ISSN 0021-9673

VOL. 174 NO. 2 JULY 1, 1979

THIS ISSUE COMPLETES VOL. 174

JOURNAL OF CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1979

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1978	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	166/1 166/2 167	168/1 168/2	169 170/1	170/2	171 172	173/1 173/2	174/1	174/2 175/1 175/2	The publication schedule for the volumes 176–180 and for further *Chromatographic Reviews* issues (Vol. 165) will be published later.				
Chromatographic Reviews				165/1			165/2						
Biomedical Applications		162/1	162/2	162/3	162/4	163/1	163/2	163/3	163/4	164/1	164/2	164/3	164/4

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews*, reviews on all aspects of chromatography, electrophoresis and related methods are published.

Submission of Papers. Papers in English, French and German may be submitted, if possible in three copies. Manuscripts should be submitted to:
The Editor of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands
or to:
The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands.
Reviews are invited or proposed by letter to the Editors and will appear in *Chromatographic Reviews* or *Biomedical Applications*. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation.

Subscription Orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, 1000 AE Amsterdam, The Netherlands. The *Journal of Chromatography, Biomedical Applications* can be subscribed to separately.

Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 19 volumes in 1979. The subscription price for 1979 (Vols. 162–180) is Dfl. 2356.00 plus Dfl. 285.00 (postage) (total *ca.* US$ 1288.00). The subscription price for the *Biomedical Applications* section only (Vols. 162–164) is Dfl. 384.00 plus Dfl. 45.00 (postage) (total *ca.* US$ 209.00). Journals are automatically sent by air mail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 161) are available at Dfl. 140.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. For customers in the U.S.A. and Canada wishing additional bibliographic information on this and other Elsevier journals, please contact Elsevier/North-Holland Inc., Journal Information Centre, 52 Vanderbilt Avenue, New York, N.Y. 10017. Tel: (212) 867-9040.

For further information, see page 3 of cover.

Printed in The Netherlands

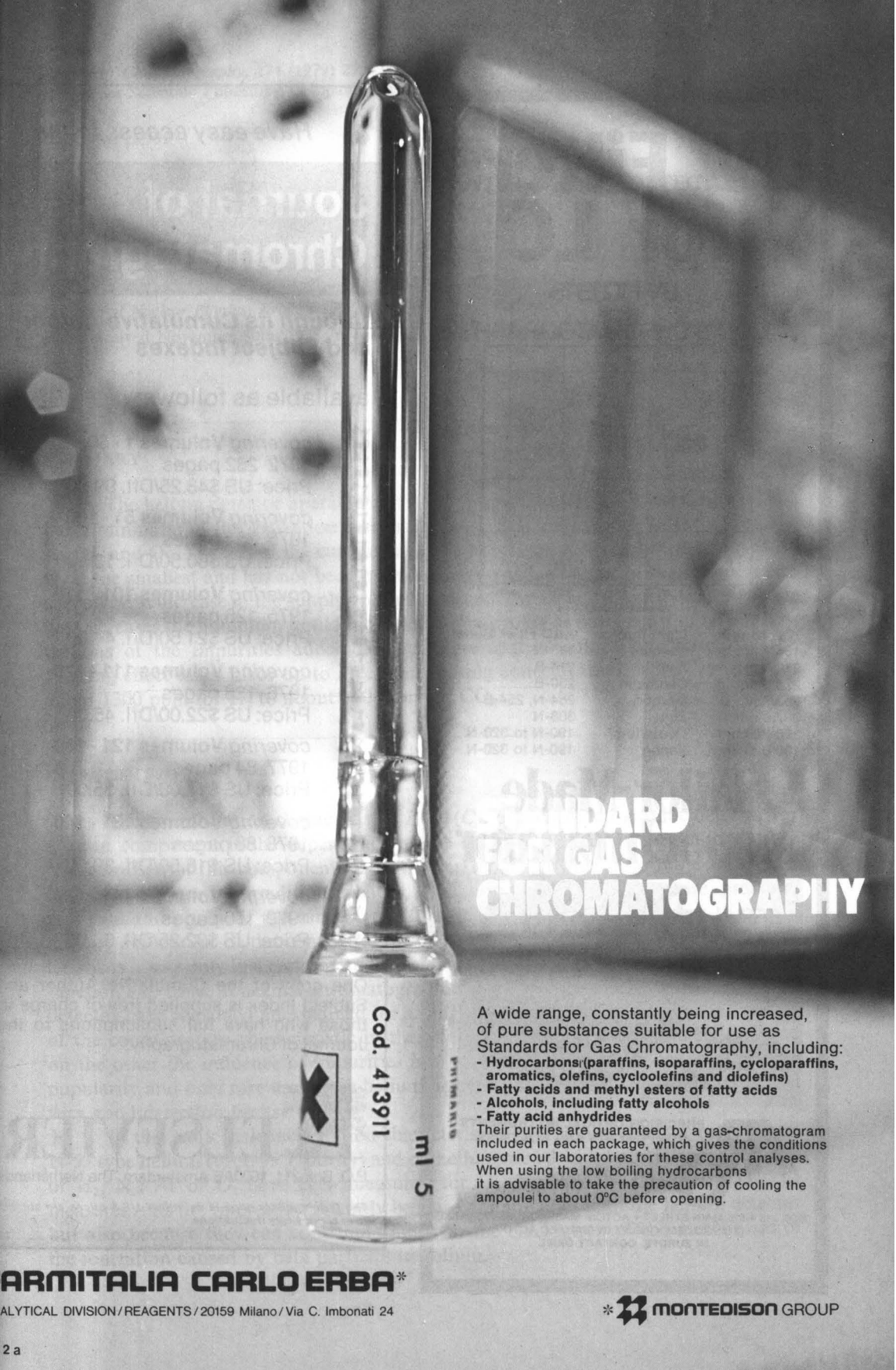
CONTENTS

(Continued overleaf)

Contents (continued)

Notes

Letters to the Editor



STANDARD FOR GAS CHROMATOGRAPHY

A wide range, constantly being increased, of pure substances suitable for use as Standards for Gas Chromatography, including:

- **Hydrocarbons (paraffins, isoparaffins, cycloparaffins, aromatics, olefins, cycloolefins and diolefins)**
- **Fatty acids and methyl esters of fatty acids**
- **Alcohols, including fatty alcohols**
- **Fatty acid anhydrides**

Their purities are guaranteed by a gas-chromatogram included in each package, which gives the conditions used in our laboratories for these control analyses. When using the low boiling hydrocarbons it is advisable to take the precaution of cooling the ampoule to about 0°C before opening.

ARMITALIA CARLO ERBA*

ALYTICAL DIVISION / REAGENTS / 20159 Milano / Via C. Imbonati 24

* MONTEDISON GROUP

Journal of Chromatography, 174 (1979) 273–282

CHROM. 11,795

CONCENTRATION CHARACTERISTICS OF THE HELIUM DETECTOR FOR GAS CHROMATOGRAPHY

EMIL BROŚ and JAN LASA
Institute of Nuclear Physics, Kraków (Poland)
(First received November 30th, 1978; revised manuscript received February 12th, 1979)

SUMMARY

The helium detector operated on N-55 type helium (5 ppm of total impurities) has a minimum in the ionization current for parts per million concentrations of H_2, Ar, N_2 and O_2. The fall in the current caused by H_2 is the largest and that caused by O_2 is the smallest and has not been previously reported. The size of the fall increases sharply with increase in the supply voltage. The addition of any impurity to the carrier gas decreases the minimum until it completely disappears at parts per million concentrations of the impurities added. The enhancement of ionization caused by the Penning effect takes place up to a certain limiting concentration, which varies from about 1300 ppm for H_2 to about 150 ppm for CO_2.

INTRODUCTION

The concentration characteristic, $I = f(C)$, of a chromatographic detector for a given component is the dependence of the detector ionization current, I, on the concentration of this component, C, in the carrier gas flowing in to the detector cell under fixed operating conditions, that is, excitation potential, temperature, pressure, flow-rate and purity of the carrier gas. This dependence should be linear to permit an easy evaluation of the concentration. However, often the concentration characteristic is linear only in a certain range, beyond which it can even be a non-monotonic function and cause abnormal chromatographic signals[1–5]. Anomalies with the helium detector have remained unexplained for a long time because on the one hand the role of the concentration characteristic in their formation has not been understood and on the other the influence of impurities has not been known. This has led to a low popularity and only rare use of the helium detector in spite of its high sensitivity and very good detection limits.

In this work the concentration characteristics of a helium detector run on pure N-55 type helium (99.9995% purity) and on helium mixed with known trace additions of H_2, N_2, Ar or O_2 have been measured for H_2, N_2, Ar, O_2, CH_4 and CO. The measurements are interesting not only because of anomalies with the helium detector but also because they can contribute much to the elucidation of the mechanism of the ionization caused by beta particles in helium.

EXPERIMENTAL

Apparatus

A Carlo-Erba Model GH gas chromatograph[4] was used but the original detector was replaced with a Model DH-73 helium detector designed and built in the Laboratory of Chromatographic Detectors of the Institute of Nuclear Physics in Kraków. Its design is shown schematically in Fig. 1. The two parallel electrodes, 12 mm in diameter, were separated by a 1.8-mm PTFE insulator. One of the electrodes was a β-ray source consisting of a stainless-steel disc supporting 238 mCi of tritium absorbed on erbium. Gas concentrations for calibration were generated with a glass exponential dilution flask[6]. N-55 type helium was used as the carrier gas and purge gas. The supplier (l'Air Liquide, Lyon, France) specified the helium impurities in the catalogue attached to the cylinders as follows: O_2, 1; H_2O, 2; N_2, 2; H_2, 0.1; CH_4, 0.1; CO, 0.1; and CO_2, 0.1 ppm (by volume). However, according to the same catalogue, the total concentration of impurities did not exceed 5 ppm.

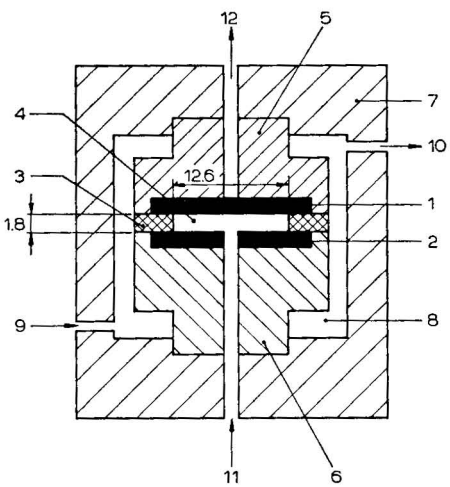


Fig. 1. Schematic diagram of the Model DH-73 helium ionization detector. 1 = Top electrode (β-ray source); 2 = bottom electrode; 3 = PTFE insulator between the electrodes; 4 = detector volume (*ca.* 0.2 cm^3); 5 and 6 = electrode insulators; 7 = detector body; 8 = guard helium atmosphere; 9 and 10 = inlet and outlet for purging helium; 11 = carrier gas inlet; 12 = carrier gas outlet.

Impurities were added to the helium carrier gas and kept at a cetrain constant level by means of the permeation tube technique[7]. The permeation chamber manufactured and calibrated for this particular application has been described elsewhere[4,5]. The volume of samples injected was 3 cm^3. The helium flow-rate through the detector was 1 cm^3/sec. The gas chromatographic column used was a 2-m stainless-steel tube (5 mm I.D., 6 mm O.D.) filled with 40–80-mesh molecular sieve 13X. The temperature of the column was kept at 80° and the temperature of the detector and all other parts of the apparatus was ambient. The pneumatic circuit was made of stainless steel, including tubing and joint connections. After every disconnection of the carrier gas circuit or a break in the detector work it was necessary to clean the system for about 1 day in order to obtain optimal performance.

Methods for determining the concentration characteristic

The concentration characteristic can be obtained by the stationary method or the pulse method.

In the stationary method a number of mixtures of helium with the investigated component are prepared and each mixture is used as the carrier gas. Having constructed the voltage–current curve for each of the mixtures the concentration characteristic can be determined. Usually the diffusion chamber is used to produce the mixtures.

In the pulse method samples containing the investigated gas in known concentrations, C_d, are introduced into the pure carrier gas and the increments of the current at the tops of the peaks, ΔI_p, are measured. Ordinates of the concentration characteristic are calculated from the simple equation

$$I = I_b + \Delta I_p \tag{1}$$

where I_b is the basic current. The concentrations corresponding to these values of the current can be found from the equation

$$C_p = \frac{C_d V_d}{t_h U} \tag{2}$$

when the carrier gas is free from the analysed component, and if it is not, from the more general equation

$$C_p = C_0 + \frac{(C_d - C_{01})\, V_d}{t_h u} \tag{3}$$

where

C_{01} = constant concentration of the analysed component present in the carrier gas as an impurity at the injection point;
V_d = volume of sample injected;
t_h = half-width of the peak;
u = volume flow-rate of the carrier gas.

Eqns. 2 and 3 are valid under the assumption that chromatographic elution curves are symmetrical and that their shapes can be approximated by a triangle.

The pulse method was mainly used in this work. The samples were taken from the exponential dilution flask and injected into the carrier gas at intervals equivalent to the time constant of dilution for the flask. Because of the low frequency of injections and the approximate validity of eqns. 2 and 3, the concentration characteristics obtained in this way are not too precise and the curves shown below serve only to illustrate the character of changes in the ionization current of the helium detector with the admixture added.

RESULTS AND DISCUSSION

The concentration characteristics of the helium detector can have two different shapes, which are shown in Fig. 2a for H_2 and in Fig. 2b for CO. The minimum at C

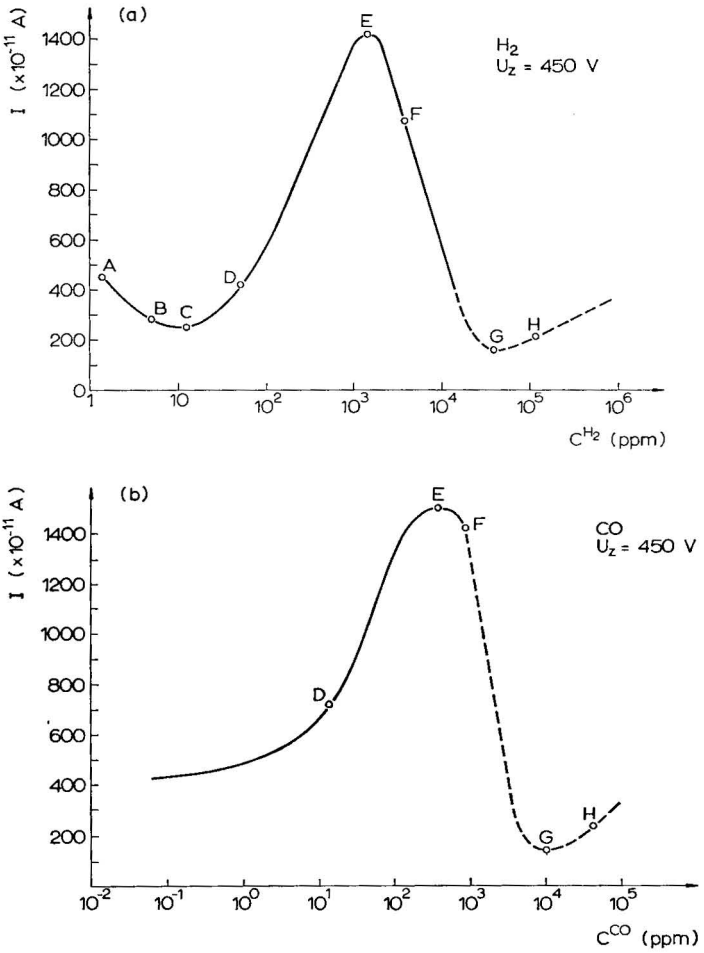


Fig. 2. Concentration characteristic, $I = f(C)$, of the He detector (a) for H_2 and (b) for CO. Carrier gas, N-55 type helium; flow-rate, 1 cm³/sec; supply voltage, $U_z = 450$ V; electrode distance, $d = 1.8$ mm; radioactive source, 238 mCi ^{3}H/Er; chromatographic column, molecular sieve 13X, 2 m long, at 90°; detector temperature, 20°.

and the maximum at E demand special consideration. The range between points C and E is called the Penning range.

Initial minimum for small concentrations

The decrease in the concentration characteristic for very small concentrations was observed for only four components: H_2, Ar, N_2 and O_2, as shown in Fig. 3. Previously this effect was reported for H_2, Ar and N_2 by Parkinson and Wilson[2,3] and for H_2 by Poy and Verga[4].

The fall in the current, $\Delta I_{\text{min.}}$, at the minimum point C, given by the equation

$$\Delta I_{\text{min.}} = I_b - I_{\text{min.}} \tag{4}$$

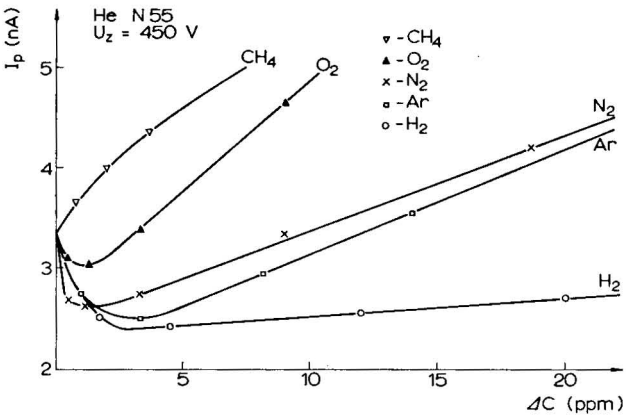


Fig. 3. Variation in helium detector current *versus* concentrations of H_2, Ar, N_2, O_2 and CH_4 for N-55 type helium as carrier gas. Operating conditions as in Fig. 2.

where $I_{min.}$ is the lowest current at point C on the curve in Fig. 2a, was found to be greatest for H_2 and smallest for O_2. Admixture of every other gas to the helium reduces the fall in current, $\Delta I_{min.}$, and above a certain impurity level the minimum disappears for all four gases. It was noticed that the initial fall of the detector current is removed by adding about 25 ppm of H_2, 10 ppm of O_2, 15 ppm of N_2 or 5 ppm of Ar to the N-55 type helium carrier gas by means of the diffusion chamber. As the impurity level is increased the initial fall in current disappears first for O_2 and then for N_2, Ar and finally H_2. Fig. 4 shows the initial parts of the concentration characteristics obtained for the same gases as in Fig. 3 but with the detector supplied with N-55 type helium containing 35 ppm of H_2.

The concentration, $C_{min.}$, at which the detector current is at a minimum can be read from the concentration characteristic. However, it was found that the value of $C_{min.}$ for H_2 depended on the method of measuring it. Concentration characteristics

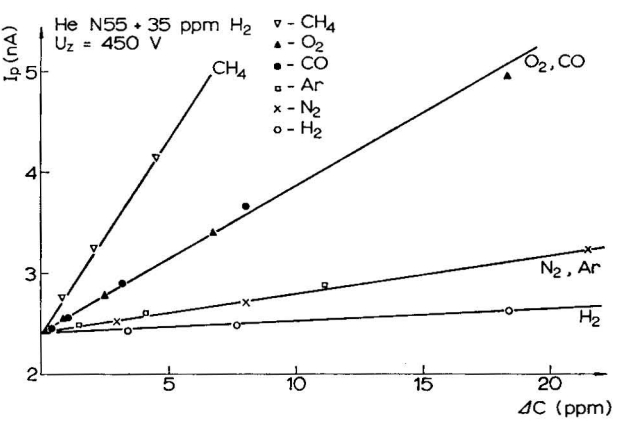


Fig. 4. Variation in helium detector current *versus* concentration of H_2, Ar, N_2, O_2, CO and CH_4 for N-55 type helium mixed with 35 ppm of H_2 as the carrier gas. Other conditions as in Fig. 2.

for H_2 obtained by three different methods are shown in Fig. 5. Although the values of $I_{min.}$ are virtually identical, the values of $C_{min.}$ differ considerably, being 5 ppm by the pulse method with the exponential dilution flask, 20 ppm by the pulse method with the diffusion chamber used to generate the concentrations needed and 30 ppm by the stationary method. These differences are not understood and they cannot be attributed to experimental errors only. They were found only for H_2.

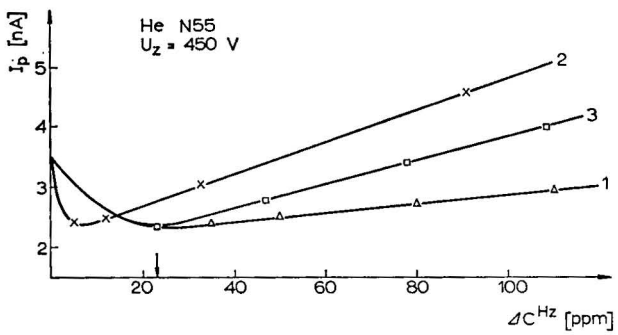


Fig. 5. Initial part of the concentration characteristic of the helium detector for H_2 obtained by (1) stationary method, (2) pulse method with the exponential dilution flask and (3) pulse method with the diffusion chamber.

The fall in the current, $\Delta I_{min.}$, depends strongly on the supply voltage, as illustrated in Fig. 6. The dependence of the detector basic current, I_b, on the voltage and the ratio $\Delta I_{min.}/I_b$ are also indicated in Fig. 6. For the voltage from the plateau of the voltage–current curve, $\Delta I_{min.}$ is distinguishable from the noise level for H_2 and Ar only. It can be seen from Fig. 6 that from 250 V $\Delta I_{min.}$ increases exponentially at a greater rate than the basic current, I_b, for pure N-55 type helium.

The value of $C_{min.}$ was found to be independent of the voltage in the tested range.

The reasons for the initial fall in the ionization current with the addition of parts per million amounts of H_2, Ar, N_2 or O_2 into the N-55 type helium carrier gas have not yet been elucidated. Some hypotheses that had been made by a few authors[1,2,8] in the past were summarized elsewhere[9]. Further investigations are needed in order to achieve a full understanding of that phenomenon.

The minimum of the concentration characteristic at point C has the disadvantage that it causes abnormal responses of the helium detector, but it can also play a positive role as an indication that the purity of a batch of helium carrier gas is sufficiently high that its further purification is not necessary. The possibility of the utilization of this phenomenon for the detection of hydrogen with a 10-fold higher sensitivity than the normal positive range of the He detector was first indicated by Parkinson and Wilson[2]. It can be seen from Fig. 7 that the sensitivity of the helium detector run on pure N-55 type helium in the negative mode is a few times better for O_2 and N_2 than the sensitivity of the detector in the positive mode after adding 35 ppm of H_2 to the carrier gas.

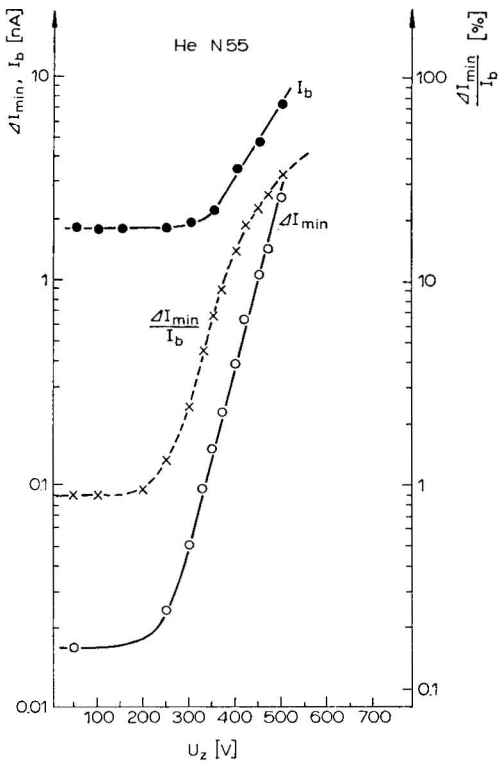


Fig. 6. Dependence of $\Delta I_{min.}$ on the supply voltage for H_2 and the ratio of $\Delta I_{min.}$ to the standing current, I_b.

Penning range

When all factors are constant, the ionization current of the helium detector between points C and E increases with addition of the test compound to the carrier gas until the maximum value, $I_{max.}$, is reached. This enhancement of the ionization is caused by the Penning effect and the differences in sensitivity for various gases are well known[1,4,10] and understood[8]. Most often just the detector operating in this range is referred to as the helium detector and is used as such in quantitative analysis.

The concentration characteristic, $I = f(C)$, does not depend directly on the carrier gas flow-rate but it does depend on it indirectly, owing to the changes in pressure inside the detector cell and to a change in the amount of impurities introduced through small leaks and diffusion into the system. An increase in the pressure decreases the current. The assertions concerning the influence of the flow-rate and pressure are valid for the whole concentration characteristic.

The sensitivity of the helium detector decreases with increase in the impurity level; however, the addition of parts per million amounts of hydrogen to N-55 type helium can slightly improve it. The deterioration of the sensitivity depends not only on the concentration of impurities but also on their type. The addition of CO_2, CO, O_2 or other gases for which the cross-section for the Penning effect is large is more harmful than the addition of H_2, for which this cross-section is the smallest.

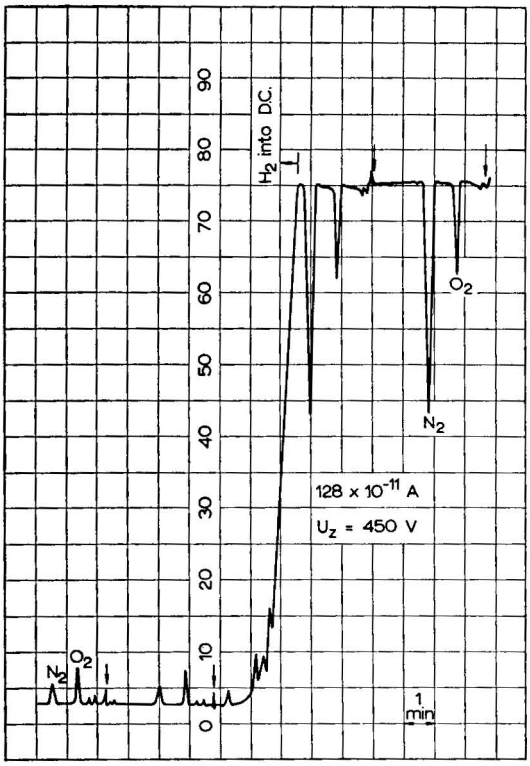


Fig. 7. Effect of the addition of 35 ppm of H_2 to N-55 type helium carrier gas on the standing current and the polarity and magnitude of the detector signal for O_2 and N_2.

The maximum of the concentration characteristic

The maximum increment of the helium detector current, $\Delta I_{max.}$, given by the equation

$$\Delta I_{max.} = I_{max.} - I_b \tag{5}$$

where $I_{max.}$ is the highest value of the current at point E, does not depend on excitation potential up to about 300 V. Above this value it increases first exponentially and then faster until a current breakdown, which takes place between 470 and 500 V depending on the compound being tested. Because electrical breakdowns in the helium detector can be damaging, the supply voltage should not exceed 450 V.

For voltages up to about 300 V the detector current does not fall with further addition of the test compound after reaching the maximum value, but levels off. The value of $\Delta I_{max.}$ varies with the compound being tested.

As the impurity level of the carrier gas increases, the value of $I_{max.}$ decreases. A 30% decrease in $I_{max.}$ was observed after addition of about 18 ppm of O_2 to the N-55 helium carrier gas.

The value of the concentration, $C_{max.}$, for which the current reaches a maximum for a given compound is a limiting value. If the impurity content of the carrier

gas is equal to or higher than this value, the detector responds with negative peaks to all of the eluted components. On the other hand, the closer the impurity level of the carrier gas approaches the limiting value, the smaller is the usable range of the detector for the analysis of samples.

Table I gives values of $C_{max.}$ evaluated by means of eqn. 2 on the basis of the data obtained during calibration of the detector at 450 V. The values of $C_{max.}$ evaluated by Berry[1], Wiseman[11] and Karmen *et al.*[12] are also quoted. These workers used a detector with cylindrical electrodes but did not mention the values of the supply voltage.

TABLE I

LIMITING CONCENTRATIONS ($C_{max.}$) IN N-55 TYPE HELIUM

Component	*Concentration (ppm by volume)*			
	Present measurements	*Berry*[1]	*Wiseman*[11]	*Karmen et al.*[12]
H_2	1300	1500	1000	—
Ar	850	1000	170	960
O_2	350	400	250	280
N_2	700	800	750	520
CH_4	400	500	100	—
CO	450	300	—	—
CO_2	150	100	170	80
H_2O	—	50	—	—

The differences between the values in Table I probably stem from differences in the contamination of the helium carrier gas used and from differences in the supply voltages. The effect of impurities is additive so that combination of impurities reduces the individual values of $C_{max.}$. $C_{max.}$ decreases slightly as the supply voltage increases. Another possible explanation is that the other workers might have evaluated the limiting concentrations in a different way.

Whatever the differences, the common factor about the values in Table I is the highest value of $C_{max.}$ for hydrogen and the lowest for CO_2 (or H_2O in one instance). Large differences between one component and another make it evident how important the composition of helium impurities is for the proper operation of the helium detector.

The $C_{max.}$ values are the upper limits of concentrations of samples analysed in the positive and monotonic range of the helium detector. For excitation potentials higher than the critical value the upper limit establishes the concentration at which the electrical breakdown occurs; the higher the voltage, the lower is this concentration.

Concentration characteristic above $C_{max.}$

The mechanism of the fall in current for concentrations higher than $C_{max.}$ has not yet been explained. It is probable that this fall is caused by the moderation of the electrons in inelastic collisions with the added molecules to such an energy level that the excitation of helium atoms to metastable states becomes impossible.

It seems that the range of the concentration characteristic between points E

and G in which the current drops could be used for quantitative analysis, but further investigations are needed.

At point G the function changes its character to a rising curve and further increases in the concentration of additives cause an increase in the current, as the detector begins to work as a cross-section detector in the gas amplified region.

CONCLUSION

The results obtained indicate that the helium carrier gas should not be too pure and should contain small amounts of impurities in order to remove the initial minimum and make the function rise monotonically to a limiting concentration beyond which the detector current begins to fall. The limiting concentrations range from about 150 ppm for CO_2 to about 1300 ppm for H_2. If the contamination of the helium carrier gas exceeds the limiting value for a given component, the ionization current decreases with the addition of any other component and it becomes impossible to run the detector in the positive range.

These results create a basis for a better understanding of the anomalous signals of the helium detector[13]. They also contribute to a further explanation of secondary processes that occur in slightly contaminated helium ionized with beta particles and standing behind the extremes of the helium detector concentration characteristics.

REFERENCES

1 R. Berry, in M. van Swaay (Editor), *Gas Chromatography 1962*, Butterworths, London, 1962, p. 321.
2 R. T. Parkinson and R. E. Wilson, *J. Chromatogr.*, 24 (1966) 412.
3 R. T. Parkinson and R. E. Wilson, *J. Chromatogr.*, 37 (1968) 310.
4 F. Poy and R. Verga, *Characteristics and Performance of a New Helium Ionization Detector GC System*, Carlo Erba, Milan, 1970.
5 J. Lasa and E. Broś, *J. Chromatogr. Sci.*, 12 (1974) 807.
6 J. E. Lovelock, *Anal. Chem.*, 33 (1961) 162.
7 A. N. O'Keeffe and G. C. Ortman, *Anal. Chem.*, 38 (1966) 760.
8 E. Broś and J. Lasa, *J. Chromatogr.*, 94 (1974) 13.
9 E. Broś, *Ph.D. Thesis*, University of Mining and Metallurgy, Kraków, 1975.
10 C. H. Hartman and K. P. Dimick, *J. Gas Chromatogr.*, 4 (1966) 163.
11 W. A. Wiseman, *Nature (London)*, 192 (1961) 964.
12 W. A. Karmen, L. Giuffrida and R. L. Bowman, *Nature (London)*, 191 (1961) 906.
13 E. Broś and J. Lasa, in preparation.

Journal of Chromatography, 174 (1979) 283–290

CHROM. 11,777

SPECTRAL-BANDWIDTH EFFECTS OF VARIABLE-WAVELENGTH ABSORPTION DETECTORS IN LIQUID CHROMATOGRAPHY

JAMES E. STEWART
Spectra-Physics, 2905 Stender Way, Santa Clara, Calif. 95051 (U.S.A.)
(First received October 17th, 1978; revised manuscript received February 5th, 1979)

SUMMARY

A theoretical discussion is given of the sensitivity and linearity of variable-wavelength absorption detectors used in liquid chromatography. The influence of the effective bandwidth of the monochromator relative to the width of the sample absorption band is considered, as well as the effect of making absorption measurements on the side of an absorption band instead of the peak. Some results of experimental measurements on biphenyl solutions are discussed.

INTRODUCTION

The influence of the shape and width of monochromator-slit functions on the shape and peak height of recorded absorption bands has been investigated many times[1], but not under conditions appropriate for the use of variable-wavelength detectors in liquid chromatography (LC). For example, it is usually assumed that quantitative analyses are made with the monochromator adjusted to place its central wavelength at the maximum of the absorption band. In LC, this is not generally the case, because the spectrometer is set in advance at a selected wavelength for which only a few (if any) of the components in the sample have absorption maxima. Also, the absorption maximum of a component is often not accessible because of wavelength limitations or solvent interference, and measurements must be confined to the side of the absorption band.

In this paper, I shall consider the effect of the monochromator bandwidth and wavelength setting on sensitivity and linearity in the analysis of static samples. The conclusions drawn from this study should be useful to chromatographers, although any complications introduced by flowing samples are not discussed at this time. In order to work with mathematically convenient forms, the monochromator and the absorbing sample are characterized by simple models.

THEORETICAL DISCUSSION

Absorption coefficient, slit function, and apparent absorbance

There are two convenient and widely used models for sample absorption. One

model assumes an absorption coefficient with a Lorentzian wavelength distribution:

$$a(\lambda) = \frac{a_0 s^2}{s^2 + 4(\lambda - \lambda_0)^2}$$

The other model has the form of a Gaussian distribution:

$$a(\lambda) = a_0 \, e^{-4 \ln 2 (\lambda - \lambda_0)^2 / s^2}$$

In both cases, a_0 is the peak absorption coefficient, λ_0 is the peak wavelength, and s is the absorption bandwidth (full width at half maximum absorption coefficient). A Lorentzian band has stronger absorption in its wings than a Gaussian band of equal width. Real absorption bands very often cannot be represented by one of these simple models because they are skewed or distorted, and, frequently, an observed spectrum is made up of a summation of overlapping, unresolved bands. These cases will not be considered in this study, nor will spectra consisting of overlapping bands from different chemical compounds.

Triangular slit functions are expected for monochromators when entrance and exit slit widths are equal and wide enough to neglect the effects of optical aberrations and diffraction. The equation for a normalized triangular slit function is:

$$\sigma(\lambda_m - \lambda) = \frac{1}{m}\left(1 - \frac{|\lambda_m - \lambda|}{m}\right), \; |\lambda_m - \lambda| < m$$

$$0 \qquad\qquad , \; |\lambda_m - \lambda| \geq m$$

Where λ_m is the monochromator wavelength setting measured from λ_0 and m is the effective bandwidth (full width at half maximum).

Variable-wavelength detectors for LC sometimes use the sample cell itself in place of a monochromator exit slit. The slit functions of these instruments are not expected to be triangular, but effective bandwidths can still be defined, and the results of measurements made with these detectors will be qualitatively similar to those from instruments with real exit slits.

The apparent spectral transmittance of a sample is the convolution of the absorption band and the slit function,

$$T_{app} = \int_{\lambda_m - m}^{\lambda_m + m} e^{-a(\lambda)} \, \sigma(\lambda_m - \lambda) \, d\lambda \qquad (1)$$

where $\alpha = a(\lambda)bc$ includes the path length b and the concentration c. The apparent absorbance is given by the usual relationship:

$$A_{app} = -\log T_{app}$$

In general, eqn. 1 is integrated numerically; for convenience in the following discussion, we take $\lambda_0 = 0$.

The ratio of effective bandwidth to absorption bandwidth is defined by the bandwidth ratio:

$$\beta = m/s$$

The monochromator setting may be given in terms of the absorption band width by

$$\gamma = | \lambda_m / s |$$

The rest of this paper treats the dependence of A_{app} on the parameters α, β and γ.

Band-shape distortion

Fig. 1 shows how Gaussian and Lorentzian bands are distorted by triangular slit functions ranging from 0 to 5 times wider than the absorption bands. Figures resembling this are well-known. They illustrate the rule-of-thumb used by spectroscopists that the effective bandwidth of the monochromator should be no greater than 10 to 20% of the absorption bandwidth for tolerable distortion and peak-height loss. Distortion becomes excessive if much wider slits are used. On the other hand, because the irradiance of the sensor is proportional to the square of the slit width, signal-to-noise ratio is sacrificed unnecessarily with narrower slits.

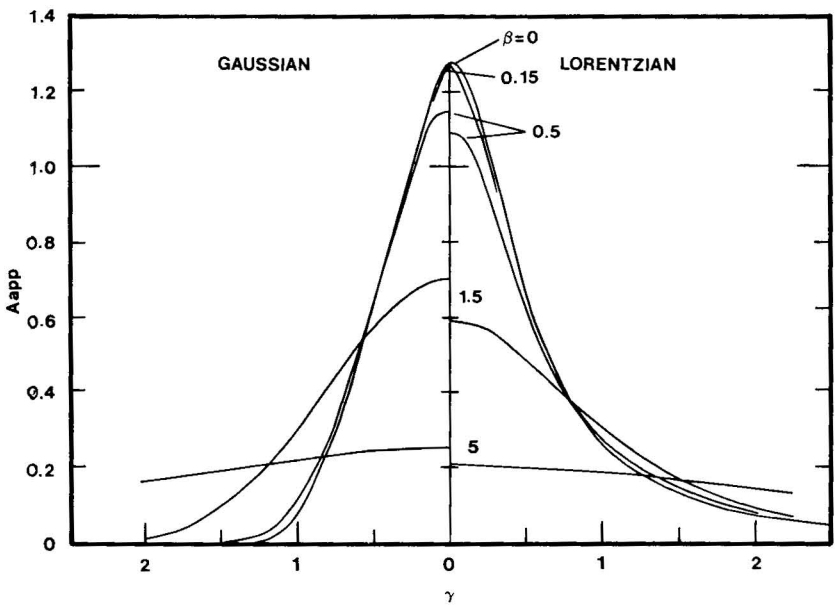


Fig. 1. Influence of the effective bandwidth of a monochromator with a triangular slit function on Gaussian- and Lorentzian-shaped absorption bands. The parameter β is the ratio of effective bandwidth to absorption bandwidth, and γ is the number of absorption bandwidths from the absorption maximum to the monochromator setting.

Many organic molecules in solution have absorption bands around 25 nm wide in the UV region. Therefore, for measurements made near the absorption maxima of such bands, the effective bandwidth should be 2.5 to 5 nm. However, the situation is different when, for whatever reason, measurements are made far from the band maximum ($0.5 < \gamma < 2$). Indeed, the apparent absorbance may even be increased by using a relatively large effective bandwidth.

Sensitivity at small absorbance

For small values of α, eqn. 1 can be simplified by approximating the exponential factor with the first two terms of a power series. The integration can then be performed, and an expression for the sensitivity of A_{app} to small changes in concentration can be written for a Gaussian band

$$\frac{1}{0.4343a_0b}\left(\frac{dA_{app}}{dc}\right) = \frac{1}{2\beta}\left\{\frac{1}{4\beta \ln 2}\left(e^{-4\ln 2(\gamma-\beta)^2} + e^{-4\ln 2(\gamma+\beta)^2} - 2e^{-4\ln 2\gamma^2}\right)\right.$$
$$+ \sqrt{\frac{\pi}{4\ln 2}}\left[\left(1-\frac{\gamma}{\beta}\right)\right.$$
$$\times (\operatorname{erf}\sqrt{4\ln 2}\gamma - \operatorname{erf}\sqrt{4\ln 2}(\gamma-\beta))$$
$$\left.\left.-\left(1+\frac{\gamma}{\beta}\right)(\operatorname{erf}\sqrt{4\ln 2}\gamma - \operatorname{erf}\sqrt{4\ln 2}(\gamma+\beta))\right]\right\} \quad (2a)$$

and for a Lorentzian band

$$\frac{1}{0.4343a_0b}\left(\frac{dA_{app}}{dc}\right) = \frac{1}{4\beta^2}\ln\left(\frac{4\gamma^2+1}{\{1+4(\gamma-\beta)^2\}^{1/2}\{1+4(\gamma+\beta)^2\}^{1/2}}\right)$$
$$-\frac{\gamma}{\beta^2}\tan^{-1}2\gamma - \frac{1}{2\beta}\left(1-\frac{\gamma}{\beta}\right)\tan^{-1}2(\gamma-\beta)$$
$$+\frac{1}{2\beta}\left(1+\frac{\gamma}{\beta}\right)\tan^{-1}2(\gamma+\beta) \quad (2b)$$

These equations are plotted in Fig. 2 as functions of β for a variety of values of γ. When measurements are made close to the absorption band centre ($\gamma \leq 0.25$), sensitivity is reduced if the effective bandwidth exceeds about 0.2 absorption bandwidth. However, signal-to-noise ratio improves with increased effective bandwidth, compensating somewhat for the lowered sensitivity. When measurements are made farther from the absorption band center ($\gamma > 0.5$), sensitivity may be increased by increasing the effective bandwidth up to several times the absorption bandwidth. The magnitude of the effect of β and γ on sensitivity is strongly dependent on the shape of the absorption band. The effect is less pronounced for a Lorentzian band than for a Gaussian because of the stronger wings of the former.

Absorbance linearity

Plots of A_{app} *versus* concentration, calculated from eqn. 1, show noticeable departure from linearity at bandwidth ratios greater than *ca.* 0.5, especially when the spectrometer is set on the side of the absorption band.

Let us define a linearity error parameter

$$E(c) = \left(c \cdot \frac{dA_{app}}{dc} - A_{app}(c)\right)\Big/ c \cdot \frac{dA_{app}}{dc}$$

which is the relative difference between the absorbance that would be predicted by

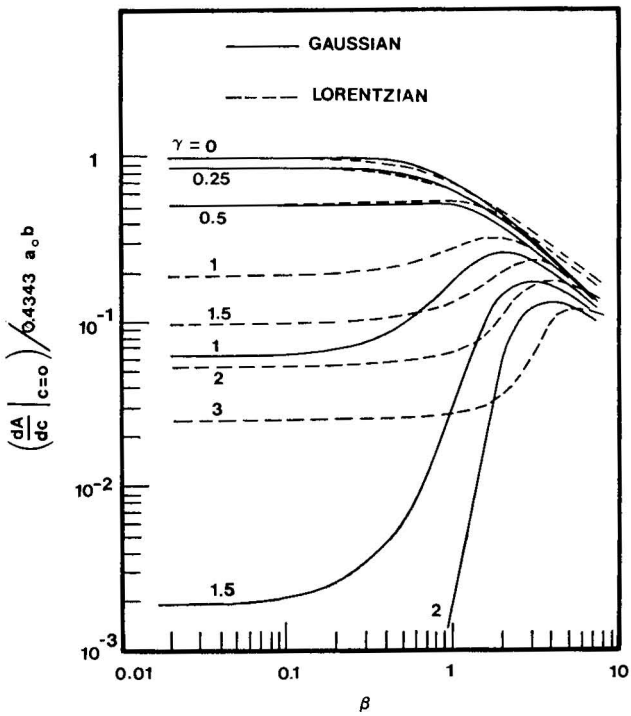


Fig. 2. The sensitivity of absorbance to concentration change at very small concentration for Gaussian- and Lorentzian-shaped bands as a function of bandwidth ratio for a series of monochromator settings.

extrapolation of the sensitivity at vanishing concentration (eqn. 2) and the apparent absorbance at concentration c.

Fig. 3 contains plots of E at $c = 1$ *versus* γ over a range of values of β for Lorentzian and Gaussian bands. The product $a_0 b$ is arbitrarily chosen to be 2.947, to give a maximum absorbance of 1.28 at $c = 1$. This is consistent with the maximum absorbance attainable by many LC detectors. It is evident that, for any bandwidth ratio, the magnitude of the linearity error first increases as the measuring wavelength is moved away from the absorption band center, then it decreases. The effects of β and γ on linearity are also strongly dependent on band shape.

EXPERIMENTAL MEASUREMENTS

A few experimental measurements were made in order to test the theoretical predictions. I used a versatile spectrometer designed in this laboratory; it has an off-axis Ebert monochromator configuration with a telescope mirror of focal length 0.25 m, and is capable of 0.1-nm resolution when a fine diffraction grating is installed, but, for the present purpose, a coarse grating (258 lines per mm) was used. Biphenyl was selected as a test compound. The spectrum of a methanolic solution (8.5 mg/l) in a 1-cm cell was measured with a spectrometer effective bandwidth of 2.4 nm. The absorbance spectrum could be fitted closely to a Gaussian function with 1.03 peak

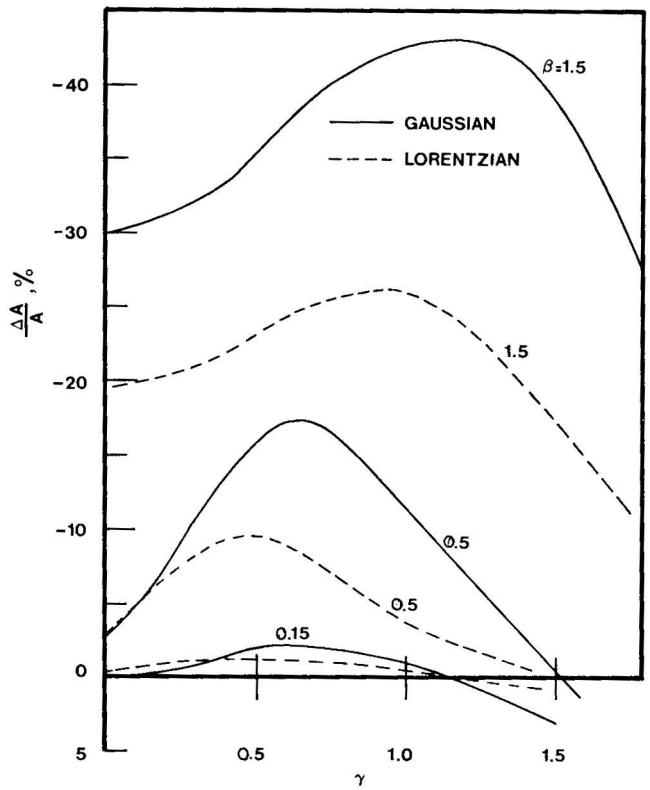


Fig. 3. Absorbance non-linearity for Gaussian and Lorentzian bands as a function of monochromator setting for several values of bandwidth ratio. $\Delta A/A$ is the relative departure of the absorbance from the small concentration value extrapolated to 1.28 absorbance units.

absorbance at 247.5 nm and a half bandwidth (corrected for the spectrometer effective bandwidth) of 33.9 nm.

In Fig. 4, absorbance values are plotted for a series of concentrations for effective bandwidths of 2.4 and 32 nm ($\beta = 0.071$ and 0.94, respectively) and for wavelength settings of 248, 264 and 280 nm ($\gamma = 0$, 0.48, and 0.96, respectively). These measurements verify the loss of sensitivity with increasing value of γ predicted in Fig. 2:

β	γ	$A'(\gamma)/A'(\gamma = 0)$	
		Calculated	*Experimental*
0.071	0.48	0.50	0.53
	0.96	0.064	0.068
0.94	0.48	0.72	0.76
	0.96	0.25	0.31

The measurements also verify the predicted behaviour of sensitivity with β for given values of γ:

	$A'(\beta = 0.94)/A'(\beta = 0.071)$	
γ	*Calculated*	*Experimental*
0	0.71	0.77
0.48	0.99	1.05
0.96	2.8	3.4

Sensitivity is lost by using the wide slit at $\gamma = 0$, it remains relatively unchanged at $\gamma = 0.48$, and it increases by a factor of 3 at $\gamma = 0.96$. Finally, the experimental curves display the predicted non-linearity in response.

