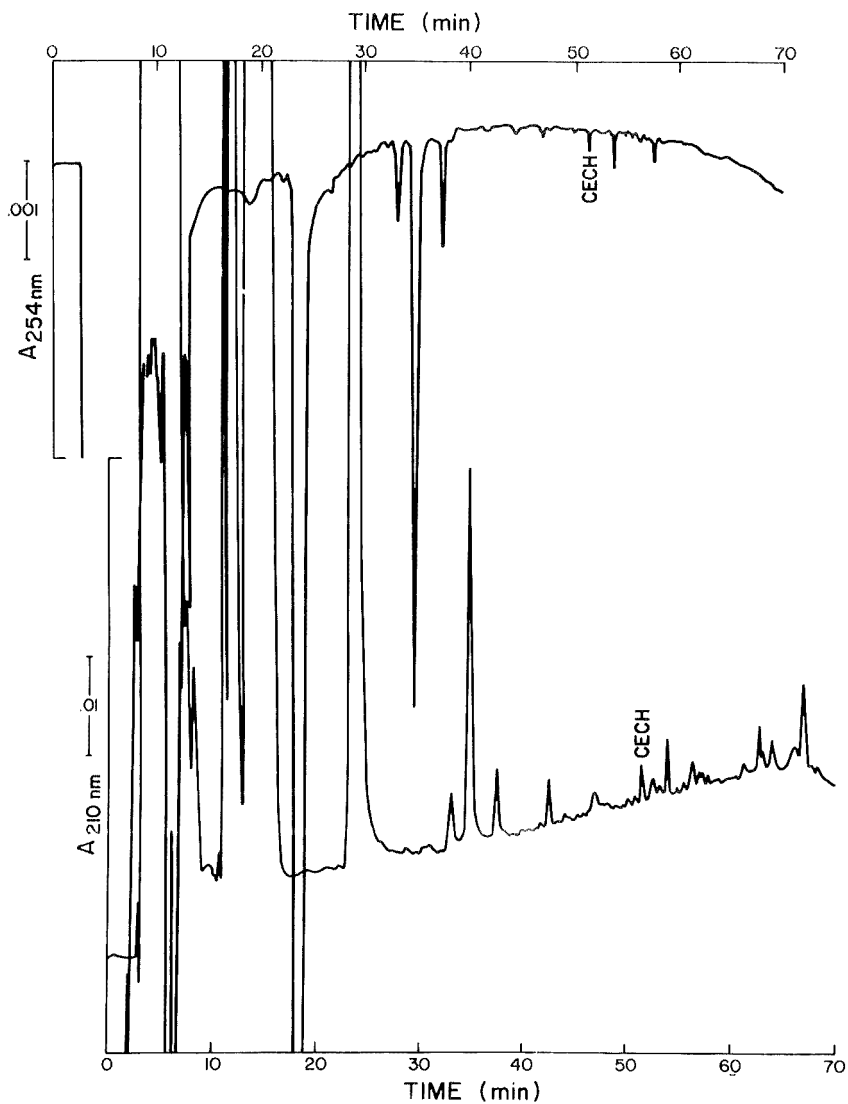
FIGURE 5. Elution profile for the isolation of CECH from the eyestalks of 32 P. pugio on the Supelcosil LC-18DB column with TFA buffer. Buffer system and hplc conditions are described in Table 1.

TABLE 3
Absorbance Ratio for Invertebrate Neuropeptides on
the Supelcosil LC-18DB-TFA and Zorbax C-8-TEAP Systems

| Peptide | Absorbance Ratio[a] | |
	Supelcosil LC-18DB -TFA[b]	Zorbax C-8- TEAP[b]
Proctolin	5.13	25.14
MCEN	5.86	21.90
LAKH	1.63	3.82
CECH (synthetic)	1.62	3.46
CECH (natural)	1.62	3.46

a. $A_{210 nm}/A_{254 nm}$ for TFA, $A_{195 nm}/A_{254 nm}$ for TEAP, $A_{210 nm}$
     and $A_{195 nm}$ at 0.1 AUFS, $A_{254 nm}$ at 0.01 AUFS.

b   Buffer and hplc conditions are described in Table 1.

from as few as 10 pairs of eyestalks (20 mg). Homogeneity of the
peak at 51.5 min was confirmed by analysis on another hplc system.
Thus the peak at 51.5 min was collected and analyzed with TEAP buffer
on a Zorbax C-8 column, after removal of acetonitrile. This column
and buffer system has been previously reported by us (21) to give
excellent resolution and sensitivity with invertebrate neuropeptides.
The resulting peak on the Zorbax C-8 column at 49.9 min was sharp
and had a retention time and absorbance peak ratio (Table 3)
consistent with a pure sample of CECH.