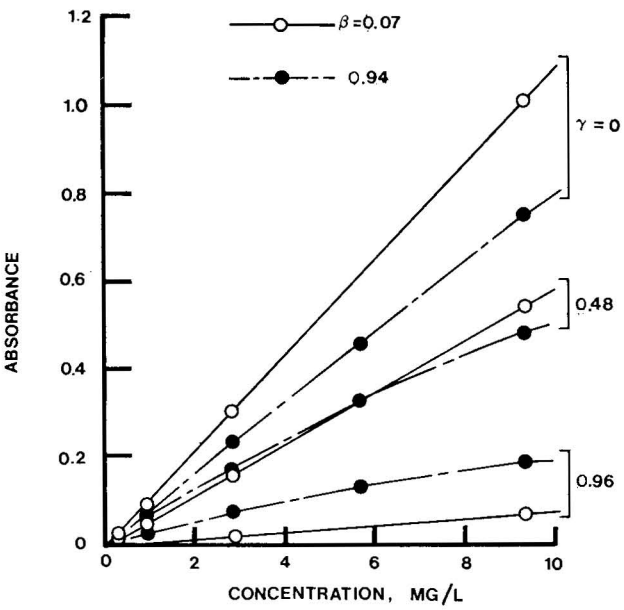


Fig. 4. Measured absorbance of biphenyl in a 1-cm cell as a function of concentration for narrow and wide spectrometer effective bandwidths and for different monochromator wavelength settings. Effective bandwidths are 2.4 and 32 nm, for which $\beta = 0.071$ and 0.94, respectively. Measurements are made at 247.5 nm at the peak of the absorption band and at 264 and 280 nm. Respective values of γ are 0, 0.48, and 0.96.

CONCLUSION

If a variety of components are to be detected in a single chromatogram, and their absorption maxima occur at different wavelengths, it is often better to use a large bandwidth ratio. This sacrifices sensitivity for the components that happen to absorb close to the monochromator setting, but improves sensitivity for others. Linearity is acceptable with a large bandwidth ratio for quantitative analysis at very small absorbance values, for which the enhanced signal-to-noise ratio afforded by the wider spectrometer slits is particularly desirable. If, on the other hand, linearity is required for effective quantitative analysis over a wide range of concentrations, the

bandwidth ratio should not in general exceed *ca.* 0.15. If there is assurance that the monochromator is tuned close to the absorption maximum of the component to be measured, it appears that the bandwidth ratio can be increased to *ca.* 0.3 if a 1% non-linearity is tolerable at 1.28 absorbance units. Rules such as these must be treated with caution because of the dependence of the numerical results on the shape of the absorption band.

REFERENCE

1 J. E. Stewart, *Infrared Spectroscopy: Experimental Methods and Techniques*, Marcel Dekker, New York, 1970, Ch. VII.

Journal of Chromatography, 174 (1979) 291–305

CHROM. 11,813

SELECTIVITY OF NUCLEOSIL 10 NH_2 AS AN ADSORBENT IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

W. E. HAMMERS, M. C. SPANJER and C. L. DE LIGNY
Laboratory for Analytical Chemistry, State University, Croesestraat 77a, Utrecht (The Netherlands)
(Received February 19th, 1979)

SUMMARY

Net retention volumes per gram of Nucleosil 10 NH_2 have been measured for a large number of mono- and disubstituted benzene derivatives and of polycyclic aromatic hydrocarbons, using *n*-hexane, dichloromethane and a mixture of both as eluents at 25°.

The retention data are interpreted in terms of the semi-empirical adsorption model, developed by Snyder for bare adsorbents, using octadecylsilylsilica as a reference adsorbent. The effects of the bound monomers on adsorbent deactivation, solute and eluent localization, change of the charge distribution in the solute molecule and adsorption mode of the solute are evaluated and discussed in terms of donor–acceptor interaction (including hydrogen bonding).

INTRODUCTION

Recent investigations on solute retention behaviour on bonded phases[1,2] have shown that the adsorption model of Snyder[3] is a valuable tool for the characterization of the adsorptive properties of these interesting adsorbents. As this model was developed to describe adsorption on bare adsorbents, it can be expected that its applicability to adsorption equilibria on chemically modified silicas will be restricted to silicas covered by moderate amounts of bound monomers. Further, the model does not account for solute–eluent interactions and hence it is not suitable for describing solute adsorption from the very polar solvents commonly used in reversed-phase and ion-pair chromatography. Finally, it can only predict the retention data of flatly adsorbed solute molecules. Despite these restrictions, it is a reliable guide for selecting and optimizing separation systems in high-performance liquid chromatographic (HPLC) practice.

The aim of this work was to evaluate (1) the change in the adsorptive properties of silica as a result of the reaction with the monomers and (2) the specific contribution of solute–monomer interactions to solute retention.

In order to distinguish both contributions, an appropriate reference bonded phase is required, and in this work octadecylsilylsilica (ODS-silica) with a relatively

low surface concentration of ODS groups was used. In order to exclude hydrophobic interaction effects, *n*-hexane, dichlomethane and a mixture of both were used as eluents. Hence, the application of Snyder's model to retention data on ODS-silica yields model parameters that characterize the adsorptive properties of silica covered by apolar monomers in such an amount that all reactive silanol groups are eliminated and only free silanol groups are present on the silica surface beneath the ODS bristles, and flat adsorption of solutes is not restricted by the anchored monomers up to solute molecular diameters of about 9 Å (*e.g.*, coronene).

This concept considerably facilitates the interpretation of solute retention on a given polar monomeric bonded phase, as a discrepancy between the value of any parameter of the polar bonded phase and of ODS-silica is related to a particular adsorptive property of the polar bonded phase in question. With respect to N-cyanoethyl-N-methylaminosilica (CNA-silica), this approach gave evidence for the following conclusions[2]: the main result of the amination by the CNA groups is a strong deactivation of the silica surface; the cyano groups are to a large extent (up to about 90% in *n*-hexane) adsorbed to the remaining silica sites; as a result, polycyclic aromatic hydrocarbons show skewed adsorption to the silica surface; the CNA layer stabilizes polar solute–adsorbent complexes; and the CNA groups show a small but significant specific interaction with (aromatic) nitrile and aldehyde groups.

This paper deals with the properties of Nucleosil 10 NH_2, a silica covered by aminobutylsilyl (ABS) groups. As the ABS group is a very strong Lewis base (electron donor), it can be expected that the ABS layer will considerably affect the acidic character of silica, and will show specific interactions with electron acceptor groups of the solute molecules.

THEORETICAL

The adsorption model of Snyder[3] and its application to monomeric bonded phases have been outlined in detail in previous papers[1,2,4]. It can be summarized with the following equations:

$$\log (V_N/W) = \log V_a + \alpha (S^0 - \varepsilon^0 A_s) \tag{1}$$

where V_N (cm^3) is the net retention volume, W (g) the weight of adsorbent in the column, α the adsorbent activity, S^0 is the solute Lewis base (or acid) strength, ε^0 the (mean) eluent strength and A_s the (effective) adsorbed surface area of the solute (expressed in units of 8.5 $Å^2$). V_a is the volume of a monolayer of adsorbed eluent per gram of adsorbent and can be estimated from

$$V_a = 35 \cdot 10^{-5} A - 0.01 \text{ wt.-\% } H_2O \tag{2}$$

Therein, A (m^2/g) is the adsorbent specific surface area.

If steric effects are excluded, essentially four effects contribute to S^0. For disubstituted benzenes S^0 is given by the equation

$$S^0 = \sum_i Q_i^0 - \beta f(Q_k^0) Q_i^0 + \delta + \varrho_k \sigma_i \tag{3}$$

The term $\sum_i Q_i^0$ accounts for the adsorptive contributions of all solute groups i of the adsorbate. The second term on the right-hand side corrects for solute localization by the strongest adsorbed group k to the strongest sites of the energetically heterogeneous adsorbent surface. The third term, δ, is related to solute–eluent interactions, which become significant as the solute and/or eluent polarity increases. The term $\varrho_k\sigma_i$ describes the contribution of intramolecular electronic effects by a solute group i on the adsorptive strength of group k in terms of the well known Hammett σ constants[5]. As the values of Q_i^0 and those of the localization function $f(Q_k^0)$ were given by Snyder (on bare silica[3]), only the parameters β, σ and ϱ_k need to be determined. Their meaning will be related to the bonded phase properties under Results and Discussion.

The S^0 values of unsubstituted polycyclic aromatic hydrocarbons can be described by only two terms:

$$S^0 = nQ^0_{-C=} - \zeta(n-6) \tag{4}$$

The first term on the right-hand side is the sum of the contributions $Q^0_{-C=}$ of the n aromatic carbon atoms of the arenes, and the second term accounts for solute localization.

The term $-\alpha\varepsilon^0 A_s$ in eqn. 1 represents the primary eluent effect, which arises as a result of the replacement of adsorbed eluent by the adsorbate molecules. The A_s values of disubstituted benzenes are given by

$$A_s = \sum_i a_i\,(\text{calc.}) + \gamma \sum_i \Delta a_i\,(SiO_2) \tag{5}$$

where a_i (calc.) is the contribution of a non-localized solute group i to A_s, which can be calculated from Van der Waals radii and bond angles. The discrepancy between experimental and calculated a_s values, $\gamma\Delta a_i$ (SiO_2), observed for localized groups on bare silica, is accounted for by the last term in eqn. 5. The parameter γ appears to be related to the extent of deactivation of bare silica by water[6,7].

The A_s values of arenes on bare silica can be estimated from

$$A_s = 6 + 0.8\,(h-6) + 0.25\,(c-h) \tag{6}$$

where c and h are the numbers of carbon atoms and protons in the arenes, respectively.

The eluent strength (ε^0) data and the Q_i^0, $f(Q_k)$, a_i and Δa_i (SiO_2) values on bare silica collected by Snyder will also apply to bonded phases. Thus, any difference between the log V_N/W data on bare silica and on the bonded phase in question will be ascribed to different values of the parameters log V_a, α, β, γ, δ, ζ and ϱ_k.

The parameters α and log V_a are determined from a plot of log V_N/W data of monosubstituted benzenes in n-hexane ($\varepsilon^0 = 0$) against their S^0 values.

When α and log V_a are known, the S^0 values of the arenes can easily be obtained from their net retention data in n-hexane, and be plotted against n to give ζ according to eqn. 4.

As α and log V_a are independent of the eluent strength, A_s values can be calculated from

$$A_s = \frac{1}{\alpha \varepsilon_E^0} \cdot \log (V_{N,H}/V_{N,E}) \tag{7}$$

where $V_{N,H}$ and $V_{N,E}$ are the net retention volumes in *n*-hexane and an eluent E with strength ε_E^0. The value of γ is estimated from eqn. 5 using A_s values of mono-substituted benzenes.

Finally, β, δ and ϱ_k values are obtained from multiple regression analysis using the equation

$$\frac{1}{\alpha} \cdot \log [V_N(i\text{-}\varnothing\text{-}k)/V_N(\varnothing\text{-}k)] - (Q_i^0 - \varepsilon_E^0 a_i) = \delta - \beta f(Q_k^0)\, Q_i^0 + \varrho_k(E)\, \sigma_i \tag{8}$$

wherein i-$\varnothing$-k represents a member of a solute series which has the strongest adsorbed group k in common. The value of a_i can be calculated from $a_i = a_i$ (calc.) $+ \gamma \Delta a_i$ (SiO_2) according to eqn. 5. From the standard error of fit, it can be judged whether Hammett σ, σ^- or σ^+ constants[8] should be used in eqn. 8. The latter are appropriate if the electron-withdrawing or -repelling action of group i is favoured by mesomerism (*i.e.*, a resonance hydrid electron configuration involving the groups i and k).

EXPERIMENTAL

Chemicals and adsorbent characterization

All solutes (obtained from Fluka, Buchs, Switzerland) were of the highest available purity and were used as received. *n*-Hexane and dichloromethane were supplied by Baker (Deventer, The Netherlands) and were dried with molecular sieve 5A before use.

Nucleosil 10 NH_2 (Macherey, Nagel & Co., Düren, G.F.R.) was examined by the BET technique according to Broekhoff and Linsen[9]. Results for specific surface area, pore volume and pore diameter are given in Table I. Elemental analyses of Nucleosil 10 NH_2 showed the same C and N contents before and after treatment with 0.1 *N* hydrochloric acid. Mean results were 4.80 wt.-% C and 1.36 wt.-% N (with respect to bare silica), which corresponds to a surface concentration of 2.54 μmole/m^2 of aminobutylsilyl groups. The mean distance of adjacent ABS anchoring places is about 9.5 Å.

Apparatus and procedure

The column (precision-bore stainless steel, length 25 cm, I.D. 2.1 mm) was packed by the viscous-slurry packing method[10]. The slurry (15 wt.-% ABS-silica, particle diameter 10 μm, in *n*-butanol) was degassed and homogenized by ultrasonic treatment and forced into the column with *n*-hexane at 350 atm. Finally, 500 ml of dry dichloromethane was flushed through the column. The weight of adsorbent in the column was 0.43 g. The HETP of an unretained solute was about 0.15 mm at a linear flow-rate of 1 cm/sec using *n*-hexane as the eluent.

TABLE I

CHARACTERISTIC ADSORBENT PROPERTIES OF ODS-, CNA- AND ABS-SILICA

Property	*ODS-silica*	*CNA-silica*	*ABS-silica*
Monomer structure	$>Si-(CH_2)_{17}CH_3$ | OH	$-N(CH_3)(CH_2)_2CN$	$>Si-(CH_2)_4NH_2$ | OH
BET specific surface area (m^2/g)	316 ± 2	263 ± 3	388 ± 2
Pore volume (ml/g)	0.60	1.05	1.44
Mean pore diameter (Å)	75	170	150
Surface concentration of monomers (μmole/m^2)	1.64	1.25	2.54
Log V_a	−1.03 ± 0.04	−0.93 ± 0.05	−0.79 ± 0.04
α	0.50 ± 0.01	0.45 ± 0.01	0.39 ± 0.01
Fraction of adsorbed monomers (*n*-hexane)	0	0.88	0.95
$\Delta\varepsilon^0_{le}$ (*n*-hexane)	0.00	0.04	0.09
$\Delta\varepsilon^0_{le}$ (dichloromethane)	0.00	0.00	0.03

The apparatus and measuring technique have been described previously[1]. The solute sample size was smaller than 25 μg (UV detection at 254 nm). The reproducibility of triplicate V_N measurements was about 10 μl or 3% for strongly adsorbed solutes. The adsorbent activity was monitored occasionally by measuring retention volumes of some polar solutes in *n*-hexane and appeared to be constant within experimental error during the whole measuring period.

The following eluents were used: *n*-hexane (H, $\varepsilon^0 = 0$), *n*-hexane–dichloromethane (65:35, v/v) (B, $\varepsilon^0 = 0.22$) and methylene chloride (C, $\varepsilon^0 = 0.32$). The eluent strength data were given by Snyder[6,7]. All measurements were made at 25°.

RESULTS AND DISCUSSION

Adsorbent activity

Experimental log (V_N/W) data for some monosubstituted benzenes and chlorobenzenes are given in Table II and are plotted against S^0 in Fig. 1. Contrary to the results on ODS- and CNA-silica, those on ABS-silica cannot be fitted accurately by eqn. 1. In order to establish which solutes behave anomalously, the log (V_N/W) data on ABS-silica are plotted against those on ODS-silica at 25° [1]in Fig. 2. The log (V_N/W) value of acetophenone at 25° on ODS-silica is not availble. It can be estimated from that at 43.5° by accounting for the effect of the slight difference of α at the two temperatures, which yields log (V_N/W) = 2.13. In Fig. 2, the data points 10 (methyl benzoate) and 11 (acetophenone) appear to deviate significantly from the straight line through the other data points. Regression analysis of the remaining log (V_N/W) data by eqn. 1 yields $\alpha = 0.39 \pm 0.01$ and log $V_a = -0.79 \pm 0.04$.

Compared with water-free bare wide-pore silica (where $\alpha = 0.83$; ref. 3) or ODS-silica (where $\alpha \approx 0.50$), the activity of ABS-silica is very small. The deactivation by the ABS groups is due to two effects. Firstly, the removal of *reactive* silanol groups by the silylation reaction reduces the value of α to about that of ODS-silica, where only free silanol groups are available for solute adsorption[1]. Secondly, the

TABLE II

LOG (V_N/W) DATA FOR MONOSUBSTITUTED BENZENES, CHLOROBENZENES, PHENOLS, ANILINES AND PYRIDINES IN *n*-HEXANE (H), *n*-HEXANE–DICHLOROMETHANE (65:35) (B) AND DICHLOROMETHANE (C) AT 25°

i	*No.*	*i*-∅			*Isomer*	*i*-∅*Cl*	*i*-∅*OH*		*i*-∅NH_2		*i*-C_5H_5N	
		H	*B*	*C*		*H*	*B*	*C*	*B*	*C*	*B*	*C*
H	1	−0.24	*	*		−0.22	1.63	0.96	0.76	−0.08	1.15	0.61
F	2	−0.25	*	*	*m*-		1.95	1.18	0.63	−0.25		
					p-		1.81	1.03	0.85	0.00		
Cl	3	−0.22	*	*	*m*-		2.01	1.22	0.63	−0.24	0.57	−0.07
					p-		1.93	1.14	0.75	−0.12		
Br	4	−0.19	*	*	*m*-		2.00	1.17	0.66	−0.24	0.57	−0.08
					p-		1.98	1.09	0.75	−0.14		
CH_3	5	−0.27	*	*	*m*-		1.57	0.73	0.73	−0.09		
					p-		1.57	0.74	0.83	0.03	1.30	0.74
SCH_3	6	0.32	*	*								
OCH_3	7	0.45	*	*	*m*-	0.38	1.92	0.94	1.01	0.03		
					p-		1.84	0.87	1.24	0.29		
NO_2	8	0.82	−0.33	*	*m*-	0.79	**	1.57	1.00	−0.13		
					p-		**	2.09	1.26	0.08		
CN	9	1.12	−0.17	−0.81	*m*-		**	1.51	1.14	−0.03	0.94	−0.06
					p-	1.07	**	1.82	1.21	0.02	1.01	0.06
CO_2CH_3	10	0.97	−0.20	−0.86	*m*-		**	1.26				
					p-		**	1.54				
$COCH_3$	11	1.38	0.07	−0.61	*m*-		**	1.52	1.45	0.26		
					p-		**	1.82	1.58	0.33		

* V_N values are very small.
** V_N values are very large.

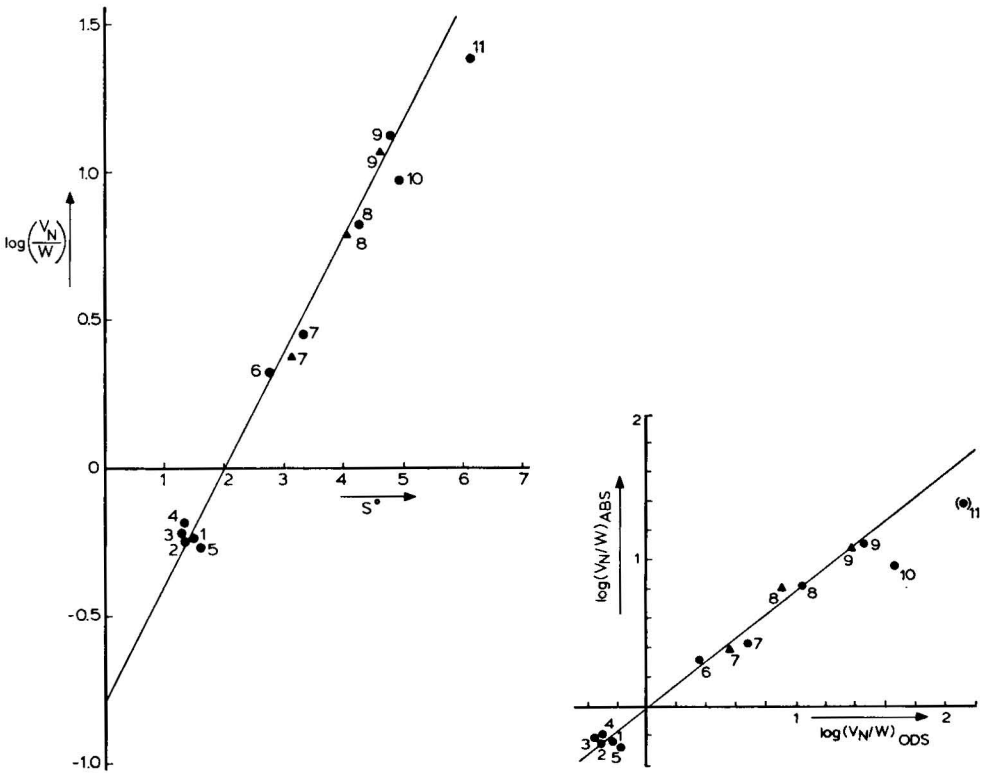


Fig. 1. Experimental log (V_N/W) data for monosubstituted benzenes (●) and *m*- and *p*-substituted chlorobenzenes (▲) on ABS-silica *versus* $S°$. Eluent: *n*-hexane (25°). Numbering of the data points according to Table II.

Fig. 2. Experimental log (V_N/W) data of monosubstituted (chloro)benzenes in *n*-hexane at 25° on ABS-silica *versus* those on ODS-silica. Numbering of the data points according to Table II.

adsorption of the ABS groups on the silica surface contributes to the eluent strength of *n*-hexane and reduces α further to its effective value of 0.39. Similar results have been obtained for CNA-silica. The fractions of adsorbed CNA and ABS groups and the effects on the eluent strength are given in Table I. The estimation of these data has been outlined previously[2]. In order to correct α and log V_a for ABS adsorption, the S^0 values must be adjusted by the term $-\varepsilon^0 A_s$. Here $\varepsilon^0 = 0.09$ (see Table I), whereas A_s is given by eqn. 5 using $\gamma = 0.61$ (value for ODS-silica). The corrected values are $\alpha = 0.47$ and log $V_a = -0.65$.

The estimated α value is close to that for ODS-silica ($\alpha = 0.50 \pm 0.01$). The log V_a value obtained, which holds for 1 g of silica plus bound monomers, must be corrected for the weight of the ABS layer before comparing it with theoretical estimates. This yields log $V_a = -0.59$. Eqn. 2 cannot be applied straightforwardly for the comparison because part of the silica surface is blocked by silyl groups (area about 25 Å^2) and part of the ABS groups. Therefore, it can be expected that log $V_a \lesssim -1.07$, *i.e.* much smaller than the corrected experimental value of -0.59

(on ODS-silica this discrepancy is only about 0.08). This may be the result of interaction of ABS groups with the phenyl nucleus of the benzenes (discussed below).

Solute–ABS interaction

Although solute–silica interaction forces are of prime importance on ABS-silica, the retention behaviour of all solute types examined shows a definite change compared with that on ODS- and CNA-silica.

As mentioned above, methyl benzoate and acetophenone have relatively small S^0 values. In order to explain these anomalous values, attention will be focused first on the experimental S^0 values of the arenes presented in Table III. These values are plotted against the number of aromatic carbon atoms in Fig. 3.

TABLE III

LOG (V_N/W) DATA FOR SOME POLYCYCLIC AROMATIC COMPOUNDS IN *n*-HEXANE (H) AND *n*-HEXANE–DICHLOROMETHANE (65:35) (B) AT 25°, THEIR EXPERIMENTAL S^0 AND A_s VALUES AND CALCULATED A_s DATA

Solute	*No.*	*H*	*B*	S^0	A_s	A_s *(calc.)*
Benzene	1	−0.24	*	1.44		6.0
Naphthalene	2	0.21	*	2.58		8.1
Acenaphthene	3	0.31	*	2.85		9.7
Diphenyl	4	0.33	*	2.89		9.7
Fluorene	5	0.52	−0.73	3.38	14.6	9.9
Bibenzyl	6	0.38	*	3.02		12.4
Anthracene	7	0.66	−0.68	3.74	15.6	10.2
Phenanthrene	8	0.73	−0.59	3.92	15.4	10.2
Fluoranthene	9	0.94	−0.43	4.46	16.0	10.7
Chrysene	10	1.17	−0.29	5.04	17.0	12.3
Triphenylene	11	1.18	−0.31	5.08	17.4	12.3
p-Terphenyl	12	0.86	−0.79	4.24	19.2	13.4
3,4-Benzopyrene	13	1.31	−0.24	5.42	18.1	12.8
Perylene	14	1.41	−0.16	5.67	18.3	12.8
Picene	15	1.57	−0.10	6.07	19.5	14.4
Coronene	16	1.63	0.01	6.23	18.9	13.8
p,p'-Quaterphenyl	17	1.37	−0.52	5.57	22.0	17.1

* V_N values are very small.

In view of the localization of arenes, one might expect at first sight that their S^0 values would be close to those on ODS-silica. However, it must be taken into account that about 95% of the ABS groups are adsorbed silanol groups. Therefore, flat adsorption of the arenes can hardly be expected and accordingly the S^0 values should be similar to (or even smaller than) those on CNA-silica, where the same phenomenon occurs. Hence, the observed large S^0 values on ABS-silica (Fig. 3) indicate strong interaction forces between the arenes and free ABS groups. Further, the S^0 values of the fused arenes appear to be larger than those of the polyphenyls with the same number of aromatic carbon atoms. The data can be described by means of eqn. 4, using $Q^0_{-C=} = 0.25$ and $\zeta = -0.036 \pm 0.009$ (fused arenes) or $\zeta = 0.023 \pm 0.006$ (polyphenyls), although it is evident that these ζ values have no physical meaning. The data points 6 (bibenzyl) and 16 (coronene) in Fig. 3 have been excluded from this calculation. The relatively small S^0 value of coronene may be a

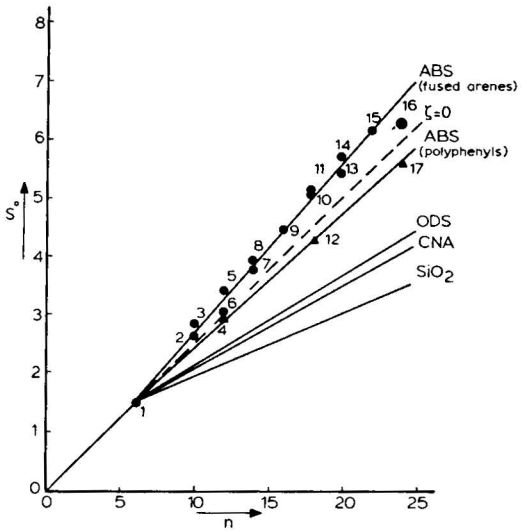


Fig. 3. Experimental S° values of polycyclic aromatic hydrocarbons on ABS-silica in *n*-hexane (25°) *versus* the number of aromatic carbon atoms (n). Numbering of the data points according to Table III. Data points on bare silica, ODS- and CNA-silica have been omitted for the sake of clarity. Dashed line: expected on the basis of the additivity concept ($\zeta = 0$).

result of pronounced skewed adsorption as its diameter (9 Å) is about equal to the mean distance of adjacent monomer silyl groups. Bibenzyl behaves anomalously because it is not delocalized ($S^0 \approx 3.0$) on ABS- or ODS-silica or bare silica. Snyder[3,11] has suggested that the distance between both phenyl groups permits simultaneous adsorption of them on silanol sites*.

The discrepancy between the ζ values of the fused arenes and of the polyphenyl cannot be due to skewed adsorption of the slightly twisted polyphenyls[12] on the silica surface, because it is not observed on ODS- and CNA-silica. Further, neither the smallness of the ζ values nor their discrepancy on ABS-silica can be ascribed to dipole-induced dipole interaction, as the dipole of an ABS amino group is only one third of that of a CNA nitrile group and, moreover, the polarizabilities of benzenes, naphthalene and diphenyl[13] are linearly related to n. Therefore, it seems logical to assume that the arene–ABS interaction is of the donor–acceptor type and that its strength increases with the extent of π-conjugation in the arene. For equal numbers of aromatic carbon atoms the extent of π-conjugation is larger in a flat fused arene than in a slightly twisted polyphenyl. We presume that the adsorbed arenes are electron acceptors towards the ABS group which is a strong n-donor[14]. Complexes between arenes and electron acceptors have been examined extensively[15]. The work of Dewar and Thompson[16] on arene–tetracyanoethylene (TCNE) complexes in chloroform is noteworthy with respect to this work. The log $K_c$values (K_c, l/mole,

* Previously, we expressed some doubt about this explanation because acenaphthene and fluorene also show relatively large S^0 values (see Fig. 2 in ref. 1). However, this argument is not correct as the cycloalkyl moiety of the latter two solutes contributes to S^0 to some extent (L. R. Snyder, personal communication), and this has not been accounted for. It is interesting that these solutes show "normal" behaviour on ABS-silica.

is the complex association constant) of some arene–TCNE complexes are plotted against n in Fig. 4. They show a similar pattern to that in Fig. 3. Presumably, on ABS-silica as well as in bulk solution and irrespective of the direction of charge transfer, the stability of the complex depends on the extent of π-conjugation in the arene. Hence we believe that on ABS-silica a "sandwich" complex of the type SiOH←arene←ABS may occur, but it need not be assumed to explain the results.

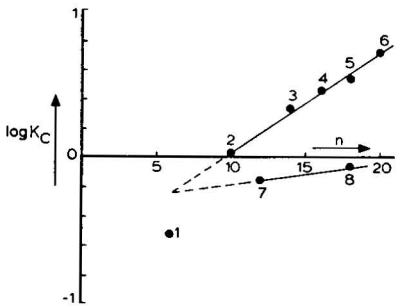


Fig. 4. Experimental log K_c values of some arene–tetracyanoethylene complexes in chloroform at 25°[16] *versus* the number of carbon atoms (n). 1 = Benzene;2 = naphthalene; 3 = phenanthrene; 4 = pyrene; 5 = triphenylene; 6 = 3,4-benzpyrene; 7 = diphenyl; 8 = *p*-terphenyl.

The small S^0 values obtained for methyl benzoate and acetophenone can be explained similarly. The bulky methyl group forces the substituent group to turn slightly out of the plane of the phenyl ring on adsorption on the small crystalline flat surface regions of a wide-pore silica, and so disturb the π-conjugation between the substituent and the phenyl ring and weakens the interaction with the ABS group. The fact that "mesomeric interactions" occur on adsorption of the planar *p*-hydroxybenzaldehyde, but not with the non-planar *p*-hydroxyacetophenone, has been explained along the same lines[1,2]. ABS–phenyl interaction is probably also the cause of the large log V_a value mentioned above.

Primary eluent effects

Experimental log (V_N/W) data for some monosubstituted benzenes in the binary eluent (B) and in dichloromethane (C) are given in Table II. Data for the arenes in the binary eluent are presented in Table III. The use of $\alpha = 0.39$, $\varepsilon_B^0 = 0.22$ and $\varepsilon_C^0 = 0.32$ in eqn. 7 yields A_s values, from which γ_i values can be obtained*.

The effective γ_i values of some monosubstituted benzenes in both eluents are given in Table IV. Previously, it has been shown that γ_i is independent of substituent i and of temperature[1,2]. Therefore, it is justifiable to compare the mean γ values of these bonded phases. On ODS- and CNA-silica the γ values are identical. Obviously, the solute and eluent molecules show approximately the same (and weak) interaction with ODS and CNA groups. So far, there is no reason to assume that γ depends on

* These γ_i values are about 0.07 too large in both eluents due to the contribution of the ABS groups to the eluent strength (and correspondingly to the α and log V_a values). This hardly significant effect will be omitted in the following discussion.

TABLE IV

γ_i VALUES FOR MONOSUBSTITUTED BENZENES ON ABS-SILICA IN *n*-HEXANE–DICHLOROMETHANE (65:35) (B) AND IN DICHLOROMETHANE (C) AND MEAN γ VALUES ON ABS-, ODS- AND CNA-SILICA

Adsorbent	*Eluent*	γ_i				$\bar{\gamma} \pm s_\gamma$
		NO_2	CN	CO_2CH_3	$COCH_3$	
ABS-silica	B	0.98	1.08	0.92	1.01	1.00 ± 0.07
	C		1.17	1.09	1.10	1.12 ± 0.04
ODS-silica	B					0.61 ± 0.05
CNA-silica	B					0.59 ± 0.03

the surface concentration of bound monomers (see Table I), provided that it is large enough to eliminate the reactive (or bound) silanol groups (0.5–1.0 μmole/m^2 on a wide-pore silica[2]) and small enough to guarantee flat adsorption of the benzenes. Therefore, it is assumed that the value of $\gamma \approx 0.60$ is characteristic for a silica surface covered merely by free silanol sites. From this point of view, the large γ values on ABS-silica should be ascribed to solute ABS interaction accompanied by the the breakdown of a relatively strong dichloromethane–ABS interaction. As the occurrence of weak donor–acceptor complexes of solvents such as chloroform and dichloromethane with amines cannot be ruled out[15], this explanation of the large γ values on ABS-silica seems plausible. The slight increase in γ with increasing dichloromethane concentration on ABS-silica is probably due to the increase in the mean number of dichloromethane molecules in the solvent shell around the ABS amino groups.

The experimental and calculated A_s values of the arenes are given in Table III and are plotted against each other in Fig. 5. The A_s values on ODS- and CNA-silica are also presented in Fig. 5 for the sake of comparison. On ODS-silica the A_s values

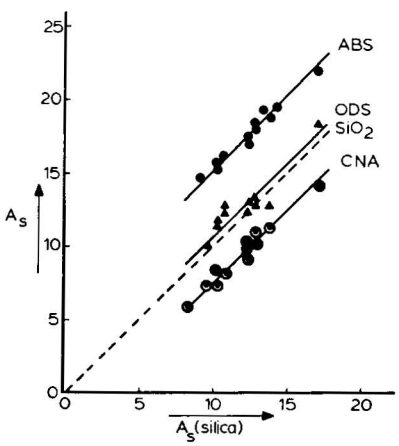


Fig. 5. Experimental A_s values of polycyclic aromatic hydrocarbons on ABS-, ODS- and CNA-silica *versus* calculated A_s (silica) values. Eluents: *n*-hexane and *n*-hexane–dichloromethane (65:35) at 25°.

are close to the calculated values, which hold for bare silica. This indicates flat adsorption on the silica surface beneath the ODS bristle layer. On CNS-silica the A_s values are about 2.5 smaller as a result of skewed adsorption, as mentioned above. The A_s values on ABS-silica are anomalously large. The mean discrepancy between the experimental and calculated values is 5.2 ± 0.1, although small A_s values would be expected in view of steric interference to flat adsorption by adsorbed ABS groups. Obviously, these steric effects are completely overruled by the competitive interaction of the arene and dichloromethane molecules with the ABS groups.

Adsorption behaviour of m- and p-substituted phenols, anilines and pyridines

Experimental log (V_N/W) values are given in Table II. An attempt to obtain retention data for substituted benzaldehydes failed, presumably because of a condensation reaction with the ABS group, catalysed by (acidic) silanol groups[17].

The phenols with strongly electron-withdrawing substituents could not be eluted with the binary eluent within reasonable time. As the remaining solutes of this series cover only small range of σ^- values, a three-parameter analysis will fail to give reliable results. Therefore, only retention data of the phenols in dichloromethane will be discussed below.

Multiple regression analyses of the data (left-hand side of eqn. 8) showed that the best fit can be achieved using σ^- and σ^+ substituent constants for the phenols and the pyridines, respectively. The data for the aniline series can be described equally well with σ or σ^- constants, because the influence of intramolecular effects on the adsorptive strength of these solutes appears to be small on ABS-silica. Optimal fit on applying σ^- or σ^+ constants indicates the possibility of direct "mesomeric interaction" between the substituent groups i and k on adsorption. Whether mesomerism causes anomalously large or small retention volumes depends on the sign of ϱ_k.

The values of β, ϱ, σ and the standard error of fit (s) in the binary eluent and n dichloromethane are given in Table V.

TABLE V

VALUES OF β, ϱ, δ AND THE STANDARD ERROR OF FIT (s) FOR *m*- AND *p*-MONOSUBSTITUTED PHENOLS, ANILINES AND PYRIDINES IN *n*-HEXANE–DICHLOROMETHANE (65:35) (B) AND DICHLOROMETHANE (C) AT 25°

Solute series		*Parameter*	*B*	*C*
Phenols	(σ^-)	$\beta \pm s_\beta$		0.56 ± 0.10
Anilines	(σ)		0.34 ± 0.15	0.20 ± 0.10
Pyridines	(σ^+)		0.22 ± 0.14	0.10 ± 0.20
Phenols	(σ^-)	$\varrho \pm s_\varrho$		2.56 ± 0.20
Anilines	(σ)		-0.24 ± 0.24	-0.46 ± 0.26
Pyridines	(σ^+)		-2.19 ± 0.31	-2.42 ± 0.45
Phenols	(σ^-)	$\delta \pm s_\delta$		0.25 ± 0.09
Anilines	(σ)		0.25 ± 0.09	0.32 ± 0.10
Pyridines	(σ^+)		-0.26 ± 0.12	-0.30 ± 0.18
Phenols	(σ^-)	s		0.26
Anilines	(σ)		0.25	0.27
Pyridines	(σ^+)		0.19	0.28

The influence of the eluent strength on β, ϱ and σ appears not to be significant on either ABS- or CNA-silica[2]. On ODS-silica, however, ϱ increases significantly with increasing eluent strength[1]. Obviously, the (polar) solute–adsorbent complexes are better stabilized in polar solvents. This stabilization is surprisingly well fulfilled by the CNA and ABS monomers, as their contribution to the strength of the binary eluent is only about 0.02 and 0.05, respectively.

The mean β and ϱ values on the three bonded phases will be discussed on the basis of the solute–adsorbent structures I, II and III presented in Fig. 6.

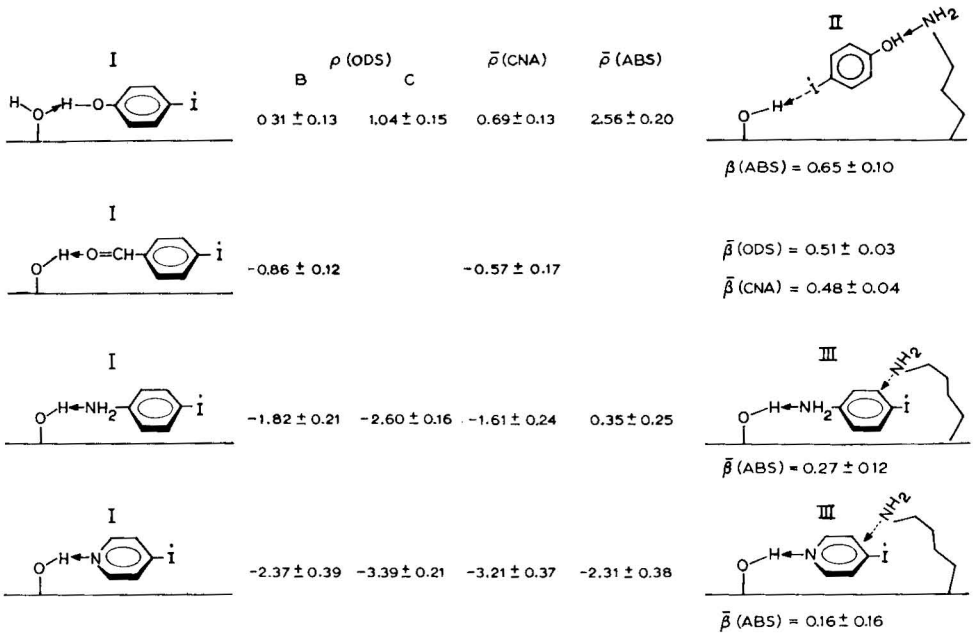


Fig. 6. Experimental ϱ and β values of *m*- and *p*-substituted phenols, benzaldehydes, analines and pyridines on ODS- (eluents B and C), CNA- and ABS-silica (mean ϱ values in eluents B and C), and the corresponding structures of the solute–adsorbent complexes on ODS- and CNA-silica (I) and on ABS-silica (phenols, I and II; anilines and pyridines, I and III).

The ϱ values on ABS-silica are considerably more positive than those on ODS- and CNA-silica, particularly in dichloromethane. Obviously, the ABS groups are involved in the adsorption process.

For the phenols, it can be assumed that the acidic OH group forms a strong hydrogen bond with the ABS group. The transferred charge from the ABS group can be withdrawn by group *i* and may even be transmitted to an available (acidic) silanol site (structure II). As σ_i (or σ_i^-) is a measure of the electron-withdrawing ability of group *i*, it can be expected that the occurrence of the complex II will contribute to the ϱ value. On ODS-silica, and presumably also on CNA-silica, merely the structure I occurs, as will be elucidated below by means of the β values on these adsorbents.

The basic anilines and pyridines will preferably form a hydrogen bond with the silanol groups on all three adsorbents. In addition, the ABS group can form a

donor–acceptor complex with the group i (or the $\varnothing$–i moiety) of these solutes (III). The associated charge transfer opposes electron withdrawal by group i and hence ϱ will shift to less negative values on ABS-silica.

The mean β values on ODS- and CNA-silica are identical within experimental error[1,2]. According to the adsorption model of Snyder, β decreases with increasing number of accessible active sites per square metre. Therefore, solute–CNA interactions, if present, are of minor importance. Obviously, the value $\beta \approx 0.50$ accounts for solute localization on a water-free silica surface covered merely by active free silanol sites.

The small β values of the anilines and pyridines indicate an increased solute localization. These molecules are adsorbed flatly on a silanol site (I) and in addition can be subject to interactions exerted by the flexible ABS sites (III).

The β value of the phenols on ABS-silica is relatively large. Probably, hydrogen bonding to the flexible ABS site is accompanied by an unfavourable position of the i–$\varnothing$ moiety to the silica surface (II). Thus, localization to the ABS group can imply delocalization of the i–$\varnothing$ group and hence an increased β value.

CONCLUSIONS

The adsorption theory of Snyder appears to be a valuable means of evaluating the adsorptive properties of Nucleosil 10 NH_2.

The effective activity of this bonded phase is small ($\alpha = 0.39$) compared with the of bare wide-pore silica ($\alpha \approx 0.83$) and ODS-silica ($\alpha = 0.50$). This is a result of preferential deactivation of reactive silanol groups and a considerable contribution of the ABS groups to the eluent strength of n-hexane.

The contribution of solute–ABS interactions can be explained in terms of donor–acceptor complexation (including hydrogen bonding).

These donor–acceptor interactions give rise to the large S^0 values of the fused arenes. Their contribution appears to depend on the extent of π-conjugation. The experimental S^0 values obtained for polyphenyls, methyl benzoate and acetophenone are accordingly relatively small: the benzenes mentioned cannot be adsorbed flatly on the silica surface, whereas the twisted polyphenyls experience strain on flat adsorption, which restricts π-conjugation.

The anomalously large contribution of eluent localization to the A_s values of the arenes and the monosubstituted benzenes [γ (binary eluent) = 1.00 and γ (dichloromethane) = 1.12] is the result of competitive solute–ABS and dichloromethane–ABS interactions.

The ϱ values of m- and p-substituted phenols are more positive and those of the anilines and pyridines are less negative than the values obtained on ODS- and CNA-silica. This shift of the ϱ values indicates hydrogen bonding between the phenolic OH and a free ABS group and to interaction of an ABS group with the electrophilic parts of the aniline and pyridine molecules. Owing to the very effective stabilization of the polar solute–adsorbent complexes by the ABS groups, the ϱ values are not significantly affected by an increase of the eluent strength. The solute localization parameters β are larger (phenols) and smaller (anilines and pyridines) than those on ODS- and CNA-silica ($\beta = 0.50$), where only free silanol sites are involved in adsorption.

ACKNOWLEDGEMENTS

The authors are indebted to Mr. P. A. Elberse and Dr. L. A. M. Hermans for their accurate BET measurements on Nucleosil 10 NH_2.

REFERENCES

1 W. E. Hammers, R. H. A. M. Janssen, A. G. Baars and C. L. de Ligny, *J. Chromatogr.*, 167 (1978) 273.
2 W. E. Hammers, C. H. Kos, W. K. Brederode and C. L. de Ligny, *J. Chromatogr.*, 168 (1978) 9.
3 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968.
4 W. E. Hammers, R. H. A. M. Janssen and C. L. de Ligny, *J. Chromatogr.*, 166 (1978) 9.
5 L. P. Hammett. *Physical Organic Chemistry*, McGraw-Hill, New York, 1940.
6 L. R. Snyder, *J. Chromatogr.*, 11 (1963) 195.
7 L. R. Snyder, *J. Chromatogr.*, 25 (1966) 274.
8 C. D. Ritchie and W. F. Sager, *Progr. Phys. Org. Chem.*, 2 (1964) 323.
9 J. C. P. Broekhoff and B. G. Linsen, in B. G. Linsen (Editor), *Physical and Chemical Aspects of Adsorbents and Catalysts*, Academic Press, London, 1970, p. 1.
10 J. Asshauer and I. Halász, *J. Chromatogr. Sci.*, 12 (1974) 139.
11 L. R. Snyder, *J. Phys. Chem.*, 67 (1963) 2622.
12 F. J. Adrian, *J. Chem. Phys.*, 28 (1958) 608.
13 A. Eucken and K. H. Hellwege (Editors), *Landolt-Börnstein, Zahlenwerte und Funktionen I (3)*, Springer-Verlag, Berlin, 1951, pp. 386 and 509.
14 R. S. Mulliken, *J. Chim. Phys.*, 61 (1964) 20.
15 R. Foster, *Organic Charge-Transfer Complexes*, Academic Press, London, 1969, p. 187.
16 M. J. S. Dewar and C. C. Thompson, Jr., *Tetrahedron, Suppl.*, 7 (1966) 97.
17 C. K. Ingold, *Structure and Mechanism in Organic Chemistry*, Cornell University Press, New York, 1969, p. 843.

Journal of Chromatography, 174 (1979) 307–313

CHROM. 11,789

MIXED SOLVENTS IN GAS–LIQUID CHROMATOGRAPHY

ACTIVITY COEFFICIENTS FOR BENZENE, CYCLOHEXANE, PENTANE AND HEPTANE IN SQUALANE–DINONYLPHTHALATE MIXTURES AT 303 °K

ANTHONY J. ASHWORTH and DAVID M. HOOKER
School of Chemistry, University of Bath, Bath (Great Britain)
(First received December 4th, 1978; revised manuscript received February 2nd, 1979)

SUMMARY

Activity coefficients at infinite dilution for benzene, cyclohexane, pentane and heptane in mixtures of squalane and dinonyl phthalate at 303 °K have been determined by the extrapolation of measurements made using a vacuum microbalance technique. Partition coefficients for the solutes at infinite dilution in the solvent mixtures have been derived and found to differ by as much as 6% from those calculated from the partition coefficients for the pure single solvents using the Purnell–Andrade equation, whereas those calculated on the basis of the Flory–Huggins solution theory differ from the experimentally derived values by no more than 1%.

INTRODUCTION

In a previous note[1] the activity coefficients at infinite dilution for hexane in squalane–dinonylphthalate (DNP) solvent mixtures were reported and used to demonstrate the deviation of the system from the relationship proposed by Purnell and Vargas de Andrade[2] to predict the behaviour of mixed solvents in gas–liquid chromatography (GLC). We have now determined activity coefficients for benzene, cyclohexane, pentane and heptane in the lower solute concentration region so that activity coefficients at infinite dilution can be derived and the deviation from the Purnell–Andrade relation examined further.

EXPERIMENTAL

Absorption isotherms were determined as before[1], using a Sartorius Model 4102 electronic vacuum microbalance in conjunction with a Texas Instruments quartz Bourdon gauge. The apparatus, the techniques and the materials have been described previously[1,3,4]. The temperature of the absorption isotherms was controlled at 303.04 ± 0.01 °K. A nominal temperature of 303 °K is used throughout.

TABLE I

ABSORPTION OF SOLUTES (A) IN SQUALANE (B)–DNP (C) MIXTURES AT 303°K

$n_B:n_C$ = solvent mole ratio; other symbols: see text.

$n_B:n_C$	*Benzene*			*Cyclohexane*		
	x_A	P_A (mmHg)	$\ln \gamma_A$	x_A	P_A (mmHg)	$\ln \gamma_A$
3:1	0.0901	6.91	−0.4290	0.0986	6.95	−0.5318
	0.1631	13.05	−0.3869	0.1639	12.06	−0.4890
	0.2258	18.77	−0.3490	0.2378	18.27	−0.4464
	0.2818	24.18	−0.3177	0.2986	23.85	−0.4081
	0.3368	29.86	−0.2856	0.3598	29.90	−0.3690
	0.3900	35.64	−0.2557	0.4143	35.66	−0.3345
	0.4419	41.72	−0.2237	0.4720	42.27	−0.2954
	0.4859	47.10	−0.1976	0.5149	47.51	−0.2658
1:1	0.0899	6.43	−0.4987	0.0876	6.86	−0.4260
	0.1626	12.21	−0.4506	0.1524	12.43	−0.3866
	0.2345	18.35	−0.4094	0.2162	18.20	−0.3551
	0.2972	24.17	−0.3715	0.2744	23.85	−0.3237
	0.3555	29.99	−0.3353	0.3322	29.83	−0.2916
	0.4101	35.81	−0.3013	0.3878	35.86	−0.2629
	0.4660	42.28	−0.2633	0.4366	41.53	−0.2348
	0.5115	47.76	−0.2351	0.4842	47.30	−0.2089
1:3	0.1152	8.02	−0.5261	0.0663	6.09	−0.2666
	0.1737	12.55	−0.4888	0.1273	11.98	−0.2430
	0.2408	18.13	−0.4483	0.1848	17.78	−0.2214
	0.3057	24.02	−0.4061	0.2389	23.52	−0.1992
	0.3687	30.19	−0.3652	0.2924	29.43	−0.1773
	0.4229	35.87	−0.3304	0.3464	35.63	−0.1562
	0.4718	41.44	−0.2957	0.3931	41.24	−0.1369
	0.5229	47.58	−0.2609	0.4421	47.29	−0.1180
	Pentane			*Heptane*		
	x_A	P_A (mmHg)	$\ln \gamma_A$	x_A	P_A (mmHg)	$\ln \gamma_A$
3:1	0.0733	29.96	−0.3668	0.0750	3.22	−0.2956
	0.1411	59.50	−0.3371	0.1401	6.18	−0.2690
	0.2052	89.35	−0.3068	0.1979	8.97	−0.2418
	0.2638	118.43	−0.2783	0.2567	11.94	−0.2166
	0.3212	148.59	−0.2502	0.3164	15.06	−0.1938
	0.3740	117.93	−0.2241	0.3673	17.85	−0.1733
	0.4248	207.68	−0.1987	0.4163	20.68	−0.1517
	0.4730	237.33	−0.1747	0.4669	23.64	−0.1331
1:1	0.0652	29.75	−0.2577	0.0607	2.96	−0.1689
	0.1292	60.51	−0.2323	0.1363	6.78	−0.1456
	0.1870	89.76	−0.2092	0.1783	9.03	−0.1328
	0.2429	119.46	−0.1869	0.2316	11.92	−0.1167
	0.2953	148.47	−0.1660	0.2835	14.78	−0.1014
	0.3474	178.74	−0.1454	0.3353	17.78	−0.0864
	0.3953	207.76	−0.1267	0.3876	20.87	−0.0719
	0.4424	237.23	−0.1087	0.4365	23.79	−0.0588
1:3	0.0581	30.86	−0.1023	0.0506	2.94	0.0063
	0.1112	60.35	−0.0860	0.1011	5.91	0.0151
	0.1620	89.35	−0.0708	0.1503	8.89	0.0233
	0.2126	119.26	−0.0559	0.2009	11.98	0.0311
	0.2604	148.41	−0.0422	0.2467	14.79	0.0376
	0.3083	178.33	−0.0289	0.2943	17.76	0.0438
	0.3547	208.02	−0.0166	0.3400	20.66	0.0489
	0.3995	237.39	−0.0053	0.3897	23.77	0.0537

RESULTS AND DISCUSSION

The absorption isotherms are presented in Table I in terms of the mole fraction x_A, of the solute absorbed in the involatile solvent mixture at a solute vapour pressure P_A. The activity coefficient of the solute γ_A was calculated from these measurements as before[1,5]. Activity coefficients at infinite dilution for these systems were determined by regarding the squalane–DNP mixture as a single component and fitting the results for an individual isotherm to the Flory–Huggins expression for a binary system[6]:

$$\ln \gamma_A = \ln (\varphi_A/x_A) + 1 - \varphi_A/x_A + (1 - \varphi_A)^2\chi \quad (1)$$

In this expression (eqn. 1) the volume fraction φ_A of the solute is defined in terms of the molar volumes of the components. The pseudo-binary interaction parameter χ, allowed to vary linearly with φ_A, calculated for the solute in a particular solvent mixture enables the activity coefficient at infinite dilution γ_A^∞ to be determined[1]. The γ_A^∞ value resulting from each isotherm is reported in Table II, together with those determined earlier[5] for the solutes with the pure single solvents.

TABLE II

ACTIVITY COEFFICIENTS γ_A^∞ AND PARTITION COEFFICIENTS K_R FOR SOLUTES (A) AT INFINITE DILUTION IN SQUALANE (B)–DNP (C) MIXTURES AT 303 °K

$n_B:n_C$ = solvent mole ratio.

$n_B:n_C$	C_6H_6		C_6H_{12}		C_5H_{12}		C_6H_{14}		C_7H_{16}	
	γ_A^∞	K_R	γ_A^∞	K_R	γ^∞	K_R	γ_A^∞	K_R	γ_A^∞	K_R
1:0	0.698*	436	0.510*	587	0.620*	98.2	0.640*	306	0.669*	929
3:1	0.622	512	0.557	561	0.669	95.2	0.697**	294	0.723	899
1:1	0.580	576	0.627	522	0.753	88.6	0.785**	273	0.829	822
1:3	0.554	633	0.745	462	0.887	79.1	0.933**	242	0.997	718
0:1	0.549*	673	0.931*	389	1.115*	66.2	1.201*	198	1.313*	574

* Values from ref. 5.
** Values from ref. 1.

A partition coefficient for the solute at infinite dilution between the solvent and the vapour phase, can be calculated using the equation

$$K_R = RT/(P_A^\bullet \gamma_{P,A}^\infty V_L) \quad (2)$$

where $P_A^\bullet$ is the vapour pressure of the pure solute, $\gamma_{P,A}^\infty$ is the uncorrected activity coefficient and V_L is the molar volume of the solvent. Values of K_R calculated for the four solutes studied here are presented in Table II, together with the values for hexane derived from the previously reported results[1]. The vapour pressures, $P_A^\bullet$, were calculated from the constants for the Antoine equation given in ref. 7. The molar volume V_L of the squalane–DNP mixture was taken as a linear function of the mole fraction composition using the molar volumes of the two pure solvents reported

TABLE III

CORRECTION USED IN CALCULATING THE SOLUTE ACTIVITY COEFFICIENT AT INFINITE DILUTION

$ln\ \gamma_A^\infty - ln\ \gamma_{P,A}^\infty$				
C_6H_6	C_6H_{12}	C_5H_{12}	C_6H_{14}	C_7H_{16}
0.0089	0.0104	0.0398	0.0167	0.0069

previously[6]. The corrections necessary to calculate the activity coefficient at infinite dilution,

$$\ln \gamma_A^\infty - \ln \gamma_{P,A}^\infty = [P_A^\bullet (V_A^\bullet - B_{AA})/RT] + [(B_{AA}P_A^\bullet)^2/2(RT)^2] \quad (3)$$

are tabulated in Table III. The second virial coefficients B_{AA} and the molar volumes $V_A^\bullet$ of the solutes employed were as given previously[5,6]. The inclusion of $V_A^\bullet$ in eqn. 3 corrects for the effect of pressure on the activity of the liquid, and the terms in B_{AA} allow for the inperfection of the vapour.

Table IV shows the partition coefficients calculated for the mixed solvents from those for the pure single solvents $K_{R(B)}$ and $K_{R(C)}$, given in Table II, using the Purnell–Andrade equation[2]:

$$K_R = \varphi_B K_{R(B)} + \varphi_C K_{R(C)} \quad (4)$$

The solvent volume fractions φ_B and φ_C, like φ_A, are defined in terms of the molar volumes of the components. The percentage deviation of the partition coefficient

TABLE IV

PARTITION COEFFICIENTS FOR THE SOLUTES AT INFINITE DILUTION IN SQUALANE (B)–DNP(C) MIXTURES AT 303 °K CALCULATED FROM THE PARTITION COEFFICIENTS IN THE PURE SOLVENTS

$n_B:n_C$ = solvent mole ratio; K_R(PA) = partition coefficient calculated from the Purnell–Andrade equation (eqn. 4); K_R(FH) = partition coefficient calculated from the Flory–Huggins theory (eqn. 7); $\%\Delta$ = percentage deviation of calculated K_R from experimentally derived value in Table II.

Solute	$n_B:n_C$	$K_R(PA)$	$\%\Delta$	$K_R(FH)$	$\%\Delta$
Benzene	3:1	487	4.9	507	1.0
	1:1	543	5.7	577	0.3
	1:3	605	4.4	637	0.6
Cyclohexane	3:1	544	3.0	564	0.5
	1:1	498	4.6	524	0.4
	1:3	446	3.5	465	0.6
Pentane	3:1	91.3	4.1	95.0	0.2
	1:1	83.7	5.5	88.7	0.1
	1:3	75.4	4.7	79.0	0.1
Hexane	3:1	283	3.7	296	0.7
	1:1	257	5.9	275	0.7
	1:3	229	5.4	242	0.0
Heptane	3:1	852	5.2	898	0.1
	1:1	769	6.4	828	0.7
	1:3	676	5.8	717	0.1

K_R(PA), thus calculated, from the value given in Table II based on the extrapolated experimental measurements is given in the adjacent column in Table IV.

The three isotherms reported here in Table I for a particular solute in the mixed solvents may be combined with the two isotherms for that solute with the pure solvents reported previously[5], and the combined results for the five isotherms fitted to the Flory–Huggins expression for a ternary system[6],

$$\ln \gamma_A = \ln (\varphi_A/x_A) + 1 - \varphi_A/x_A + (1 - \varphi_A)(\varphi_B\chi_{AB} + \varphi_C\chi_{AC}) - \varphi_B\varphi_C\chi_{BC} \quad (5)$$

In the curve-fitting procedure the interaction parameters χ_{AB} and χ_{AC} are allowed to vary linearly with φ_A, thus

$$\chi_{AB} = \chi^{\circ}_{AB} + \varphi_A\chi'_{AB} \quad (6)$$

and similarly χ_{AC}, while the parameter χ_{BC} for the two solvents is assumed to be constant. The isothermal results for a particular solute in the three solvent mixtures and the two pure solvents are thus represented by these parameters, which are reported in Table V. The interaction parameter for the two solvents χ_{BC} can be used to calculate the partition coefficients in the mixed solvent phase from those in the pure solvent phase by using the relation pointed out by Perry and Tiley[8], namely that

$$\ln K_R = \varphi_B \ln K_{R(B)} + \varphi_C \ln K_{R(C)} + \varphi_B\varphi_C\chi_{BC} \quad (7)$$

Note that our χ_{BC} is equivalent to the product of the solute molar volume and the interaction parameter used by Perry and Tiley. The partition coefficients K_R(FH) derived on this basis are shown in Table IV. The percentage deviation of the partition coefficients calculated using the Flory–Huggins theory from those based on the experimental measurements is given in the adjacent column. The deviation of the partition coefficients calculated from the Purnell–Andrade equation, which basically assumes that the two solvents are immiscible, can be seen to be much greater than that using eqn. 7 based on conventional Flory–Huggins solution theory. The success of the Flory–Huggins theory in representing these systems can be seen by examination

TABLE V

FLORY–HUGGINS INTERACTION PARAMETERS DETERMINED FROM ANALYSIS OF THE COMBINED ISOTHERMS (THREE TERNARY—TABLE I AND REF. 1 AND TWO BINARY—REF. 5) FOR EACH SOLUTE (A) IN SQUALANE (B)–DNP (C) MIXTURES AT 303 °K

V^*_A = solute molar volume.

Solute	χ^{0}_{AB}	χ'_{AB}	χ^{0}_{AC}	χ'_{AC}	χ_{BC}	χ_{BC}/V^*_A *(mol dm^{-3})*
C_6H_6	0.5698	0.0824	0.1998	−0.0851	0.3416	3.797
C_6H_{12}	0.1135	0.0512	0.5626	−0.0460	0.2900	2.651
C_5H_{12}	0.2454	0.0250	0.6981	−0.0277	0.3076	2.628
C_6H_{14}	0.1908	−0.0093	0.6810	0.0223	0.3658	2.760
C_7H_{16}	0.1524	0.0365	0.6919	0.0154	0.4122	2.778

of the interaction parameter for the mixed solvents per unit molar volume of the solute $\chi_{BC}/V_A^\bullet$, shown in the last column of Table V. These values are in good agreement with each other and do not differ greatly from those found earlier for the higher concentration range[6], except that for benzene. This may be an indication that benzene is involved in molecular complex formation. Should such complexing occur in these systems, then the benzene–dinonyl phthalate system is that in which it is most likely.

CONCLUSION

Partition coefficients for benzene, cyclohexane, pentane, hexane and heptane at infinite dilution in squalane–DNP mixtures differ from the values calculated by the Purnell–Andrade equation by as much as 6%. Values calculated on the basis of the Flory–Huggins solution theory, allowing for interaction between the two solvents, differ from the experimental values by no more than 1%. This supports the previous conclusion[1] that the equation proposed by Purnell and Vargas de Andrade may be a useful approximation for predicting the behaviour of mixed solvents in GLC, but the ternary systems we have studied conform more to conventional theory of liquid mixtures rather than to a "micro-partitioning" theory.

APPENDIX

Derivation of $\ln K_R = \varphi_B \ln K_{R(B} + \varphi_C \ln K_{R(C)} + \varphi_B\varphi_C\chi_{BC}$ (eqn. 7)

$$Q_A = \frac{x_A V_A^\bullet}{x_A V_A^\bullet + (1 - x_A) V_L}$$

$$\therefore \quad \lim_{x_A \to 0} \left(\frac{\varphi_A}{x_A}\right) = \frac{V_A^\bullet}{V_L}$$

$$\therefore \quad \text{from eqn. 5} \quad \ln \gamma_A^\infty = \ln \frac{V_A^\bullet}{V_L} + 1 - \frac{V_A^\bullet}{V_L} + \varphi_B\chi_{AB} + \varphi_C\chi_{AC} - \varphi_B\varphi_C\chi_{BC}$$

$$\therefore \quad \text{from eqn. 2} \quad \ln K_R = \ln \frac{RT}{P_A^\bullet} + (\ln \gamma_A^\infty - \ln \gamma_{P,A}^\infty) - \ln V_A^\bullet - 1 + \frac{V_A^\bullet}{V_L}$$

$$- \varphi_B\chi_{AB} - \varphi_C\chi_{AC} + \varphi_B\varphi_C\chi_{BC}$$

Applying this result to the binary systems:

$$\ln K_{R(B)} = \ln \frac{RT}{P_A^\bullet} + (\ln \gamma_A^\infty - \ln \gamma_{P,A}^\infty) - \ln V_A^\bullet - 1 + \frac{V_A^\bullet}{V_B^\bullet} - \chi_{AB}$$

and

$$\ln K_{R(C)} = \ln \frac{RT}{P_A^\bullet} + (\ln \gamma_A^\infty - \ln \gamma_{P,A}^\infty) - \ln V_A^\bullet - 1 + \frac{V_A^\bullet}{V_C^\bullet} - \chi_{AC}$$

where $V_B^\bullet$ and $V_C^\bullet$ are the molar volumes of the pure B and C, and where ($\ln \gamma_A^\infty - \ln \gamma_{P,A}^\infty$) is, according to eqn. 3, independent of the solvent.

Since $\varphi_B + \varphi_C = 1$ at infinite dilution it follows that

$$\varphi_B \ln K_{R(B)} + \varphi_C \ln K_{R(C)} = \ln \frac{RT}{P_A^\bullet} + (\ln \gamma_A^\infty - \ln \gamma_{P,A}^\infty) - \ln V_A^\bullet - 1$$

$$+ V_A^\bullet \left(\frac{\varphi_B}{V_B^\bullet} + \frac{\varphi_C}{V_C^\bullet}\right) - \varphi_B \chi_{AB} - \varphi_C \chi_{AC}$$

But at infinite dilution $\varphi_B = x_B V_B^\bullet / V_L$ and $\varphi_C = (1 - x_B) V_C^\bullet / V_L$ where x_B is the mole fraction of B.

$$\therefore \quad \frac{\varphi_B}{V_B^\bullet} + \frac{\varphi_C}{V_C^\bullet} = \frac{1}{V_L}$$

Hence, comparing with the expression for $\ln K_R$ above, eqn. 7 follows.

REFERENCES

1 A. J. Ashworth and D. M. Hooker, *J. Chromatogr.*, 131 (1977) 399.
2 J. H. Purnell and J. M. Vargas de Andrade, *J. Amer. Chem. Soc.*, 97 (1975) 3585.
3 A. J. Ashworth, *Prog. Vac. Microbalance Tech.*, 1 (1972) 313.
4 A. J. Ashworth and D. M. Hooker, *Prog. Vac. Microbalance Tech.*, 3 (1975) 330.
5 A. J. Ashworth, *J. Chem. Soc., Farad. Trans. I*, 69 (1973) 459.
6 A. J. Ashworth and D. M. Hooker, *J. Chem. Soc., Farad. Trans. I*, 72 (1976) 2240.
7 R. R. Dreisbach, *Adv.an in Chem. Ser.*, No. 15 (1955) and No. 22 (1959).
8 R. W. Perry and P. F. Tiley, *J. Chem. Soc., Farad. Trans. I*, 74 (1978) 1655.

Journal of Chromatography, 174 (1979) 315–323

CHROM. 11,790

MOLEKÜLSTRUKTUR UND RETENTIONSVERHALTEN

XII. ZUR RETENTION VON ALKYLNAPHTHALINEN BEI DER GAS-VERTEILUNGS- UND GAS-ADSORPTIONS-CHROMATOGRAPHIE*,**

W. ENGEWALD, L. WENNRICH und E. RITTER

Sektion Chemie der Karl-Marx-Universität Leipzig, Liebigstrasse 18, 701 Leipzig (D.D.R.)

(Eingegangen am 9. März 1978; geänderte Fassung eingegangen am 9. Februar 1979)

SUMMARY

Molecular structure and retention behaviour. XII. Retention behaviour of alkylnaphthalenes by gas–liquid and by gas–solid chromatography

The retention indices of 1-alkyl- and 2-alkylnaphthalenes, of isomeric dimethylnaphthalenes, of some trimethylnaphthalenes and methylazulenes have been determined by gas–liquid chromatography (GLC) on glass capillaries of different polarity (methylsilicone OV-1, Ucon LB 550 X and Ucon 50 HB 280 X polar) and by gas–solid chromatography (GSC) on graphitized thermal carbon black, respectively. The retention within homologous series of straight chain 1-alkyl- and 2-alkylnaphthalenes, of isomeric butylnaphthalenes and dimethylnaphthalenes is discussed by means of homomorphic factors and index differences and compared with corresponding values of alkylbenzenes. The different retention behaviour of the isomeric dimethylnaphthalenes by GLC and by GSC can be described by an incremental approach.

EINLEITUNG

Die gaschromatographische Analyse von Alkylnaphthalinen ist Gegenstand von Arbeiten verschiedener Autoren (vgl. Lit. 1–5 und dort zitierte Literatur), in denen besonders die Trennung der sich in ihren Eigenschaften nur geringfügig unterscheidenden Positionsisomeren untersucht wurde. Da die Auftrennung der Ethylnaphthaline sowie der isomeren Di- und Trimethylnaphthaline auf konventionellen Säulen Schwierigkeiten bereitet, wurden neben leistungsfähigen Trennkapillaren[1] auch "geometrieselektive" Phasen, wie z.B. Benton 34 [2,5], graphitierter Russ[2,3] oder kristalline Flüssigkeiten[4,5] angewandt. Dagegen existieren nur wenige Angaben über an ver-

* XI. Mitteilung: Lit. 9.

** Auszugsweise vorgetragen auf dem Analytiktreffen 1976 "Fortschritte in der Gas- und Flüssigkeits-Chromatographie", Leipzig, 1976.

schiedenen stationären Phasen gemessene Retentionsindices[6,7], die für die Individuenanalyse isomerer Alkylaromaten in komplexen Gemischen von Bedeutung sind.

In Fortsetzung unserer Untersuchungen über das gaschromatographische Verhalten von Alkylbenzolen[8,9] beschäftigten wir uns daher auch mit der Retention von Alkylnaphthalinen bei der Gas-Verteilungs-Chromatographie (GVC) und Gas-Adsorptions-Chromatographie (GAC). Es wurden die Retentionsindices geradkettiger und verzweigter 1- bzw. 2-Alkylnaphthaline, der isomeren Dimethylnaphthaline sowie einiger Trimethylnaphthaline bei der GVC an Glaskapillaren unterschiedlicher Polarität und bei der GAC an graphitiertem thermischen Russ (GTR) gemessen. In die Untersuchungen wurde auch das dem Naphthalin isoelektronische Azulen und einige Methylazulene einbezogen.

EXPERIMENTELLES

GVC

Die Bestimmung der Retentionsindices erfolgte an folgenden Glaskapillaren, deren Herstellung und Säulenpolaritäten in Lit. 8 und 10 näher beschrieben sind: 50 m × 0.23 mm I.D. OV-1; 70 m × 0.23 min I.D. Ucon LB 550 X; 70 m × 0.23 mm I.D. Ucon 50 HB 280 X polar.

Die Messungen wurden an einem Eigenbau-Kapillargaschromatographen bzw. einem Gerät Moduline 2740 (Varian, Palo Alto, Calif., U.S.A.) im Temperaturbereich von 120–150° durchgeführt; die Totzeit wurde aus den Bruttoretentionszeiten aufeinanderfolgender *n*-Alkane ermittelt.

GAC

Die Bestimmung der Retentionsindices an GTR erfolgte an einer mit GTR Sterling MT (Phase Separations, Sohlingen, B.R.D.) der Korngrösse 0.20–0.25 mm gefüllten Säule von 1.2 m Länge und einem Innendurchmesser von 1.9 mm bzw. an einer Mikropack-Säule[11,12] von 1.5 m × 0.8 mm I.D., gefüllt mit GTR-Pellets der Grösse 0.16–0.20 mm. Beide Säulen wurden jeweils über einen Eingangssplitter in den Gaschromatographen Moduline 2740 eingebaut.

ERGEBNISSE UND DISKUSSION

In Tabelle I sind die an den stationären Phasen OV-1 (I^{OV}), Ucon LB 550 X (I^{LB}), Ucon 50 HB 280 X polar (I^{HB}) und GTR (I^{GTR}) gemessenen Retentionsindices der untersuchten Kohlenwasserstoffe zusammengestellt. Die Tabelle enthält ebenfalls die an Ucon LB und Ucon HB erhaltenen Temperaturkoeffizienten ($\partial I/\partial T$) und die Indexdifferenzen $\Delta I^{HB-OV} = I^{HB} - I^{OV}$.

In Anlehnung an vorangegangene Arbeiten[8,9] soll im vorliegenden Beitrag das Retentionsverhalten anhand von Homomorphiefaktoren* H^{OV} und H^{GTR} sowie von ΔI-Werten der unsubstituierten Kohlenwasserstoffe, der homologen Reihen von geradkettigen 1- und 2-Alkylnaphthalinen, der verzweigten 1- und 2-Butylnaphthaline und der isomeren Dimethylnaphthaline bei der GVC und GAC diskutiert und mit der Retention entsprechender Benzole verglichen werden.

* Als homomorphe Kohlenwasserstoffe werden die *n*-Alkane gleicher C-Zahl betrachtet.

TABELLE I

RETENTIONSINDICES VON ALKYLNAPHTHALINEN UND METHYLAZULENEN BEI DER GVC AN OV-1 (I^{OV}), UCON LB 550 X (I^{LB}), UCON 50 HB 280 X POLAR (I^{HB}) UND BEI DER GAC AN GTR (I^{GTR})

GVC: $T_{Saule} = 130°$.

Verbindung	*GVC*					*GAC*		
	I^{OV}	I^{LB}	$\frac{\partial I}{\partial T} \cdot 10$	I^{HB}	$\frac{\partial I}{\partial T} \cdot 10$	ΔI^{HB-OV}	I^{GTR}	T_{Saule} (°C)
Naphthalin	1183.7	1361.0	7.7	1468.2	8.5	284.5	1091	270
1-Methyl-	1301.7	1483.0	7.5	1592.5	7.5	290.8	1238	270
2-Methyl-	1286.8	1463.9	7.2	1568.0	9.1	281.2	1252	270
1-Ethyl-	1379.2	1552.2	8.0	1658.6	8.3	279.4	1288	270
2-Ethyl-	1377.4	1552.2	8.0	1655.0	7.8	277.6	1301	270
1-*n*-Propyl-	1460.2	1624.2	8.9	1728.8	8.3	268.6	1378	320
2-*n*-Propyl-	1465.2	1631.8	7.9	1734.8	8.0	269.6	1395	320
2-*iso*-Propyl-	1434.8	1599.3	6.4	1701.2	6.7	266.4	1307	320
1-*n*-Butyl-	1555.2	1719.7	7.7	1825.0	7.2	269.8	1469	320
2-*n*-Butyl-	1564.1	1731.8	7.4	1835.0	8.4	270.9	1487	320
1-*iso*-Butyl-	1500.8	1653.5	7.6	1753.1	8.6	252.3	1418	320
2-*iso*-Butyl-	1512.6	1669.3	8.1	1769.8	8.5	257.2	1442	320
2-*sec*-Butyl-	1520.7	1674.6	7.6	1776.1	8.0	255.4	1349	320
2-*tert*-Butyl-	1502.0	1655.8	7.5	1757.5	8.5	255.5	1332	320
2,6-Dimethyl-	1388.2	1563.4	7.0	1668.1	7.8	279.9	1412	270
2,7-Dimethyl-	1390.1	1566.6	7.6	1670.4	8.9	280.3	1414	320
1,3-Dimethyl-	1402.3	1591.2	7.6	1691.8	8.6	289.5	1392	270
1,6-Dimethyl-	1402.2	1590.8	7.7	1691.2	8.5	289.0	1393	270
1,7-Dimethyl-	1409.5	—	—	1699.8	—	290.3	1403	320
1,4-Dimethyl-	1419.0	1607.3	8.1	1711.7	9.6	292.7	1384	270
2,3-Dimethyl-	1419.6	1603.8	7.3	1712.8	8.9	293.2	1421	320
1,5-Dimethyl-	1420.7	1603.8	7.2	1713.1	9.5	292.4	1382	270
1,2-Dimethyl-	1432.0	1617.5	8.6	1729.1	9.9	297.1	1405	270
1,8-Dimethyl-	1449.1	1641.8	8.4	1756.8	10.1	307.7	1378	270
1,3,7-Trimethyl-	1500.8	1680.3	7.5	1784.6	8.7	283.8	1558	320
2,3,6-Trimethyl-	1522.0	1702.9	7.3	1809.8	8.9	287.8	1585	320
2,3,5-Trimethyl-	1533.4	1718.8	7.3	1827.4	8.3	294.0	1569	320
Azulen	1295.9	1501.0	8.2	1624.0	9.5	328.1	—	
1-Methyl-	1401.4	1603.0	10.1	1723.8	10.0	322.4	—	
5-Methyl-	1400.0	1605.5	9.4	1726.3	10.0	326.3	—	
6-Methyl-	1409.7	1616.3	9.9	1739.6	10.4	329.9	—	
4,6,8-Trimethyl-	1637.6	1855.1	11.7	1986.3	10.6	348.7	—	

Retention von Benzol, Naphthalin und Azulen

Die im Vergleich zum Benzol ($H^{OV} = 64$; $\Delta I = 158$ Indexeinheiten (IE) bei 100°) bedeutend höheren Homomorphiefaktoren H^{OV} und ΔI-Werte von Naphthalin ($H^{OV} = 184$; $\Delta I = 284.5$ IE) und Azulen ($H^{OV} = 296$; $\Delta I = 328$ IE) weisen auf die relativ grosse Fähigkeit der 10-π-Elektronensysteme zu zwischenmolekularen Wechselwirkungen mit unpolaren und polaren Trennflüssigkeiten hin. Für den höheren ΔI-Wert des Azulens ist die für einen Kohlenwasserstoff beträchtliche Polarität dieses nichtalternierenden aromatischen Systems verantwortlich, die durch eine π-Elek-

tronenverschiebung vom 7- zum 5-Ring bewirkt wird und auch in einem Dipolmoment von 1.1 D zum Ausdruck kommt.

Auch bei der GAC an GTR werden für kondensierte Aromaten relativ hohe Retentionswerte gefunden. Während Benzol vor dem *n*-Hexan eluiert wird, besitzen Naphthalin, Anthracen und Phenanthren positive H^{GTR}-Werte (Azulen wurde an GTR nicht eluiert), obwohl mit Vergrösserung des kondensierten Ringsystems die Anzahl der H-Atome und damit deren inkrementeller Beitrag zur Potentialenergie der Adsorption relativ zu den *n*-Alkanen abnimmt (vgl. Lit. 13).

Homologe Reihen der 1- und 2-Alkylnaphthaline

In Fig. 1a sind die an OV-1 erhaltenen Homomorphiefaktoren der unverzweigten 1- bzw. 2-Alkylnaphthaline sowie der entsprechenden Alkylbenzole als Funktion der C-Zahl *n* der Seitenkette dargestellt. Es ist zu erkennen, dass die bei den Alkylbenzolen diskutierten Befunde[8], (1) Erhöhung der Homomorphiefaktoren bei Methylsubstitution und Erniedrigung bei Substitution durch grössere Alkylgruppen und (2) relativ starke Abnahme der *H*-Werte bis zur Propylgruppe, auch für die 1- und 2-Alkylnaphthaline zutreffen.

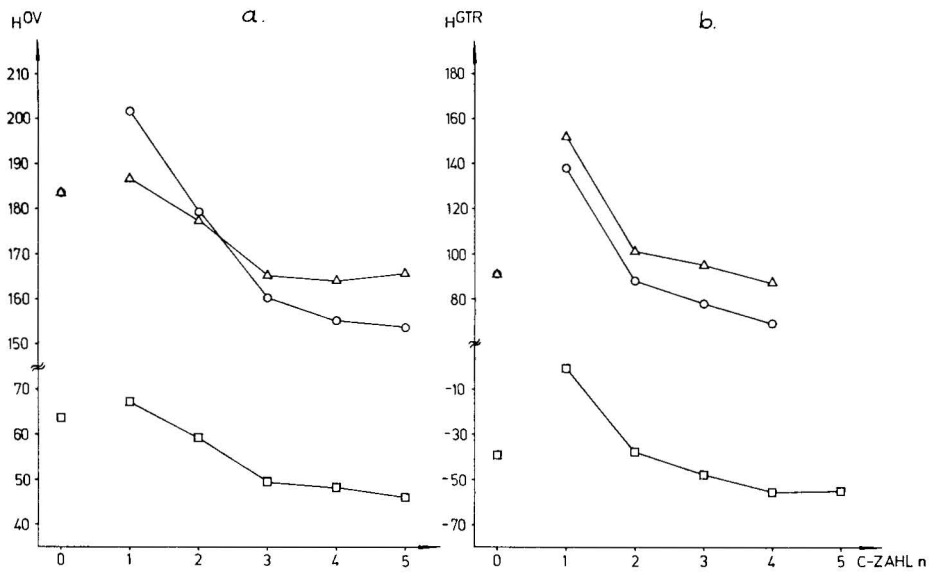


Fig. 1. Abhängigkeit der Homomorphiefaktoren H^{OV} (a) bzw. H^{GTR} (b) unverzweigter 1-Alkylnaphthaline (○), 2-Alkylnaphthaline (△) und Alkylbenzole (□) von der C-Zahl *n* der Alkylgruppe.

Entsprechend ihrer grösseren thermodynamischen Stabilität und den damit verbundenen niedrigeren Siedepunkten werden 2-Methyl- und 2-Ethylnaphthalin vor den 1-Isomeren eluiert; bei den höheren Homologen ist die Siedepunkts- und Elutionsreihenfolge umgekehrt. Bei der GAC an GTR eluieren die unverzweigten Alkylnaphthaline ebenfalls nach den *n*-Alkanen gleicher C-Zahl (Fig. 1b). Im Vergleich dazu werden bei Alkylbenzolen positive H^{GTR}-Werte nur dann gefunden, wenn mehrere Methylgruppen im Molekül vorhanden sind[9]. Der hohe Beitrag der Methylgruppe zur Retention (α-Me: $\delta H^{GTR} = 47$ IE; β-Me: $\delta H^{GTR} = 62$ IE) und die Ab-

nahme der H^{GTR}-Werte mit Vergrösserung der Seitenkette entspricht den bei Alkylbenzolen erhaltenen Resultaten. An diesem Adsorbens besitzen 2-Alkylnaphthaline stets grössere Retentionswerte als die 1-Isomeren. Da die Retentionsunterschiede zwischen 1- und 2-substituierten Naphthalinen an GTR ausnahmslos grösser sind als bei der GVC, lassen sich diese Positionsisomeren an kurzen, mit GTR gepackten Säulen trennen (Fig. 2).

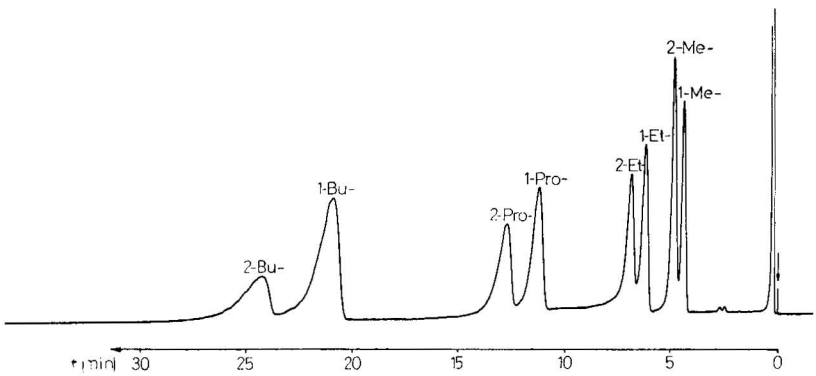


Fig. 2. Trennung positionsisomerer Alkylnaphthaline an einer mit GTR Sterling MT (Korngrösse 0.16–0.20 mm) gefüllten Mikropack-Säule von 1.5 m Länge und 0.8 mm I.D. bei 320°.

In Fig. 3 sind die Indexdifferenzen ΔI^{HB-OV} von Alkylnaphthalinen und Alkylbenzolen als Funktion der C-Zahl der Alkylgruppe dargestellt. Daraus ist zu entnehmen, dass in Analogie zu anderen cyclischen Systemen[8,14,15] auch Alkylnaphthaline eine geringere gaschromatographische Polarität besitzen als der unsubstituierte Kohlenwasserstoff. Eine Ausnahme bildet lediglich 1-Methylnaphthalin, dessen im Vergleich zum Naphthalin höherer ΔI-Wert mit der sterischen Spannung dieses Moleküls im Einklang steht, die durch eine gehinderte Rotation der Methylgruppe infolge Wechselwirkungen mit dem peripheren H-Atom[16,17,20] hervorgerufen wird. (Interessanterweise verändert auch im Azulensystem eine Methylgruppe je nach ihrem Substitutionsort die gaschromatographische Polarität in unterschiedlicher Weise: Methylgruppen in den Positionen 1 und 5 führen zu einer Erniedrigung, in den Positionen 4 und 6 zu einer Erhöhung der ΔI-Werte im Vergleich zum Grundkörper. Als Ursache kann die alternierende Ladungsverteilung in diesen nichtbenzoiden Aromaten angesehen werden.)

Verzweigte Butylnaphthaline

Mit zunehmender Verzweigung der Alkylgruppe nimmt die Retention sowohl bei der GVC als auch bei der GAC ab. Das Retentionsverhalten der isomeren 2-Butylnaphthaline weist mit dem der Butylbenzole folgende Gemeinsamkeiten auf:

(1) Abnahme der Retention bei der GVC an den verwendeten Trennflüssigkeiten in der gleichen Reihenfolge $I_n > I_{sec} > I_{iso} > I_{tert}$ und in der gleichen Grössenordnung (z.B. gleiche Retentionsintervalle für OV-1 mit 62 IE).

(2) Gleiche Elutionsreihenfolgen bei der GAC an GTR: $I_n > I_{iso} > I_{sec} > I_{tert}$.

Für die isomeren 2-Butylnaphthaline ist das Retentionsintervall an GTR mit

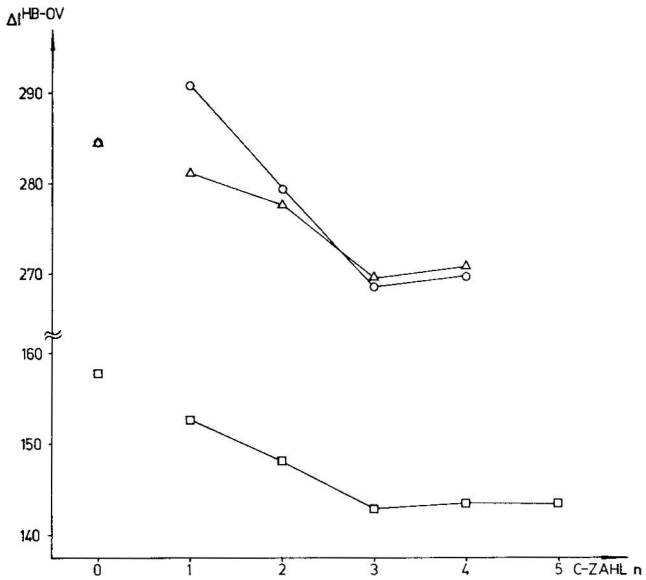


Fig. 3. Abhängigkeit der Indexdifferenzen ΔI^{HB-OV} unverzweigter 1-Alkylnaphthaline (○), 2-Alkylnaphthalene (△) und Alkylbenzole (□).

155 IE wesentlich grösser als bei der GVC und übertrifft auch das der Butylbenzole (99 IE). An diesem Adsorbens ist der grosse Retentionsunterschied zwischen *iso*- und *sec*-Butylnaphthalin bemerkenswert.

Dimethylnaphthaline (DMN)

Aus den Retentionsindices in Tabelle I ist ersichtlich, dass an schwach polaren Trennflüssigkeiten keine Auftrennung der drei schwierig zu trennenden Isomerengruppen, 2,6-/2,7-DMN, 1,3-/1,6-DMN und 1,4-/1,5-/1,2-DMN möglich ist.

Zum Verständnis des Retentionsverhaltens der DMN bei der GVC muss man in Betracht ziehen, dass die bei den isomeren Methylnaphthalinen und Polymethylbenzolen abgeleiteten Struktur-Retentionsbeziehungen (1) grössere Retention für Methylgruppen in α-Position, (2) grössere Retention für benachbarte Methylgruppen sowie elektronische und geometrische Faktoren in komplexer Weise zusammenwirken. Dementsprechend besitzen an OV-1 die Isomeren mit zwei β-ständigen Methylgruppen (2,6- und 2,7-DMN) die kleinsten Retentionswerte, wobei die etwas geringere Retention des 2,6-DMN durch die grössere Symmetrie der Ladungsverteilung plausibel wird. Es folgen die Dimethylnaphthaline mit einer Methylgruppe in α-Position (1,6-, 1,3- und 1,7-DMN), von denen das 1,7-DMN entsprechend seiner grösseren Unsymmetrie der Ladungsverteilung eine grössere Retention besitzt. Zuletzt werden die Dimethylnaphthaline mit zwei Methylgruppen in α-Position (1,4-, 1,5- und 1,8-DMN) bzw. mit benachbarten Methylgruppen (2,3- und 1,2-DMN) eluiert. [Diese Reihenfolge kommt auch in dem von Altenburg[19] zur Beschreibung des Retentionsverhaltens isomerer Kohlenwasserstoffe verwendeten Molekülparameter n_3, d.h. der Anzahl der durch drei C-C-Bindungen voneinander getrennten Kohlenstoffatome, zum Ausdruck (vgl. Lit. 19): Für Dimethylnaphthaline mit zwei β-Methylgruppen ergibt sich $n_3 = 22$,

mit einer α- und einer β-Methylgruppe 23 und mit zwei Methylgruppen in α-Position ist $n_3 = 24$. Bei benachbarten Methylgruppen erhöht sich die n_3-Zahl um 1 (Tabelle II).] Auch innerhalb dieser Gruppe bewirkt die Unsymmetrie der Ladungsverteilung eine Erhöhung der Retention von 1,2- und 1,8-DMN gegenüber 1,4- und 1,5-DMN. Als wesentliche Ursache für die hohe Retention des 1,8-DMN ist die sterische Hinderung der periständigen Methylgruppen anzusehen.

TABELLE II

INKREMENTELLE BERECHNUNG DER RETENTION ISOMERER DIMETHYL- UND TRIMETHYLNAPHTHALINE BEI DER GVC UND GAC

Substanz	*Methylgruppeninkrement*			n_3	I^{OV}		$\Delta I^{HB\text{-}OV}$		I^{GTR}	
	α-Me	*β-Me*	*vic. Me*		*theor.*	*exp.*	*theor.*	*exp.*	*theor.*	*exp.*
2,6-DMN	0	2		22	1390	1388	278.5	279.9	1413	1412
2,7-DMN	0	2		22	1390	1390	278.5	280.3	1413	1414
1,6-DMN	1	1		23	1405	1402	287.5	289.0	1399	1393
1,3-DMN	1	1		23	1405	1402	287.5	289.5	1399	1392
1,7-DMN	1	1		23	1405	1410	287.5	290.3	1399	1403
1,4-DMN	2	0		24	1420	1419	296.5	292.4	1385	1384
2,3-DMN	0	2	1	23	1416	1420	291	293.2	1423	1421
1,5-DMN	2	0		24	1420	1421	296.5	292.4	1385	1382
1,2-DMN	1	1	1	24	1429	1432	300	297.1	1409	1405
1,8-DMN	2	0	1	24	1444	1449	309	307.7	1395	1378
1,3,7-TMN	1	2	–	25	1508	1501	284.5	283.5	1560	1558
2,3,6-TMN	–	3	1	25	1517	1522	288.0	287.8	1584	1585
2,3,5-TMN	1	2	1	26	1532	1533	297.0	294.0	1570	1569

Es wurde versucht, die Indices der Dimethylnaphthaline aus Inkrementen für die α- und β-ständige Methylgruppe $\delta H_{\alpha-Me} = 18$ IE und $\delta H_{\beta-Me} = 3$ IE sowie einem Inkrement II. Ordnung (vgl. Lit. 18) für benachbarte Methylgruppen $H^{II}_{vic} = 24$ IE, das aus I^{OV}-Werten der positionsisomeren Dimethylbenzole abgeleitet wurde, zu berechnen:

$$I_{DMN} = 100\,m + H_{Naph.} + \Sigma\delta H_{Me}$$

$$\Sigma\delta H_{Me} = \delta H_{\alpha-Me} + \delta H_{\beta-Me} + H^{II}_{vic}$$

wobei m = C-Zahl des Moleküls.

Da der Substituentenabstand in perisubstituierten Naphthalinen mit den in entsprechenden *o*-substituierten Aromaten vergleichbar bzw. sogar geringer ist[16], erschien es berechtigt, das Inkrement II. Ordnung für benachbarte Methylgruppen auch beim 1,8-DMN anzuwenden.

Nach der gleichen Methode wurden die ΔI-Werte der Dimethylnaphthaline inkrementell berechnet ($\delta\Delta I_{\alpha-Me} = 6$ IE, $\delta\Delta I_{\beta-Me} = -3$ IE, $\Delta I^{II}_{vic} = 12.5$ IE):

$$\Delta I_{DMN} = \Delta I_{Naph.} + \Sigma\delta\Delta I$$

In Tabelle II sind die berechneten I^{OV}- und ΔI-Werte den experimentellen Daten gegenübergestellt. Daraus geht hervor, dass die berechneten Werte recht gut mit den

experimentellen übereinstimmen. Die Abweichungen sind kleiner als 5 IE und lassen sich u.a. auf die Vernachlässigung der oben diskutierten Einflüsse der Ladungsverteilung bei dieser Abschätzung erklären.

Auf die im Vergleich zur GVC unterschiedliche Retentionsreihenfolge von Dimethylnaphthalenen bei der GAC an GTR wurde bereits von Fryčka[2] und Gonnord *et al.*[3] aufmerksam gemacht. Von den letzteren Autoren konnte die Retentionsfolge einiger isomerer Dimethylnaphthaline durch molekularstatistische Berechnungen richtig beschrieben werden.

Die Retentionsfolge an GTR wird ebenfalls verständlich, wenn man von der höheren Retention des 2-Methylnaphthalins sowie von den Retentionsregeln bei Polymethylbenzolen (grösste Retention bei Isomeren mit benachbarten Methylgruppen) ausgeht. Demzufolge werden zuerst die Dimethylnaphthaline mit zwei α-ständigen Methylgruppen (1,8-, 1,5- und 1,4-DMN) eluiert, es folgen die Isomeren mit einer α- und einer β-ständigen Methylgruppe (1,3-, 1,6-, 1,7- und 1,2-DMN) und zuletzt eluieren die Dimethylnaphthaline mit beiden Methylgruppen in β-Position. Innerhalb dieser Gruppen besitzen jeweils die Isomeren mit benachbarten Methylgruppen (1,2- bzw. 2,3-DMN) die grössten I^{GTR}-Werte. Der Retentionsbereich ist für die Dimethylnaphthaline an GTR mit 43 IE kleiner als bei der GVC (61 IE für OV-1).

In Analogie zur GVC lassen sich die I^{GTR}-Werte ebenfalls aus Inkrementen für die Methylgruppe in α- bzw. β-Position (47 bzw. 61 IE) sowie einem aus den I^{GTR}-Werten der isomeren Dimethylbenzole[9] abgeleiteten Inkrement für benachbarte Methylgruppen ($H^{II}_{vic} = 10$ IE) berechnen. Aus Tabelle II wird deutlich, dass mit Ausnahme des 1,8-Dimethylnaphthalins die inkrementell berechneten Werte gut mit den experimentellen Retentionsindices übereinstimmen. Die Tabelle veranschaulicht weiterhin, dass die in der GVC schwierig zu trennenden Substanzpaare sich auch in ihren die GTR-Retention bestimmenden Strukturmerkmalen kaum unterscheiden, so dass selbst bei Verbesserung der Trennleistung an diesem Adsorbens keine vollständige Auftrennung aller isomeren Dimethylnaphthaline erreicht werden kann.

Mit den zur Beschreibung der Retention von Dimethylnaphthalinen angewandten Beziehungen kann auch das Retentionsverhalten der untersuchten Trimethylnaphthaline erklärt werden, deren Retentionswerte sich sowohl bei der GVC als auch der GAC durch Addition der entsprechenden Inkremente abschätzen lassen (Tabelle II).

SCHLUSSFOLGERUNGEN

Alkylnaphthaline und Alkylazulene zeichnen sich durch relativ hohe Homomorphiefaktoren, ΔI-Werte und Temperaturkoeffizienten aus, die sich zur gaschromatographischen Charakterisierung dieser aromatischen Kohlenwasserstoffe heranziehen lassen. Innerhalb der homologen Reihen der monosubstituierten Alkylnaphthaline folgt die Retention den gleichen Beziehungen wie die der Alkylbenzole.

Die durch Wechselwirkung der α-Methylgruppe mit dem H-Atom bzw. einer CH_3-Gruppe in *peri*-Stellung verursachte sterische Spannung führt im Vergleich zur β-Methylgruppe bei der GVC zu erhöhten, bei der GAC an GTR zu erniedrigten Retentionswerten. Die Anzahl α- und β-ständiger sowie vicinaler Methylgruppen sind daher bestimmend für die unterschiedlichen Elutionsfolgen isomerer Dimethyl- und Trimethylnaphthaline bei der GVC und GAC.