     The simultaneous monitoring at two wavelengths, 254 and 195 nm
(for TEAP) or 210 nm (for TFA) enables the measurement of absorbance
peak ratios. The calculation of these ratios has been described
(25) as perhaps the most useful of all spectroscopic procedures in
liquid chromatography. Absorbance peak ratios for the invertebrate
neuropeptides on the Zorbax C-8 and Supelcosil LC-18DB columns for
the TFA and TEAP buffers are listed in Table 3.

     Retention times of the invertebrate neuropeptides proctolin,
MCEN, LAKH, AND CECH were repeatable within 1 min over the course
of this study. However, as expected, there was some deterioration
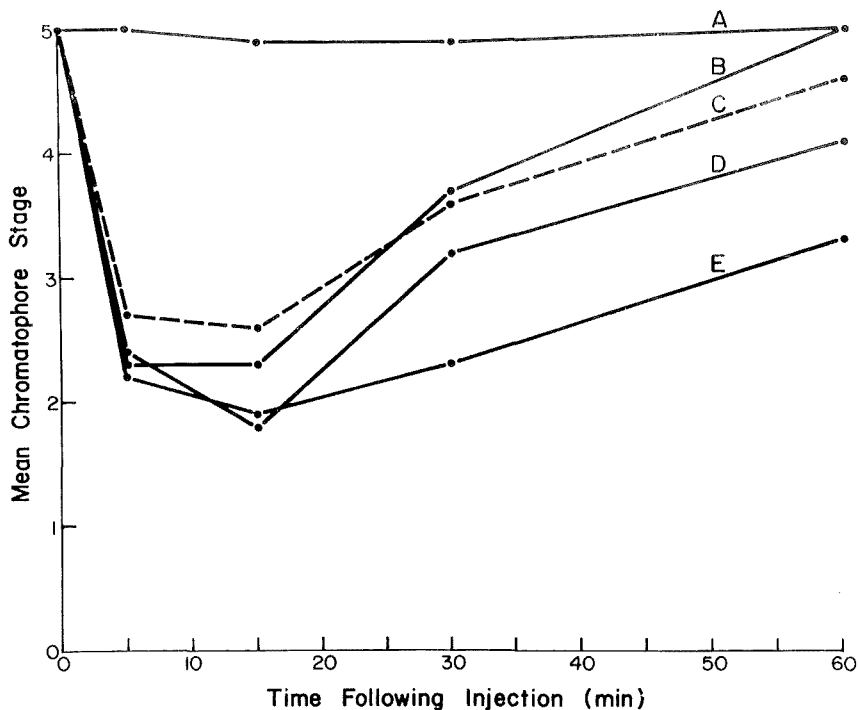of the columns with use as evidenced by peak broadening.

FIGURE 6.   Effects of CECH on the erythrophores of P. pugio.   Each
point represents the mean response of at least 5 animals to
10 μl injections of the following samples: A.   Control (2.7%
NaCl), B.   0.11 pmol synthetic CECH, C.   Natural CECH (1/20 of the
collected CECH peak in Fig. 5 after lyophilization), D.   0.55 pmol
synthetic CECH and E.   2.20 pmol synthetic CECH.

As shown in Figure 6, the bioassay for erythrophore concentra-
ting activity in P. pugio detects as little as 0.1 pmol of CECH.
The peak at 51.5 min shown in Figure 5 was determined by our
standard load response curve (Figure 4) to correspond to 28 ng of
CECH.   Bioassay of this collected peak after lypholization was
positive with 1/20 of this material giving an unequivocal positive
response (Figure 6).

In order to test the recovery of peptides in a biological
sample, known quantities of commercially available peptides were

TABLE 4

Recovery of Synthetic Invertebrate Neuropeptides by Hplc
from Lobster Nerve Tissue Homogenates

Peptide[a]	% Recovery[b]
Proctolin	59
LAKH	74
CECH	78

a. Lobster homogenates were spiked with 1.0 µg of each peptide
    and worked-up by the method described above.
b. Spiked samples were analyzed on the Supelcosil LC-18DB
    column with TFA buffer as described above.

added to brain and eyestalk tissue of the lobster, H. americanus,
and the samples processed by our method. Recoveries shown in
Table 4 were 59-78%. As an additional test of our methods,
lobster brain tissue samples were processed by our method and a
fraction collected between 50-60 min was examined for CECH activity
by bioassay. Results were positive indicating the presence of
CECH-like activity in that fraction. No attempt was made to
further isolate or purify any hormone from the fraction.

    We believe our hplc method of isolation of CECH from shrimp
eyestalks to be a distinct improvement over previously described
methods of isolation of the hormone in that we start with much
smaller amounts of tissue and utilize only one highly efficient
chromatographic separation to yield hplc-pure material.
By contrast, isolation of CECH from eyestalks of Pandalus borealis
by Fernlund and Josefsson required 100 g of freeze-dried eyestalks
and four time consuming Sephadex separations to yield 20 µg of pure
hormone (26).