ZUSAMMENFASSUNG

Die Retentionsindices von 1- und 2-Alkylnaphthalinen bis C_{14}, der isomeren Dimethylnaphthaline sowie einiger Trimethylnaphthaline und Methylazulene werden bei der Gas-Verteilungs-Chromatographie (GVC) an Glaskapillaren unterschiedlicher Polarität (OV-1, Ucon LB 550 X und Ucon HB 280 X polar) sowie bei der Gas-Adsorptions-Chromatographie (GAC) an graphitiertem thermischen Russ gemessen. Anhand der daraus abgeleiteten Homomorphiefaktoren und Indexdifferenzen wird die Retention innerhalb homologer Reihen geradkettiger 1- und 2-Alkylnaphthaline, isomerer Butylnaphthaline und Dimethylnaphthaline diskutiert und mit den entsprechenden Werten von Alkylbenzolen verglichen.

Das unterschiedliche Retentionsverhalten der isomeren Dimethylnaphthaline bei der GVC und GAC lässt sich durch eine inkrementelle Betrachtung der retentionsbestimmenden Faktoren gut beschreiben.

DANK

Frau Dr. I. Topalova von der Universität Sofia danken wir für die Bereitstellung einiger Alkylnaphthaline.

LITERATUR

1 J. Mostecky, M. Popl und J. Kříž, *Anal. Chem.*, 42 (1970) 1132.
2 J. Fryčka, *Chromatographia*, 8 (1975) 413.
3 M.-F. Gonnord, C. Vidal-Madjar und G. Guiochon, *J. Chromatogr. Sci.*, 12 (1974) 839.
4 S. Wasik und S. Chesler, *J. Chromatogr.*, 122 (1976) 451.
5 K. Tesarik, J. Frycka und S. Ghyczy, *J. Chromatogr.*, 148 (1978) 223.
6 D. I. Papazova und M. C. Pankova, *J. Chromatogr.*, 105 (1975) 411.
7 M. C. Pankova, R. S. Milina, R. P. Belcheva und A. S. Ivanov, *J. Chromatogr.*, 137 (1977) 198.
8 W. Engewald und L. Wennrich, *Chromatographia*, 9 (1976) 540.
9 W. Engewald, L. Wennrich und J. Pörschmann, *Chromatographia*, 11 (1978) 434.
10 I. Stopp, W. Engewald, H. Kühn und Th. Welsch, *J. Chromatogr.*, 147 (1978) 21.
11 Th. Welsch, W. Engewald und J. Pörschmann, *J. Chromatogr.*, 148 (1978) 143.
12 Th. Welsch, W. Engewald und J. Pörschmann, *J. Prakt. Chem.*, 320 (1978) 493.
13 H.-J. Hofmann, W. Engewald, D. Heidrich, J. Pörschmann, K. Thieroff und P. Uhlmann, *J. Chromatogr.*, 115 (1975) 299.
14 C. E. Döring, D. Estel und R. Fischer, *J. Prakt. Chem.*, 316 (1974) 1.
15 G. Schomburg, *J. Chromatogr.*, 14 (1964) 157.
16 V. Balasubramaniyan, *Chem. Rev.*, 66 (1966) 567.
17 J. Galli, J. Fruwert und G. Geiseler, *Z. Chem.*, 16 (1976) 57.
18 G. Schomburg, *Chromatographia*, 4 (1971) 286.
19 K. Altenburg, in H. G. Struppe (Herausgeber), *Gas Chromatographie 1968*, Akademie-Verlag, Berlin, 1968, S. 1.
20 V. V. Voronenkov und Ju. G. Osokin, *Usp. Khim.*, 41 (1972) 1366.

Journal of Chromatography, 174 (1979) 325–339

CHROM. 11,801

GAS–LIQUID CHROMATOGRAPHY OF SOME ALKYL DERIVATIVES OF 5-FLUOROURACIL

ANDRÉ P. DE LEENHEER and M. Cl. COSYNS-DUYCK

Laboratoria voor Medische Biochemie en voor Klinische Analyse, Faculteit van de Farmaceutische Wetenschappen, Rijksuniversiteit, 135 De Pintelaan, B-9000 Gent (Belgium)

(Received February 16th, 1979)

SUMMARY

Various procedures for converting 5-fluorouracil into its methyl, butyl and hexyl derivatives are described. Structures were established as the N,N′-dialkyl derivatives using mass spectrometry or combined gas–liquid chromatography–mass spectrometry.

The reaction conditions, *i.e.*, the amount of derivatization reagents and reaction time, were optimized. Gas–liquid chromatographic characteristics of the derivatives were investigated on different stationary liquid phases, and 2% or 3% SP-2250, 5% XE-60 and 5% OV-1 were found to be superior. With 5-chlorouracil as the internal standard a linear response for the various derivatives was observed in the microgram range. The applicability of the different dialkyl derivatives in the measurement of 5-fluorouracil in biological materials is discussed.

INTRODUCTION

5-Fluorouracil (5-FU), an antimetabolite of uracil, plays an important role in the chemotherapy of certain forms of cancer[1–4]. A specific and sensitive assay for 5-FU in biological fluids is needed for the evaluation of its chemotherapeutic regimens.

In recent years, a few gas–liquid chromatographic (GLC) assays for 5-FU have been described using flame-ionization detection (FID)[5,6], electron-capture detection[7] and multiple-ion detection[8–10]. Most of these techniques are based on detection of the silyl derivative of the drug[5,8] and on the use of internal standards such as anthracene[6] or thymine[7,9]. Both substances appear to be inappropriate for determination of 5-FU in biological samples, as anthracene is structurally far removed from 5-FU and thymine may occur endogenously.

This paper describes some rapid, simple and virtually quantitative alkylation methods for 5-FU, yielding stable derivatives that are amenable to GLC and to mass fragmentographic analysis[10]. 5-Chlorouracil (5-ClU) is proposed as an internal standard for quantitative measurements.

EXPERIMENTAL

Materials

Sources of reference compounds and reagents are as follows: 5-FU, Sigma (St. Louis, Mo., U.S.A.); 5-ClU, Calbiochem (Lucerne, Switzerland); *n*-alkanes, Poly-Science Corp. (Evanston, Ill., U.S.A.); dried dimethyl sulphoxide (DMSO), Merck (Darmstadt, G.F.R.); N,N-dimethylacetamide (DMA), >99%, Aldrich-Europe (Beerse, Belgium); tetramethylammonium hydroxide (TMeOH), 20% methanolic solution, Aldrich (Milwaukee, Wisc., U.S.A.).

Tetrapentylammonium ($TPeA^+$) and tetrahexylammonium ($THeA^+$) counter ion solutions (about 0.15 and 0.01 *M*, respectively) were prepared as described previously[11].

The methylsulphinyl carbanion reagent, potassium *tert.*-butoxide in dried DMSO (6 g per 100 ml), was prepared according to De Leenheer and Gelijkens[12].

Tetrahexylammonium hydroxide (THeAOH) (0.2 *M* solution in methanol) was prepared as follows. A 9.6-g amount of tetrahexylammonium iodide (Eastman-Kodak, Rochester, N.Y., U.S.A.) was dissolved into 100 ml of methanol, 3.5 g of finely divided silver oxide were added and the solution was mixed for at least 1 h at room temperature to precipitate the iodide. The mixture was filtered and the filtrate, consisting of 0.2 *M* THeAOH, stored in well stoppered, amber-glass bottles. This solution is stable for at least 3 months when stored at room temperature.

All other chemicals were of analytical-reagent grade and were used without further purification.

Apparatus

GLC was performed on a Hewlett-Packard (Avondale, Pa., U.S.A.) Model 5750G or 5830A instrument equipped with dual flame-ionization detectors and a Hewlett-Packard 3370B integrator or a built-in recorder/integrator (HP 18850A GC terminal), respectively. Spiral silanized glass columns (1.8 or 2.3 m × 2 mm I.D.) were packed with the following stationary liquid phases (Supelco, Bellefonte, Pa., U.S.A.): 3 or 5% OV-1 (methyl silicone polymer), 2 or 3% SP-2250 (methyl phenyl silicone polymer), 3% OV-17 (methyl phenyl silicone polymer), 3% OV-25 (methyl phenyl silicone polymer), 2 or 3% QF-1 (fluoroalkyl silicone polymer), 3 or 5% XE-60 (nitrile, silicone gum), 3% Dexsil-300 (carborane–methyl silicone polymer) and 3 or 1% FFAP (reaction product of Carbowax 20M and *m*-nitroterephthalic acid). All liquid phases were coated on 80–100 or 100–120 mesh Gas-Chrom Q (Supelco) using the filter/fluidizing technique of Horning *et al.*[13] and Kruppa *et al.*[14]. The injector and detector (FID) block temperatures were maintained 10° higher than the oven temperature except for the flash-alkylation technique, where the injector heater was at 270°. Nitrogen was used as the carrier gas at a linear velocity of about 7 cm/sec. The hydrogen and air flow-rates were adjusted so as to give optimal sensitivity and good stability.

Mass spectrometric (MS) analysis was carried out on an LKB 9000S instrument (LKB, Bromma, Sweden). The electron energy was 70 eV, the ionization current 60 μA, the accelerating voltage 3.5 kV and the ion source temperature 270°. Crystallized products were introduced into the mass spectrometer using the direct inlet system. The analysis was performed at the evaporation temperature of the

compound. The hexyl derivatives of 5-FU and 5-ClU were prepared by flash alkylation and analysed in the GLC–MS mode. Therefore, derivatives were separated on a 3% Dexsil-300 column (Gas-Chrom Q, 100–120 mesh, 1.8 × 2 mm I.D.) using helium at a flow-rate of 30 ml/min as the carrier gas. The injector block and oven temperatures were 260° and 230°, respectively.

Analytical derivatization study

Extractive methylation

To 1.0 ml of dichloromethane eluate[11] containing 100 μg of 5-FU was added 1.0 ml of 0.14 *M* $TPeA^+$ solution. When $THeA^+$ was used as counter ion, the methylation conditions were as follows: to 1.0 ml of an aqueous solution of 5-FU (25 μg/ml), previously adjusted to pH 10.0 with 0.1 *M* sodium hydroxide, was added 1.0 ml of 0.005 *M* $THeA^+$ solution. In both instances methyl iodide and 2 ml of dichloromethane were added and the mixture was shaken for several hours. This procedure was studied by adding 5, 10, 25, 50, 75 and 100 μl of methyl iodide and by shaking the mixture for 0.25, 0.5, 0.75, 1, 2 and 4 h. The phases were separated and the organic layer was washed with an equal volume of a saturated solution of silver sulphate in 0.1 *M* sulphuric acid. The organic layer was dried over sodium sulphate and evaporated to dryness at 40° under a stream of nitrogen. When $THeA^+$ was used as counter ion, the residue obtained after evaporation was redissolved in 1 ml of water and the derivative extracted into 2 ml of diethyl ether. The ether phase was washed with two 1-ml portions of water, dried over sodium sulphate and evaporated to dryness at 30° under a stream of nitrogen. The residue obtained by the $TPeA^+$ and $THeA^+$ procedures was dissolved in 200 μl of dichloromethane to which 0.2 mg of n-C_{24}/ml was added. A 1–2-μl aliquot was submitted to GLC analysis.

Wet alkylations

Methylation. A volume of 50 μl of methylsulphinyl carbanion reagent was added to 0.5 ml of a 5-FU solution in dried DMSO (200 μg/ml). After a few minutes, 100 μl of methyl iodide were added and the reaction mixture was kept in the dark for a given time. The reaction was stopped by adding 5 ml of water, 100 μg of crystallized N,N′-dimethyl-5-ClU were added and the derivatives were extracted into 5 ml of chloroform. The organic layer was washed with three 5-ml portions of water, dried over sodium sulphate and evaporated to dryness under a stream of nitrogen at 50°. The residue was dissolved in 100 μl of chloroform and 1 μl was injected on to the top of the GLC column. The influence of the amount of methylsulphinyl carbanion was checked by carrying out the same experiment as described above using 3:4, 2:4, 3:8, 1:4 and 1:10 dilutions of the stock methylsulphinyl carbanion reagent in dried DMSO. The methylsulphinyl carbanion and the methyl iodide reaction times were varied from 30 sec to 5 min and from 0 (after adding methyl iodide and mixing, water was added) to 2 h, respectively.

Butylation. An amount of 50 μg of 5-FU was dissolved in a mixture of 50 μl of DMA and 15 μl of 2% TMeAOH in methanol, 20 μl of *n*-butyl iodide were added and the solution was thoroughly mixed. After a certain time, 1 ml of water was added and the derivative was extracted into 2 ml of diethyl ether. The organic layer was dried over sodium sulphate and evaporated to dryness at 30° under a stream of

nitrogen. The residue was dissolved in 50 μl of methanol and a 1-μl aliquot was submitted to GLC analysis.

Optimization experiments were carried out following the procedure described using 50 μg of 5-ClU and varying the reaction time between 1 and 30 min. An amount of 50 μg of N,N'-dibutyl-5-FU was added to the reaction mixture as an internal standard before the ether extraction step.

Flash hexylation

A volume of 0.2 ml of a methanolic solution of 5-FU (50 μg/ml) placed in a glass tube and the solvent removed by evaporation. The residue obtained was dissolved in 25 μl of 0.2 *M* THeAOH in methanol and an aliquot was introduced directly into the injection port of the gas chromatograph.

The reproducibility of this gas-phase reaction was investigated by repeated injections of 1-μl aliquots of a solution containing 4 μg of 5-FU and 5 μg of 5-ClU in 25 μl of 0.2 *M* THeAOH in methanol.

Preparative derivatization procedures

Extractive methylation

An amount of 5 mg of 5-FU was extracted as an ion pair using a cellulose column[11]. To the eluate were added 5 ml of 0.14 *M* $TPeA^+$ and 0.5 ml of methyl iodide. When using $THeA^+$ as counter ion, 5 mg of 5-FU was dissolved in 40 ml 0.005 *M* $THeA^+$ and shaken for 2 h with a mixture of 20 ml of dichloromethane and 0.5 ml of methyl iodide. The solutions were further purified as described above.

Wet alkylations

Methylation. The procedure was carried out as outlined above using 50 mg of 5-FU (or 5-ClU), 5 ml of dried DMSO, 4 ml of methylsulphinyl carbanion reagent and 2 ml of methyl iodide. The reaction was stopped by adding 10 ml of water and the derivative was extracted into 20 ml of chloroform. The organic layer was subsequently washed with equal volumes of 0.5 *M* sodium thiosulphate and water.

Butylation. An amount of 10 mg of 5-FU (or 5-ClU) was dissolved in a mixture of 200 μl of 20% TMeAOH in methanol, 0.8 ml of methanol and 4 ml of DMA. Subsequently 0.5 ml of *n*-butyl iodide was added. After keeping the mixture in the dark at room temperature for 2 h, 5 ml of water was added and the derivative was extracted into 20 ml of diethyl ether.

For all instances described, the organic layer was evaporated to dryness in a water-bath under a stream of nitrogen. The residue obtained was dissolved in a minimal volume of diethyl ether and light petroleum (b.p. 40–60°) was added until the solution became turbid. The product was crystallized by refrigeration overnight and the crystals were separated and dried under vacuum.

Linearization experiments

The different alkylation reactions were carried out in the range 0–100 (25, 50, 75, 100), 0–50 (10, 20, 30, 40, 50), 0–25 (5, 10, 15, 20, 25) and 0–10 (2, 4, 6, 8, 10) μg of 5-FU using optimal reaction conditions as discussed below. Depending on the FID response, an appropriate amount of crystallized corresponding dialkyl deriv-

ative of 5-ClU was added to the mixture after stopping the reaction. For the flash hexylation procedure 5-ClU was added directly to the 5-FU solutions. A linearity check was made by plotting peak-height or peak-area ratios of 5-FU to 5-ClU against the amount of 5-FU.

RESULTS AND DISCUSSION

Identification of derivatives

The results for the MS and GLC–MS analysis are given in Table I. Fig. 1 proposes a rationale for a generalized mass fragmentation pattern for the compounds studied. All of these data strongly indicate the formation of the corresponding N,N'-dialkyl derivatives.

Dibutyl and dihexyl derivatives exhibit an ion intensity pattern which differs significantly from that of the underivatized substances and the dimethyl derivatives. On lengthening the alkyl chain, abundant ions originate from fragmentation of the alkyl chain and are superimposed on the entire mass spectra.

Extractive methylation

The extractive methylation process can be described as a two-step reaction, in which the anionic form of 5-FU (HFU^-) is first extracted as an ion pair with a suitable quaternary ammonium ion ($TPeA^+$, $THeA^+$–Q^+) into an organic phase where methylation takes place.

$$HFU^- + Q^+ \rightleftharpoons HFU^-Q^+_{org} \qquad \text{extraction step} \qquad (1)$$

$$HFU^-Q^+_{org} + CH_3I \longrightarrow CH_3HFU_{org} + QI_{org} \qquad \text{alkylation step} \qquad (2)$$

The first step was discussed in detail in a previous paper[11]. Extraction of 5-FU as an ion pair with $TPeA^+$, yielding a high recovery, is possible only by using a cellulose column. In this instance the dichloromethane eluate (HFU^-TPeA^+) should be used instead of the aqueous solution of 5-FU. From eqn. 2 the formation of the monomethyl derivative seems obvious. However, spectrometric analysis proved the formation of the dimethyl derivative. This is explained by assuming a pK_a value for CH_3HFU that is lower than the pK_{a_2} value of the underivatized base. Under these circumstances the monomethyl derivative should be extractable as an ion pair with $TPeA^+$ and further methylated to the dimethyl derivative. To prove this hypothesis, the monomethyl derivative should be synthesized, and its pK_a value determined, a task which appears to be difficult.

Fig. 2 shows a gas chromatogram obtained after six repetitive injections of the dimethyl derivative of 5-FU synthesized according to the extractive methylation procedure. The large tailing solvent peak and fluctuations of the baseline are due to thermodegradation of tetraalkylammonium iodide formed during the derivatization reaction[15]. As shown in Fig. 3, this effect is successfully eliminated by extracting the organic phase with a saturated solution of silver sulphate in 0.1 *M* sulphuric acid. The sulphate is the preferred anion because of the low extraction constant of sulphate ion pairs[16]. For the $THeA^+$ reaction, however, the sulphate ion pair possesses still too high an extraction constant owing to the more lipophilic character of the hexyl in comparison with the pentyl chain. Therefore, under these circumstances a successful

TABLE I

IONS FORMED BY MASS SPECTROMETRIC FRAGMENTATION OF 5-FU, 5-ClU AND SOME OF THEIR ALKYL DERIVATIVES

Substituent	*5-FU*		*5-ClU*		*N,N'-Dimethyl-5-FU*		*N,N'-Dimethyl-5-ClU*		*N,N'-Dibutyl-5-FU*		*N,N'-Dibutyl-5-ClU*		*N,N'-Dihexyl-5-FU*		*N,N'-Dihexyl-5-ClU*	
X	F		Cl		F		Cl		F		Cl		F		Cl	
R_1	H		H		CH_3		CH_3		C_4H_9		C_4H_9		C_6H_{13}		C_6H_{13}	
R_2	H		H		CH_3		CH_3		C_4H_9		C_4H_9		C_6H_{13}		C_6H_{13}	
*Fragment ion**	*m/z*	*I(%)***	*m/z*	*I(%)***	*m/z*	*I(%)***	*m/z*	*I(%)***	*m/z*	*I(%)***	*m/z*	*I(%)***	*m/z*	*I(%)***	*m/z*	*I(%)***
M^+	130	100.0	146	88.4	158	78.1	174	70.0	242	20.4	258	6.5	298	7.1	314	9.2
I	87	48.0	103	100.0	101	31.8	117	62.1	143	14.4	159	17.5	171	9.3	187	7.8
I + H	88	5.7	104	10.8	102	3.9	118	7.1	144	80.0	160	57.0	172	7.1	188	4.1
II }	59	10.0	75	6.6	73	41.9	89	45.0	115	7.0	131	6.0	143	7.0	159	4.3
VI }					59	0.8	75	1.4	59	6.2	75	2.5	59	3.6	75	2.1
III	86	2.6	102	1.6	100	5.3	116	4.3	142	2.5	158	2.5	170	1.8	186	1.1
IV	58	6.5	74	10.8	72	15.3	88	3.6	114	51.0	130	26.0	142	1.4	158	1.2
V	28	93.0	28	116.3	42	100.0	42	100.0	84	6.0	84	7.0	112	2.8	112	2.3
VII	60	46.0	76	82.5	74	3.6	90	3.6	116	5.3	132	8.7	144	46.4	160	43.9

* Roman figures refer to the fragment ions presented in Fig. 1.

** I = relative intensity of fragment ions (70 eV).

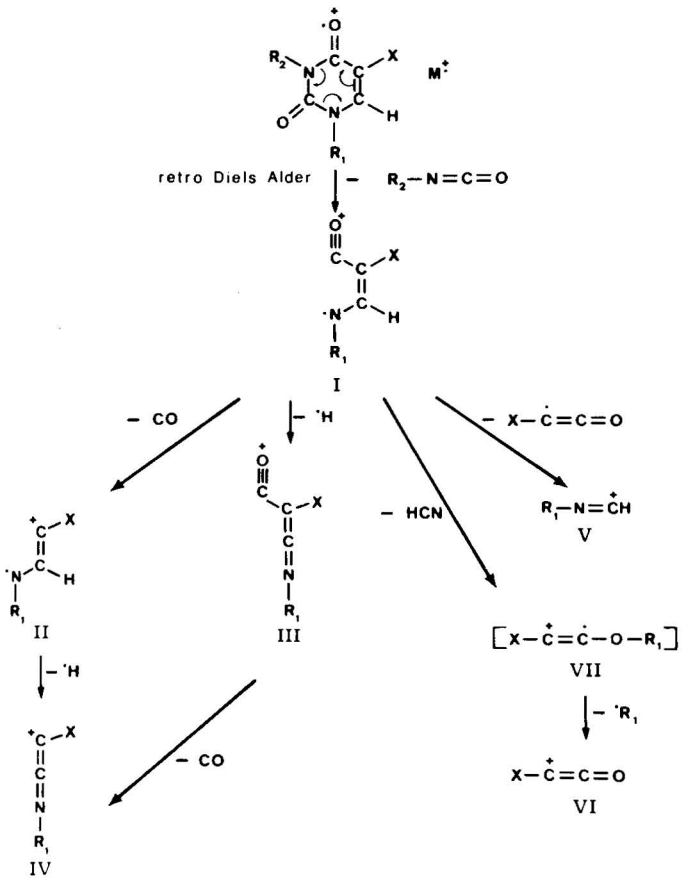


Fig. 1. Proposed mass fragmentation pattern of 5-FU, 5-ClU and some of their alkyl derivatives.

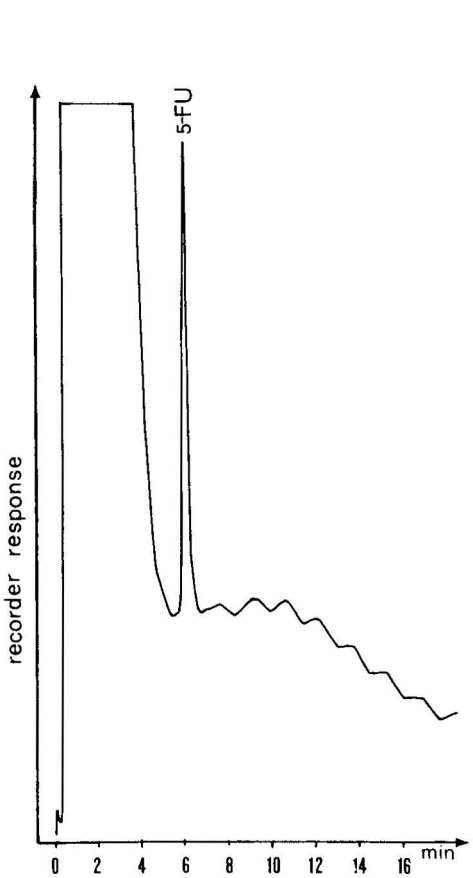
clean-up can only be performed by an additional back-extraction step with diethyl ether.

To optimize the reactions conditions quantitative gas chromatography is performed using n-C_{24} as the internal standard. The effects of the amount of methyl iodide and the reaction time are illustrated in Figs. 4 and 5, respectively. From these experiments we conclude that extractive methylation of 5-FU in the range from 0 to 100 μg yielding optimal recovery is achieved by using 5 μl of methyl iodide and a reaction time of 1 h.

Wet alkylations

Methylation

Reaction conditions were studied using N,N′-dimethyl-5-ClU as the internal standard. As we always used the same batch of methylsulphinyl carbanion reagent, we investigated the influence of the amount of methylsulphinyl carbanion by diluting the stock reagent (Fig. 6). Tubes in which the methylsulphinyl carbanion or methyl iodide reaction time was varied yielded the same recovery for 5-FU. It also was found that the amount of methyl iodide is not critical provided that a large excess is



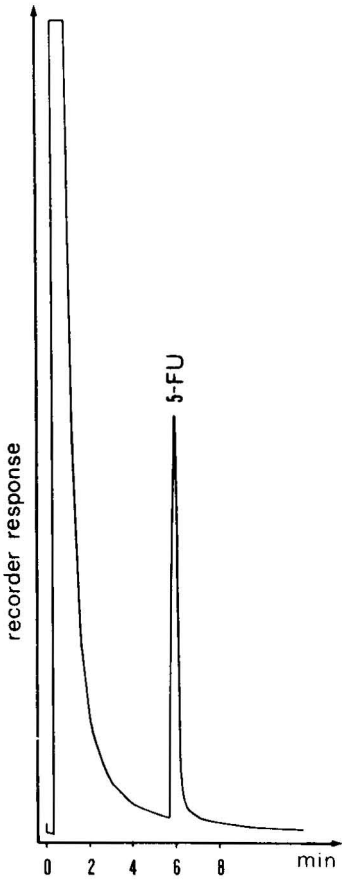


Fig. 2. GLC (FID) of the dimethyl derivative of 0.5 μg of 5-FU prepared by extractive methylation obtained after six repetitive injections of the reaction mixture (2% SP-2250; oven temperature, 135°; linear velocity of nitrogen, 7 cm/sec).

Fig. 3. GLC (FID) of the dimethyl derivative of 0.5 μg of 5-FU prepared by extractive methylation after clean-up with silver sulphate (2% SP-2250; oven temperature, 135°; linear velocity of nitrogen, 7 cm/sec).

used. Moreover, the latter is easily removed by evaporation and does not give rise to interferences in the gas chromatographic process itself.

In summary, the recovery was shown to be optimal with a 1:4 dilution of the methylsulphinyl carbanion (proton abstractor) stock reagent, and the chemical reactions occurred almost immediately.

Butylation

In this method, the acidic compound 5-FU was converted into a soluble salt (ion pair) with the organic base TMeAOH. The reaction was carried out in methanolic DMA, a highly polar solvent system. The salt thus formed reacted further with an excess of *n*-butyl iodide, yielding the corresponding dibutyl derivative. TMeAI precipitated slowly as a side-reaction product. Both 5-FU and 5-ClU were butylated and

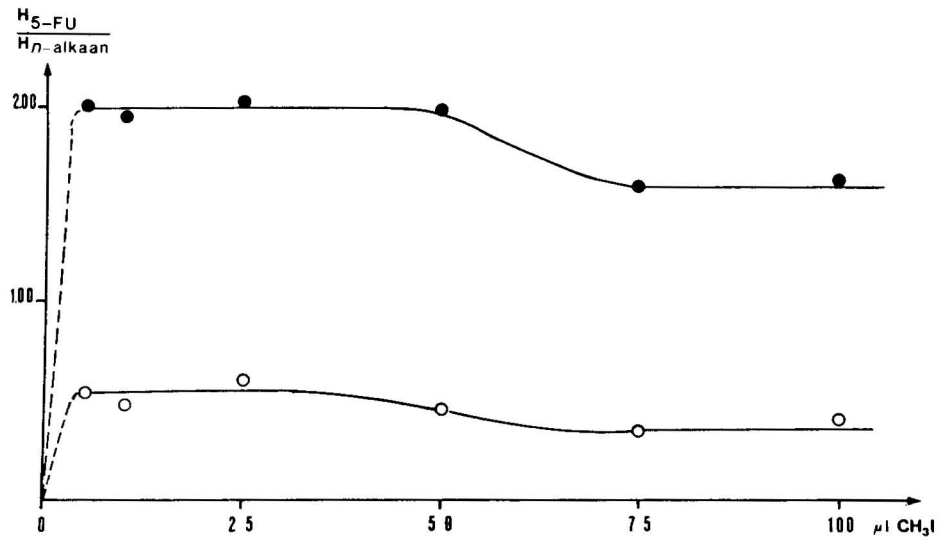


Fig. 4. Influence of the amount of methyl iodide using the extractive methylation procedure (5% XE-60; oven temperature, 165°; linear velocity of nitrogen, 7 cm/sec). ●, 100 µg of 5-FU, $TPeA^+$ as counter ion; ○, 25 µg of 5-FU, $THeA^+$ as counter ion.

a longer period for the precipitation of TMeAI was observed for 5-ClU. The reaction conditions were optimized for 5-ClU, and N,N'-dibutyl-5-FU was used as the internal standard. From these experiments, we conclude that under the circumstances outlined a reaction time of about 10 min is satisfactory (Fig. 7).

Flash hexylation

The hexylation reaction occurring in the injection port of the gas chromato-

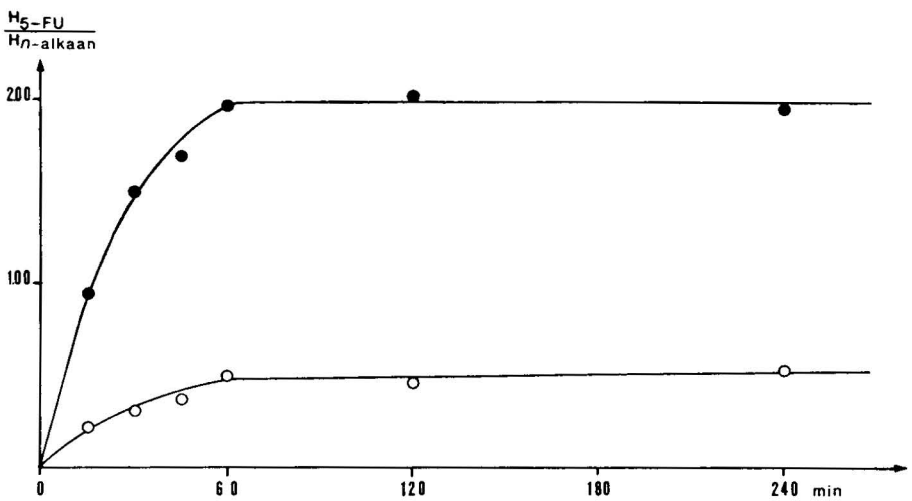


Fig. 5. Influence of the reaction time using the extractive methylation procedure (5% XE-60; oven temperature, 165°; linear velocity of nitrogen, 7 cm/sec). ●, 100 µg of 5-FU, $TPeA^+$ as counter ion; ○, 25 µg of 5-FU, $THeA^+$ as counter ion.

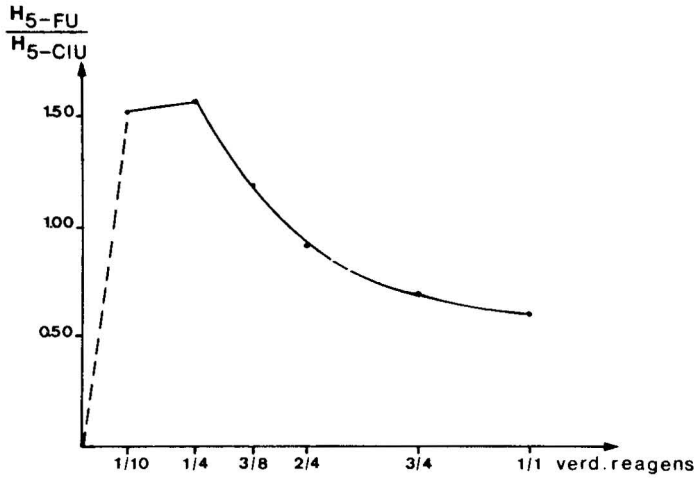


Fig. 6. Influence of the amount of methylsulphinyl carbanion on the N-permethylation reaction (5% XE-60; oven temperature, 165°; linear velocity of nitrogen, 7 cm/sec).

graph is based on the Hofmann degradation[17]. A typical gas chromatogram is shown in Fig. 8. We originally thought that peaks 1 and 1′ correspond to the monohexyl derivatives of 5-FU and 5-ClU, respectively. However, mass spectrometry did not confirm this assumption. Some fragment ions characteristic of N-alkylamines were found but no further identification could be achieved.

Reproducibility of the hexylation reaction was demonstrated by repeated injections of 5-FU and 5-ClU (internal standard), into the gas chromatograph under the conditions described. Measurement of peak-height ratios of 5-FU to 5-ClU afforded

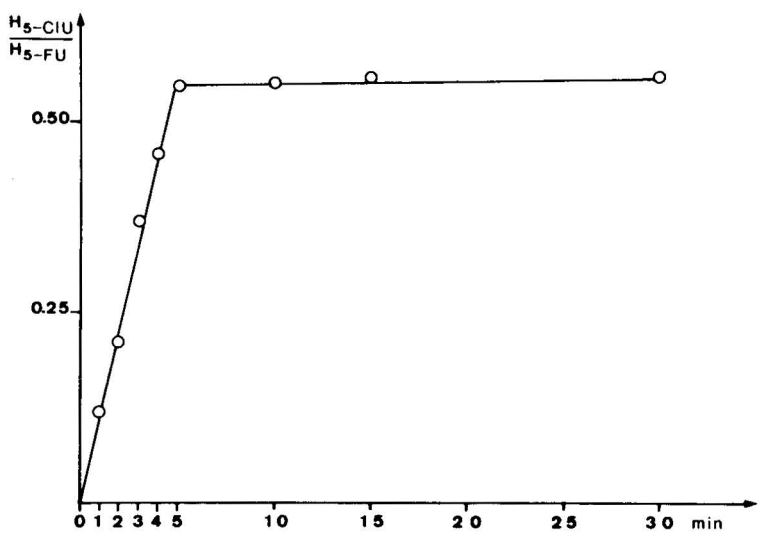


Fig. 7. Influence of the reaction time on the butylation reaction (3% FFAP; oven temperature, 200°; linear velocity of nitrogen, 7 cm/sec).

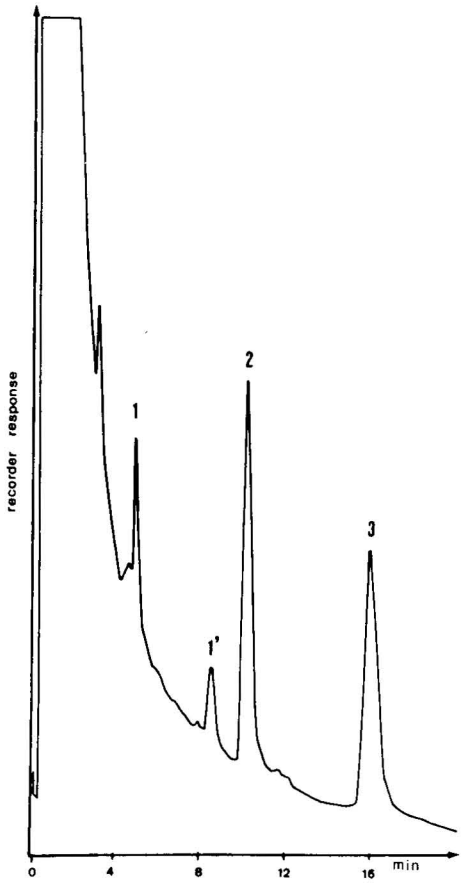


Fig. 8. GLC (FID) of the hexyl derivative of 5-FU and 5-ClU (5% XE-60; oven temperature, 210°; linear velocity of nitrogen, 7 cm/sec). 1 and 1', N-alkylamines; 2, 0.4 μg of derivatized 5-FU; 3, 0.6 μg of derivatized 5-ClU.

a coefficient of variation (CV) of 3.97% ($n = 10$, 160 ng of 5-FU and 200 ng of 5-ClU injected).

Gas–liquid chromatography

All derivatives were tested for gas chromatographic behaviour on different stationary liquid phases, as reported in Table II. The best results in terms of a small theoretical plate height and peak symmetry were obtained on the 2% or 3% SP-2250 and 5% XE-60 column systems. For the dibutyl derivatives 5% OV-1 also gave satisfactory results.

For quantitative purposes it was obvious to choose a uracil derivative as an internal standard. In view of their possible application, dimethyl derivatives of 5-chloroucacil (5-ClU), 5-bromouracil (5-BrU) and 5-iodouracil (5-IU) were synthesized and chromatographed on a 3% OV-17 column (1.8 m × 2 mm I.D.; oven temperature, 145°; linear velocity of nitrogen carrier gas, 7 cm/sec). These compounds had retention times of 15.0, 23.8 and 35.6 min, respectively, compared with 6.3 min

TABLE II

GAS-LIQUID CHROMATOGRAPHIC PROPERTIES OF SOME ALKYL DERIVATIVES OF 5-FU AND 5-ClU

t_R = retention time; r = relative retention time (t_R of 5-FU/t_R of 5-ClU; H = column plate height; asym. = peak asymmetry factor.

Liquid phase	*Gas-Chrom Q: mesh size*	*Column length (m)*	*Oven temperature (°C)*	*N,N'-Dimethyl-5-FU*			*N,N'-Dibutyl-5-ClU*			*N,N'Dibutyl-5-FU*			*N,N'-Dihexyl-5-ClU*			*N,N'-dihexyl-*		
				t_R (min)	H (mm)	Asym.	t_R (min)	H (mm)	Asym.	r	H (mm)	Asym.	t_R (min)	H (mm)	Asym.	r	H (mm)	Asym.
3% OV-1	80–100	1.8	150	1.64	1.44	1.09	—	—	—	—	—	—	—	—	—	—	—	—
5% OV-1	100–120	2.3	200	—	—	—	12.80	0.48	1.09	0.54	0.53	1.05	—	—	—	—	—	
2% SP-2250	80–100	1.8	135	6.01	0.43	0.97	—	—	—	—	—	—	—	—	—	—	—	
3% SP-2250	100–120	2.3	210	—	—	—	—	—	—	—	—	—	12.13	0.53	1.09	0.55	0.62	1.00
3% OV-17	80–100	1.8	140	8.00	0.45	0.94	—	—	—	—	—	—	—	—	—	—	—	
	80–100	1.8	205	—	—	—	—	—	—	—	—	—	17.50	0.56	1.16	0.56	0.64	1.09
3% OV-25	100–120	2.3	150	6.27	0.93	1.09	—	—	—	—	—	—	—	—	—	—	—	
2% QF-1	100–120	2.3	135	10.29	0.55	1.13	—	—	—	—	—	—	—	—	—	—	—	
3% QF-1	80–100	1.8	140	9.92	0.59	1.15	—	—	—	—	—	—	—	—	—	—	—	
			210	—	—	—	—	—	—	—	—	—	5.39	0.88	1.14	0.77	1.04	1.07
3% XE-60	80–100	1.8	165	5.40	0.97	1.24	—	—	—	—	—	—	—	—	—	—	—	
5% XE-60	100–120	1.8	165	8.90	0.47	1.07	—	—	—	—	—	—	—	—	—	—	—	
			210	—	—	—	—	—	—	—	—	—	15.70	0.46	1.06	0.63	0.53	0.97
			180	—	—	—	13.13	0.47	1.15	0.58	0.56	1.09	—	—	—	—	—	
3% Dexsil-300	80–100	1.8	230	—	—	—	—	—	—	—	—	—	14.45	0.78	1.17	0.61	0.79	1.26
	100–120	1.8	210	—	—	—	10.54	0.45	1.71	0.51	0.51	1.45	—	—	—	—	—	
3% FFAP	100–120	2.3	200	—	—	—	17.80	0.70	1.23	0.63	0.82	1.17	—	—	—	—	—	—
1% FFAP	80–100	1.8	180	—	—	—	8.67	1.60	2.20	0.65	1.21	0.53	—	—	—	—	—	—

for N,N′-dimethyl-5-FU as a reference. From these results it was apparent that only the chlorine-substituted substance 5-ClU is acceptable as the others (for reasons of too drastic an increase in molecular weight) possess too long retention times. Crystallized N,N′-dimethyl-5-ClU, N,N′-dibutyl-5-ClU and 5-ClU were used as internal standards to examine the linearity of the methylation, butylation and hexylation derivatization reactions, respectively. We always found a linear relationship between the peak-height or peak-area ratios of 5-FU to 5-ClU *versus* the amount of 5-FU submitted to the alkylation. As an example, Fig. 9 shows the linearization for the butyl derivatives covering the range 0–10 μg of 5-FU.

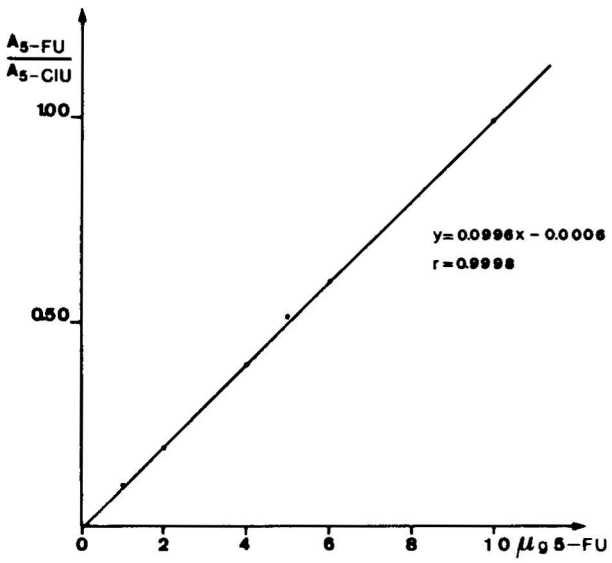


Fig. 9. Linearization of the butylation reaction in the range from 0 to 10 μg of 5-FU (5% OV-1; oven temperature, 200°; linear velocity of nitrogen, 7.5 cm/sec).

Biological applicability

Direct application of ion-pair extraction or extractive methylation to plasma samples in combination with GLC seemed impossible because of the interfering effect of the tetraalkylammonium iodide formed during the derivatization reaction[11]. Fig. 10 shows a gas chromatogram from a methylated plasma extract using the methylsulphinyl carbanion reagent and methyl iodide. From this we conclude that the methyl derivative of 5-FU is unsuitable for the quantitative analysis of plasma samples as many co-extracted compounds co-chromatograph with N,N′-dimethyl-5-FU. However, this problem was solved by increasing the retention of 5-FU by lengthening the alkyl chain in its derivative. Thus, hexylation of 5-FU and 5-ClU gave rise to compounds whose retention volumes are favourable for quantitative analysis of 5-FU in biological samples[18]. On the other hand, the reagent used for the flash hexylation was found to be extremely deleterious to the gas chromatographic column. In fact, this technique tends to strip the liquid phase from the solid support, whereas the high recommended injection block temperature of 270° causes

rapid deterioration of the column packing near the injection port. When operated daily, the latter needs to be replaced after about 1 month. In addition, as the hexyl derivatives are formed in the injection port of the gas chromatograph, preliminary purification by solvent extraction is impossible.

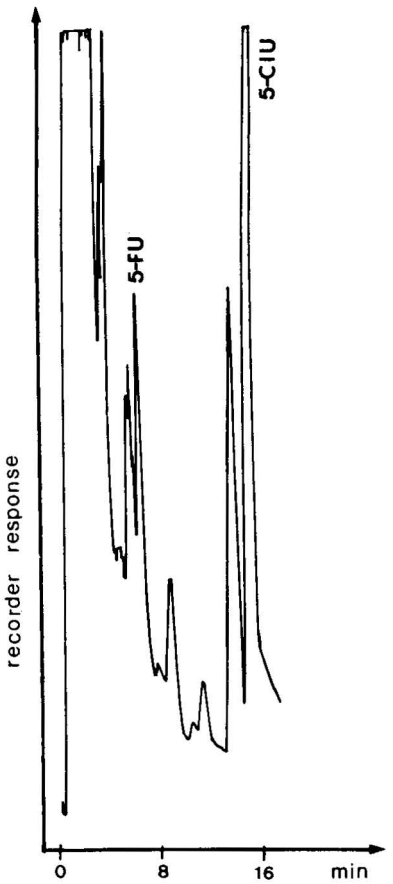


Fig. 10. GLC (FID) of a methylated extract of 1.0 ml of plasma spiked with 4 μg of 5-FU (3% OV-25; oven temperature, 150°; linear velocity of nitrogen, 7 cm/sec).

Another derivatization procedure for the sensitive detection of 5-FU in plasma samples[10] is compulsory. Derivatization with TMeAOH and *n*-butyl iodide as outlined above is more successful as the destructive quaternary ammonium salt (TMeAI) is removed by precipitation during the derivatization itself. The derivative can be purified further before chromatographic analysis by an additional clean-up with diethyl ether.

Mass fragmentography based on the detection of the dibutyl derivative of 5-FU allows quantitation of nanogram amounts of 5-FU in plasma[10].

ACKNOWLEDGEMENTS

This work was supported by the National Medical Research Foundation (F.G.W.O.) through grants 20210, 20452 and 3.0004.76. The authors are grateful to Dr. A. A. M. Cruyl, of the N.F.W.O. for running the mass spectra.

REFERENCES

1 F. J. Ansfield, J. M. Schroeder and A. R. Curreri, *J. Amer. Med. Ass.*, 181 (1962) 295.
2 C. Heidelberger and F. J. Ansfield, *Cancer Res.*, 23 (1963) 1226.
3 E. M. Jacobs, W. J. Reeves, D. A. Wood, R. Pugh, J. Braunwald and J. R. Bateman, *Cancer*, 27 (1971) 1302.
4 S. R. Lahiri, G. Boileau and T. C. Hall, *Cancer*, 28 (1971) 902.
5 J. J. Windheuser, J. L. Sutter and E. Auen, *J. Pharm. Sci.*, 61 (1972) 301.
6 J. L. Cohen and P. B. Brenan, *J. Pharm. Sci.*, 62 (1973) 572.
7 H. W. van den Berg, R. F. Murphy, R. Hunter and D. T. Elmore, *J. Chromatogr.*, 145 (1978) 311.
8 C. Finn and W. Sadée, *Cancer Chemother. Rep.*, 59 (1975) 279.
9 B. L. Hillcoat, M. Kawai, P. B. McCulloch, J. Rosenfeld and C. K. O. Williams, *Brit. J. Clin. Pharmacol.*, 3 (1976) 135.
10 A. P. De Leenheer, M. Cl. Cosyns-Duyck and A. A. M. Cruyl, in A. P. De Leenheer, R. R. Roncucci and C. van Peteghem (Editors), *Quantitative Mass Spectrometry in Life Sciences II, Proceedings of Second International Symposium on Quantitative Mass Spectrometry in Life Sciences, Gent, June 13–16, 1978*, Elsevier, Amsterdam, Oxford, New York, 1978, p. 399.
11 A. P. De Leenheer, M. Cl. Cosyns-Duyck and P. M. Van Vaerenbergh, *J. Pharm. Sci.*, 66 (1977) 1190.
12 A. P. De Leenheer and C. F. Gelijkens, *Anal. Chem.*, 48 (1976) 2203.
13 E. C. Horning, E. A. Moscatelli and C. C. Sweeley, *Chem. Ind. (London)*, 78 (1959) 751.
14 R. F. Kruppa, R. S. Henly and D. L. Smed, *Anal. Chem.*, 39 (1967) 851.
15 J. MacGee and K. G. Allen, *Anal. Chem.*, 42 (1970) 1672.
16 A. Tilly, *Acta Pharm. Suecica*, 10 (1973) 111.
17 A. W. Hofmann, *Ber. Deut. Chem. Ges.*, 14 (1881) 494.
18 A. P. De Leenheer and M. Cl. Cosyns-Duyck, in preparation.

Journal of Chromatography, 174 (1979) 341–349

CHROM. 11,812

DETERMINATION OF BROMOCRIPTINE IN PLASMA: COMPARISON OF GAS CHROMATOGRAPHY, MASS FRAGMENTOGRAPHY AND LIQUID CHROMATOGRAPHY

NIELS-ERIK LARSEN

Clinical Pharmacological Laboratory, Department of Clinical Chemistry, Glostrup Hospital, DK-2600 Glostrup (Denmark)

ROLF ÖHMAN and MARGARETA LARSSON

Psychiatric Department III, University of Göteborg, Lillhagen's Hospital, S-422 03 Hisings Backa (Sweden)

and

EIGILL F. HVIDBERG

Department of Clinical Pharmacology, Rigshospitalet, University of Copenhagen, DK-2100 Copenhagen Ø (Denmark)

(Received February 20th, 1979)

SUMMARY

Gas chromatographic, mass fragmentographic and liquid chromatographic techniques for the determinations of bromocriptine (2-bromo-α-ergocriptine; Parlodel) in human plasma are described. These methods were found to be suitable for determining concentrations of bromocriptine down to 0.5, 1.0 and 10.0 μg/l, respectively. Accuracy, specificity and analytical capacity were satisfactory for all three methods.

Gas chromatography was compared with liquid chromatography, and the two methods were demonstrated to give identical results in patients treated with bromocriptine for Parkinson's disease. Gas chromatography was also compared with mass fragmentography, and the results from these two assays were also in agreement.

INTRODUCTION

Bromocriptine (BCT) (2-bromo-α-ergocriptine; Parlodel) has been demonstrated to have therapeutic potency in the treatment of hyperprolactinaemia[1–3], and in high doses has been shown to have significant anti-Parkinson effects[4–6]. Treatment with BCT might be improved by monitoring plasma concentrations, but the first prerequisite is reliable analytical procedures for its determination.

More than one method might be suitable, and it is of importance to compare different assays. The aim of this paper is to describe a comparison of three different

methods for the determination of BCT in plasma from patients undergoing ordinary treatment with this drug. The gas chromatographic (GC) and mass fragmentographic (MF) methods were developed at Glostrup Hospital, and the liquid chromatographic (LC) method was developed at Lillhagen's Hospital. The GC method has recently been used in an investigation of the pharmacokinetic properties of BCT in patients with Parkinson's disease[7].

MATERIALS AND METHODS

Chemicals

Concentrated borate buffer (pH 12) of analytical reagent grade was obtained from E. Merck (Darmstadt, G.F.R.), hexamethyldisilazane from Pierce (Rockford, Ill., U.S.A.) and 1-heptanesulphonic acid (PIC-B7) from Waters Assoc. (Gothenburg, Sweden). Other commercial chemicals and solvents used were of analytical-reagent grade.

Stock solutions (1 g/l) of BCT and of the internal standards ergotamine and dihydroergocristine were prepared in ethanol. When kept in a refrigerator at 4° they were stable for at least 1 year.

Gas chromatography

Extraction procedure. To a 500-μl plasma sample were added 100 μl of borate buffer (pH 12) and 10 ng of dihydroergocristine (DHEC) as internal standard. Toluene (6 ml) was added and the sample mixed for 5 min at 20 rpm on a rotary mixer. The specimen was then contrifuged for 3 min at 1000 *g*, after which 5 ml of the organic phase were transferred into another centrifuge tube containing 1 ml of 1 *M* sulphuric acid. The contents were mixed for 5 min, centrifuged, and the organic phase was discarded. The original plasma phase was re-extracted by mixing with a further 6 ml toluene for 5 min. After centrifugation, 5 ml of this toluene phase was transferred to the sulphuric acid phase, mixed and centrifuged, then the toluene phase was discarded. A volume of 400 μl of 6 *M* sodium hydroxide solution was added. Toluene (3 ml) was added to the aqueous phase and mixed for 5 min. After centrifugation for 1 min, the organic phase was transferred into a tapered tube moistened with hexamethyldisilazane (HMDS) in ethanol (0.5, w/w). The toluene phase was evaporated to dryness under a stream of nitrogen. The aqueous phase was re-extracted with 3 ml of toluene, centrifuged, and the toluene transferred into the tapered tube and evaporated to dryness. The final residue was dissolved in 50 μl of a mixture of HMDS in ethanol (0,5, w/w). A few microlites of this solution were injected into the chromatograph.

Apparatus and conditions. A Pye Series 104, Model 74, gas chromatograph equipped with an electron-capture detector was used. A glass column (0.9 m $\times$ 4 mm I.D.) was used. The stationary phase was 1% (w/w) OV-17 on Celite JJ CQ (100–120 mesh B.S.). The amount of column filling was 7 g. The column was conditioned at 350° for 48 h. The column temperature was 245°, injection block temperature 250°. detector temperature 350°, carrier gas (argon–methane, 90:10) flow-rate 50 ml/min and quench gas flow-rate 10 ml/min. The detector was run in a pulsed mode (150 μsec). The attenuation of the recorder was $5 \cdot 10^2$. The retention time of BCT was 2 min.

Calculations. The concentrations were read from a calibration graph constructed on the basis of a series of standard samples containing known amounts of BCT. The ratio of the peak height of BCT to that of DHEC was plotted against concentration.

Mass fragmentography

Extraction procedure. The procedure was similar to that used in the GC assay except that ergotamine (EGT) was used as the internal standard.

Apparatus and conditions. An LKB 9000 mass spectrometer was used. A glass column (0.5 m × 2 mm I.D.) was used. The stationary phase was 3% (w/w) OV-101 on Celite JJ CQ (100–120 mesh B.S.). The amount of column filling was 1.5 g. The column was conditioned at 350° for 48 h. The column temperature was 180°, injection block temperature 200°, molecule seperator temperature 260°, ion source temperature 290°, carrier gas (helium) flow-rate 20 ml/min, electron energy 27 eV and trap current 60 μA. The mass fragments used were 308 and 314 for BCT and EGT, respectively. The retention time of BCT was 2 min.

Calculations. The concentrations were read from a calibration graph constructed on the basis of a series of standard samples containing known amounts of BCT. The ratio of the peak height of BCT to that of EGT was plotted against concentration.

Liquid chromatography

Extraction procedure. A 2-ml volume of serum or plasma was used for each analysis. BCT (10, 20 and 30 ng/ml) was added to standard samples of pooled blank serum, 400 μl of 2.5 *M* potassium carbonate solution and 1.5 ml of diethyl ether were added and the sample was mixed for 30 sec on a rotary mixer, followed by centrifugation for 5 min at 1000 *g*. The ether phase was withdrawn and the aqueous phase re-extracted with 1 ml of diethyl ether. A 100-μl volume of 0.05 *M* sulphuric acid was added to the combined organic phase (2 ml), followed by mixing and centrifugation. A 75-μl volume of the aqueous phase was collected and injected for liquid chromatography.

Apparatus and conditions. A Waters Assoc. liquid chromatograph equipped with a Model 440 UV detector (254 nm) was used, with a U6K injector a 6000A pump and a Tarkan 600 recorder. The column (30 cm × 3.9 mm I.D.) was packed with μBondapak C_{18}. The mobile phase was methanol–water (65:35) that was 0.01 *M* in 1-heptanesulphonic acid). The column flow-rate was 1.5 ml/min, sensitivity 0.005 a.u.f.s., full recorder deflection 2 mV and recorder speed 0.5 cm/min. The retention time of BCT was 7 min.

Calculations BCT peaks were identified and peak heights plotted against concentration of BCT on a standard graph.

Plasma samples

Venous blood samples were drawn from patients undergoing continuous treatment with BCT for Parkinson's disease. The plasma was immediately separated by centrifugation and stored at −20° until required for analysis. Samples were transported between laboratories in containers packed with dry-ice, which kept the samples deep frozen at all times.

RESULTS

Chromatograms

Fig. 1 illustrates chromatograms of plasma samples. Fig. 1a and b demonstrate the GC of plasma extracts containing 0 and 20 μg of BCT per litre of plasma, respectively. Under the conditions described BCT and DHEC appeared 2 and 5 min after the injection, respectively. Fig. 1c and d demonstrate the LC of plasma extracts containing 0 and 10 μg of BCT per litre of plasma, respectively, and Fig. 1e and f demonstrate the MF of plasma extacts containing 0 and 4 μg of BCT per litre of plasma, respectively.

Fig. 2a is a calibration graph constructed on the basis of plasma samples analysed by GC containing various concentrations of BCT in the range from 0 to 30 μg/l. A linear graph was obtained. Fig. 2b and c are similar graphs for plasma samples analysed by LC and MF, respectively.

Sensitivity

For all three analytical procedures the sensitivity was defined as the lowest concentration giving a peak at least 10 times higher than the noise on the baseline. In this way the lowest concentrations giving reliable safe quantitation were 0.5 (GC), 1.0 (MF) and 10.0 μg/l (LC).

Accuracy and reproducibility

For clinical use both accuracy and reproducibility were found to lie within acceptable limits for all three methods (Table I).

Specificity

Tests were not carried out by adding different drugs to the samples. However, plasma samples used for the comparison of the methods were drawn not only from patients receiving BCT as the sole medication, but also from patients on concomitant medication with L-dopa and diazepam. None of these drugs interfered with the determination owing to complete separation from BCT and the applied internal standards in the chromatographic systems. Furthermore, no interfering peaks were detected in blank plasma samples. Finally, the specificity was confirmed by MF (see below).

Capacity

As illustrated in Fig. 1, the chromatogram from a sample can be run with an interval of 8 min in all three assays. This gives a (theoretical) maximal capacity of about seven injections into the chromatographic system per hour.

Inter-method correlations

Ten plasma samples drawn from patients undergoing commonly applied dose regimens with BCT (for Parkinson's disease) were analysed both by GC and LC. The correlation between the results of the two methods is demonstrated in Fig. 3. The methods gave almost identical results for the concentration range between *ca.* 12 and 26 μg/l. As one sample deviated significantly, the correlation coefficient between the two methods is 0.862. If the result for this sample was dis-

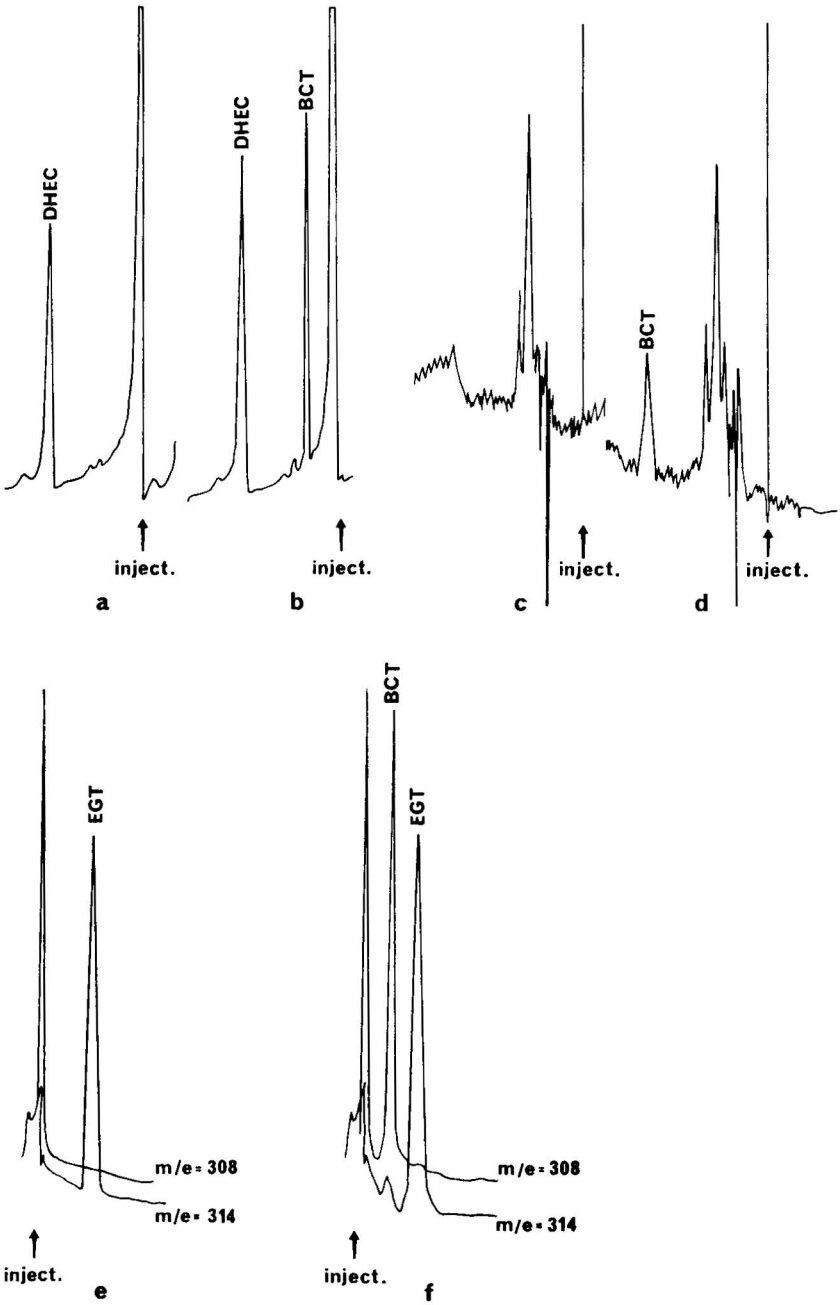


Fig. 1. Gas chromatograms, liquid chromatograms and mass fragmentograms from plasma samples containing different concentrations of bromocriptine (BCT). Concentrations in plasma: GC, (a) 0 and (b) 20 μg/l; LC, (c) 0 and (d) 10 μg/l; MF, (e) 0 and (f) 4 μg/l.

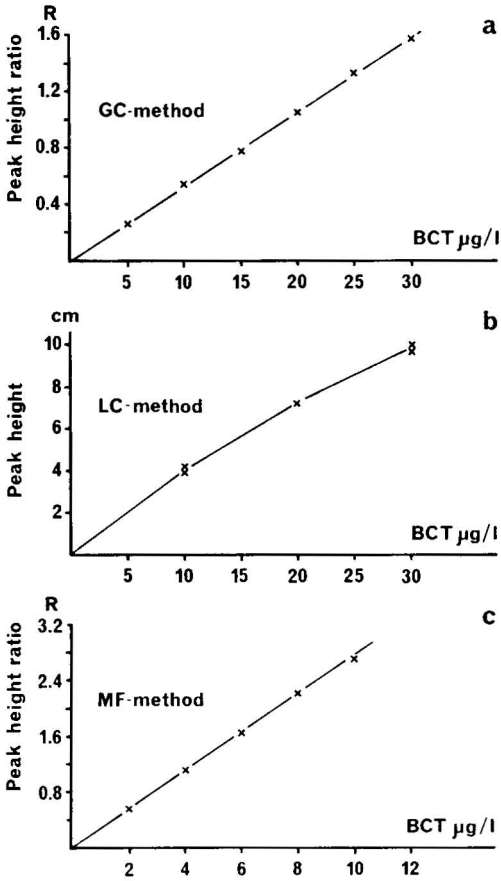


Fig. 2. Calibration graphs. Ordinate: (a) GC, the ratio (R) of the peak height of BCT to that of DHEC; (b) LC, the peak height of BCT in centimetres; (c) MF, the ratio (R) of the peak height of BCT to that of EGT.

TABLE I

ACCURACY OF BROMOCRIPTINE DETERMINATIONS

Method	*Concentration added (µg/l)*	*Number of samples*	*Calculated mean concentration (µg/l)*	*Coefficient of variation (%)*
GC	1.00	10	1.02	6.8
	5.00	10	4.95	1.8
	10.00	10	9.91	1.0
	20.00	10	20.15	0.6
	30.00	10	30.11	0.6
MF	2.00	10	2.01	2.0
	4.00	10	3.98	2.3
	6.00	10	6.02	0.5
	8.00	10	8.00	0.3
	10.00	10	10.01	0.6
LC	10.0	10	10.0	5.4
	20.0	10	19.9	6.0
	30.0	6	29.9	3.0

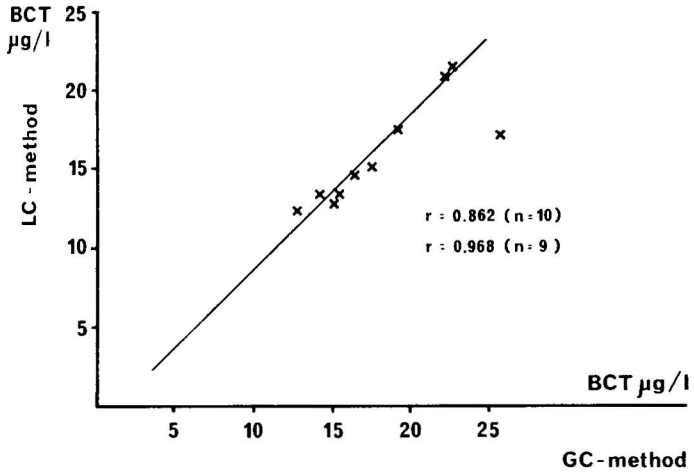


Fig. 3. Correlation between the GC and LC procedures. Ten plasma samples measured by each assay.

regarded ($n = 9$), a correlation coefficient of 0.968 was obtained. In order to verify further the specificity of the GC assay, another ten plasma samples from BCT-treated patients were analysed both by GC and MF. The selected fragments in the MF method were $m/e = 308$ and 314 for BCT and EGT, respectively. As demonstrated in Fig. 4, they produced identical results ($r = 0.996$) for the concentration range 1.5–8.3 μg/l.

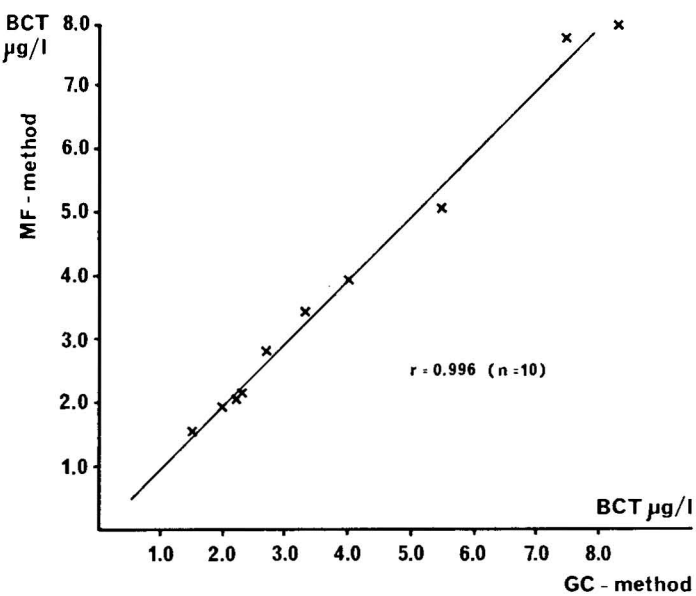


Fig. 4. Correlation between the GC and MF procedures. Ten plasma samples measured by each assay.

The GC method was also compared with a radioimmunoassay (RIA) method developed by Price *et al.*, and ten plasma samples were analysed by both methods. The comparison revealed some deviations, particularly for concentrations exceeding 4–5 μg/l. This might be due to the fact that in the RIA method different plasma was used for the calibration and for the measurements (Fig. 5).

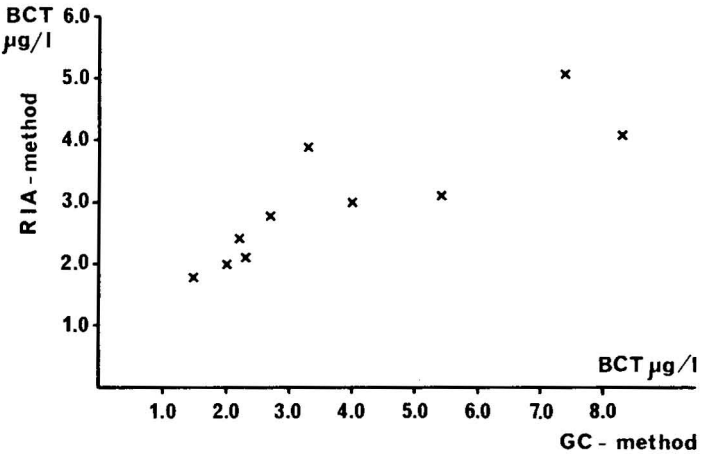


Fig. 5. Relationship between the GC procedure and a radioimmunoassay (RIA) method. Ten plasma samples measured by each assay.

DISCUSSION

The development of the three different assays for BCT in plasma revealed some interesting data. The retention time of BCT in the GC method, using a column temperature of 245°, is only 2 min (Fig. 1). This is surprisingly short considering the molecular structure of BCT, which is a tripeptide alkaloid with a molecular weight of 654.5. α-Ergocriptine demonstrated, under identical conditions, an identical retention time in spite of the lack of a bromine atom. This strongly indicates cleavage of the BCT molecule when exposed to the column temperature. Therefore, the peak representing BCT on the chromatogram is probably only the tripeptide moeity. Such a rearrangement would explain the apparently short retention time of BCT. Injection of EGT revealed a much longer retention time compared with that of BCT, which supports the above theory of the cleavage of BCT. For the MF method the fragments $m/e = 308$ and 314 for BCT and EGT, respectively, were selected. These fragments correspond exactly to the tripeptide moiety for the two drugs. Further, the mass spectrum of BCT did not indicate the presence of a bromine atom, with equal intensity for M and M + 2.

None of the three methods can be claimed to be superior to the others when concentrations above 10 μg/l have to be measured, which are relevant to plasma levels (therapeutic levels) seen during BCT treatment in Parkinson's disease[7]. Each method has particular advantages, and the best choice depends on the specific use and on the facilities and experience of the laboratory. As the results of these three assays are comparable, the outcome of different investigations can be compared.

Figs. 3 and 4 show that in our hands these analytical procedures give almost identical results when the assays are applied to the same plasma samples. The RIA method is incomparable to the others as the method does not seem to be fully developed and further comparisons have to be done. As investigations carried out with different methods that do not give comparable results are bound to cause confusion, inter-laboratory comparisons of methods for drugs determinations are desirable, especially when assays are being developed.

REFERENCES

1 G. M. Besser, L. Parke, C. R. W. Edwards, I. A. Forsyth and A. S. McNeilly, *Brit. Med. J.*, 3 (1972) 669.
2 M. D. Thorner, A. S. McNeilly, C. Hayen and G. M. Besser, *Brit. Med. J.*, 2 (1974) 419.
3 H. G. Bohnet, H. G. Dahlén, W. Wuttke and H. P. G. Schneider, *J. Clin. Endocrinol. Metab.*, 42 (1976) 132.
4 D. B. Calne, P. F. Teychenne, L. E. Claveria, R. Eastman, J. K. Greenacre and A. Petrie, *Brit. Med. J.*, 4 (1974) 442.
5 P. F. Teychenne, D. B. Calne, P. N. Leigh, J. K. Greenacre, J. L. Revel, A. Petrie and A. N. Bamji, *Lancet*, 2 (1975) 473.
6 U. Grøn, *Acta Neurol. Scand.*, 56 (1977) 269.
7 M. L. Friis, U. Grøn, N.-E. Larsen, H. Pakkenberg and E. F. Hvidberg, *Eur. J. Clin. Pharmacol.*, (1978) in press.
8 P. Price, A. Debono, J. D. Parkes, C. D. Marsden and J. Rosenthaler, *Brit. J. Clin. Pharmacol.*, 6 (1978) 303.

Journal of Chromatography, 174 (1979) 351–360

CHROM. 11,786

CONTRIBUTION DE LA CHROMATOGRAPHIE EN PHASE LIQUIDE À HAUTE PERFORMANCE À LA SÉPARATION DES LANTHANIDES TRIVALENTS SUR RÉSINE CATIONIQUE EN PRÉSENCE D'EDTA

I. SYNTHÈSE ET PROPRIÉTÉS GÉNÉRALES DES RÉSINES PELLICULAIRES POLYSTYRÈNE–DIVINYLBENZÈNE SULFONATES

F. SCHOEBRECHTS, E. MERCINY et G. DUYCKAERTS
Laboratoire de Chimie Analytique et Radiochimie, Université de Liège, B-4000 Sart Tilman (Belgique)
(Reçu le 12 février 1979)

SUMMARY

High-performance liquid chromatographic separation of trivalent lanthanides on cationic exchanger with EDTA. I. Synthesis and general properties of polystyrene–divinylbenzene sulphonate pellicular ion exchangers

The synthesis of pellicular ion exchangers by incomplete sulphonation of a styrene–divinylbenzene polymer following the method described by Hansen and Gilbert is controlled by scanning electron microscopy. The thickness of the ion-exchange shell is obtained by measuring the exchange capacity and the swelling due to the sulphonation. The exchange properties of homogeneous and thick-shell ion exchangers are similar if referred to the weight of exchanger. On the contrary, for thin shells, the behaviour seems to be that of a homogeneous ion exchanger of higher crosslinking.

INTRODUCTION

La chromatographie en phase liquide à haute performance (HPLC) s'est surtout développée en chimie organique et en biochimie et les performances qu'elle permet d'atteindre en ont fait un outil de choix pour l'analyste organicien[1]. En chimie minérale, par contre, les applications sont assez rares[2]. Notre travail a pour but de mettre en évidence les possibilités de cette technique pour la séparation des lanthanides trivalents sur échangeur cationique. On peut constater que la plupart des publications de chromatographie sur échangeur d'ions concernent essentiellement l'étude de nouveaux agents complexants plus sélectifs ou cinétiquement plus favorables. La HPLC sur une phase stationnaire constituée soit de grains de petit diamètre soit de résine pelliculaire[3] ouvre de nouvelles perspectives qu'il était intéressant d'étudier dans le cas des lanthanides trivalents.

Il n'existe pas actuellement des résines échangeuses d'ions pelliculaires com-

merciales de différentes épaisseurs de pellicule. Or, certains travaux ont montré qu'il existe, pour un système chromatographique donné, une épaisseur de pellicule optimale[4-6].

Avant d'exposer les résultats obtenus en HPLC sur résine pelliculaire dans la séparation des lanthanides, nous nous proposons, dans cette première partie, de décrire la synthèse et les principales propriétés de ces résines.

PARTIE EXPÉRIMENTALE

Synthèse

Le polymère Bio-Beads SX-8 (Bio-Rad Labs., Richmond, Calif., U.S.A.), de pureté pour analyse, est tamisé pour ne retenir que les particules dont le diamètre est compris entre 45 et 50 μm.

Parmi les deux méthodes de sulfonation du polystyrène–divinylbenzène décrites dans la littérature, nous avons choisi celle par l'acide sulfurique concentré à 100° en présence de sel d'argent[7]; elle ne semble pas donner lieu à des réactions secondaires importantes comme dans le cas de la méthode utilisant le complexe SO_3–dioxane.

On introduit 0.5 g de polymère dans un mélange contenant 150 mg de Ag_2SO_4 dissous dans 100 ml d'H_2SO_4 concentré. Le mélange réactionnel est maintenu à 100° $\pm$ 1° durant 2 à 60 min suivant les cas. On filtre alors rapidement sur verre fritté, on lave d'abord avec H_2SO_4 9 *M* afin d'éviter le contact exothermique avec l'eau, puis à l'eau.

Détermination de la capacité

Le polymère est séché à 100° durant 24 h, sous vide durant 48 h, puis pesé.

Deux méthodes ont été utilisées pour obtenir la valeur la plus exacte possible de la capacité d'échange ionique: dans la première, on fait passer sur le polymère sous forme acide une solution de NaCl 1.0 *M* et l'on recueille quantitativement les ions H^+ libérés qui sont dosés par titrage pH-métrique à la soude; dans la seconde, on pèse le polymère, séché dans les mêmes conditions, avant et après sulfonation; la différence de poids représente le poids de groupements SO_3H fixés et permet le calcul de la capacité d'échange pour autant que la sulfonation n'ait pas donné lieu à des réactions secondaires et que le polymère pesé ne contienne plus d'eau résiduelle.

Mesure du volume moyen des grains

Le volume moyen des grains assimilés à des sphères est obtenu à partir de la mesure du diamètre d'une centaine d'entre eux. Cette mesure s'effectue à l'aide d'un micromètre Neubeur gradué à 25 μm et d'un microscope optique muni d'un oculaire à règlette mobile. Le mouvement de celle-ci peut être ajusté par une vis se déplaçant le long d'une échelle de 100 divisions (précision: 0.5 μm).

Mesure du degré d'hydration

Après avoir séché la résine pelliculaire dans les conditions décrites précédemment, on en introduit un poids connu dans une enceinte où l'on fait circuler un courant d'air saturé en vapeur d'eau à 25°. On pèse régulièrement la résine jusqu'à poids constant.

Mesure des coefficients de distribution

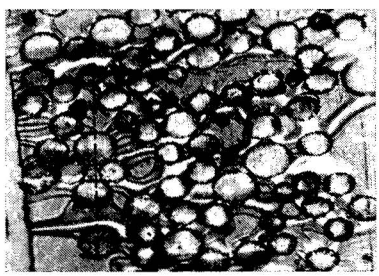
Les coefficients de distribution des cations sont obtenus par mesure de l'activité spécifique dans les deux phases radiochimiquement après équilibration durant 24 h d'un poids connu de résine sèche (0.2 g environ) avec 10 ml de phase aqueuse contenant l'ion à étudier en dose traceur. Les résines ont été, au préalable, équilibrées avec une solution de composition identique à celle utilisée au cours de l'échange ionique.

Les coefficients de distribution ont été déterminés à pH = 2.00 dans des solutions respectivement 0.1 *M*, 0.8 *M* et 1.5 *M* en NH_4Cl dans le cas de ^{137}Cs, ^{60}Co et $^{152,154}Eu$.

RÉSULTATS ET DISCUSSION

Examen des résines pelliculaires synthétisées

Une coupe de 1 μm d'épaisseur, obtenue au microtome dans un grain de résine pelliculaire saturé en Fe^{2+}, a été examinée à la microsonde électronique de Castaing[8]. Le cliché de microscopie électronique (Fig. 1) montre clairement que la sulfonation a bien eu lieu sur la pellicule extérieure du grain, en accord avec les observations de Wheaton et Harrington[9]. L'analyse à la microsonde électronique par balayage du soufre et du fer suivant la direction 1-1′ montre de façon indiscutable que le procédé de sulfonation utilisé fournit bien des résines pelliculaires.



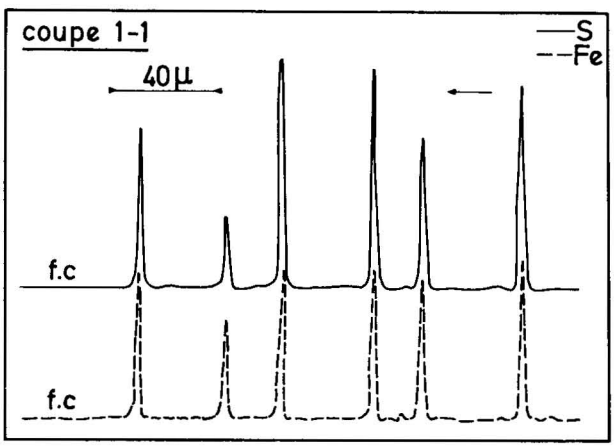


Fig. 1. Enregistrement de la composition en S et Fe le long du trajet parcouru dans une coupe de résine pelliculaire (1-1′).

TABLEAU I

CARACTÉRISTIQUES DE RÉSINES PELLICULAIRES OBTENUES APRÈS DIFFÉRENTES DURÉES DE SULFONATION

Poids de polymère vierge sec (g)	*Poids de polymère pesé après sulfonation (g)*	*Poids d'H_2O résiduelle dans le polymère sulfoné (g)*	*Poids de polymère sulfoné anhydre (g)*	*Capacité obtenue par différence de poids (ion g/g de polymère vierge)*	*Capacité obtenue par titrage (ion g/g de polymère vierge)*	*Capacité retenue (ion g/g de polymère vierge)*
0.5352	0.9510	0.019	0.936*	$9.3\ 10^{-3}$	$9.1\ 10^{-3}$	$9.1\ 10^{-3}$
0.5895	1.0207	0.018*	1.00*	$8.8\ 10^{-3}$	$8.5\ 10^{-3}$	$8.5\ 10^{-3}$
0.2757	0.4025	0.005*	0.398*	$5.5\ 10^{-3}$	$5.5\ 10^{-3}$	$5.5\ 10^{-3}$
0.7433	0.8350	0.004*	0.831*	$1.5\ 10^{-3}$	$1.5\ 10^{-3}$	$1.5\ 10^{-3}$
1.8025	1.8470	0.002*	1.845*	$3.0\ 10^{-4}$	$3.2\ 10^{-4}$	$3.1\ 10^{-4}$

* Valeurs calculées.

Volume, capacité, épaisseur et poids moyen de la pellicule

L'une des caractéristiques essentielles d'une résine pelliculaire en chromatographie est l'épaisseur de la pellicule échangeuse d'ions. Puisqu'il est établi que la sulfonation s'effectue à saturation de l'extérieur vers l'intérieur du grain, il devient possible de connaître l'épaisseur en comparant la capacité de la résine pelliculaire à celle de la résine homogène correspondante compte tenu de l'acroissement de volume du polymère résultant de la sulfonation.

Le Tableau I donne les valeurs de la capacité obtenue, d'une part par titrage des ions H^+ déplacés par Na^+ et, d'autre part, par pesée du polymère avant et après sulfonation, pour les différentes résines pelliculaires préparées. Dans la seconde méthode, le poids de résine sulfonée doit être corrigé du poids d'eau résiduelle demeurant dans le polymère sulfoné séché dans les conditions que nous avons décrites; on peut, en effet, montrer par titrage de Karl Fischer qu'une résine pelliculaire séchée contient encore en moyenne 0.2 molécule d'eau par groupement sulfonate. On con-

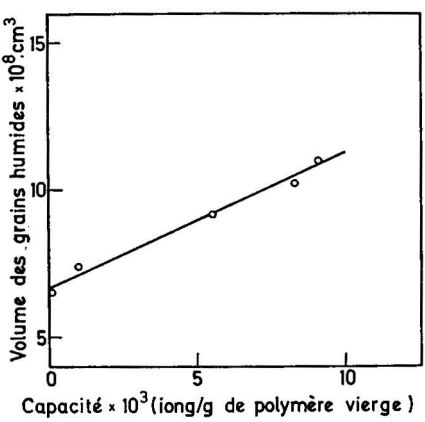


Fig. 2. Variation du volume moyen des grains humides en fonction de la capacité des résines pelliculaires.

state que les résultats obtenus par ces deux méthodes sont assez concordants sauf pour les deux résines les plus sulfonées où la capacité obtenue par pesée est plus élevée. La cause réside peut-être dans la formation de produits de réactions secondaires en quantité plus importante suite au séjour beaucoup plus long du polymère dans l'acide sulfurique concentré et chaud. Dans ces conditions, nous retenons, pour la suite des calculs, les valeurs de la capacité déterminées par titrage.

La Fig. 2 permet de constater que le volume moyen des grains de résine humide augmente linéairement avec la capacité d'échange: un grain complètement sulfoné est ainsi en moyenne 1.7 fois plus volumineux que le grain de départ.

Connaissant les capacités de la résine pelliculaire et de la résine homogène correspondante d'une part et, d'autre part, l'accroissement de volume résultant de la sulfonation, il devient possible de calculer le pourcentage en volume de la pellicule sulfonée, le pourcentage du diamètre sulfoné et enfin le poids de la pellicule sulfonée au moyen des relations suivantes:

$$\%\text{ en volume de la pellicule} = \left(\frac{\text{Capacité de la résine pell.}}{\text{Capacité de la résine homog.}} \times \frac{\text{Volume moyen grain homog.}}{\text{Volume moyen grain pell.}}\right) \times 100 \quad (1)$$

$$\%\text{ rayon sulfoné} = \left(1 - \sqrt[3]{\frac{100 - \%\text{ en volume occupé par la pellicule}}{100}}\right) \times 100 \quad (2)$$

$$\text{Poids de la pellicule} = \text{Pds polymère sulfoné} - \text{Pds polymère de départ} \times \frac{(100 - \%\text{ vol. sulf.})}{100} \quad (3)$$

Les résultats de ces calculs sont repris dans le Tableau II. Leur précision est limitée à la fois par la précision de la détermination expérimentale de la capacité et par celle du volume moyen des grains sulfonés.

Comportement particulier des résines à fines pellicules

Les résines à pellicules fines ou épaisses se différencient sous deux aspects:

(1) Le nombre de molécules d'eau d'hydratation par groupement SO_3H n'est pas le même: placées dans une atmosphère à 95% d'humidité relative, les résines à pellicules épaisses fixent, comme les résines homogènes correspondantes[10], entre 9.5 et 10 molécules d'eau par groupement SO_3H contre 6.7 seulement pour les résines à fines pellicules (Tableau III). Si l'on compare ces résultats à ceux obtenus pour des résines homogènes de différents degrés de réticulation[10], on peut conclure que les résines à fines pellicules se comportent, sous cet aspect, comme deux fois plus réticulées que la résine de départ.

(2) On a mesuré, par équilibration statique, les coefficients de distribution qui ont été rapportés au poids de polymère sulfoné (K_d*) pour trois ions de charge différente, à savoir Cs^+, Co^{2+} et Eu^{3+} (Tableau IV). On constate pour Cs^+ et Co^{2+} que K_d* reste constant quelle que soit l'épaisseur de la couche sulfonée si l'on excepte la valeur légèrement inférieure obtenue avec la résine la moins sulfonée pour Co^{2+}. Pour Eu^{3+}, par contre, les différences sont beaucoup plus nettes et se reproduisent aussi bien en essais statique que dynamique[11]; le coefficient de distribution pour les résines à fines pellicules est supérieur à celui observé avec les résines homogènes.

TABLEAU II

DÉTERMINATION DES CARACTÉRISTIQUES PHYSIQUES DES RÉSINS PELLICULAIRES: VOLUME, POIDS ET ÉPAISSEUR DE LA PARTIE SULFONÉE

Résine homogène signifie sulfonée à saturation.

Poids de polymère vierge (g)	*Capacité (ion g/g de polymère vierge)*	*Rapport entre le volume de résine homogène et pelliculaire*	*Volume sulfoné en %*	*Rayon sulfoné (%)*	*Poids du polymère sulfoné anhydre (g)*	*Poids du volume non sulfoné (g)*	*Poids de la pellicule sulfonée (g)*
1.0000	$9.1\ 10^{-3}$	1.0	100	100	1.75	0.00	1.75
1.0000	$8.5\ 10^{-3}$	(1.0 ± 0.1)	$(96 \pm 7)^*$	$(60 \pm 20)^*$	1.70	$(0.04 \pm 0.07)^*$	$(1.66 \pm 0.07)^*$
1.0000	$5.5\ 10^{-3}$	(1.2 ± 0.1)	$(71 \pm 5)^*$	$(34 \pm 2)^*$	1.44	$(0.29 \pm 0.05)^*$	$(1.15 \pm 0.05)^*$
1.0000	$1.5\ 10^{-3}$	(1.5 ± 0.1)	$(24 \pm 2)^*$	$(9.1 \pm 0.6)^*$	1.12	$(0.76 \pm 0.02)^*$	$(0.36 \pm 0.02)^*$
1.0000	$3.1\ 10^{-4}$	(1.6 ± 0.1)	$(5.4 \pm 0.3)^*$	$(1.9 \pm 0.1)^*$	1.03	$(0.966 \pm 0.003)^*$	$(0.074 \pm 0.003)^*$

* Valeurs calculées.

TABLEAU III

DÉTERMINATION DU NOMBRE DE MOLÉCULES PAR GROUPEMENT SO_3H POUR LA RÉSINE HOMOGÈNE ET POUR LES RÉSINES PELLICULAIRES

Capacité (ion g/g de polymère vierge)	*Capacité (ion g/g de polymère sulfoné sous fomre H^+)*	*Poids de résine conditionnée sous forme H^+ sèche (g)*	*Poids de résine conditionnée sous forme H^+ anhydre (g)*	*Poids de résine humide (g)*	*Poids d'H_2O fixe (g)*	*Poids d'H_2O par ion g H^+ (g)*	*Nombre de molécules d'H_2O par groupement sulfonate*
$9.1\ 10^{-3}$	$5.2\ 10^{-3}$	0.0886	0.087	0.1673	0.080	177	9.8
$8.5\ 10^{-3}$	$5.0\ 10^{-3}$	0.1151	0.113*	0.2090	0.096	170	9.4
$5.5\ 10^{-3}$	$3.8\ 10^{-3}$	0.0998	0.099*	0.1662	0.067	178	9.9
$1.5\ 10^{-3}$	$1.3\ 10^{-3}$	0.0838	0.083*	0.0957	0.013	120	6.7
$3.1\ 10^{-4}$	$3.0\ 10^{-4}$	0.3600	0.360*	0.3729	0.013	120	6.7

* Valeurs calculées.

Notons cependant que, dans la gamme des capacités étudiées, il n'y a pas d'inversion dans l'ordre d'affinité avec la charge de l'ion comme l'ont observé Skafi et Lieser[12]; si l'on normalise, en effet, les résultats obtenus pour une même concentration en contre-ion NH_4^+ (0.1 *M* par exemple), on conserve bien l'ordre d'affinité $Eu^{3+} > Co^{2+} > Cs^+$ quelle que soit l'épaisseur de la pellicule (Tableau V). De même, contrairement aux résultats obtenus par ces auteurs, nous n'observons des valeurs de K_d* pour les résines pelliculaires fines supérieures à celles des résines homogènes que dans le cas de Eu^{3+}. Il faut toutefois signaler que la comparaison entre leurs résultats et les nôtres est dangereuse étant donné que ces auteurs ont travaillé avec des résines pelliculaires beaucoup plus fines. Dans ces conditions, il semble prématuré de vouloir tirer des conclusions générales sur le comportement comparatif des résines pelliculaires et homogènes; notons simplement que, dans le cas de Eu^{3+}, le coefficient de distribution supérieur pour une résine pelliculaire fine semble à nouveau indiquer que son comportement se rapproche de celui d'une résine homogène de degré de réticulation plus important; il est, en effet, bien établi, dans le cas de résines homogènes, que le coefficient de distribution augmente avec la réticulation[13].

Si l'on tente d'expliquer ces deux comportements particuliers des résines pelliculaires, il semble bien que l'on doive exclure toute participation de la partie non-sulfonée du polymère tant dans le phénomène d'hydration que dans celui de distribution ionique: d'une part, en effet, des essais ont montré que le polymère non-sulfoné placé dans une atmosphère à 95% d'humidité relative reste parfaitement anhydre ce qui, du reste, est en parfait accord avec le caractère hydrophobe du polystyrène–divinylbenzène. D'autre part, l'analyse à la microsonde électronique de Castaing[8] sur une résine pelliculaire saturée en ions ferreux montre qu'aucune fixation n'a lieu en dehors de la pellicule active (Fig. 1).

Dans ces conditions, nous pensons plutôt pouvoir attribuer les phénomènes observés à un effet mécanique: les résines sulfonées sur une fine pellicule ne peuvent gonfler dans l'eau que de façon restreinte, la majeure partie du grain demeurant hydrophobe. Il n'est pas étonnant, dès lors, que cette contrainte conduise à un gonflement analogue à celui d'une résine homogène plus réticulée.

Néanmoins, ni le degré de réticulation des échangeurs d'ions homogènes, ni la diminution de l'épaisseur de la pellicule sulfonée dans le domaine exploré ne modifient de façon importante l'équilibre d'échange ionique. Toutefois, Boyd et Soldano[14] ont montré, dans le cas des résines homogènes, que le coefficient de diffusion d'un lanthanide dans la résine diminue fortement lorsque le degré de réticulation augmente; il diminue d'un facteur six en passant d'une résine X-8 à une résine X-16.

Dans ces conditions, en passant d'une résine homogène à une résine pelliculaire de même réticulation et de même granulométrie, on doit s'attendre à une diminution du coefficient de diffusion du lanthanide et, par conséquent, la diminution simultanée du coefficient de diffusion et de l'épaisseur de la couche de diffusion dans la résine pelliculaire doivent exercer des effets opposés dans la contribution du terme de diffusion particulaire à la hauteur équivalente à un plateau théorique. Les résultats expérimentaux qui seront discutés dans la seconde partie montreront le gain réel réalisé avec les résines pelliculaires.

TABLEAU IV

DÉTERMINATION DU COEFFICIENT DE DISTRIBUTION K_d* RAPPORTÉ AU POIDS DE RÉSINE SULFONÉE POUR Cs^+, Co^{2+}, Eu^{3+} À pH = 2.00 ENTRE UNE SOLUTION AQUEUSE RESPECTIVEMENT 0.1 *M*, 0.8 *M* ET 1.5 *M* EN NH_4Cl ET DES RÉSINES PELLICULAIRES DE DIFFÉRENTES CAPACITÉS À 25° ± 0.1, K_d: coefficient de distribution rapporté au poids total de la phase solide à l'état sec.

Les expériences ont été effectuées en équilibrant 10 ml de solution contenant le traceur radioactif avec un poids p_1 de résine partiellement sulfonée. K_d ($ml \cdot g^{-1}$) = $(a_4 \times 10)/(p_1 \times a_3)$. K_d* ($ml \cdot g^{-1}$) = = $(a_4 \times 10)/(p_2 \times a_3)$.

	Capacité ion g/g de polymère vierge	*Poids de résine sulfonée (g) (p_1)*	*Poids de la fraction sulfonée (g) (p_2)*	*Activité initiale en solution (coups) (a_1)*	*Activité à l'équilibre en solution (coups) (a_2)*	*Activité corrigée du fond continu (coups) (a_3)*	*Activité fixée sur la résine (coups) (a_4)*	*Log K_d*	*Log K_d**
Cs^+	$3.1\ 10^{-4}$	0.1435	0.0103	733191	689018	675018	44173	0.66	1.8
	$1.5\ 10^{-3}$	0.2686	0.087	786177	505777	491177	280400	1.33	1.8
	$5.5\ 10^{-3}$	0.1862	0.129	867790	444171	430171	423619	1.72	1.8
	$9.1\ 10^{-3}$	0.1448	0.1448	622075	314992	300992	307083	1.85	1.8
Co^{2+}	$3.1\ 10^{-4}$	0.1165	0.0084	424360	418139	403139	6221	0.12	1.3
	$1.5\ 10^{-3}$	0.2276	0.074	514645	441304	426304	75341	0.89	1.4
	$5.5\ 10^{-3}$	0.1534	0.123	448184	342608	327608	105576	1.32	1.4
	$9.1\ 10^{-3}$	0.1488	0.1488	457589	333392	318392	124197	1.42	1.4
	Résine commerc.	0.2105	0.2105	500381	329494	314494	170887	1.41	1.4
Eu^{3+}	$3.1\ 10^{-4}$	0.1169	0.0084	281618	253281	238281	28337	1.01	2.1*
	$1.5\ 10^{-3}$	0.1847	0.060	350362	213561	198561	137001	1.57	2.1*
	$5.5\ 10^{-3}$	0.1150	0.092	330593	197770	182770	132823	1.80	1.9*
	$9.1\ 10^{-3}$	0.1435	0.1435	268896	146591	131591	122305	1.81	1.8

* Valeurs calculées.

TABLEAU V

VALEURS DES COEFFICIENTS DE DISTRIBUTION DU Cs^+, Co^{2+}, Eu^{3+} POUR UNE RÉSINE PELLICULAIRE DE CAPACITÉ 3.1 10^{-4} ET UNE RÉSINE HOMOGÈNE DE CAPACITÉ 9.1 10^{-3} CALCULÉES DANS LES CONDITIONS NORMALISÉES DE 1.0 *M* EN NH_4Cl ($t = 25° \pm 0.1$)

Ion	Cs^+	Co^{2+}	Eu^{3+}
Log K_d* (capacité 3.1 10^{-4})	1.8	3.1	5.6
Log K_d* (capacité 9.1 10^{-3})	1.8	3.2	5.3

CONCLUSIONS

Il est possible de préparer des résines pelliculaires cationiques à base de polystyrène–divinylbenzene sulfonates par sulfonation d'un polystyrène–divinylbenzène dans l'acide sulfurique concentré et chaud en présence de sel d'argent, en arrêtant la sulfonation après un temps fixé en fonction de l'épaisseur pelliculaire recherchée. Certaines propriétés, comme la teneur en eau ou le coefficient de distribuition, sont comparables à celles des résines homogènes correspondantes (mêmes réticulation et granulométrie) à condition toutefois de rapporter les grandeurs au poids de la pellicule sulfonée.