    Our method, illustrated by the isolation of CECH, should be
adaptable to the identification of a wide range of peptides from
invertebrate nerve tissue. In the case where hplc separation is
insufficient to resolve the desired peptide from closely eluting
peaks, two consecutive hplc separations on different systems could
be employed. The first could utilize the non-volatile TEAP buffer,

followed by the TFA buffer from which isolation of the peptide by
lyophilization would be possible. The same or different columns
could be employed for the hplc separations.

In summary, a technique is described which allows the rapid
and routine identification and purification of nanogram amounts
of peptide hormones from mg amounts of invertebrate tissue. The
method is based on the superior separation power of hplc, is fast,
simple, and should be adaptable to a wide range of peptide hormones.

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LETTER:

ON THE PROBLEM OF MEASURING t_o

Dear Sir,

We read with much interest the recent paper of Neidhart et.al. which appeared recently in this Journal (1). The problem of t_o, the so called "void time" or "inert time" is indeed a difficult one. Several reports have appeared recently discussing the measurement of t_o (e.g.2). The paper by Neidhart et.al.(1), however, raises some questions as to its validity. Take,for example,the dead time determination using the Nucleosil 10-C_{18} column. From the given dimensions of the column - 25 cm length and 2.9 mm i.d- its empty volume is calculated to be 1.65 mL. Yet, the t_o calculated for that column was about 2.05 min.,which at a flow rate of 1 mL/min (as indicated by the authors), means a void volume of 2.05 mL. It is hard to imagine a column whose total porosity is greater than 1 ! Similar calculations for the other columns show that,while the void volume is less now than that of the unpacked columns, the porosities are much too high, e.g. 0.9. These findings require an explanation. Could it be that the dead volume of the system was very large ?

We tried to apply the approach of Neidhard et al. to the data of Vigh and Varga-Puchony (3). Table IV of reference 3 shows that ΔH values of n-hexanal-dinitrophenylhydrazone and 2-n-hexanone-dinitophenylhydrazone are almost the same - 4.17 kcal/mole vs-4.15 kcal/mole. For such a system, t_o should be obtainable via equation 6 of ref. 1. Since Vigh and Varga-Puchony did not report t_R values, we assumed t_o of 1 and have calculated "appeerent t_R" values from the given capacity ratios. When these t_R values are used in conjunction with equation 6 of ref. 1,

t_o of 1 should result. However, the value obtained depends on the two temperatures chosen . For instance if T_1 is 20°C and T_2 60°C, then t_o is .786; if T_2 is 43.6°C, then t_o is 0.404, last, if T_1 is 43.6°C and T_2 60°C, then t_o is 1.101. The problem lies in the assumption used to arrive at equation 6 of ref. 1. If the ΔS values of the two solutes are very similar then, using the notation of ref. 1, we have:

$$t_{RA} (T_1) \approx t_{RB} (T_1)$$

and

$$t_{RA} (T_2) \approx t_{RB} (T_2)$$

In such a case equation 6 of ref. 1 demands very high precision of the measured t_R values, and its practicality diminishes. When the ΔH and ΔS values of the solutes are the same, the equation cannot be used. In that connection, it is not clear to us why the retention times in Fig.1 of ref. 1 are so different when the reported ΔH and k_o (a measure of ΔS) are so close in magnitude.

   In summary, the method given in ref. 1 should be used, if at all, only after careful examination of the chromatographic parameters.

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LETTER:

THE PRINCIPLE OF MEASURING t_O REMAINS UNCHANGED

Dear Sir,

We acknowledge the preceeding comment of Grushka et.al.
on our paper concerning the determination of t_O by tempe-
rature dependent RP-HPLC (1) which reveals, that the pro-
blem of measuring t_O is still far from being completely
solved on a pure theoretical basis. This means that all
methods for measuring t_O, known so far, are more or less
restricted concerning their validity. The limits of vali-
dity of each single method are difficult to predict and
therefore still discussed controversively (see e.g. ref. 2
of ref.1). At any rate, it must be taken into account that
mathematical equations, which have been derived under de-
fined assumptions are only used for data handling if the
stated assumptions are fulfilled.

If the ΔS values of the two solutes are very similar, it
is obvious that eq.6 of ref. 1 cannot be used as the fac-
tors a and b in eq. 5 of ref. 1 are equal, which turns the
denominator of eq. 6 to zero. In order to check eq. 6 of
ref. 1, Grushka et.al. have used by chance the data of two
compounds which in the system of Vigh and Varga-Puchony (2),
have almost identical ΔS values ($^{1}k_O$ / $^{2}k_O$ = 1.04). In the
system noradrenaline - adrenaline which we used, the ratio
$^{1}k_O$ / $^{2}k_O$ ranged between $\sim$ 2.5 and $\sim$3.5. Furthermore eq.4
of ref. 1 should only be used if eq. 1 of ref. 1 is valid
within a wide temperature range, taking into consideration
the condition $T_1 = \dfrac{2\,T_2 \cdot T_3}{T_2 + T_3}$ from ref. 5 of ref. 1.