Dans le cas des lanthanides qui nous intéressera dans la suite, il semble néanmoins se produire un léger effet sur le coefficient de distribution qui laisse prévoir une diminution du coefficient de diffusion particulaire de nature à faire perdre en partie le bénéfice résultant de la diminution de l'épaisseur de la couche de diffusion.

REMERCIEMENTS

Il nous est agréable de remercier l'Institut Interuniversitaire des Sciences Nucléaires pour les subsides accordés à notre laboratoire.

RÉSUMÉ

L'analyse par la microsonde à balayage des résines polystyrène–divinylbenzène, Bio-Beads SX-8 (45–50 μm), partiellement sulfonées selon la méthode décrite par Hansen et Gilbert montre clairement l'obtention d'échangeurs d'ions pelliculaires. L'épaisseur de la péllicule sulfonée a été déterminée à partir de la capacité d'échange et du gonflement dû à la sulfonation. L'étude de l'évolution des propriétés de ces résines en fonction de leur capacité montre qu'à l'exception des résines à fines pellicules qui se comportent comme plus réticulées que le polymère de départ, les propriétés sont comparables à celles des résines homogènes, à condition toutefois de les rapporter au poids de la pellicule sulfonée.

BIBLIOGRAPHIE

1 J. J. Kirkland, *Chromatographie en Phase Liquide*, Gauthier-Villars, Paris, 1973.
2 E. P. Horwitz et C. A. A. Bloomquist, *J. Chromatogr. Sci.*, 12 (1974) 11.
3 J. A. Marinsky et Y. Marcus, *Ion Exchange and Solvent Extraction*, Vol. 5, Marcel Dekker, New York, 1973, p. 207.

4 L. C. Hansen, *Theoretical and Experimental Study of Resin Design for H.P.L.C.*, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1973.
5 L. C. Hansen et T. W. Gilbert, *J. Chromatogr. Sci.*, 12 (1974) 458.
6 L. C. Hansen et T. W. Gilbert, *J. Chromatogr. Sci.*, 12 (1974) 464.
7 G. Hild, A. Haeringer et P. Rempp, *C.R. Acad. Sci., Ser. C* (1975) 1405.
8 C. R. Castaing, *Thèse de doctorat*, Office National d'Études et de Recherches Aéronautiques, Châtillon-sous-Bagneux (Seine), Publication No. 55, 1951.
9 R. M. Wheaton et D. F. Harrington, *Ind. Eng. Chem.*, 44 (1952) 1796.
10 G. E. Boyd et B. A. Soldano, *Z. Electrochem.*, 57 (1953) 162.
11 F. Schoebrechts, E. Merciny et G. Duyckaerts, *J. Chromatogr.*, soumis pour publication.
12 M. Skafi et K. H. Lieser, *Z. Anal. Chem.*, 250 (1970) 306.
13 O. D. Bonner et L. L. Smith, *J. Phys. Chem.*, 62 (1958) 250.
14 G. E. Boyd et B. A. Soldano, *J. Amer. Chem. Soc.*, 75 (1953) 6091.

Journal of Chromatography, 174 (1979) 361–368

CHROM. 11,822

TEMPERATURE EFFECTS IN AFFINITY CHROMATOGRAPHY OF ALANINE AMINOTRANSFERASE

T. K. KORPELA and E. MÄKINEN
Department of Biochemistry, University of Turku, SF-20500 Turku 50 (Finland)
(Received December 28th, 1978)

SUMMARY

The effects of temperature on the elution parameters of alanine aminotransferase and albumin were studied on substituted agaroses designated for the affinity chromatography of the enzyme.

The elution volume of alanine aminotransferase depended logarithmically and and the elution volume of albumin linearly on temperature. Both decreased when the temperature increased. It was concluded that the observed elution volume of alanine aminotransferase was due to two types of retardation mechanisms: specific (the logarithmic mode) and non-specific (the linear mode), both of which were additive. Thermodynamic parameters were estimated for the specific mode and the calculation resulted in ΔH^0 and ΔS^0 values of *ca.* −40 kJ/mole and −140 J/mole·°K, respectively.

INTRODUCTION

Despite the significant temperature effects in affinity chromatography, very few papers have considered the topic[1–5]. In adsorption chromatography temperature will usually exert a strong influence on the equilibrium between the mobile and stationary phases[6]. At present there are no profound differences between biospecific and non-biospecific adsorptions, and therefore some aspects of adsorption chromatography are worth utilizing in affinity systems. Usually heat is given off during adsorption, and a temperature rise will therefore usually lead to a faster migration of the solutes[6]. In contrast to the ionic forces, the hydrophobic interaction increases with increase in temperature[7]. Thermodynamic parameters describe the sorption process both qualitatively and quantitatively but, unfortunately, much effort is generally required to obtain them.

We have previously examined the affinity purification of alanine aminotransferase (AlaAT*) at 8°C on various agaroses substituted with substrates or inhibitors of

* Abbreviations: AlaAT = alanine aminotransferase; AE-agarose = ethylenediamine coupled to cyanogen bromide-activated agarose; 2-oxoglutaric-AE-agarose = 2-oxoglutarate coupled to AE-agarose; CS-agarose = cycloserine coupled to cyanogen bromide activated agarose.

the enzyme[8,9]. It was suggested that the separation was due to a combination of specific and non-specific affinities[8]. Gradient elution, commonly used in affinity chromatography, greatly increases the complications of any theoretical treatment of the elution process[10]. The enzyme AlaAT was elutable from the column using normal (isocratic) elution when the buffer composition was properly selected. Therefore, and because of the heat stability of AlaAT, the affinity system of AlaAT was suitable for studying temperature effects. As the present study contained much routine work, the analyses were automated.

The results were consistent with the high negative enthalpy of sorption. A new approach for calculating thermodynamic parameters from elution volumes produced results that were parallel to those previously obtained from binding constants[1–3].

EXPERIMENTAL

Materials

Alanine aminotransferase (EC 2.6.1.2) in 1.8 *M* ammonium sulphate solution (93 U/mg protein) from pig heart, lactate dehydrogenase (LDH), salt-free powder (880 U/mg protein), L-alanine, 2-oxoglutaric acid, reduced β-nicotinamide-adenine dinucleotide (NADH), grade III, bovine albumin, fraction V and Sepharose 4B 200 were purchased from Sigma (St. Louis, Mo., U.S.A.) Ultrogel AcA 44 was obtained from LKB (Stockholm, Sweden).

The substrate solution contained 37 m*M* L-alanine, 25 m*M* disodium 2-oxoglutarate, 40 μ*M* pyridoxal 5′-phosphate, 0.3 m*M* NADH and 4400 units of LDH per litre in 0.1 *M* sodium phosphate solution (pH 7.6).

The sample to be chromatographed (190 μl) usually contained 1 μl of the AlaAT solution and 5 mg of albumin dissolved in 1 ml of elution buffer (25 m*M* sodium phosphate, pH 6.0, supplemented with 0.1 *M* sodium chloride).

The preparation of the affinity gels and the methods used for their analyses have been reported earlier[8].

Apparatus

An appartus for automatically recording enzyme activity and protein (absorbance at 280 nm) was constructed. Fig. 1 shows the shceme of the device. Elution buffer of constant composition was introduced at a flow-rate of about 30 ml/h with an Ismatec mp-ge peristaltic pump via a sample applicator[11] into a jacketed glass column of 12 mm I.D., made in our laboratory workshop. The gel volumes were usually 50 ml in the packed state, except that of Ultrogel AcA 44, which was used in a volume of about 90 ml. The temperature in the jacket was maintained by circulating water–ethylene glycol (2:1) (8 l/min) from a Lauda K2 thermostat. The thermostat was provided with a suitable circulation of ethanol at −20°C through its cooling coil. The temperature of the solution entering the column was pre-adjusted so that about 40 cm of the feeding tube was coiled on the jacket.

The eluate was divided after the column into two parts, one moving through an Isco UA-2 UV analyser (280 nm) and the other being taken for enzyme analysis by the pump at a flow-rate of 8 ml/h. An equal volume of the substrate solution was mixed with the flow of enzymic sample and the solution was then

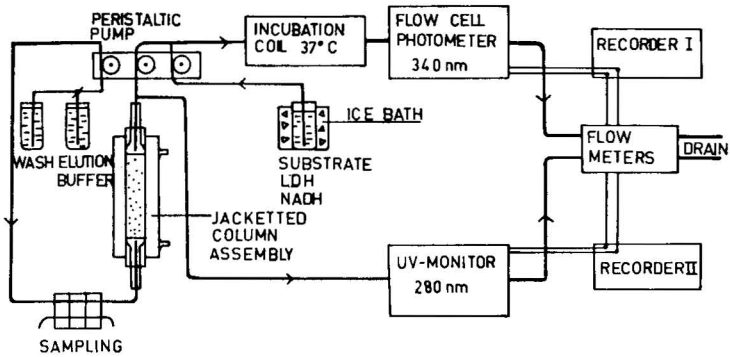


Fig. 1. Apparatus used for studying the effeets of temperature on the chromatography of alanine aminotransferase.

introduced through an incubation coil (PTFE, 1 mm I.D., internal volume 12 ml) into a flow cell (volume 0.5 ml) which was continuously monitored by a Beckman DB spectrophotometer. The linear transmission output of the Beckman DB was changed to linear absorbance with an Optilab Multianalog 201 converter. The flow-rates of the enzyme and protein channels were separately monitored semi-continuously by two 10-ml siphon-operated flow meters[12]. The photometers were synchronized to the same time scale by following the absorbance of albumin at 280 nm in both channels. In such runs NADH and pyridoxal phosphate were omitted from the substrate solution. The elution volumes were calculated according to the time delay and according to the check points obtained by the flow meters.

The chromatographic runs were carried out from lower to higher temperatures. The gel was not washed between runs because of the virtually 100% recoveries of the proteins. When the temperature of the column was changed, it was allowed to equilibrate for 1 h before applying a new sample.

The elution volumes of 0.5% Blue Dextran 2000 and tritium oxide were separately measured at temperatures of 3, 25 and 37°C, collecting fractions which were analysed by measuring the absorbance at 265 nm and radioactivity.

RESULTS

The temperature effects were studied on three substituted agaroses, all of which separated AlaAT from albumin at 8°C[8]. The elution buffer was the same as described earlier[8], except that in one experiment on AE-agarose the pH of the buffer was changed from 6.0 to 7.4.

Fig. 2A–C shows that on all three gels an increase in temperature only slightly decreased the elution volume of albumin, but that the decrease was large with AlaAT. The elution volume of albumin was a linear function of the temperature (about −1 ml per 10°C), while that of AlaAT was logarithmic. As estimated from Fig. 2A–C, the change in the elution volume of AlaAT between 5 and 15°C was about 15 ml. At pH 7.4 the curve was of the same form, but the change was only 7 ml.

Stabilities of the gels and AlaAT

Curves similar in form to those in Fig. 2 were reproducible when the runs

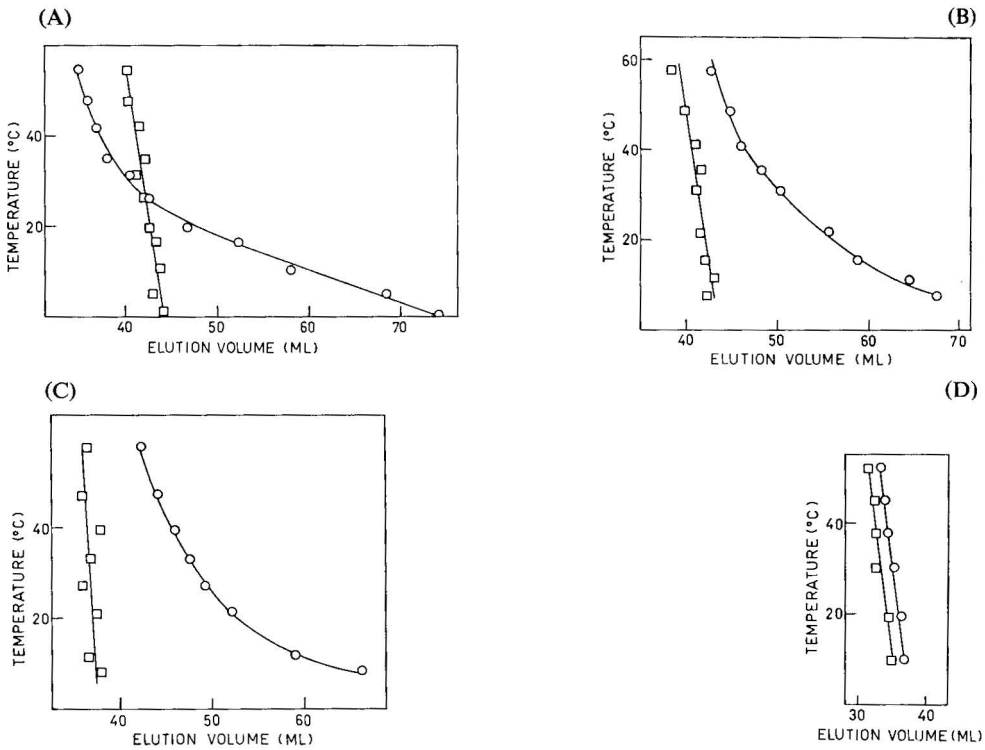


Fig. 2. Effect of temperature on elution volumes of alanine aminotransferase (○) and albumin (□). A = on AE-agarose; B = on 2-oxoglutaric-AE-agarose; C = on CS-agarose; D = on unsubstituted agarose. The gel volume was 50 ml. The elution was carried out with 25 m*M* sodium phosphate supplemented with 0.1 *M* sodium chloride.

were repeated. However, a small decrease in the gel volume (about 1–2%) was observed after exposure to the highest temperatures, which were higher than the manufacturer's recommendations (up to about 40°C). According to amino acid analyses before and after the run cycle with CS-agarose, the ligand concentration (per settled volume of gel) did not change during the cycle. Especially with CS-agarose, leakage of the ligand should have led to significant enzyme inhibition, but this was not detectable. The results thus suggest that the whole matrix either leaked or irreversibly melted at the highest temperatures.

As can be estimated from Fig. 2A–C, the enzyme remained in the column at the highest temperatures for about 1.5 h. The area of the enzyme activity peak was constant to 57–60°C. When a separate enzymic sample was kept in a water-bath of 60°C for 1.5 h it lost 40% of its activity. Hence the protein–gel interaction shielded the enzyme from denaturation. With affinity gels capable of hydrophobic interactions, the shielding may be even larger because the adsorption is stronger at higher temperatures owing to the endothermic nature of the binding[7].

Isothermal runs

No systematic study was made of the effects of the proteins on their elution

volumes because the automatic system was inconvenient for this purpose. On AE-agarose a three-fold increase in the concentrations did not alter the elution volumes at 5 or 35°C.

Basis for dividing the retardation of AlaAT into specific and non-specific modes

Fig. 2D shows that the elution volumes of albumin and AlaAT changed linearly and in a parallel manner on unsubstituted agarose. On Ultrogel AcA 44 the changes were also linear, but not exactly parallel (Fig. 3). There are arguments based on the results presented in Figs. 2 and 3 that the elution volume of AlaAT approaches asymptotically a line parallel to that of albumin. If this assumption is correct, the retardation of AlaAT can be divided into two additive components, one logarithmically dependent on temperature, which probably originates from adsorption, and the other, linearly dependent, originating from a gel chromatographic separation. We shall hereafter call these specific and non-specific contributions, respectively. Nishikawa *et al.*[13] came to similar conclusions when deriving their equation for affinity separation:

$$V_e = V_0 + K_{av}V_i + K_pV_g \quad (1)$$

where V_e = observed elution volume, V_0 = void volume, V_i = internal volume, V_g = volume taken by the gel itself and K_p and K_{av} are the distribution coefficients for adsorption and gel chromatography, respectively.

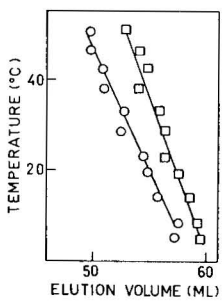


Fig. 3. Effect of temperature on elution volumes of alanine aminotransferase (○) and albumin (□) on Ultrogel AcA 44. The gel volume was 90 ml. Experimental conditions as in Fig. 2.

Calculation of the thermodynamic parameters from elution volumes

Assuming that the difference between the specific (V_e^s) and non-specific (V_e^n) elution volumes of AlaAT at 60°C was 1 ml and that V_e^n is parallel to the V_e of albumin in Fig. 2A–C, log (V_e—V_e^n) plotted against temperature produced a straight line for all runs, which obeyed, at pH 6.0, the equation log (V_e—V_e^n) = $-0.27\,t + 1.6$ where t (°C) is temperature. From this line one can calculate that an increase of 10°C in temperature caused a 1.9-fold decrease in V_e^s.

On the basis of eqn. 1 and with the assumptions made above, $V_e^n = V_0 + K_{av}V_i$ for AlaAT and thus $K_p = (V_e - V_e^n)/V_g$. For the thermodynamic values V_g must be known. Because the elution volume of tritium oxide shows the sum of

TABLE I

THERMODYNAMIC PARAMETERS FOR THE CHROMATOGRAPHY OF ALANINE AMINOTRANSFERASE ON DERIVATIZED AGAROSES

The parameters were calculated from elution volumes for the "specific binding mode" of the enzyme with the assumptions described in the text. The column headed V_g gives the volume taken by the gel at 25°C when the total volume was 50 ml.

Gel	ΔH° *(kJ/mole)*	ΔS° *(J/mole·°K)*	$\Delta G_{298\,^\circ K}$ *(kJ/mole)*	$K^{298\,^\circ K}$	*Correlation coefficient (r)*	V_g *(ml)*
AE-agarose, pH 6.0	−48.3 ± 1.4	−157 ± 5	−1.40 ± 0.08	1.76	0.995	4.4
AE-agarose, pH 7.4	−43.5 ± 1.8	−147 ± 6	0.36 ± 0.09	0.86	0.995	4.3
2-Oxoglutaric-AE-agarose, pH 6.0	−46.7 ± 2.8	−151 ± 9	−1.67 ± 0.15	1.96	0.980	4.2
CS-agarose, pH 6.0	−35.7 ± 0.9	−115 ± 3	−1.47 ± 0.05	1.81	0.997	5.3

$V_0 + V_i$ (ref. 14), $V_g = V_t - (V_0 + V_i)$, where V_t = total gel volume. Table I shows V_g values (at 25°C), which were used as constants in calculations of the thermodynamic values. The void volume appeared to be essentially independent of the temperature on 2-oxoglutaric-AE- and AE-agaroses (about 16 ml), while the V_e value of tritium oxide increased linearly (about 0.5 ml per 10°C) on increasing the temperature, thus indicating that the water–gel interaction increases with increase in temperature. This agrees with the fact that the protein–gel interaction decreased (about −1 ml per 10°C) with temperature. We could not measure the V_0 of CS-agarose because of the strong adsorption of Blue Dextran.

A plot of ln K_p against $1/T$ gives ΔH° and ΔS° values[15]. Fig. 4 shows three such plots. Both lines obtained with AE-agarose (pH 6.0 and 7.4) indicate points of discontinuity at temperatures of about 20°C and 30–35°C. The line with 2-oxoglutaric-

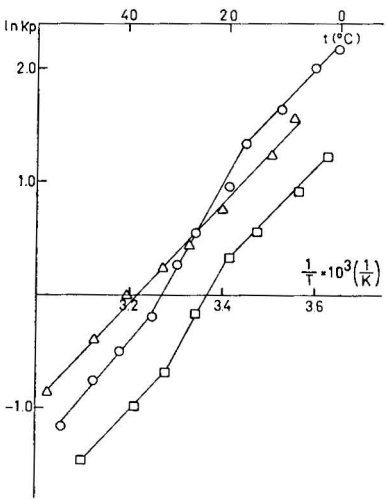


Fig. 4. Van 't Hoff plots obtained on AE-agarose, at pH 6.0 (○), on the same gel at pH 7.4 (□) and on CS-agarose at pH 6.0 (△). The distribution coefficients (K_p) were calculated for the "specific binding mode" with the assumptions described in the text.

AE-agarose (omitted from Fig. 4 for clarity) showed one point of discontinuity at 20°C. Fig. 4 shows that the experimental points on CS-agarose fit the line well. Owing to the assumptions made in the calculations, the points of discontinuity may be artefacts, and therefore only one set of values for $\Delta H°$ and $\Delta S°$ are given in Table I. It is interesting, however, that in two reports[2,3] points of discontinuity near 20°C have been described, which might suggest a common property of the agarose.

Table I also shows $\Delta G°$ and extrapolated K_p values at 25°C obtained from the correlation $\Delta G° = RT\ln K_p = \Delta H° - T\Delta S°$. In spite of the assumptions made above, the thermodynamic values correspond with those found in earlier studies on binding constants[1,2]. This suggests that the assumptions were correct. Because the true effective volume of the stationary phase is only approximated by V_g, the values of the standard entropies, and hence $\Delta G°$ and K_p, are also approximate. They may, however, be useful for comparative purposes.

DISCUSSION

The temperature effects give valuable information about the forces acting between solutes and the matrix. In the context of affinity chromatography, such studies are particularly important for a deeper understanding of the mechanism. An estimate of the temperature effects is also important for practical chromatography.

Even now in many affinity systems the only rational indication of biospecificity is an "exceptional" power to purify a protein. It is indeed very difficult to trace the precise source of separation in affinity chromatography, if for example, an unknown regulatory or "social site"[16] or another unknown effector site takes part in the separation. Only in very succesful affinity systems has biospecificity been proved fairly clearly. In our opinion, there is no absolute method by which biospecificity can be verified. It is not, for example, sufficient to prove that an enzyme is elutable from the column with a substrate, because it is now clear that proteins can be eluted even from ion exchangers using specific elution[17]. The elutability with substrate may be one indication, but the co-occurrence of a few indications of specificity may be considered convincing. When a suitable amount of information has been accumulated concerning the effects of temperature on affinity chromatography, they may be a useful way of describing the degree of biospecificity.

On the gels in Fig. 2A–C the elution volumes of AlaAT relative to albumin at 8°C were 1.4, 1.5 and 1.8, respectively[8]. Based on the present study it seems that AlaAT was best separated on CS-agarose because the non-specific contribution was most favourable on it. Gel chromatography on AcA 44 did not support the suggestion that there would have been great alterations in the structure of AlaAT at different temperatures (*e.g.*, subunit equilibria) which could explain the similar behaviour of AlaAT on different gels. Each ligand of the gels tested contained specificity elements of the substrates of AlaAT. It is known in enzymology that activation energy is more characteristic of the enzyme than of the substrate[18]. This could explain the similarity. Webb[19] has discussed the effects of temperature on enzyme inhibition.

In the course of studying the affinity chromatography of AlaAT, it has been shown that the elution volume of AlaAT is very sensitive to salt concentration, pH and temperature, in contrast to the elution volume of albumin, which is an "inert" reference protein. According to our results, albumin does not show

special chromatographic properties on substituted agaroses and therefore it is probably a suitable reference protein[20].

ACKNOWLEDGEMENTS

We are indebted to the Instrumentariumin Tiedesäätiö (Instrumentarium Foundation) for financial support of the work.

REFERENCES

1 C. R. Lowe and P. D. G. Dean, *Biochem. J.*, 133 (1973) 515.
2 M. J. Harvey, C. R. Lowe and P. D. G. Dean, *Eur. J. Biochem.*, 41 (1974) 353.
3 H-W. Hsu, D. S. Davis, C. H. Wei and W.-K. Yang, *Anal. Biochem.*, 73 (1976) 513.
4 H. P. Jennisen, *J. Chromatogr.*, 159 (1978) 71.
5 J. Turková, *Affinity Chromatography*, Elsevier, Amsterdam, Oxford, New York, 1978, pp. 71–75.
6 J. C. Giddings, in E. Heftman (Editor), *Chromatography*, Van Nostrand, London, 3rd ed., 1975, p. 30.
7 S. Hjertén, J. Rosengren and S. Påhlman, *J. Chromatogr.*, 101 (1974) 281.
8 T. Korpela, A. Hinkkanen and R. Raunio, *J. Solid-Phase Biochem.*, 1 (1977) 215.
9 T. Korpela, *J. Chromatogr.*, 143 (1977) 519.
10 C. J. O. R. Morris and P. Morris, *Separation Methods in Biochemistry*, Pitman Publishing, London, 1976, p. 33.
11 T. Korpela, *J. Chromatogr.*, 152 (1978) 531.
12 T. Korpela and E. Mäkinen, *J. Chromatogr.*, 166 (1978) 268.
13 A. Nishikawa, P. Bailon and A. Ramel, *J. Macromol. Sci.*, A10 (1976) 149.
14 J. Reiland, *Methods Enzymol.*, 22 (1971) 296.
15 R. P. Scott, in C. F. Simpson (Editor), *Practical High Performance Liquid Chromatography*, Heyden, London, 1976, p. 66.
16 P.-Å. Albertsson, *J. Chromatogr.*, 159 (1978) 111.
17 R. K. Scopes, *Biochem. J.*, 161 (1977) 253.
18 M. Dixon and E. C. Webb, *Enzymes*, Longmans, London, 2nd ed., 1965, p. 158.
19 J. L. Webb, *Enzyme and Metabolic Inhibitors*, Vol. 1, Academic Press, London, 1963, pp. 747–799.
20 T. Korpela, E. Kukko and A. Hinkkanen, *J. Solid-Phase Biochem.*, in press.

Journal of Chromatography, 174 (1979) 369–377

CHROM. 11,773

DETERMINATION OF (*R*)- AND (*S*)-EPIMERS AT C-1 IN RESIDUAL AMOUNTS OF (±)-*cis,trans*-PERMETHRIN AND CYPERMETHRIN BY GAS–LIQUID CHROMATOGRAPHY*

RALPH A. CHAPMAN and CAROL R. HARRIS

Agriculture Canada Research Institute, University Sub-Post-Office, London, Ontario N6A 5B7 (Canada)

(Received February 5th, 1979)

SUMMARY

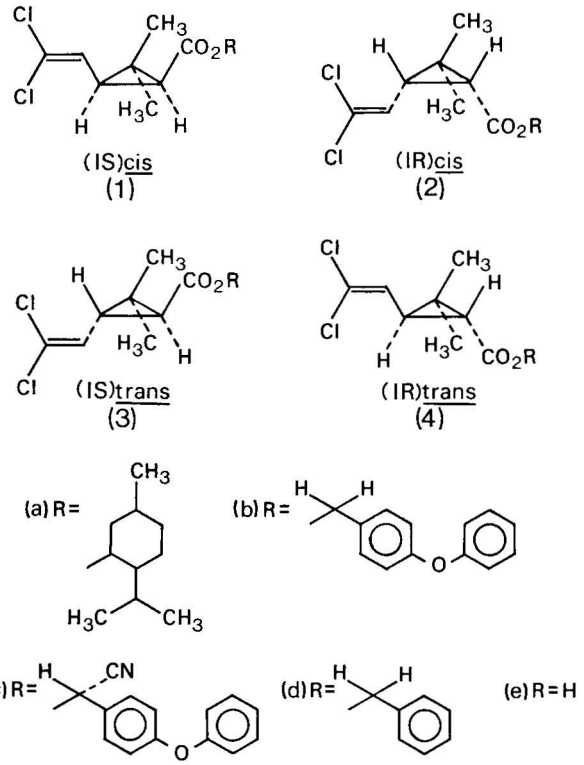
Treatment of (1*R*)*cis*-, (1*S*)*trans*- and (1*R*)*trans*-permethrin with sodium *l*-menthylate at room temperature produced the *l*-menthyl esters of the corresponding 2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylic acids without epimerization at C-1 or C-3. These three esters, or the mixture of four esters produced by similar treatment of (±)-*cis,trans*-permethrin were readily separated by gas–liquid chromatography on OV-210. Minimum detectable amounts of each ester were *ca.* 0.1 ng using a pulsed ^{63}Ni electron-capture detector. The *l*-menthyl esters corresponding to the permethrin isomers could also be prepared in high yield and without epimerization by alkaline hydrolysis at room temperature to the respective cyclopropanecarboxylic acids, crown-ether-catalyzed conversion to the benzyl esters and subsequent room-temperature trans-esterification of this ester with sodium *l*-menthylate. Using the longer procedure, cypermethrin could be converted into the same four-component mixture of *l*-menthyl esters that was obtain from permethrin. The application of these procedures to the determination of the individual (*R*)- and (*S*)-epimers (at C-1) in residual amounts of the parent pyrethroids is discussed.

INTRODUCTION

The significance of total residues of the pyrethroid insecticides permethrin (1b–4b) and cypermethrin (1c–4c) is difficult to evaluate because they are mixtures of isomers with different toxicological properties. In general, high insecticidal activity is associated with the (*R*)-configuration of the chiral cyclopropyl carbon, C-1, adjacent to the carboxyl group [2 (b and c); 4 (b and c)], whereas the enantiomer having the opposite configuration [1 (b and c); 3 (b and c)] is only slightly toxic[1,2]. In addition, enantiomers of cypermethrin differing in the configuration at C-α of the *m*-phenoxybenzyl alcohol portion of the molecules also differ in toxicity[2,3]. The synthetic methods used commercially to prepare these two insecticides do not involve chiral reagents, and equal quantities of the (*R*)- and (*S*)-configuration at C-1 and C-α are produced but degradation by enzymes or other biologically active systems in the

* Contribution No. 741, Research Institute, London, Ontario N6A 5B7, Canada.

environment will involve natural chiral reagents, which may preferentially degrade one enantiomer[4,5]. A quantitative determination of each of the optical isomers is required to describe fully the degradation of commercial mixtures or to assess the importance of residues.



Gas–liquid chromatography (GLC) has proven to be a valuable quantitative technique for both the direct separation of enantiomers on chiral stationary phases and their indirect separation on non-chiral phases after conversion into diastereoisomers; short reviews have been included in several publications[6,7,8]. The separation of diastereoisomers has been successfully applied to the C-1 enantiomers of chrysanthemic acid[9], a number of pyrethroids containing this acid[8] and, more recently, to the 2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylic acids (1e–4e) derived from permethrin at the level of 30–60 mg[10]. We wish to describe procedures for the quantitative preparation of the four *l*-menthyl 2,2-dimethyl-3-(2,2-dichlorovinyl)-cyclopropanecarboxylates (1a–4a) from permethrin or cypermethrin at the microgram level, and the separation and analysis of these esters by GLC with electron-capture detection to provide quantitative determination for the four optical isomers of permethrin and four of the eight possible optical isomers of cypermethrin at levels down to 0.05 ppm in soil.

MATERIALS AND METHODS

Instrumentation, chemicals and reagents

A Tracor MT 220 gas chromatograph fitted with a U-shaped glass column (60 cm × 4 mm I.D.) packed with 2.5% of OV-210 on Varaport 30 (100–120 mesh) and equipped with a ^{63}Ni electron-capture detector was used for analysis of the *l*-menthyl esters. The first 7 cm of the column were left unpacked to provide a glass liner for the injection port (maintained at 200°); glass wool was not used at the injector end of the column. Argon–methane (95:5) at *ca.* 70 ml/min was used as carrier gas. The detector was maintained at 290°, with a 55-V polarizing voltage applied at a 4-μsec pulse width and 240-μsec pulse rate. The detector signal was fed to a strip-chart recorder (sensitivity of 255 mm/mV) via an electrometer operating at 1.6 nA/mV. The column was maintained at 125°, and, under these conditions, the retention times of *l*-menthyl (1*S*)*cis*-, (1*R*)*cis*-, (1*S*)*trans*- and (1*R*)*trans*-2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylates were 4.9, 5.3, 6.5 and 7.6 min, respectively. Typical sensitivity is shown in Fig. 1. Under these conditions, the *cis*-benzyl esters (1d and 2d) were eluted in 4.7 min, and their *trans*-analogues (3d and 4d) in 6.0 min. In preliminary experiments, analyses for permethrin or cypermethrin were carried out as described previously[11].

Permethrin [3-phenoxybenzyl (±)-*cis,trans*-2,2-dimethyl-3-(2,2-dichlorovinyl)-cyclopropanecarboxylate] (93.9%) was supplied by Chipman (Stoney Creek, Canada). Cypermethrin [(±)-α-cyano-3-phenoxybenzyl (±)-*cis,trans*-2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylate] (96%) was provided by Shell Research (Woodstock Agricultural Research Centre, Sittingbourne, Great Britain). The samples of (1*R*)-*cis*- (2b), (1*S*)*trans*- (3b) and (1*R*)*trans*-permethrin (4b) were from the laboratory of Dr. M. Elliott (Department of Insecticides and Fungicides, Rothamsted Experimental Station, Harpenden, Great Britain). Benzyl bromide, *l*-menthol and the crown ether, dicyclohexyl-18-crown-6, were from Aldrich (Milwaukee, Wisc., U.S.A.).

Hexane (Code No. 641100, Shell Canada, Burlington, Canada), benzene (ACS reagent, Fisher Chemical Co., Don Mills, Canada) and chloroform (ACS reagent, Caledon Labs., Georgetown, Canada) were distilled in glass in our laboratory, the benzene and hexane from potassium permanganate and sodium–lead alloy (dri-Na, Baker) and the chloroform from permanganate only. Benzene used in the preparation of sodium *l*-menthylate solutions was redistilled from sodium. Absolute ethanol was from Consolidated Alcohols (Toronto, Canada) and was used as received. Silica gel (100–200 mesh, Grade 923) (Fisher) containing 4.2% of moisture was used as received. The glass chromatography columns were of 1.5 cm I.D. and were fitted with a coarse fritted-glass disc at the bottom and a ground-glass joint at the top to permit attachment of a reservoir.

Solutions of dicyclohexyl-18-crown-6 (0.02 *M*) and benzyl bromide (0.2 *M*) were prepared by dissolving 0.37 g and 2.4 ml, respectively, of chemical in 50 and 100 ml of benzene; benzyl bromide solution was prepared freshly each month. The ethanolic 5% potassium hydroxide used was prepared by dissolving 5 g of 85% pellets in 5 ml of water and diluting with 95 ml of absolute ethanol.

The 0.01 and 0.05 *M* solutions of sodium *l*-menthylate in benzene were prepared by treating 13.4 g of *l*-menthol dissolved in 50 ml of anhydrous benzene with 0.15 and 0.60 g of finely cut sodium, respectively, and refluxing the mixture until the

sodium was dissolved (*ca.* 24 h), while maintaining anhydrous conditions. Before cooling, the apparatus was purged with nitrogen, and the solution was allowed to cool to room temperature in this atmosphere. For storage, the reagent was transferred to a glass container that was closed by a PTFE-lined cap after being purged with nitrogen. The solutions of sodium *l*-menthylate in benzene were susceptible to hydrolysis and (particularly the more concentrated solutions) to oxidation. Oxidation was minimized by allowing the prepared reagent to cool in a relatively inert atmosphere and by storing it under nitrogen in a tightly closed container. With these precautions, the more concentrated solution was usable for several days. Samples that had undergone considerable oxidation produced unacceptable levels of interference in the chromatograms. The more dilute solution was apparently more susceptible to hydrolysis, which was observed as a gradual loss of activity in trans-esterification; its useful life was several weeks (depending on the frequency of opening the container). Reagent-blank and standard solutions should be processed with the unknowns to permit assessment of these problems should they arise.

The effects of benzene, chloroform and dicylclohexyl-18-crown-6 on human physiological processes have not been fully examined, and appropriate care should be taken to keep exposure to the absolute minimum until these effects have been carefully evaluated.

Hydrolysis of permethrin and cypermethrin and recovery of the 2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylic acids (1e–4e)

Permethrin or cypermethrin (up to 200 μg) in 2–3 ml of ethanol, or the pyrethroid-containing benzene–hexane (80:20) fraction from Florisil chromatography of a soil extract as described previously for crops[12] and solvent-exchanged three times to a similar volume of ethanol, was treated with 10 ml of ethanolic 5% potassium hydroxide in a 250-ml flask and allowed to stand overnight (*ca.* 16 h) at room temperature. The solvent was then evaporated to dryness at 52° on a rotary evaporator under vacuum (the large flask contains any spattering of the solid residue). The residue was rinsed with hexane (2 × 10 ml), which was transferred to a 60-ml separating funnel. The rinsed residue was dissolved in 10 ml of water, and the aqueous solution was rinsed into the separating funnel with water (2 × 5 ml). The aqueous phase was extracted with the hexane to remove neutral material, and the hexane was discarded. The aqueous phase was then acidified (pH *ca.* 2) with 1 ml of concentrated hydrochloric acid, returned to the separating funnel with water rinses (bringing the volume to *ca.* 35 ml), saturated with sodium chloride (10–12 g) and extracted with chloroform (4 × 25 ml), each extract being dried by passing it through anhydrous sodium sulphate in a funnel; a further two rinses of the sodium sulphate with chloroform completed extraction of the cyclopronanecarboxylic acids.

Crown-ether-catalyzed formation of the benzyl esters (1d–4d) of 2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylic acids

The chloroform extract from the hydrolysis was evaporated to 2–3 ml and solvent-exchanged three times with 15-ml portions of benzene, which was finally evaporated to 2–3 ml. This residue was transferred quantitatively with rinsing (final volume 5–7 ml) to a culture tube (200 × 25 mm) with a PTFE-lined cap. To this, 1 ml of benzyl bromide reagent, 1 ml of dicyclohexyl-18-crown-6 reagent, 0.3 g of

potassium bicarbonate and a small glass-covered magnetic stirring-bar were added. The tube was capped tightly and heated for 1 h in a water bath at 80° on a magnetic stirrer providing vigorous stirring. After cooling, the mixture was rinsed quantitatively onto a chromatographic column packed with 2 g of anhydrous sodium sulphate and 5 g of silica gel and topped with another 2 g of sodium sulphate. Sufficient benzene was used in the rinsing to provide *ca.* 40 ml of eluate, which was evaporated to *ca.* 2 ml and then made up to volume in a 10-ml volumetric flask.

Trans-esterification of benzyl 2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylates and permethrin with sodium l-menthylate

A 3-ml portion of the benzene solution of the benzyl esters was treated with 1 ml of the 0.05 *M* sodium *l*-menthylate in a culture tube (120 × 5 mm) with a PTFE-lined cap, and the mixture was allowed to stand for 2 h at room temperature. Water (4 ml) was added, and the mixture was shaken vigorously, then centrifuged on a clinical centrifuge to speed the separation. The aqueous phase was removed with a disposable pipette, and the benzene was dried by adding anhydrous sodium sulphate before the solution was used for analysis.

Permethrin standards or permethrin in a benzene–hexane (80:20) solution from soil extracts cleaned up by columnn chromatography on Florisil as mentioned earlier for cypermethrin (volume up to 5 ml) were treated with 1 ml of 0.01 *M* sodium *l*-menthylate reagent for 2 h at room temperature and worked up as described above for the benzyl ester trans-esterification.

DISCUSSION

Of the two general techniques for the separation of enantiomers to which GLC is applicable, direct separation on a chiral stationary phase is the more desirable, especially when one of a number of chiral centres is lost in the preparation of the diastereoisomeric derivative required for application of the second technique. Unfortunately, the low volatility of the pyrethroids, the limited thermal stability of most chiral GLC phases and the apparently general requirement for a capillary column make application of the direct technique difficult. For this reason, we directed our efforts to the development of a GLC method that would at least determine the amounts of C-1 epimers present in residues of permethrin and cypermethrin based on the separation of diastereoisomeric esters of the constituent 2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylic acids.

The formation of disastereoisomeric esters of these acids with *l*-menthol[13] and *d*- and *l*-2-octanol[10] has been reported. Also, as previously reported[7], we found that ester yields were not quantitative when microgram amounts of the acids were converted into the acid chloride with thionyl chloride and subsequently treated with an alcohol, (in our work, *l*-menthol) as described by Elliott *et al.*[13]. Esterification of the acids at the microgram level by treatment with thionyl chloride and *l*-menthol simultaneously as described[7] produced high yields of the esters, but numerous interfering components were present from the reagents when the reaction mixture was analyzed by GLC using an electron-capture detector. These components could not be removed easily (by, *e.g.*, washing with water or Florisil chromatography), and their presence would not permit analysis at the levels required.

The trans-esterification of esters with solutions of alkali-metal alkoxides is a well-known reaction. The treatment of (1*R*)*cis*-, (1*S*)*trans*- and (1*R*)*trans*-permethrin and (±)-*cis*,*trans*-permethrin with 0.01 *M* sodium *l*-menthylate in benzene or benzene–hexane mixtures at room temperature rapidly converted the permethrin isomers into the corresponding *l*-menthyl esters. Most of the parent *cis*-isomers (the least reactive) had disappeared after 1 h and none could be detected after 2 h when the hexane extract of the reaction mixture was analyzed for permethrin. That the isomers were not racemized during this treatment with anhydrous alkali is shown by the fact that a single product was formed from each isomer (see Fig. 1, A, B and C). Murano reported that no racemization of the chrysanthemic acids occurred on refluxing in aqueous alkali[9]. The stability of these enolizable materials under the alkaline conditions is presumably due to the existence of an energy barrier to racemization in the cyclopropyl carbanion[14]. The sensitivity of the electron-capture detector to these esters and the absence of interfering responses from the reagent is also shown by Fig. 1. Fig. 2 shows typical results from the analysis of permethrin-treated soil. Although some of the cyclopropanecarboxylic acids potentially present will be recovered from the soil by the extraction procedure used, they will not interfere with the analysis, as they are unreactive in the trans-esterification reaction.

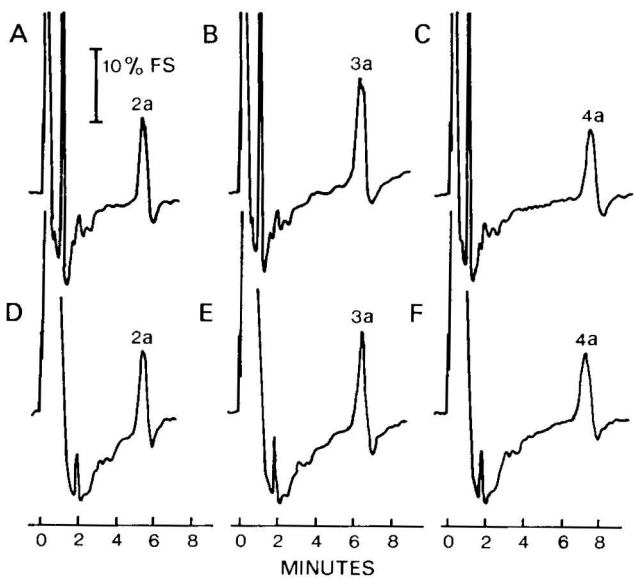


Fig. 1. Gas chromatograms of *l*-menthyl 2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylate. A, B and C, from direct trans-esterification of permethrin isomers; D, E and F, from hydrolysis, benzyl ester formation and trans-esterification of permethrin isomers. [2a = (1*R*)*cis*; 3a = (1*S*)-*trans*; 4a = (1*R*)*trans*]. Responses are equivalent to 0.7, 0.6 and 0.6 ng of the original permethrin isomer, respectively.

Cypermethrin disappeared rapidly in 0.01 *M* sodium *l*-menthylate, but the *l*-menthyl esters of the 2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylic acids were not formed. The failure of this reaction, presumably because of competing reactions of the α-cyano group, necessitated development of alternative procedures

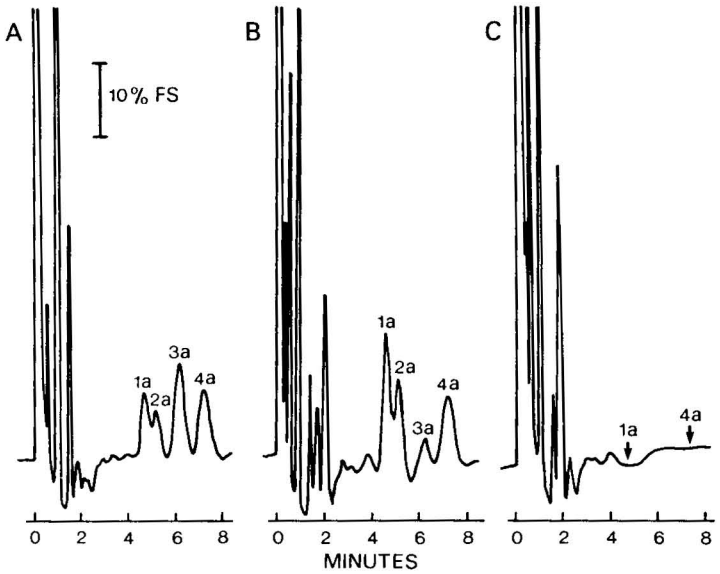


Fig. 2. Gas chromatograms of *l*-menthyl esters derived from permethrin residues in soil by direct trans-esterification. A, Immediately after treatment (2.8 ng of permethrin; 0.3 mg of soil); B, after 32 weeks (2.8 ng of permethrin; 1.6 mg of soil); C, control (1.6 mg of soil).

for determining the isomeric composition of residual cypermethrin. The hydrolysis of cypermethrin (and permethrin) was found to be complete after 16 h at room temperature in 5% potassium hydroxide in 95% ethanol, and good yields of the cyclopropanecarboxylic acids were recovered as determined by GLC analysis of methyl or trimethylsilyl esters. To make use of the relatively interference-free trans-estification procedure used for permethrin, a simple method of esterifying the recovered acids was required. Methyl esters were readily prepared, but trans-esterification proceeded too slowly with 0.01 *M* sodium *l*-menthylate at room temperature to be of practical value, and heating produced intolerable interference. The more reactive benzyl esters, prepared by the reaction of the acids with benzyl bromide in the presence of potassium bicarbonate and catalytic amounts of dicyclohexyl-18-crown-6[15], were found to be completely trans-esterified within 2 h on treatment with 0.05 *M* sodium *l*-menthylate in benzene at room temperature, so providing the required alternative route to the *l*-menthyl esters. Conversion of (1*R*)*cis*-, (1*S*)*trans*-, (1*R*)*trans*- and (±)-*cis*,*trans*-permethrin into the corresponding *l*-menthyl esters using the three-step procedure proceeded without fractionation or racemization. The yields of esters were 95–100% of those observed in the single-step trans-esterification, and there was no complication of the chromatograms by components in the reagents. The results are shown in Fig. 1 (D, E and F). Separate experiments also demonstrated that the isomeric cyclopropanecarboxylic acids were stable for at least 4 days at room temperature and for at least 4 h at reflux temperature in the ethanolic potassium hydroxide used. The crown-ether-catalyzed benzyl ester formation was found to proceed at room temperature, with vigorous sturring, but only 17% of the *cis*- and 25% of the *trans*-isomers had reacted after 1 h. Thus, room-temperature reaction was considered to be impractical for our purposes. For the analysis of cypermethrin isomer ratios in soil using this

procedure, it was necessary to remove any of the corresponding cyclopropanecarboxylic acids from the extract before starting the analysis. The Florisil chromatography procedure originally reported for crops[12] and used in the preliminary clean-up of soil extracts removed at least 50 μg of the acids from a hexane extract. Typical analyses of cypermethrin-treated soil are shown in Fig. 3.