The latter condition of equidistant $1/T$ values was, to our excuse, mixed up in equation 4 of ref. 1. Concerning the precision of measuring t_O , using the intersecting point method of ref. 1, the graphic evaluation of the data is recommended for a better rating of the errors.

Comming from the more practical side of HPLC, we have determined, calculated, and compared t_O values of the whole chromatographic system, including the dead volumes, which indeed led to "apparent t_O" values ($\varepsilon > 0.9$). This fact, however, was of no consequence concerning the aim of the study, which was the development and proof of a new method for the determination of t_O values, which should not replace but supply the already known methods.

We agree with Grushka et.al. that the method described in ref. 1 (like all other methods for measuring t_O) should be used only after careful examination of the chromatographic parameters, and we add, that it should be used only by those who have great practical experience in HPLC.

Bernd NEIDHART
Klaus-Peter KRINGE
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REFERENCES

(1) B. Neidhart, K.-P. Kringe, and W. Brockmann, J. Liq. Chromatogr. 4, 1875-1886 (1981)

(2) Gy Vigh and Z. Varga-Puchony, J. Chromatogr. 196, 1-9 (1980)

LC NEWS
————————

GEL PERMEATION CHROMATOGRAPHY (GPC) SOFTWARE PACKAGE provides fast, accurate and automatic calculation of molecular weight averages and distributions. An interactive CRT display simplifies system operation and speeds analytical procedures by guiding an operator with English proms. Analytical data can be archived on floppy disks, allowing future recalculation with reinjection. This package also proves extensive polymer peaks processing, including baseline corrections. Varian Instrument Group, JLC/82/7, 611 Hansen Way, Palo Alto, CA, 94303, USA.

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Division, Milton Roy Co., JCL/82/7, P. O. BOX 440,
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Spectra-Physics, JLC/82/7, 3333 North First
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JLC/82/7, Mack Rd., Woburn, MA, 01801, USA.

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process, with aromatic counter-ions to make
inorganic anions detectable at the sub-ppm level
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0.7 ml/min (21 degrees C.). Waters Associates,
Inc., JLC/82/7, 34 Maple Street, Milford, MA,
01757, USA.

LC CALENDAR

1982

JUNE 28 - JULY 2: Forensic Sciences Symposium, New York City. Contact: New York University Post-graduate Medical School, 550 First Avenue, New York, NY, 10016, USA.

JULY 12 - 16: 2nd Int'l. Symposium on Macromolecules-IUPAC, University of Massachusetts, Amherst, MA, USA. Contact: J. C. W. Chien, Dept. of Polymer Science & Engineering, Univ. of Mass., Amherst, MA, 01003, USA.

JULY 12 - 16: 8th Int'l. Conf. on Organic Coatings Science & Technology, Athens, Greece. Contact: A. V. Patsos, Science Bldg., SUNY, New Paltz, NY, 12561, USA.

JULY 19 - 22: 23rd Prague Microsymposium on Macromolecules: Selective Polymeric Sorbents - IUPAC, Iinst. of Macromolecular Chem., Prague, Czechoslovakia. Contact: P. M. M. Secretariat, Inst. of Macromolecular Chem., 162-06 Prague, Czechoslovakia.

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 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 - 18: Eastern Analytical Symposium, Statler-Hilton Hotel, New York. Contact: Dr. H. Issaq, 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 Biannual TLC Symposium-Advances in TLC,   Hilton Hotel, Parsippany, NJ. Contact: J.C. Touchstone, Hospital of the University   of Pennsylvania, Philadelphia, PA, 19104, USA.

1983

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 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 Kingdom.

     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.

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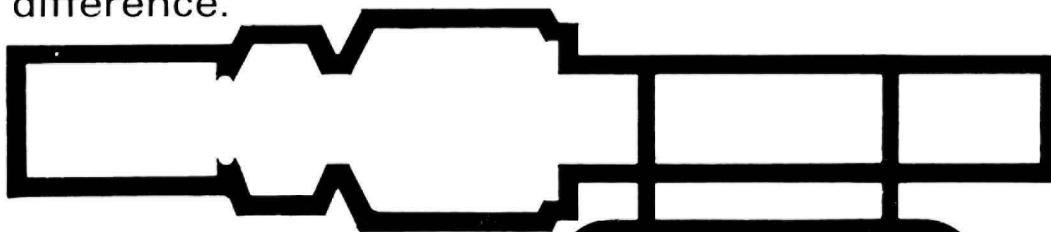
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