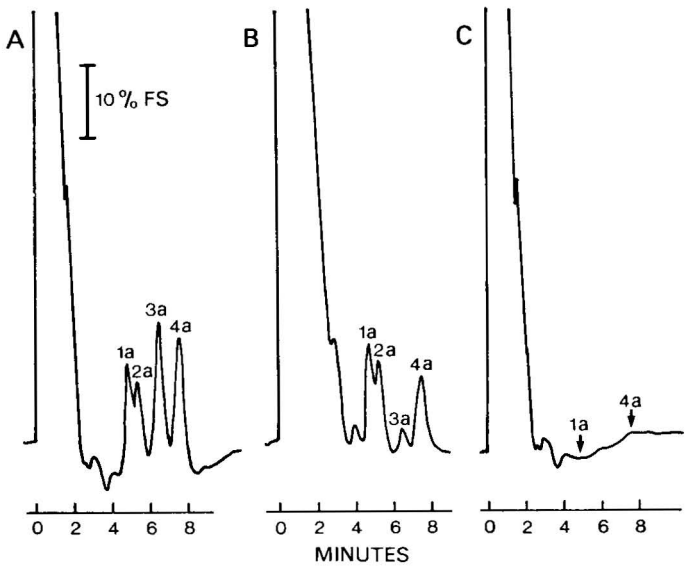


Fig. 3. Gas chromatograms of *l*-menthyl esters derived from cypermethrin residues in soil by the three-step procedure. A, Immediately after treatment (10 ng of cypermethrin; 1.5 mg of soil); B, after 6 weeks (6.4 ng of cypermethrin, 3.0 mg of soil); C, control (1.5 mg of soil).

Murano[9] investigated the GLC separation of diastereoisomeric esters of chrysanthemic acid and concluded that QF-1 gave the best separation. The *l*-menthyl (1*R*) and (1*S*)*cis*-chrysanthemates could not be separated in this study, and the optically active 2-octyl esters were used in subsequent work. The *l*-menthyl 2,2-dimethyl-3-(2,2-dichlorovinyl)cyclopropanecarboxylates were sufficiently well separated on 60-cm columns containing 2.5% of OV-210 (or QF-1) to permit satisfactory analysis for our purposes. Other esters or GLC conditions were not examined.

CONCLUSION

The procedures described provide a method of analysis for the C-1 enantiomers in residual amounts of permethrin and cypermethrin; this method has been used successfully in our laboratory over the past 2 years to measure the C-1 enantiomer ratio in residues as low as 0.05 ppm in soil. No attempt was made to use the procedure for fenvalerate, as the constituent 2-(4-chlorophenyl)-3-methylbutyric acids should racemize readily under the conditions used. Further investigation of the separation of chiral pyrethroid isomers is in progress.

ACKNOWLEDGEMENTS

We wish to acknowledge the assistance of Dr. M. Elliott in providing the samples of the individual permethrin isomers.

REFERENCES

1 P. E. Burt, M. Elliott, A. W. Farnham, N. F. Janes, P. H. Needham and D. A. Pulman, *Pestic. Sci.*, 5 (1974) 791.
2 M. Elliott, in M. Elliott (Editor), *Synthetic Pyrethroids*, ACS Symposium Series, No. 42, American Chemical Society, Washington, D.C., 1976, Ch. 1.
3 M. Elliott, A. W. Farnham, N. F. Janes and D. M. Soderlund, *Pestic. Sci.*, 9 (1978) 12.
4 G. T. Brooks, S. E. Lewis and A. Harrison, *Nature (London)*, 220 (1968) 1034.
5 P. W. Lee, R. Allahyari and T. R. Fukuto, *Pestic. Biochem. Physiol.*, 8 (1978) 158.
6 M. Hasegawa and I. Matsubara, *Anal. Biochem.*, 63 (1975) 308.
7 A. Murano, *Agr. Biol. Chem.*, 36 (1972) 2203.
8 S. Caccia, C. Chiabrando, P. DePonte and R. Fanelli, *J. Chromatogr. Sci.*, 16 (1978) 543.
9 A. Murano, *Agr. Biol. Chem.*, 36 (1972) 917.
10 M. Horiba, A. Kobayashi and A. Murano, *Agr. Biol. Chem.*, 41 (1977) 581.
11 R. A. Chapman and H. S. Simmons, *J. Ass. Offic. Anal. Chem.*, 60 (1977) 977.
12 R. A. Chapman and C. R. Harris, *J. Chromatogr.*, 166 (1978) 513.
13 M. Elliott, N. F. Janes, D. A. Pulman, L. C. Gaughan, T. Unai and J. E. Casida, *J. Agr. Food Chem.*, 24 (1976) 270.
14 H. M. Walborsky, *Rec. Chem. Progr.*, 23 (1962) 75.
15 H. D. Durst, M. Milano, E. J. Kikta, Jr., S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.

Journal of Chromatography, 174 (1979) 379–384

CHROM. 11,791

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 2,6- AND 2,4-DIAMINOTOLUENE, AND ITS APPLICATION TO THE DETERMINATION OF 2,4-DIAMINOTOLUENE IN URINE AND PLASMA

PETER D. UNGER and MARVIN A. FRIEDMAN
Allied Chemical Corporation, Corporate Medical Affairs, Morristown, N.J. 07960 (U.S.A.)
(Received February 13th, 1979)

SUMMARY

2,4-Diaminotoluene is used for the production of industrial dyes, and along with the 2,6-isomer, as an intermediate in the production of polyurethane foams. 2,6- and 2,4-diaminotoluene were resolved as sharp peaks by normal-phase high-performance liquid chromatography in 3 min by an acetonitrile–water-saturated chloroform elution solvent (8:2, v/v) with detection by ultraviolet absorbance at 250 nm. The relationship between peak height and amount injected was linear over a range of 0.025–2 μg for both compounds. Retention times and peak heights were highly reproducible. Detection was very sensitive, allowing quantitation of 1–2 ng of either compound. Quantitative recovery of 2,4-diaminotoluene from spiked urine and plasma samples was obtained by extraction with methylene chloride.

INTRODUCTION

2,4-Diaminotoluene (2,4-TDA), an industrial intermediate, is used in the production of polyurethane, industrial dyes and, until 1971, in hair-dye formulations. Most of the diaminotoluene that is produced in the U.S.A. is in the form of a mixture of unisolated isomers, primarily 2,6-TDA (*ca.* 20%) and 2,4-TDA (80%). In the production of polyurethane, the mixture of isomers is first phosgenated to the corresponding toluene diisocyanate (TDI). In 1975, an estimated 265–290 million kg of 2,4-TDA was produced in the U.S.A. and Western Europe. Figures for the production of 2,6- and 2,4-TDA as an unisolated mixture are not available, but in 1975 seven U.S. companies produced 217.4 million kg of a mixture of TDI isomers (20% 2,6-isomer and 80% 2,4-isomer)[1].

2,4-TDA has been reported to be mutagenic in the Ames test after *in vitro* metabolic activation[2], transforming to secondary hamster embryo cells[3], and carcinogenic to rodents[4–7]. Ito *et al.*[7] reported hepatocellular carcinomas in rats fed diets containing 0.06 or 0.1% 2,4-TDA for 30–36 weeks. Umeda[5] reported that weekly subcutaneous injections of 0.5 ml of a 4% solution of 2,4-TDA in propylene glycol produced rhabdosarcomas in all rats surviving more than 8 months (9 of 20).

Several procedures for the detection of 2,4-TDA have been published, including

thin-layer chromatography (TLC)[8–11] paper chromatography[12], and gas chromatography[10]. Gas chromatography has been used to separate the diaminotoluene isomers[13–15]. Guthrie and McKinney[16] have recently reported a sensitive TLC procedure for the determination of 2,6- and 2,4-TDA in urethane foams using fluorimetric detection. No high-performance liquid chromatography (HPLC) procedure for quantitation of diaminotoluenes, however, has been described. The present study, therefore, was undertaken to develop a sensitive and reliable HPLC procedure for the determination of 2,6- and 2,4-TDA, adaptable to biomonitoring and metabolic studies.

EXPERIMENTAL

Standards

2,6- and 2,4-TDA, of greater than 98% purity, were obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Crystalline 2,6- and 2,4-TDA were weighed and dissolved in the appropriate volume of the elution solvent to yield standards containing 0.0025, 0.005, 0.01, 0.05, 0.1, and 0.2 μg/μl. Standard solutions were analyzed immediately after preparation, and kept on ice to retard amine oxidation.

Equipment

Normal-phase chromatography was performed using an LDC Constametric IIG HPLC system including an LDC Spectromonitor II variable wavelength absorbance detector (Laboratory Data Control, Riviera Beach, Fla., U.S.A.). Samples were injected onto the column using a Valco N60 fixed-volume (10 μl) loop injector (Valco Instruments, Houston, Texas, U.S.A.). Separations were achieved with a Lion Technology (Dover, N.J., U.S.A.) Silarex SRI silica adsorption column (10 μm particle size) at an elution rate of 3 ml/min. 2,6- and 2,4-TDA were detected at 250 nm, with the absorbance detector at sensitivities of 0.01–1.28 absorbance units full scale (AUFS). Solvent programming (Gradient Master, LDC) was used to establish optimum solvent ratios.

Elution solvent

The elution solvent consisted of glass-distilled acetonitrile and water-saturated chloroform (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Water-saturated chloroform was prepared by adding 100 ml of distilled water to 1 l of chloroform, shaking four times (*ca.* 1 min each time), and allowing the mixture to stand overnight. A ratio of 8 parts of acetonitrile to 2 parts of water-saturated chloroform was found to give good resolution of 2,6-TDA from 2,4-TDA, and adequate separation of 2,4-TDA from interfering peaks in plasma and urine extracts.

Extraction of 2,4-TDA from rat urine and plasma

Rat urine and plasma samples were extracted twice with four volumes of methylene chloride (Burdick & Jackson) by vortexing for 1.5 min each time. The methylene chloride extracts were pooled, evaporated to dryness under a steady stream of helium, and the residue dissolved in the elution solvent for HPLC analysis. To determine the linearity of 2,4-TDA recovery, 5, 10, 20, and 100 μg of 2,4-TDA were added to 0.5 ml urine and plasma samples and extracted as above.

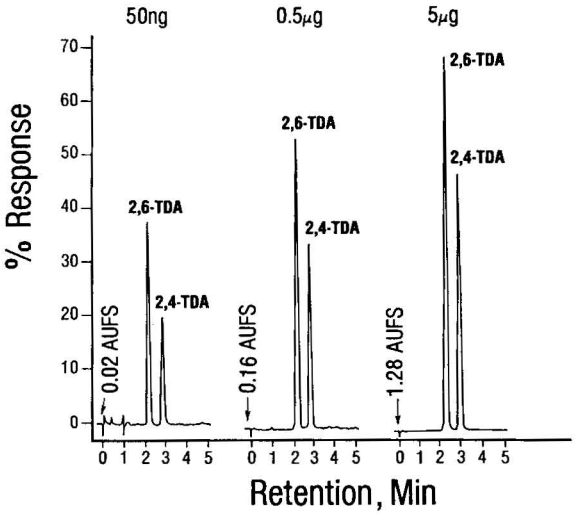


Fig. 1. HPLC resolution of 2,6- and 2,4-TDA on Silarex SRI column; elution solvent acetonitrile–water-saturated chloroform (8:2, v/v); flow-rate 3.0 ml/min; 0.05, 0.5 and 5 μg of each compound injected.

RESULTS AND DISCUSSION

Retention time

At a flow-rate of 3 ml/min, using acetonitrile–water-saturated chloroform (8:2, v/v) as the elution solvent, 2,6- and 2,4-TDA eluted as sharp, symmetrical peaks with baseline resolution (Fig. 1). The retention times were highly reproducible with the elution solvent and column used; ten injections of each compound over a period of several days gave mean retention times of 130 and 180 sec, with coefficients of variation of 0.58 and 1.52% for 2,6- and 2,4-TDA, respectively (Table I).

Linearity

The relationship between peak heights and amount of 2,6- and 2,4-TDA injected was linear over a 0.025–2.0 μg range for both compounds (Fig. 2). The peak heights were converted to a common sensitivity and plotted against the quantity injected.

TABLE I

REPRODUCIBILITY OF RETENTION TIME FOR 2,6- AND 2,4-TDA BY HPLC

Statistic	*2,6-TDA*	*2,4-TDA*
Injections, N*	10	10
Retention time (sec)		
range	129–132	177–184.5
mean	130.6	179.7
standard deviation (sec)	0.76	2.73
coefficient of variation**	0.58	1.52

* Successive injections of a mixture of 0.25 μg 2,6-TDA and 0.375 μg 2,4-TDA.

** Standard deviation/mean × 100.

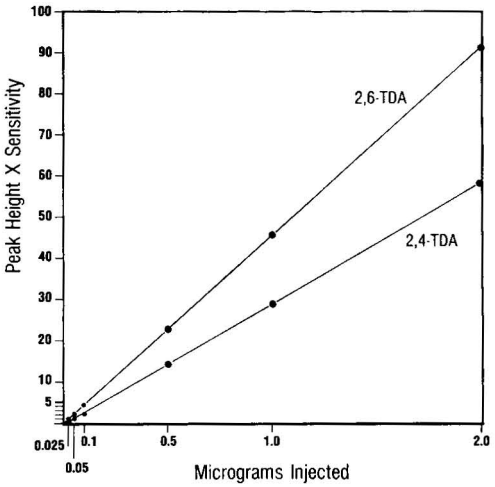


Fig. 2. Linearity, peak height *vs.* amount of 2,6- and 2,4-TDA injected.

Precision and sensitivity

Precision was evaluated by injecting ten 10-μl aliquots of a mixture of 2,6- and 2,4-TDA containing 0.25 and 0.375 μg, respectively. Reproducibility of peak height was good, with coefficients of variation of 1.003–1.18%, representing the combined errors of HPLC resolution, injection and detection (Table II).

TABLE II

PEAK HEIGHT REPRODUCIBILITY FOR 2,6- AND 2,4-TDA BY HPLC

Statistic	*2,6-TDA*	*2,4-TDA*
Injections, N*	10	10
Peak height (mm)		
range	128.5–132.0	124–128.0
mean	130.4	126.4
standard deviation (mm)	1.31	1.49
coefficient of variation (%)**	1.003	1.18
sensitivity (mm peak height/ng)***	8.35	5.39

* Successive 10-μl injections of 0.25 μg 2,6-TDA and 0.375 μg 2,4-TDA, at a sensitivity of 0.08 a.u.f.s.

** Standard deviation/mean × 100.

*** Calculated to a maximum usable sensitivity of 0.005 a.u.f.s.

The mean sensitivity of detection (millimetres of peak height/nanogram of 2,6- and 2,4-TDA; Table II) and the chromatograms (Fig. 1) indicate that 1–2 ng of either compound can easily be detected.

Recovery of 2,4-TDA from rat urine and plasma

Good recovery of 2,4-TDA was obtained from spiked rat urine and plasma samples (Table III). Chromatogram tracings of methylene chloride extracts of rat

TABLE III

RECOVERY OF 2,4-TDA FROM SPIKED URINE AND PLASMA SAMPLES

2,4-TDA added (μg/ml)	*Recovery (%), mean ± S.E.*	
	Urine	*Plasma*
0	–	–
10	90.31 ± 1.56	89.02 ± 0.83
20	92.36 ± 2.61	90.39 ± 1.02
40	89.53 ± 0.26	90.31 ± 5.27
200	101.64 ± 0.99	87.71 ± 3.52

urine and plasma samples are shown in Fig. 3. Nine different extraction solvents were tested, including hexane, cyclohexane, methylene chloride, ethyl acetate, chloroform and various ratios of a mixture of acetonitrile and ethyl acetate. The poorest recoveries were obtained with hexane and cyclohexane (<13%). Extraction with ethyl acetate yielded much higher recoveries (60–75%). The highest recoveries, however, were obtained by extraction with methylene chloride.

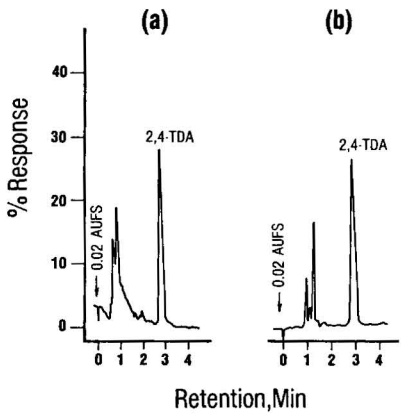


Fig. 3. Chromatogram tracing of extract of urine (a) and plasma (b) spiked with 2,4-TDA to 10 μg/ml. See text for column and conditions.

The procedures described here, with the inclusion of an extraction step for the quantitative determination of known 2,4-TDA metabolites, are currently being used in this laboratory for the study of the metabolism of 2,4-TDA in the rat.

REFERENCES

1 U.S. International Trade Commission, *Synthetic Organic Chemicals, U.S. Production and Sales, 1975*, USITC publication 804 (1977) 22, 36, 42, 49, 60, 62, 65, and 74.
2 B. N. Ames, H. O. Kammen and E. Yamasaki, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 2423.
3 R. J. Pienta, M. J. Shah, W. B. Lebherz and A. W. Andrews, *Cancer Lett.*, 3 (1977) 45.
4 S. Watanabe, *Trans. Jap. Pathol., Soc.*, 27 (1937) 421.
5 M. Umeda, *Gann*, 46 (1955) 597.
6 M. Umeda, *Gann*, 47 (1956) 153.
7 N. Ito, Y. Hiasa, Y. Konishi and M. Marugami, *Cancer Res.*, 29 (1969) 1137.

8 C. Kotteman, *J. Ass. Offic. Anal. Chem.*, 49 (1966) 954.
9 G. F. Macke, *J. Chromatogr.*, 36 (1968) 537.
10 T. Glinsukon, T. Benjamin, P. Grantham, E. Weisburger and P. Roller, *Xenobiotica*, 5 (1975) 475.
11 T. Glinsukon, T. Benjamin, P. Grantham, N. Lewis and E. Weisburger, *Biochem. Pharmacol.*, 25 (1976) 95.
12 R. H. Waring and A. E. Pheasant, *Xenobiotica*, 6 (1976) 257.
13 C. E. Boufford, *J. Gas Chromatogr.*, 6 (1968) 438.
14 L. E. Brydia and F. Willeboordse, *Anal. Chem.*, 40 (1968) 110.
15 F. Willeboordse, Q. Quick and E. T. Bishop, *Anal. Chem.*, 40 (1968) 1455.
16 J. L. Guthrie and R. W. McKinney, *Anal. Chem.*, 49 (1977) 1676.

Journal of Chromatography, 174 (1979) 385–392

CHROM. 11,814

SEPARATION OF PEPTIDES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY FOR THE IDENTIFICATION OF A HEMOGLOBIN VARIANT

W. A. SCHROEDER, JOAN B. SHELTON and J. ROGER SHELTON

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, Calif. 91125 (U.S.A)*

and

DARLEEN POWARS

Department of Pediatrics, University of Southern California School of Medicine, Los Angeles, Calif. 90033 (U.S.A.)

(Received February 23rd, 1979)

SUMMARY

High-pressure liquid chromatography on a reversed-phase column has been used to separate the tryptic peptides of a human hemoglobin variant which was then identified as hemoglobin E.

INTRODUCTION

High-pressure liquid chromatography (HPLC) is a procedure that has become increasingly important in all phases of chromatographic work, whether in routine analysis or in research. Because of the amphoteric character of peptides, either cation- or anion-exchange materials have had widespread use in their separation. However, the availability of reversed-phase HPLC packings provides a different mode of separation for peptides, which is dependent more on hydrophobicity and, hence, may be somewhat more akin to the mechanisms that obtain in peptide separations by paper or thin-layer chromatography. The increasing literature on the separation of peptides by HPLC[1–10] has been devoted largely to devising methodology and to the separation of artificial mixtures of peptides. This report describes the application of reversed-phase HPLC to the separation of the aberrant peptide in a complex mixture of tryptic peptides from a hemoglobin (Hb) variant that was detected during screening of cord blood for such variants.

MATERIALS AND METHODS

General methods

Blood samples were collected in Vacutainers, with EDTA as anticoagulant. Hematological data were obtained with a Coulter Counter (Model S).

* Contribution No. 5978.

Electrophoresis was carried out on cellulose acetate (Helena Labs., Beaumont, Texas, U.S.A.) at pH 8.4 or on starch gel at pH 9.0.

Cord-blood screening for Hb variants involved use of the method of Schroeder *et al.*[11]. The Hb composition of samples was determined by micro-chromatographic procedures[12] or use of larger-scale analytical columns[13,14]. A scaled-up version of a micro-chromatographic method to be described below was used for isolation of the Hb variant.

Osmotic fragility was determined by the procedure that is described by Huisman and Jonxis[15].

For amino acid analysis, samples were hydrolyzed *in vacuo* in redistilled 6 *M* hydrochloric acid at 110° for 24 h. Analysis was done with a Beckman 120 B amino acid analyzer that had been modified to take 12-mm flow-cells (MER Chromatographic, Mountain View, Calif., U.S.A.) and a single column (43 × 0.6 cm) of Beckman AA-15 resin with buffer flow-rate of 40 ml h^{-1}. The integrator was an Infotronics CRS 309 (International Technical Instruments, Boulder, Colo.,U.S.A.).

Isolation of the Hb variant

The erythrocytes were washed three times with normal saline and hemolyzed for 20 min at room temperature with 1.5 times their volume of water and 0.4 volume of carbon tetrachloride. After centrifugation to remove cell debris, the solution was dialyzed overnight against water at 4°.

The chromatographic separation was made on a column (22.5 × 2.5 cm) of DEAE-cellulose DE-52 (Whatman, Clifton, N.J., U.S.A.) that had been equilibrated in 0.2 *M* glycine–0.01% potassium cyanide solution to pH 7.19 as described by Schroeder *et al.*[12]. The sample was 650–800 mg of Hb in 10–14 ml of water. Each chromatogram was developed with 0.2 *M* glycine–0.015 *M* sodium chloride–0.01% potassium cyanide at a flow-rate of 80 ml h^{-1}. Under these conditions, the Hb variant moved rapidly through the column. Before it began to pass into the eluate, the chromatogram was stopped and the upper part of the column with the other Hb types was removed. The variant was eluted from the remaining column with 2% potassium cyanide solution, dialyzed and concentrated on CM-Sephadex[16] before removal of the heme with acid acetone at −20°. Under these conditions, the variant is mixed with HbA_2. A total of 480 mg of globin was available.

Tryptic hydrolysis and preparation of the sample

A 25-mg sample of the globin was dissolved in 1.9 ml of water, and the pH was adjusted to 8.8 with 0.1 *M* sodium hydroxide. After the sample volume had been adjusted to 2.0 ml, 4.0 mg of ammonium hydrogen carbonate (Mallinckrodt, St. Louis, Mo., U.S.A.) were added to give 0.025 *M* concentration, and the pH was 8.1. Following addition of 0.25 mg of trypsin (Trypsin TCPK; Worthington, Freehold, N.J., U.S.A.) in 0.125 ml of 0.001 *M* hydrochloric acid, the suspension was stirred for 4 h at 37°; when the pH (which was 8.6 at room temperature) was lowered to 2.0, all the precipitate dissolved. This solution was filtered through a 0.5-μm fluoropore filter with an inorganic pre-filter (Millipore, Bedford, Mass., U.S.A.) in a Swinney filter-holder and then freeze-dried. The 26 mg of dry material was dissolved in 0.5 ml of 0.01 *M* ammonium acetate of pH 6.07 (see below), and the solution was filtered as before. A 100-μl sample was injected into the HPLC system.

HPLC of the tryptic digest

The HPLC equipment consisted of a single Waters 6000A solvent delivery system, U6K universal injector, and column (300 × 3.9 mm) of μBondapak C_{18} (10 μm) (Waters Assoc., Milford, Mass., U.S.A.), an Altex/Hitachi Model 155-10 UV–Vis variable wavelength detector (Altex, Berkeley, Calif., U.S.A.), and a single-channel recorder (Linear Instruments Corp., Irvine, Calif., U.S.A.). The linear gradient was formed with a two-vessel system of the type described by Bock and Ling[17]; each vessel had an I.D. of 2.2 cm.

The chemicals used were ammonium acetate (Allied Chemical, Morristown, N.J., U.S.A.; B and A reagent grades), and acetonitrile (gold label 99+%, spectrophotometric grade; Aldrich, Milwaukee, Wisc., U.S.A.), which was filtered through an 0.5-μm FH organic filter (Millipore). Deionized water was filtered through an 0.45-μm HA aqueous filter (Millipore). The following solvents were made up 24 h before use. A solution of 0.01 *M* ammonium acetate was adjusted to pH 6.07 with dilute acetic acid; acetonitrile and 0.01 *M* ammonium acetate were mixed to give 40% (v/v) of acetonitrile, and the pH was adjusted to 6.07. Both solutions were filtered through 0.45-μm HA aqueous filters. De-gassing was done only to the extent that this occurred during filtering under vacuum.

The HPLC column was equilibrated with 40 ml of 0.01 *M* ammonium acetate (pH 6.07) at a flow-rate of 1.5 ml min^{-1} at room temperature. After the flow-rate had been reduced to 1 ml min^{-1}, the sample as prepared above was injected with a Precision Sampling pressure-lok liquid syringe (Series B-110). Development was made with a linear gradient of 50 ml of 0.01 *M* ammonium acetate (pH 6.07) in the mixer and 50 ml of 40% acetonitrile in 0.01 *M* ammonium acetate (pH 6.07) in the reservoir; the pressure was 1500 p.s.i., the absorbance was recorded at 220 nm at 1.0 a.u.f.s. with a chart speed of 40 cm h^{-1}, and 0.5-ml fractions were collected.

A few millilitres before the end of the gradient, 2 ml of acetonitrile were injected and passed through the system with the last of the gradient. There followed then, in sequence, 40 ml of water, a 30-ml linear gradient from water to acetonitrile and, finally, 25 ml of acetonitrile. Before the next usage, equilibration was carried out with 0.01 *M* ammonium acetate as already noted.

RESULTS

Case report

Although the basic aim of cord-blood screening at the Los Angeles Sickle Cell Center has been the detection at birth of infants with sickle cell disease[11,18], variants other than HbS or HbC are also detectable. Thus, in addition to HbF and HbA, the blood of a newborn black infant contained a third component with properties different from those of HbS or HbC. This component had the electrophoretic behavior of HbC at alkaline pH on cellulose acetate or starch gel. The chromatographic behavior was that of HbC on DEAE-cellulose[14] and approximated that of HbS on CM-cellulose[13]; this is the behavior of HbE and probably also of HbC_{Harlem}. The Hb composition at birth and at 10 weeks of age is listed in Table I. When the family (which consisted of the parents and two siblings) was examined, the variant was present in the mother and both siblings; quantitative data are in Table I.

TABLE I

HEMOGLOBIN (Hb) COMPOSITION* AND HEMATOLOGICAL DATA ON THE FAMILY

Hb type or other parameter	*Propositus*		*Father*	*Mother*	*Sib I (9 years)*	*Sib II (3 years)*
	Birth	*10 weeks*				
HbX + HbA_2 (%)	4.1	19.8	2.1	32.1	30.8	30.0
HbA (%)	12.5	43.2	86.6	59.6	61.3	60.6
HbF (%)	66.7	26.0	3.5*	2.3*	2.3*	3.6*
HbA_1 + F_1 (%)	16.7	11.0	7.8	6.0	5.6	5.8
RBC ($\times 10^{12}$ l^{-1})		4.51	5.22	4.68	5.57	4.51
Hb (g dl^{-1})		12.6	15.6	12.7	12.9	11.6
PCV(ll^{-1})		0.37	0.46	0.38	0.39	0.34
MCV (fl)		83	86	83	73	76
MCH (pg)		27.8	29.6	27.1	24.0	25.6
MCHC (g dl^{-1})		33.6	33.9	33.1	32.9	34.0

* The Hb percentages were determined by microchromatography[12]. In this method, the tail of HbA carries over into the position of HbF. These percentages of HbF are in the normal range of this method.

HPLC separation of peptides: Identity of the variant

Fig. 1 depicts the chromatographic separation of the peptides. All zones were pooled, blown dry with air, hydrolyzed, and analyzed for amino acid composition. Because each zone had either one or two peptides, it was possible to identify the

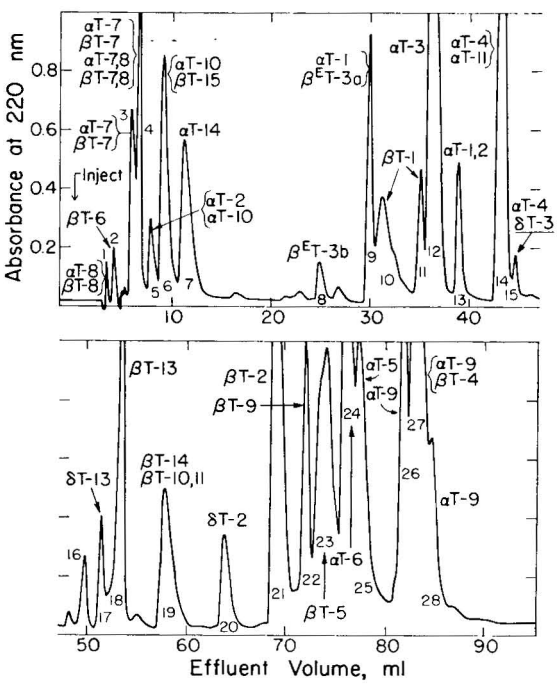


Fig. 1. Separation of the tryptic peptides of the α- and β-chains of HbE on a reversed-phase column as described in the text.

components, as the composition of all tryptic peptides from the α- and β-chains is well-known. The identity of the peptide(s) in each zone is given in Fig. 1. Thus, it was apparent from the analyses in Table II that parts of the aberrant peptide were present in Zones 8 and 9. Zone 8 had the amino acid composition of residues 27–30 of the β-chain, and Zone 9 contained αT-1 and residues 18–26 of the β-chain, with the substitution of lysine for glutamic acid. The Hb variant, therefore, is HbE.

TABLE II

AMINO ACID COMPOSITIONS OF PEPTIDES IN ZONES 8 AND 9

Amino acid	*Zone 8*			*Zone 9**				
	nmoles	*β27–30*		*nmoles**	*αT-1*		*β-18–26*	
		*Found***	*Expected***		*Found***	*Expected***	*Found***	*Expected***
Lys				60.3	1.00	1	0.94	0
Arg	41.4	0.96	1					
Asp				106.8	1.00	1	2.09	2
Ser				21.5	0.95	1		
Glu				43.6			1.08	2
Pro				20.4	0.91	1		
Gly	44.1	1.02	1	85.9			2.12	2
Ala	43.1	1.00	1	26.1	1.16	1		
Val				134.3	1.00	1	2.77	3
Leu	43.5	1.01	1	22.7	1.01	1		

* In these calculations, 22.5 nmoles of the appropriate amino acid is assumed to be part of αT-1; the remainder is associated with β18–26.

** In residues.

On this chromatogram, β^{A}T-3 would be expected between Zones 13 and 14.

DISCUSSION

Although HbE is not common in the black race, its detection in another black family is of minor consequence; this example is presented to show the power of HPLC for the separation of peptides. Parenthetically, it may be noted that the hematological data of the carriers for HbE (Table I) in this black family, with the possible exception of Sib I, agree with accepted age-related values[19,20]. In contrast, Fairbanks *et al.*[21] report microcytosis in HbE carriers from Southeast Asia and Northern Europe.

The 5-mg sample for the chromatogram is of the order commonly applied in fingerprinting methods and could be reduced if more sensitive amino acid analysis were used. If material were required for sequencing a peptide, it could be isolated by pooling the appropriate zone from a succession of chromatograms or, if the desired peptide was well separated, by increasing the load.

The size of the peak is unrelated to the quantity of material and is determined by the number of residues (hence, the absorbance related to the C=O bond) and the presence of an aromatic group. Thus, βT-15 (Tyr-His; the major peptide of Zone 6) appears to be greater in quantity than many others; likewise, Zone 20 with δT-2 is exaggerated because of the tryptophyl residue.

As mentioned, this preparation contained HbA_2. Despite the complexity of the chromatogram, three δ-chain peptides are evident in Zones 20, 15, and 17.

Peptides αT-12, αT-13, βT-10, βT-11, and βT-12 were not detected on this chromatogram, presumably because the mercapto groups were not blocked in this globin. On the other hand, all peptides were detected when a tryptic hydrolysate of an aminoethylated β-chain was chromatographed.

Because of the different mechanism of the reversed-phase column as compared with ion-exchange columns, the order of emergence of peptides is very different by the two methods. For example, peptides αT-1 and αT-11 (which are similar in length and composition) separate with some difficulty on cation-exchange columns: here they are far apart (zones 9 and 14). The reverse is also true: peptides well separated by ion-exchange chromatography may be coincident on this column. Peptides, es-

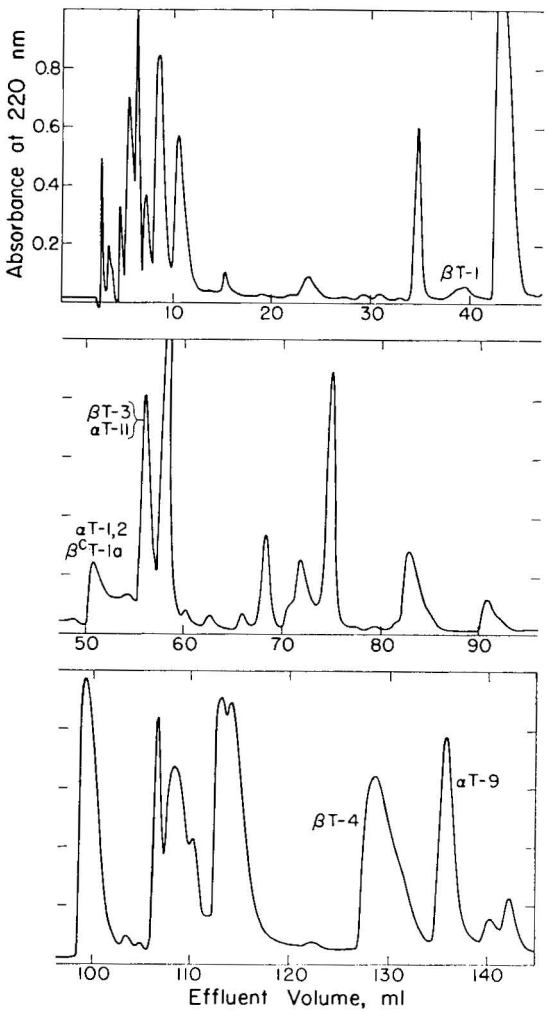


Fig. 2. Separation of the tryptic peptides of the α- and β-chains of HbC on a reversed-phase column under modified conditions as described in the text.

pecially hydrophobic ones (such as βT-4), are easily apparent in good quantity in contrast to their poor yield from ion-exchanger materials.

The reason why βT-1 is present in two zones (10 and 11) is unknown and has not been observed in other chromatograms. This peptide forms a surprisingly broad zone. In a few other instances, for reasons that are not understood, there has been some evidence that a peptide may partition between two zones.

This chromatogram, which was meant to be a pilot run in the study of this abnormal Hb, quickly provided the identification of a well-known variant. Consequently, as an example, this chromatogram by no means shows all that can be achieved by HPLC. Especially at the end of the chromatogram, the zones are close together and this development is only marginally satisfactory. By changing the slope of the gradient or by using isocratic development during part of the chromatogram, marked improvement in separation can be achieved. Fig. 2 is a chromatogram of a 24-h tryptic hydrolysate of the globin of HbC($+A_2$), which was developed with the following sequence: (i) a linear gradient initially between 50 ml of 0.01 *M* ammonium acetate at pH 6.07 in the mixer and 50 ml of 25% acetonitrile in 0.01 *M* ammonium acetate at pH 6.07 in the reservoir, (ii) 20 ml of 25% acetonitrile in 0.01 *M* ammonium acetate isocratically, and (iii) a linear gradient between 15 ml of 25% and 15 ml of 35% acetonitrile in 0.01 *M* ammonium acetate; the chromatogram required 2.5 h for completion. The positions of βT-1 (= δT-1) from the δ-chain of HbA_2, of β^CT-1a from the β-chain of HbC, and of several other peptides are marked. By comparison with Fig. 1, some separations are clearly improved (that of βT-4 from αT-9, for example), whereas, in other instances, separation may be somewhat decreased.

We are currently making a detailed examination of chromatographic conditions not only for the separation of the peptides from pairs of chains, but also of peptides from the individual chains in order to devise good conditions for separations and to learn more exactly the parameters that control them. By a study of known abnormalities, we hope to learn the influence of sequence and composition on a peptide's behaviour. Modifications such as aminoethylation or carboxymethylation of mercapto groups, or oxidation of mercapto groups and methionyl residues, may produce useful characteristics in the modified peptides. The speed with which chromatography can be accomplished and the small scale of operations means that a pilot run can be made to detect the difference in a variant Hb. With the knowledge so gained, a more sophisticated protocol of development can be devised if necessary.

ACKNOWLEDGEMENTS

We thank Colleen Warford for isolating the hemoglobins. These studies were supported in part by grants HL-O2558 and HL-15162 from the National Institutes of Health, U.S. Public Health Service.

REFERENCES

1 W. S. Hancock, C. A. Bishop and M. T. W. Hearn, *FEBS Lett.*, 72 (1976) 139.
2 A. N. Radhakrishnan, S. Stein, A. Licht, K. A. Gruber and S. Udenfriend, *J. Chromatogr.*, 132 (1977) 552.

3 J. J. Hansen, T. Greibrokk, B. L. Currie, K. N.-G. Johansson and K. Folkers, *J. Chromatogr.*, 135 (1977) 155.
4 G. W.-K. Fong and E. Grushka, *J. Chromatogr.*, 142 (1977) 299.
5 I. Molnar and C. Horvath, *J. Chromatogr.*, 142 (1977) 623.
6 E. Lundanes and T. Greibrokk, *J. Chromatogr.*, 149 (1978) 241.
7 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 153 (1978) 391.
8 C. E. Dunlap, III, S. Gentleman and L. I. Lowney, *J. Chromatogr.*, 160 (1978) 191.
9 W. S. Hancock, C. A. Bishop, L. J. Meyer, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 161 (1978) 291.
10 G. D. Efremov, J. B. Wilson and T. H. J. Huisman, *Biochim. Biophys. Acta*, in press.
11 W. A. Schroeder, T. H. J. Huisman, D. Powars, L. Evans, E. C. Abraham and H. Lam, *J. Lab. Clin. Med.*, 86 (1975) 528.
12 W. A. Schroeder, L. A. Pace and T. H. J. Huisman, *J. Chromatogr.*, 145 (1978) 203.
13 W. A. Schroeder, L. A. Pace and T. H. J. Huisman, *J. Chromatogr.*, 118 (1976) 295.
14 E. C. Abraham, A. Reese, M. Stallings and T. H. J. Huisman, *Hemoglobin*, 1 (1976) 27.
15 T. H. J. Huisman and J. H. P. Jonxis, *The Hemoglobinopathies: Techniques of Identification*, Marcel Dekker, New York, 1977, p. 80.
16 W. A. Schroeder, T. H. J. Huisman, J. R. Shelton and J. B. Wilson, *Anal. Biochem.*, 35 (1970) 235.
17 R. M. Bock and N.-S. Ling, *Anal. Chem.*, 26 (1954) 1543.
18 D. Powars, W. A. Schroeder and L. White, *Pediatrics*, 55 (1975) 630.
19 M. M. Wintrobe, G. R. Lee, D. R. Boggs, T. C. Bithell, J. W. Athens and J. Foerster, *Clinical Hematology*, Lea and Febiger, Philadelphia, Pa., 7th ed., 1974, p. 1797.
20 J. Hows, S. Hussein, A. V. Hoffbrand and S. N. Wickramasinghe, *J. Clin. Pathol.*, 30 (1977) 181.
21 V. F. Fairbanks, G. S. Gilchrist, B. Brimhall, J. A. Jareb and E. C. Goldston, *Blood*, 53 (1979) 109.

Journal of Chromatography, 174 (1979) 393–400

CHROM. 11,788

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF COBALAMINS

EUGENE P. FRENKEL*

Department of Internal Medicine (Evelyn L. Overton Hematology-Oncology Research Laboratory), The University of Texas Health Science Center at Dallas, Southwestern Medical School and Veterans Administration Medical Center, Dallas, Texas (U.S.A.)

RICHARD L. KITCHENS

Department of Internal Medicine, Southwestern Medical School, Dallas, Texas (U.S.A.)

and

RUSSELL PROUGH

Department of Biochemistry, Southwestern Medical School, Dallas, Texas (U.S.A.)

(First received December 28th, 1978; revised manuscript received February 13th, 1979)

SUMMARY

Physiological cobalamins were separated by means of high-performance liquid chromatography (HPLC). Optimal conditions for elution of methylcobalamin, adenosylcobalamin, hydroxycobalamin and cyanocobalamin were determined. Excellent separation and resolution of these physiological cobalamins by HPLC were achieved. In addition, several cobalamin analogues were also studied and shown to be separable from the physiological forms. HPLC provides a rapid, sensitive, reproducible means of characterizing physiological cobalamins.

INTRODUCTION

During the past two decades remarkable knowledge has accumulated regarding the biochemistry and biology of vitamin B_{12}. As evidence of the physiologic forms of B_{12} (methylcobalamin[Me-Cbl], adenosylcobalamin[Ado-Cbl], hydroxycobalamin [OH-Cbl], cyanocobalamin[CN-Cbl]) developed, our ability to examine these individual cobalamins was hindered by the lack of simple separation techniques. Linnell and coworkers[1–5] have provided evidence that the pattern of quantitative and qualitative changes in the cobalamin fractions has significant biological importance. Unfortunately, their contributions to the characterization of these fractions have required very tedious and complex chromatographic and bioautographic methods[1],

* To whom correspondence should be addressed, at the following address: Department of Internal Medicine, Southwestern Medical School, 5323 Harry Hines Boulevard, Dallas, Texas 75235, U.S.A.

not easily applicable to extensive studies and not generally adaptable to other laboratories.

The potential speed, sensitivity and resolution of high-performance liquid chromatographic (HPLC) technology appeared to provide an ideal approach for the investigation of the cobalamin fractions under varying pathophysiologic states. Data suggesting such feasibility for cyanocobalamin, which may not be a true physiologic moiety, were recently reported[6,7]. The present report describes the application of HPLC to the separation for characterization and quantification of the cobalamin fractions known to exist in man.

EXPERIMENTAL

Cobalamin preparations

Crystalline cobalamin standards (hydroxycobalamin and cyanocobalamin) were obtained from Sigma (St. Louis, Mo., U.S.A.); coenzyme-B_{12} and methyl-B_{12} were from Calbiochem (La Jolla, Calif., U.S.A.). Solutions were standardized by radioisotopic assay[8,9] and by spectrophotometric assay using the known extinction coefficients[10].

Sulfito-cobalamin was prepared from hydroxycobalamin by preparing a 10 mg/ml solution in 0.1 *M* sodium bisulfite[11]. The wavelength spectrum of the compound was characterized by a double maximum at 312 and 364 nm. This double peak reverted to a single maximum at 351 nm after photolysis; this was identical with authentic OH-Cbl.

Mixed monobasic acids of CN-Cbl and anilide derivatives of the acids[12] were provided by Dr. W. F. J. Cuthbertson of Glaxo Laboratories, Stoke Poges, Great Britain. The alkanolamine analogue of CN-Cbl, 2-amino-2-methylpropanol-B_{12} ("S-102") was provided by Dr. W. Friedrich, Universität Hamburg, G.F.R.[13].

Protection of cobalamins from photolysis

All extracts and solutions containing the photosensitive organocobalamins (*e.g.* Ado-Cbl, Me-Cbl, and CN-Cbl) were handled in the dark under the illumination of a photographic safelight (Foto 2001 Kindermann globe safelight with Varigam filter; 15 watt bulb, Ehrenreich Photo-Optical Industries, Garden City, N.Y., U.S.A.). Under these conditions no photolysis of endogenous ^{57}Co-labeled Ado-Cbl or Me-Cbl was observed for at least 2.5 h at room temperature at a distance of 25 cm from the safelight.

HPLC separation of cobalamins

Applicability of HPLC to the separation of the physiologic cobalamins was evaluated with two separate instruments under differing conditions. For clarity, these are denoted by temporal relationships.

In the initial studies, the cobalamins were separated by reverse-phase chromatography on a pre-packed stainless-steel column (30 cm × 3.9 mm I.D.) of μBondapak C_{18}, 10 μm particle size (Waters Assoc., Milford, Mass., U.S.A.) in a Model ALC-202/401 liquid chromatograph (Waters Assoc.) equipped with a Model 660 solvent programmer, and a Model 440 ultraviolet absorbance detector operating at a 254 nm wavelength. A reservoir of 0.05 *M* sodium acetate buffer, pH 4.0, was

connected to pump A and a reservoir of methanol ("distilled in glass", Burdick & Jackson Labs., Muskegan, Mich., U.S.A.) containing 0.05 *M* sodium acetate buffer, pH 4.0, to pump B; the buffers were prepared by mixing 2.9 ml of glacial acetic acid and 2.6 ml of 3.5 *M* sodium hydroxide with 1 l of glass-distilled water or methanol, and filtered through millipore. A solvent-inert filter (Millipore, Bedford, Mass., U.S.A.) was used for the methanol. The initial solvent concentration for the chromatography was 27% methanol. With sample injection an upward concave gradient (program No. 7 on the Model 660 programmer) was begun and completed in 10 min at final concentration of 95% methanol. The solvent flow-rate was 1.8 ml/min. Initial conditions were achieved by a linear program reversal of 5 min, followed by an equilibration period of 5 min at initial conditions. The column effluent was monitored spectrophotometrically at 254 nm and collected in (0.4 min) fractions for gamma counting.

In a second approach the cobalamins were separated by means of a DuPont Model 850 liquid chromatograph equipped with a temperature controlled column oven (DuPont, Wilmington, Del., U.S.A.). In these studies solvent intake A was 0.05 *M* NaH_2PO_4 and solvent intake B was methanol. Removal of gas from the solvents was accomplished with continuous helium sparging. The columns used were the μBondapak C_{18}, 10 μm particle size (Waters Assoc.) and the Zorbax ODS, 4.6 mm × 25 cm, 6 μm particle size (DuPont). Flow-rate was 1.8 ml/min and column oven temperature was 40°. The initial solvent concentration was 23% methanol. Following the sample injection the initial conditions were maintained for 2 min. Then, a concave gradient (exponent +2) was begun with methanol concentrations of 23 to 70% spanning 10 min. After holding at 70% methanol for 1 min, "initial conditions" were obtained by a linear reversal of methanol concentrations from 70 to 23% over a 3-min period, followed by 5 min of equilibration at these "initial" conditions.

Cobalamin assay

The cobalamins were quantitated on pooled fractions by radioisotopic competitive inhibition assay, as previously described[8,14].

RESULTS

Separation of cobalamins

Complete separation of authentic cobalamin standards (OH-Cbl, CN-Cbl, Ado-Cbl, and Me-Cbl) was accomplished in approximately 11 min with the patterns shown in Fig. 1. Trials employing acetate in the solvent system produced significant "trailing" of the peaks. Substitution of phosphate for acetate resulted in a lesser retention on the columns, thereby permitting use of a lower concentration of methanol in the eluant. These changes eliminated the "trailing" phenomenon in the studies performed on the Waters μBondapak C_{18} columns (Fig. 1A). In studies on the DuPont Zorbax ODS columns (Fig. 1B) the trailing was reduced by the phosphate substitution, but not completely eliminated, particularly in the hydroxycobalamin peak. The minor peaks seen between OH-Cbl and CN-Cbl are corrinoid contaminants of OH-Cbl of an unknown nature. Treatment with cyanide increased the retention times of these contaminants as well as OH-Cbl. The small, irregularly-shaped peaks at the end of the separation are solvent artifacts.

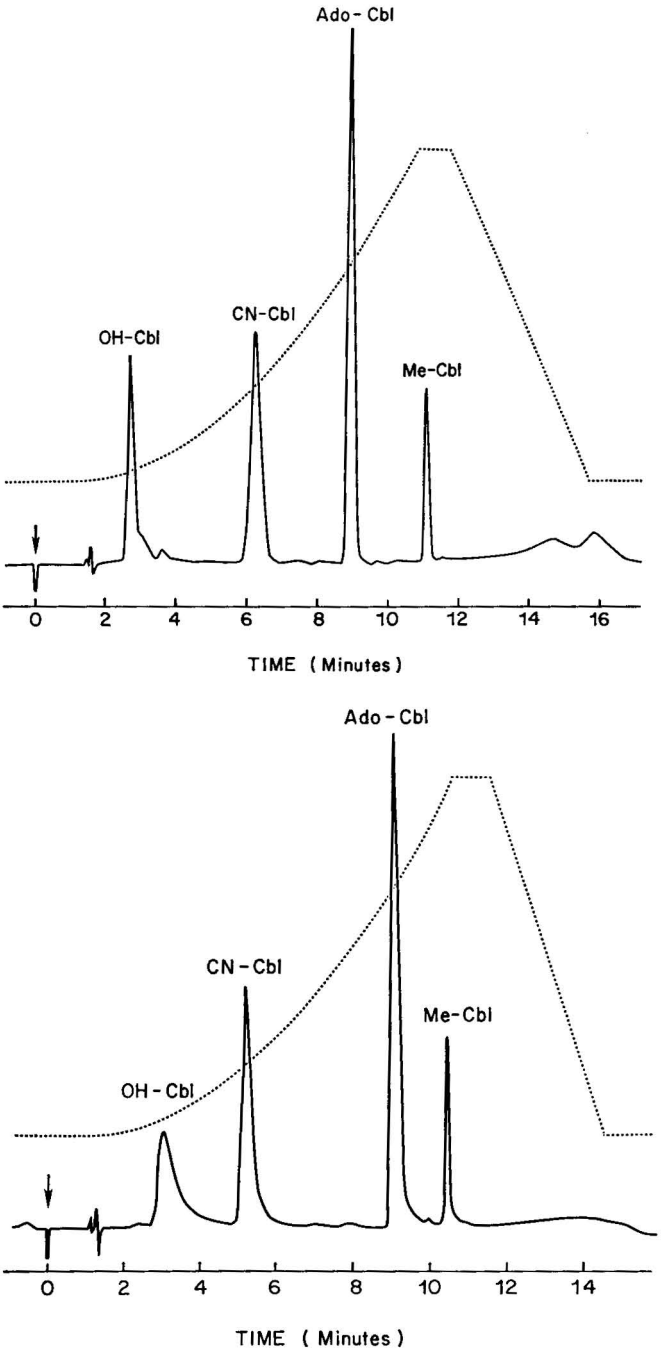


Fig. 1. HPLC separation of authentic cobalamin standards. A mixture of the cobalamins (hydroxycobalamin [OH-Cbl] 7.5 μg; cyanocobalamin [CN-Cbl] 9.8 μg; deoxyadenosylcobalamin [Ado-Cbl] 6.8 μg; and methyl cobalamin [Me-Cbl] 3.4 μg) in a 15 μl volume was injected. The effluent was monitored by ultraviolet absorption at 254 nm wavelength and 0.5 a.u.f.s. deflection. The dashed line represents the methanol concentration of the eluant. (A) The separation on the Waters μBondapak C_{18} column. (B) The separation on the DuPont Zorbax ODS column.

In studies not shown other solvent system trials demonstrated an interesting pattern when the cobalamins eluted with distilled water rather than the acid buffer. Under these conditions a reversal of the sequence of separation was seen with Me-Cbl being eluted first and OH-Cbl last. Unfortunately, a greater trailing artifact and incomplete elution precluded the application of these conditions for quantitative studies.

A variety of cobalamin analogues were also examined and their separation is shown in Fig. 2. Sulfitocobalamin (HSO_3-Cbl) was clearly identified by both systems and separated from OH-Cbl. On the Waters μBondapak C_{18} columns the HSO_3-Cbl had a longer retention time than OH-Cbl, whereas on the DuPont Zorbax ODS columns its retention was shorter than OH-Cbl. It is of interest that in earlier studies at ambient temperature using the acetate–methanol system the HSO_3-Cbl was not separable from the OH-Cbl.

The monobasic acids of CN-Cbl were resolved into two major and two minor peaks eluting beyond CN-Cbl (Fig. 2). The resolution of these peaks was clearer on the DuPont Zorbax ODS columns. When these analogues are admixed with CN-Cbl good resolution of the analogue M-1 from the CN-Cbl was achieved only when the acetate buffer system was employed. The S-102 and anilide analogues of CN-Cbl were well resolved from CN-Cbl in each of the systems studied (Fig. 2). The minor unlabeled peaks seen on Fig. 2 are contaminants of the analogue preparations.

Reproducibility of retention times and resolution of peaks were excellent regardless of the sample composition. A range of injection volumes of 2–100 μl displayed identical retention and resolution of cobalamins. Sample volumes in excess of 100 μl slightly decreased the sharpness of the peak. Reproducibility of the separation of a cobalamin extract (day to day and/or week to week) showed less than 2% variation.

The recovery of cobalamins injected onto the HPLC system was virtually 100% ($99 \pm 1\%$); *ca.* 0.2–0.3% was eluted on the subsequent run.

Quantitation of eluted cobalamins

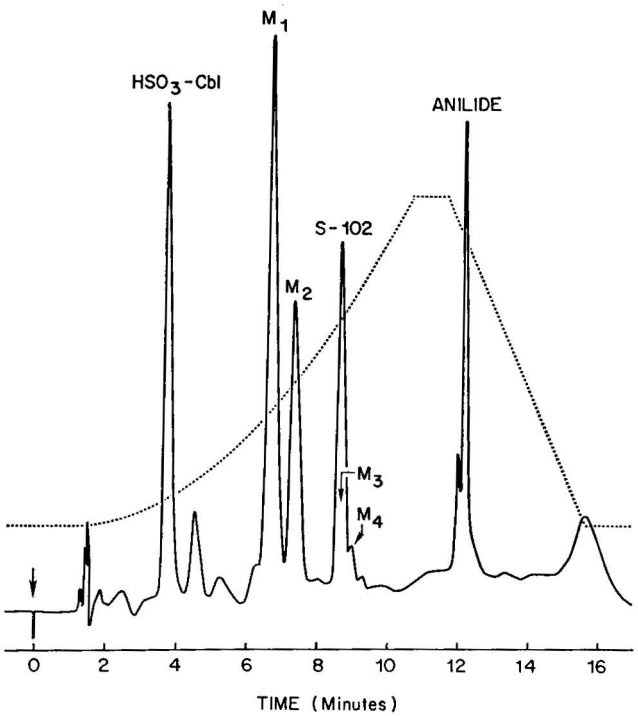
Standard curves were prepared for each cobalamin by plotting known quantities of the cobalamin injected against the peak area obtained from the spectrophotometric detector. For each cobalamin a clear linear quantitative relationship was obtained between 100 ng and 10 μg of the injected material. At any given concentration the standard deviation of the points on the standard curve was less than 2%.

Profile of photolysis of cobalamins

Experiments on the photolytic cleavage of the physiologic cobalamins are shown in Fig. 3. Photolysis of both Ado-Cbl and Me-Cbl is clearly demonstrable by HPLC techniques with the expected corresponding increase in OH-Cbl. No significant photolysis of the CN-Cbl was seen under these conditions of light exposure.

DISCUSSION

The present studies demonstrate the applicability of HPLC technology to the separation and fractionation of individual cobalamins. HPLC provides excellent resolution of the cobalamins, adequate sensitivity for application for tissue cobalamin



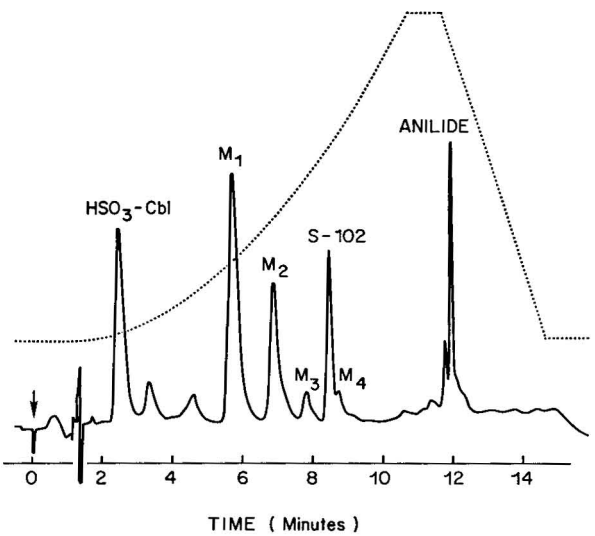


Fig. 2. HPLC separation of cobalamin analogues. Under the conditions noted in Fig. 1, 50 μl volumes (sulfito-cobalamin [HSO_3-Cbl]; mixed isomers of monobasic acids of cyanocobalamin [M_1, M_2, M_3, M_4]; alkanolamine analogue of cyanocobalamin [S-102]; and anilide derivative of the monobasic acids [anilide]) were injected. The full scale deflection was 0.2 a.u. (A) The separation on the Waters μBondapak C_{18} column. (B) The separation on the DuPont Zorbax ODS column.

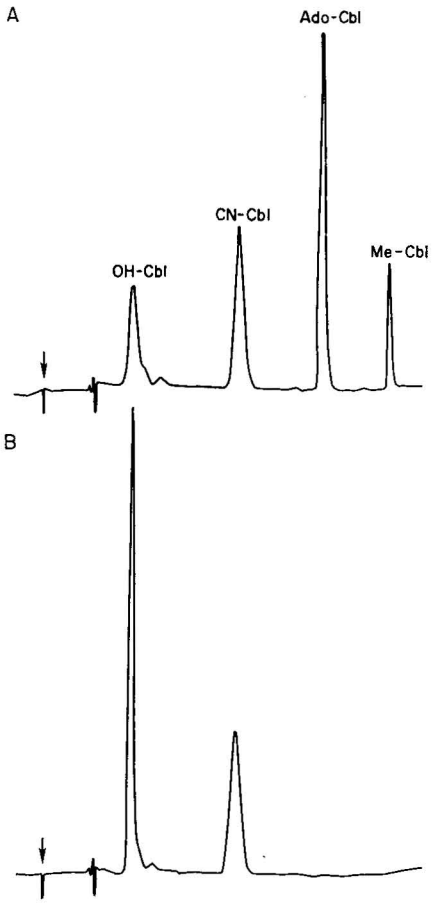


Fig. 3. HPLC of cobalamins (A) before and (B) following light exposure to high intensity light for 15 min at 4°. Photolysis of Ado-Cbl and Me-Cbl was complete, as shown in (B).

analysis enhanced by coupling with a B_{12} competitive inhibition assay[8,9,14], the technical advantage of ease and rapidity of analyses, and documented reproducibility. Studies are now in progress to characterize the fractions in tissues in a variety of clinical states.

Pathophysiological or clinical correlative relationships to individual cobalamin fractions have had only preliminary exploration[2–5], because of the serious technological limitations in separating and quantifying these cobalamins (*i.e.* methylcobalamin, adenosylcobalamin and cyanocobalamin). Although the data are limited, evidence suggests that B_{12} deprivation results in an asymmetric decline in the individual cobalamins[4,5], and that other metabolic changes (*e.g.* alcohol) have different effects on methylcobalamin and adenosylcobalamin[15] function. Thus, critical need exists for the characterization of the cobalamin fractions under a variety of clinical states. HPLC offers technological promise for a direct approach to these important pathophysiological issues.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant No. 1-P01-CA23115-01; Veterans Administration Hospital MRIS 1450; the Heddens-Good Foundation; the McDermott Foundation, and the Robert A. Welch Foundation (Grant I-616). Dr. Prough was a USPHS Research Career Development Awardee HLCA 00255.

REFERENCES

1 J. C. Linnell, H. M. Mackenzie, J. Wilson and D. M. Matthews, *J. Clin. Path.*, 22 (1969) 545.
2 J. C. Linnell, A. V. Hoffbrand, T. J. Peters and D. M. Matthews, *Clin. Sci.*, 40 (1971) 1.
3 I. L. Craft, D. M. Matthews and J. C. Linnell, *J. Clin. Path.*, 24 (1971) 449.
4 J. C. Linnell, A. V. Hoffbrand, H. A.-A. Hussein, I. J. Wise and D. M. Matthews, *Clin. Sci. Molec. Med.*, 46 (1974) 163.
5 E. V. Quadros, D. M. Matthews, I. J. Wise and J. C. Linnell, *Biochim. Biophys. Acta*, 421 (1976) 141.
6 R. A. Beck and J. J. Brink, *Environ. Sci. Technol.*, 10 (1976) 173.
7 R. A. Beck, *Anal. Chem.*, 50 (1978) 200.
8 E. P. Frenkel, S. Keller and M. S. McCall, *J. Lab. Clin. Med.*, 68 (1966) 510.
9 E. P. Frenkel, M. S. McCall and J. D. White, *Amer. J. Clin. Path.*, 55 (1971) 58.
10 H. P. C. Hogenkamp, in B. M. Babior (Editor), *Cobalamin: Biochemistry and Pathophysiology*, Wiley-Interscience, New York, 1975, p. 53.
11 J. A. Hill, J. M. Pratt and R. J. P. Williams, *J. Theoret. Biol.*, 3 (1962) 423.
12 E. L. Smith, *Vitamin B_{12}*, Methuen, London, Wiley, New York, 3rd ed., 1965, Ch. 10.
13 W. Friedrich, H. C. Heinrich, E. Konigh and P. Schulze, *Ann. N.Y. Acad. Sci.*, 112 (1964) 601.
14 E. P. Frenkel, M. S. McCall and J. D. White, *Amer. J. Clin. Path.*, 53 (1970) 891.
15 E. P. Frenkel and R. L. Kitchens, *Blood*, 50 (Suppl. 1) (1977) 78.

Journal of Chromatography, 174 (1979) 401–407

CHROM. 11,805

ANALYSIS OF ERGOT ALKALOIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. CYCLOL ALKALOIDS (ERGOPEPTINES)

M. WURST, M. FLIEGER and Z. ŘEHÁČEK
Institute of Microbiology, Czechoslovak Academy of Sciences, Prague 4 (Czechoslovakia)
(Received January 31st, 1979)

SUMMARY

A high-performance liquid chromatographic method has been developed for the separation and quantitative analysis of a mixture of cyclol ergot alkaloids. The method utilizes silica gel treated with alkylamine (LiChrosorb NH_2) as the stationary phase, and isocratic and gradient elution with diethyl ether–ethanol as the mobile phase. Individual alkaloids are detected with a UV detector and determined using the method of internal normalization.

INTRODUCTION

Cyclol ergot alkaloids (CEA) are produced by the pyromycete *Claviceps purpurea*. The attention of pharmacologists is concentrated on cyclol derivatives of L-lysergic acid (designated by the suffix -ine), as derivatives of isolysergic acid (designated by the suffix -inine) are biologically almost inactive. Problems of the separation, isolation, identification and determination of these fungal metabolites have become more urgent with their extended clinical use.

For the analysis of ergot alkaloids (EA), chromatographic methods, mainly paper and thin-layer chromatography[1–14], were applied, utilizing either a spectrophotometric determination (*in situ*) on a thin layer[4,7–9] or measurement of the fluorescence of solutions obtained after extraction from chromatograms[5] and fluorodensitometric measurements[13]. The detection sensitivity can be increased by, *e.g.*, π-acceptors. Gas chromatography, either alone[15–19] or in combination with mass spectrometry[20–22], is less frequently applied in ergot studies, mainly because CEA already have a high molar mass, low vapour pressure and are not stable to heat. In addition, gas chromatography does not give satisfactory results when stereoisomers of lysergic acid are separated[20]. However, in combination with mass spectrometry it appears to be promising for the determination of degradation products of the peptide moiety of CEA[21,22].

Recently, high-performance liquid chromatography (HPLC) has been applied in the analysis of EA[23–33], followed by UV or fluorescence detection. In the HPLC

method, silica gel[23,24,32] and silica gel modified with alkylamine[31] have been used as the stationary phase and reversed phase[25–27,30,32,33], using both isocratic and gradient elution[28,29,31].

In this paper, which extends our previous work[31], an HPLC method is described for the separation and determination of a CEA mixture.

EXPERIMENTAL

Chloroform, isopropanol, diethyl ether and ethanol for UV spectroscopy (Lachema, Brno, Czechoslovakia, all of analytical-reagent grade) served as mobile phases for the separation of the CEA mixture. Reference CEA samples were obtained from Prof. H. G. Floss (Purdue University, Lafayette, Ind., U.S.A.), Prof. C. Spalla (Farmitalia, Milan, Italy) and Dr. E. Udvardy (Gedeon Richter Ltd., Budapest, Hungary).

Analyses of the CEA mixture were performed on a Varian 8500 liquid chromatograph, in which the concentration gradient of the mobile phase can be programmed. The instrument is equipped with a Variscan LC UV detector, A25 line recorder and 485 integrator (Varian Aerograph, Walnut Creek, Calif., U.S.A.). Chromatographic columns were 25 cm long with an I.D. of 2 mm. Silica gel (LiChrosorb Si 60, pore size 10 μm), modified with alkylamine served as the stationary phase. One column (A) was obtained from Varian (MicroPak NH_2; number of theoretical plates for agroclavine $N = 280$); the other (B) was packed in this laboratory (LiChrosorb NH_2, $N = 1070$; Applied Science Labs., State College, Pa., U.S.A.).

The temperature of the columns was 25°, the pressure on column A was 3.0–8.5 MPa (according to the system used and ratio of the components of the mobile phase), and that on column B was 1.5 MPa. Three solvent systems served as mobile phases: chloroform–isopropanol (90:10), diethyl ether–isopropanol (60:40) and diethyl ether–ethanol (84:16, 88:12 and 93:7). The flow-rates of the mobile phase during isocratic elution were 1 and 0.67 ml·min^{-1} on column A and B, respectively.

Gradient elution was performed with the system diethyl ether–ethanol. The separation was first run for 7 min with a constant ratio of the two components of the mobile phase (97.5:2.5), then the concentration of ethanol was increased at a rate of 0.3%·min^{-1} and the final stage of the separation proceeded at the final composition of the solvents, *i.e.* diethyl ether–ethanol (95:5). The flow-rate and pressure were the same as during isocratic elution on column B.

Qualitative analysis of CEA mixtures was performed by comparing the elution volumes of the components with those of reference CEA samples. The method of internal normalization was used for quantitative analysis. Peak areas were evaluated by using the integrator. Correction factors were calculated from values of the absorption coefficients (absorption coefficient of the analysed sample/absorption coefficient of the reference sample) obtained from UV spectra (Table I) measured in a Cary 118 C spectrometer (Varian Aerograph) with a wavelength range of 210–350 nm and a spectral bandwidth of 0.1 nm; the photometric accuracy at 1.0 absorbance was less than 0.001 absorbance. The cuvettes were 1 cm wide. All spectra were measured in the system diethyl ether–ethanol (80:20) at 25°. The concentration of the measured solutions varied within the range $1 \cdot 10^{-6}$–$2 \cdot 10^{-5}$ g·ml^{-1}. In this range, a linear relationship was found between absorbance and CEA concentration in all instances.

TABLE I

SPECIFIC DECADIC ABSORPTION COEFFICIENTS OF CYCLOL ERGOT ALKALOIDS

Alkaloid	*Absorption coefficient*				
	225 nm	*240 nm*	*254 nm*	*282 nm*	*310 nm*
Ergocryptine	343	320	165	66	143
Ergocryptinine	332	342	225	57	151
Ergocornine	325	304	153	59	134
Ergocorninine	409	420	277	73	188
Ergocristine	365	318	161	61	139
Ergocristinine	301	295	197	51	134
Ergosine	335	345	228	59	154
Ergosinine	286	288	184	55	126
Ergostine	426	361	186	73	159
Ergostinine	343	323	208	56	140
Ergotamine	394	329	172	67	139
Ergotaminine	303	292	187	52	130
Ergobasine	507	502	258	103	232
Ergobasinine	508	569	352	108	257
Ergine	786	786	385	157	363
Erginine	673	730	449	138	329
Agroclavine*	1175	130	102	302	3.1

* Reference sample.

RESULTS AND DISCUSSION

Satisfactory results were obtained when analysing clavines and simple derivatives of lysergic acid on silica gel modified with alkylamine as the stationary phase. Therefore, separations were carried out on both columns with the same stationary phase: MicroPak NH_2 and LiChrosorb NH_2. According to our previous experience with the mobile phases used, the best separation of the CEA mixture was obtained with the system diethyl ether–ethanol (Table II). This solvent mixture makes it possible to make measurements within the whole range of absorption maxima of all EA, particularly clavines, gives relatively stable solutions and is hence suitable for use in quantitative analysis. The isocratic elution of CEA with diethyl ether–ethanol as the mobile phase yielded good results. The optimal separation of the CEA mixture was obtained with ratios of these components of 88:12 and 93:7 on columns A and B, respectively. Column B (packed in this laboratory) was three times more effective for agroclavine than column A (obtained commercially). A composition of the mobile phase of 84:16 is given in Table II for comparison of the elution data of CEA with the elution values of clavines and simple derivatives of lysergic acid.

The chromatographic separation of CEA stereoisomers is made possible probably owing to the steric arrangement of the amide group and hydrogen at position C-8 of the ergolene ring. A change of configuration at position C-8 influences most pronouncedly their elution volumes (Fig. 1). The elution volumes of "-ines" are substantially higher than those of "-inines". It follows that the amide group of "-ines" is in the equatorial position with respect to the D-ring of the ergolene nucleus, whereas in the "-inines" its position is axial and is stabilized by an intramolecular hydrogen

TABLE II

RELATIVE RETENTIONS OF CYCLOL ERGOT ALKALOIDS

Column: (A) MicroPak NH_2; (B) LiChrosorb NH_2. Detection: UV Variscan LC detector; wavelength 310 nm. Flow-rate: (A) 1 ml · min^{-1}; (B) 0.67 ml · min^{-1}. Pressure: (A) 3.0–8.5 MPa; (B) 1.5 MPa.

Alkaloid	*Molar mass*	*Mobile phase*				
		Diethyl ether–ethanol			*Diethyl ether–isopropanol, 60:40(A)*	*Chloroform–isopropanol, 90:10(A)*
		93:7(B)	*88:12(A)*	*84:16(A)*		
Ergocryptine	575.7	2.82	0.50	0.47	0.31	0.13
Ergocryptinine	575.7	1.59	0.25	0.21	0.14	0.03
Ergocornine	561.7	3.06	0.64	0.56	0.32	0.12
Ergocorninine	561.7	2.00	0.34	0.30	0.18	0.05
Ergocristine	609.7	3.76	0.89	0.78	0.45	0.15
Ergocristinine	609.7	2.35	0.45	0.38	0.26	0.04
Ergostine	595.7	7.38	1.85	1.30	0.82	0.33
Ergostinine	595.7	3.15	0.75	0.57	0.37	0.05
Ergosine	547.6	8.47	1.83	1.21	0.71	0.47
Ergosinine	547.6	3.09	0.63	0.46	0.28	0.07
Ergotamine	581.7	12.44	2.25	1.94	1.08	0.53
Ergotaminine	581.7	4.56	0.94	0.88	0.52	0.07
Ergobasine	325.4	12.82	5.12	4.16	2.55	4.93
Ergobasinine	325.4	3.82	1.05	1.00	0.57	0.98
Ergine	267.3	—	—	2.90	1.85	4.00
Erginine	267.3	—	—	0.83	0.64	0.58
8-Hydroxy-ergotamine	597.7	8.88	—	—	—	—
Agroclavine	238.3	1.00	1.00	1.00	1.00	1.00
Retention volume of agroclavine (ml)	—	1.10	6.00	4.67	3.50	2.67

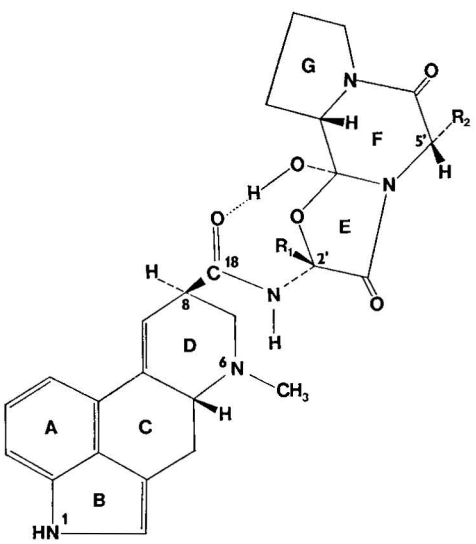


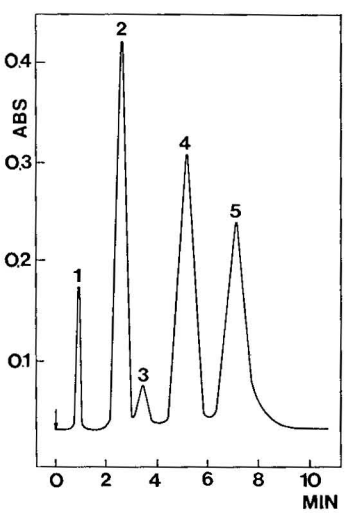
Fig. 1. Structure of cyclol ergot alkaloids.

bond between the basic nitrogen in position N-6 and the hydrogen of the amide group in position C-18.

Aromatic or aliphatic groups (benzyl, isobutyl and isopropyl) at position C-5′, together with an alkyl group (methyl, ethyl and isopropyl) at position C-2′, bound to the cyclic peptide of CEA influence considerably the elution volumes (Fig. 1). The elution volumes of CEA with a benzyl group at position C-5′, together with an alkyl group at position C-2′, are higher than those of CEA with alkyl groups in both positions. Free π-electrons of the benzene nucleus play a role in the interaction between the analysed compound and the stationary phase. The smaller the alkyl group in position C-2′, the higher are the elution volumes. In the series ergocristine, ergostine ergotamine, the elution volume increases with decreasing molar mass (Table II).

CEA with aliphatic groups at positions C-2′ and C-5′ have elution volumes lower than those of CEA with a single aromatic group at position C-5′ and it also holds here that the larger the alkyl groups in both positions the lower are the elution volumes. Substitution of the aromatic group by a branched alkyl group at position C-5′ and of the methyl group at position C-2′ by ethyl and especially isopropyl produces greater steric hindrance on the peptide moiety of the alkaloids for interaction with the stationary phase. In the series ergosine, ergocornine, ergocryptine, the elution volume decreases with increasing molar mass (Table II).

The elution volume of 8-hydroxyergotamine, which has a tertiary hydroxyl group at position C-8, is lower than that of ergotamine, which has only hydrogen at position C-8 (Table II, Fig. 1). The effect of the hydrogen bond between the



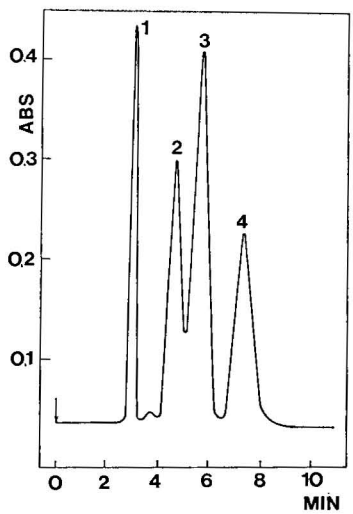


Fig. 2. Chromatogram of a mixture of cyclol ergot alkaloids of the ergotamine type. Column, LiChrosorb NH_2. Mobile phase, diethyl ether–ethanol (93:7). Flow-rate of mobile phase, 0.67 ml · min^{-1}. Pressure, 1.5 MPa. Detector, Variscan UV at 310 nm. Peaks: 1 = solvent; 2 = ergosinine; 3 = ergotaminine; 4 = ergosine; 5 = ergotamine.

Fig. 3. Chromatogram of a mixture of cyclol ergot alkaloids of the ergotoxine type. Column, LiChrosorb NH_2. Mobile phase, diethyl ether–ethanol (97.5:2.5). Flow-rate of mobile phase, 0.67 ml · min^{-1}. Pressure, 1.5 MPa. Detector, Variscan UV at 310 nm. Peaks: 1 = ergocryptinine; 2 = ergocristinine; 3 = ergocryptine; 4 = ergocristine.

oxygen atom of the carbonyl group at position C-18 and the hydrogen atom of the hydroxyl group at position C-8 is involved here. Therefore, the sorption of 8-hydroxyergotamine is less pronounced than that of ergotamine. The same also holds true for the sorption of setoclavine and lysergine[31]. In simple derivatives of lysergic acid, *e.g.*, ergine, the elution volume is influenced by the primary amino group at position C-18 and in ergobasine by the primary hydroxyl group on isopropyl in the same position (Fig. 1, Table II).

The analysis of CEA at the optimal ratio of the components of the diethyl ether–ethanol mobile phase (93:7) made it possible to separate very well CEA stereoisomers and mixtures of the ergotamine type (Fig. 2). CEA mixtures of the ergotoxine type were separated best in the system diethyl ether–ethanol (97.5:2.5) (Fig. 3). Elution with a programmed concentration gradient of the mobile phase was more advantageous for a CEA mixture than isocratic elution. Under these conditions it was

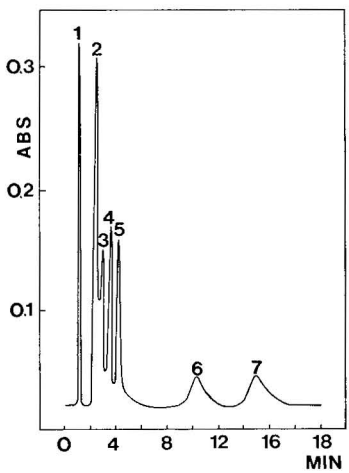


Fig. 4. Chromatogram of mixture of cyclol ergot alkaloids. Column, LiChrosorb NH_2. Gradient elution: 7 min diethyl ether–ethanol (97.5:2.5), 10 min with increasing ethanol concentration at 0.3% · min^{-1}, 5 min with diethyl ether–ethanol (95:5). Flow-rate of mobile phase, 0.67 ml · min^{-1}. Pressure, 1.5 MPa. Detector, Variscan UV at 310 nm. Peaks: 1 = solvent; 2 = ergocorninine; 3 = ergocristinine; 4 = ergocornine; 5 = ergocristine; 6 = ergosine; 7 = ergotamine.

TABLE III

DETERMINATION OF MIXTURES OF CYCLOL ERGOT ALKALOIDS USING THE HPLC METHOD

Alkaloid	*Amount (wt.-%)*		*Standard deviation*	
	Weighed	*Determined**	*Absolute*	*Relative*
Ergocorninine	11.8	10.8	0.21	1.94
Ergocristinine	27.8	27.9	0.32	1.15
Ergocornine	21.1	22.9	0.56	2.45
Ergocristine	13.7	12.8	0.39	3.05
Ergotamine	25.6	25.6	0.52	2.03

* A total of 8 determinations were performed.

possible to analyse simultaneously CEA of both the ergotamine and ergotoxine types. Programmed elution of the mobile phase was performed with the same solvent mixture as was used in isocratic elution. An increase in the ethanol content from 2.5% to 5% gave good results (Fig. 4).

The method was verified with a mixture of standard CEA and the results are presented in Table III. Deviations of the values determined for individual CEA varied within the range commonly observed in most chromatographic analyses.

The method described is suitable for the determination of ergot alkaloids in fermentation media with emerged and submerged cultures of *Claviceps purpurea*, *C. paspali*, *Aspergillus flavus*, *Penicillium* sp., etc., and for determination of these alkaloids in natural materials and in semi-synthetic and pharmaceutical commercial preparations.

REFERENCES

1 J. Kolsek, *Microchim. Acta*, 10 (1956) 1500.
2 J. L. McLaughlin, J. E. Goyan and A. G. Paul, *J. Pharm. Sci.*, 53 (1964) 306.
3 S. Agurell, *Acta Pharm. Suecica*, 2 (1965) 357.
4 K. Genest, *J. Chromatogr.*, 19 (1965) 531.
5 L. A. Dal Cortivo, J. R. Broich, A. Dihrberg and B. Newman, *Anal. Chem.*, 38 (1966) 1959.
6 A. Kornhauser, M. Perpar and L. Gasperut, *Arch. Pharm. (Weinheim)*, 303 (1970) 882.
7 A. Cavallaro, G. Rossi and G. Elli, *Boll. Lab. Chim. Prov.*, 21 (1970) 303.
8 T. Niwaguchi and T. Inoue, *J. Chromatogr.*, 59 (1971) 127.
9 S. Keipert and R. Voigt, *J. Chromatogr.*, 64 (1972) 327.
10 R. Fowler, P. J. Gomm and D. A. Patterson, *J. Chromatogr.*, 72 (1972) 351.
11 J. Reichelt and S. Kudrnáč, *J. Chromatogr.*, 87 (1973) 433.
12 E. Eckart and S. Walter, *Planta Med.*, 27 (1975) 58.
13 M. Prošek, E. Kučan, M. Katič and M. Bano, *Chromatographia*, 9 (1976) 273 and 325; 10 (1977) 147.
14 G. Rücker and A. Taha, *J. Chromatogr.*, 132 (1977) 165.
15 C. A. Radecka and J. C. Nigam, *J. Pharm. Sci.*, 55 (1966) 861.
16 M. Lerner, *Bull. Narcot.*, 19 (1967) 39.
17 A. R. Sperling, *J. Chromatogr. Sci.*, 12 (1974) 265.
18 K. D. Barrow and F. R. Quigley, *J. Chromatogr.*, 105 (1975) 393.
19 G. Szepesi and M. Gazdag, *J. Chromatogr.*, 122 (1976) 479.
20 S. Agurell and A. Ohlsson, *J. Chromatogr.*, 61 (1971) 339.
21 J. W. F. van Mausvelt, J. E. Greving and R. A. De Zeeuw, *J. Chromatogr.*, 151 (1978) 113.
22 T. A. Plomp, J. G. Leferink and R. A. A. Maes, *J. Chromatogr.*, 151 (1978) 121.
23 J. D. Wittwer Jr. and J. H. Kluckholm, *J. Chromatogr. Sci.*, 11 (1973) 1.
24 R. A. Heacock, K. R. Langille, J. D. MacNeil and R. W. Frei, *J. Chromatogr.*, 77 (1973) 425.
25 I. Jane and B. B. Wheals, *J. Chromatogr.*, 84 (1973) 181.
26 R. V. Vivilecchia, R. L. Cotter, R. J. Limpert, N. Z. Thimot and J. N. Little, *J. Chromatogr.*, 99 (1974) 407.
27 J. Christie, M. W. White and J. M. Wiles, *J. Chromatogr.*, 120 (1976) 496.
28 H. Bethke, B. Delz and K. Stich, *J. Chromatogr.*, 123 (1976) 193.
29 F. Erni, R. W. Frei and W. Lindner, *J. Chromatogr.*, 125 (1976) 265.
30 J. Dolinar, *Chromatographia*, 10 (1977) 364.
31 M. Wurst, M. Flieger and Z. Řeháček, *J. Chromatogr.*, 150 (1978) 477.
32 L. Szepesy, I. Fehér, G. Szepesi and M. Gazdag, *J. Chromatogr.*, 149 (1978) 271.
33 V. Hartmann, M. Rödniger, W. Ableidinger and H. Bethke, *J. Pharm. Sci.*, 67 (1978) 98.

Journal of Chromatography, 174 (1979) 409–419

CHROM. 11,794

ION-EXCHANGE SEPARATION OF NUCLEIC ACID CONSTITUENTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

EDWARD H. EDELSON and JAMES G. LAWLESS
Extraterrestrial Biology Division, Ames Research Center, NASA, Moffett Field, Calif. 94035 (U.S.A.)
and
C. TIMOTHY WEHR and SETH R. ABBOTT
Varian Associates, Walnut Creek, Calif. 94598 (U.S.A.)
(Received February 14th, 1979)

SUMMARY

The high-performance liquid chromatographic separation of a large variety of nucleic acid constituents on a silica-based, weak-anion exchange column was accomplished. Using this technique it was possible to achieve some relatively difficult separations, such as the separation of 2′-, 3′-, and 5′-AMP, and the separation of a mixture of ribo- and deoxyribo-nucleosides and -nucleotides. A number of other separations are demonstrated by isocratic or gradient elution. These include the separation of a mixture of nucleoside monophosphates, the separation of a mixture of nucleoside mono-, di-, and triphosphates, the separation of a mixture of nucleosides and bases, and the separation of a mixture of nucleotide oligomers. These chromatographic separations were accomplished using relatively simple experimental procedures at ambient temperatures and involved relatively short analysis times. Excellent separations were obtained, in most cases, by adjustment of buffer concentration and pH, or by addition of an organic modifier. In some cases, it was necessary to use gradient elution to achieve optimum resolution.

INTRODUCTION

The analysis of nucleic acid constituents by high-performance liquid chromatography (HPLC) has been reviewed by several workers[1–3]. The need for efficient chromatographic analysis of these compounds has developed, in part, in order to more fully understand the central role of nucleic acids in heredity and cell function. Intracellular patterns of nucleotides and nucleosides provide information on the metabolic state of the cell. Altered levels of these compounds in biological fluids can indicate changes in nucleic acid metabolism in tissues associated with certain disease states or drug responses. Separation of nucleosides, nucleotides, and free bases is necessary in the determination of base composition of nucleic acids, the synthesis of specific polynucleotides, and the elucidation of gene sequences, while studies of the prebiotic evolution of nucleic acids require the separation of a different subset of these

compounds. Because of the similarities in their physical and chemical properties, separation of nucleoside monophosphate isomers has been a difficult analytical problem and in the past has been achieved through use of radio-labeling techniques combined with chromatography (*i.e.*, paper chromatography or electrophoresis)[4,5]. In this paper, we describe a rapid method for the separation of the 2′-, 3′-, and 5′-isomers of AMP. In addition, we describe the rapid separation of nucleosides, nucleotides, oligonucleotides, and bases using a silica-based,bonded-phase,weak-anion exchanger. It is particularly useful in the separation of nucleoside monophosphate isomers and separation of ribo- from deoxyribo-nucleosides or -nucleotides.

EXPERIMENTAL

Anion-exchange chromatography of synthetic mixtures of nucleosides, nucleotides, purines, and pyrimidines was carried out on 4 mm × 30 cm MicroPak AX-10 column (Varian, Walnut Creek, Calif., U.S.A.). The AX-10 column is a difunctional weak anion-exchange bonded phase prepared on 10-μm Lichrosorb Si-60 silica, and has an exchange capacity of ~2 mequiv./g (5 mequiv. per column as determined by titration with orthophosphoric acid). Nucleosides, nucleotides, and bases were obtained from ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.), Sigma (St. Louis, Mo., U.S.A.), and P-L Biochemicals, (Milwaukee, Wisconsin, U.S.A.); all were chromatographically pure with the exception of 3′-AMP (ICN Pharmaceuticals,) which contained some 2′-AMP as an impurity. Standard solutions of these compounds (10^{-3} *M*) were prepared and aliquots of the standard solutions were then taken and used to make up mixtures. One drop of chloroform was added to each standard solution to inhibit bacterial growth. Glass-distilled acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and pesticide quality acetonitrile was obtained from MC&B Manufacturing Chemists (Los Angeles, Calif., U.S.A.). Analytical reagent grade potassium dihydrogen phosphate was obtained from linckrodt (Phillipsburg, N.J., U.S.A.). Buffers were prepared in triply distilled water or in HPLC water, obtained from J. T. Baker (Phillipsburg, N.J., U.S.A.). The pH of buffers was adjusted using ortho-phosphoric acid or sodium hydroxide solution and pH was measured using a Corning Digital 110 expanded scale pH meter equipped with a combination pH electrode. In the case of acetonitrile-containing eluents, the phosphate buffer was first prepared by dissolving KH_2PO_4 in water to give the desired concentration, followed by adjustment of the pH to the appropriate value, and acetonitrile was then added to give the reported acetonitrile content, by volume.

Gradient separations and some isocratic separations were performed using a Varian Model 5020 liquid chromatograph (Varian Aerograph) equipped with three solvent proportioning valves and a Valco manual loop injector. Eluents were monitored by UV absorbance at 254–270 nm using a Varichrom variable wavelength detector.

Isocratic separations were also performed using an LC System Support Unit I (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) equipped with a manual loop injector (Glenco Scientific, Houston, Texas, U.S.A.). Eluents were monitored by UV absorbance at 254 or 259 nm using a Schoeffel Spectroflow monitor SF770 in conjunction with a Schoeffel GM770 monochrometer (Schoeffel Instrument, Westwood, N.J., U.S.A.). Data recording and integration were performed using a Hewlett-

Packard 3380A integrator-recorder (Hewlett-Packard, Palo Alto, Calif., U.S.A.). All chromatographic separations were performed at room temperature. Typically, 25-μl injections were made and solute concentrations were *ca.* 10^{-4} *M*. Flow-rates ranged from 0.8 to 2.0 ml/min.

Perchloric acid extract of Balb-C mouse liver (prepared by the method of Khym[6]) was kindly provided by Dr. F. Klein, University of California Medical School, San Francisco, Calif., U.S.A.

RESULTS AND DISCUSSION

A variety of columns and experimental conditions have been used for the separation of nucleoside monophosphate isomers by HPLC. Partial resolution of AMP isomers using anion-exchange chromatography with gradient elution has been reported[7], as well as an unsuccessful attempt to separate 2'-AMP and 3'-AMP on a silica-based strong cation exchanger[8]. More recently, the successful separation of the 2'- and 3'-isomers of AMP and GMP on a strong anion-exchange resin was reported[9].

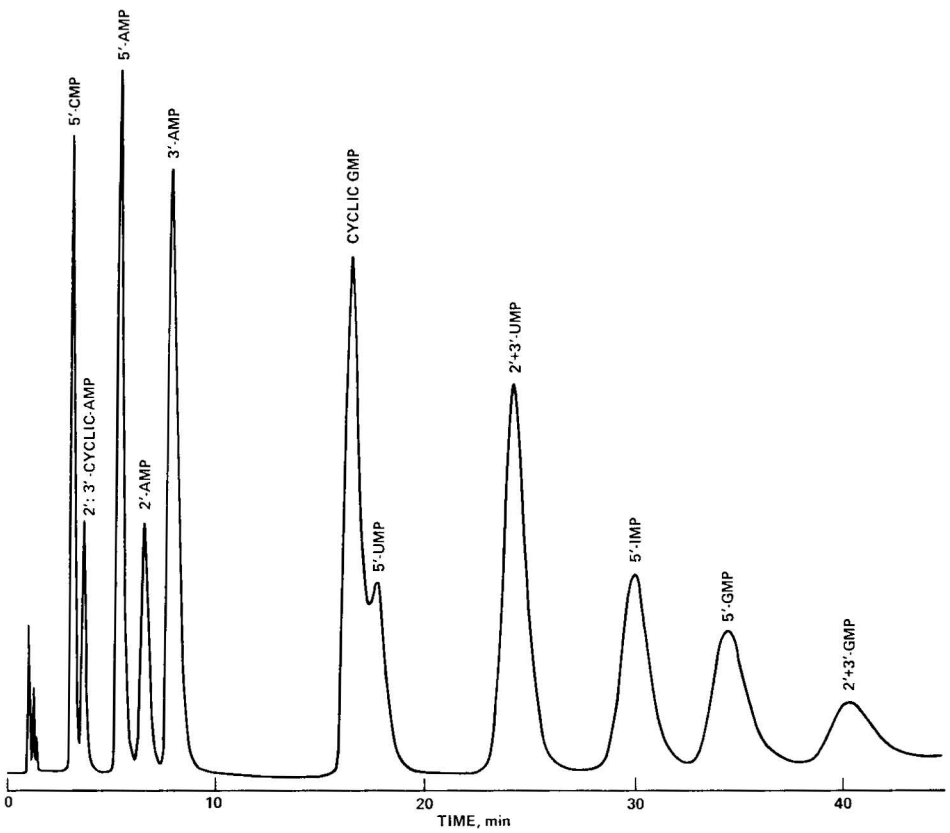


Fig. 1. HPLC separation of nucleoside monophosphates by isocratic elution with 0.01 *M* KH_2PO_4 (pH 3.0). Flow-rate, 120 ml/h; detection, 254 nm, 0.08 AUFS. The three peaks eluting at 1–2 min are nucleoside impurities contained in some of the nucleotides.

TABLE I

ISOCRATIC ELUTION TIME OF A VARIETY OF NUCLEIC ACID CONSTITUENTS AS A FUNCTION OF BUFFER pH AND ACETONITRILE CONTENT

Compound	Buffer pH or composition[a]						
	pH 2.25	pH 2.85	pH 3.52	pH 5.56	pH 7.0	Mixed buffer No. 1 (80% CH_3CN)[b]	Mixed buffer No. 2 (20% CH_3CN)[c]
2'-AMP	7.52	14.23	45.60				18.21
3'-AMP	8.56	16.42	52.66				20.36
5'-AMP	7.52	11.70	23.10			>120	15.34
Adenine		2.56	3.11	4.04	5.25	5.06	
Adenosine		2.56	3.15	3.61	4.00	4.24	
ADP	6.36	11.82	32.01				
ATP	>120	>120					
ApA[d]		3.75					
ApApA[d]		25.71					
2'-CMP	7.08	9.95	28.09				13.30
3'-CMP	6.13	9.95	27.40				11.87
5'-CMP	5.70	6.95	14.94				8.09
Cytosine		2.44	2.57	3.44	4.72	9.48	
Cytidine		2.44	2.67	3.30	3.99	10.69	
2'-UMP	34.03	50.42					
3'-UMP	37.46	50.60					
5'-UMP	28.23	38.46					
Uracil		3.56	3.76	3.75	3.84	2.49	
Uridine		3.40	3.48	3.48	3.60	3.36	
UDP	>120	>120					
UTP	>120	>120					
2'-GMP	36.48	66.00					
3'-GMP	35.07	65.30					
5'-GMP	22.57	59.94					
Guanine		3.10	4.03	4.53	4.90	5.61	
Guanosine		3.65	3.90	4.00	4.26	6.28	
deoxy-5'-AMP	6.57	16.12	33.07				14.53
deoxy-5'-CMP	5.02	6.74	14.19				7.22
deoxy-5'-UMP	28.81	40.78					
deoxy-5'-GMP		55.96					
2'-deoxy-3'-AMP	7.47	14.99	40.45				
2'-deoxy-3':5'-cyclic AMP		11.08	20.25				10.30
3':5'-cyclic AMP	6.21	9.78	17.51				9.64

[a] Unmixed buffers are prepared from 0.01 M KH_2PO_4 and adjusted to indicated pH by addition of H_3PO_4. Flow rates are 0.8 ml/min unless otherwise noted.

[b] 80% CH_3CN - 20% (0.01 M KH_2PO_4 adjusted to pH 2.85). Flow rate, 1.5 ml/min.

[c] 20% CH_3CN - 80% (0.01 M KH_2PO_4 adjusted to pH 2.85). Flow rate, 1.0 ml/min.

[d] Flow rate, 1.33 ml/min.

The complete resolution of 2′-, 3′-, and 5′-CMP has been achieved[4] in about 15 h using a Dowex-1 (formate) column. It has been suggested[10] that 2′-, 3′-, 5′-isomer separations can also be obtained for other nucleotides using similar conditions, but analysis times are prohibitively long. Typically, the separation of the 5′-isomer from the 2′- and 3′-isomers is readily accomplished, whereas the separation of the 2′- and 3′-isomers has proven to be difficult[11].

As shown in Fig. 1, an excellent separation of a mixture of nucleoside monophosphates was obtained under isocratic conditions using 0.01 *M* KH_2PO_4 by adjustment of buffer pH and flow-rate. With the mobile phase pH adjusted to 3.0, 2′-, 3′-, 5′-, and 2′:3′-cyclic AMP were completely separated within 10 min. Most of the other nucleoside monophosphates are also separated under these conditions, with the exception of the 2′-, 3′-combinations. Attempts were made to achieve a better separation for 2′- and 3′-UMP, 2′- and 3′-CMP, and 2′- and 3′-GMP by varying the buffer pH from 2.25 to 7. It was not possible to resolve any of these conditions (Table I). By using the same mobile phase adjusted to pH 2.85, it was possible to separate a mixture of adenosine, 5′-AMP, ApA and ApApA (Fig. 2). Such separations of low-molecular-weight polynucleotides are useful in monitoring the synthesis or degradation of nucleotide oligomers, and in the study of precursor interactions in prebiotic evolution of nucleic acids.

Rapid HPLC analysis of nucleosides has commonly been performed using reversed-phase methods[12–14]. However, it is often of interest in biological samples to analyze bases, nucleosides, and nucleotides simultaneously, which necessitates the use

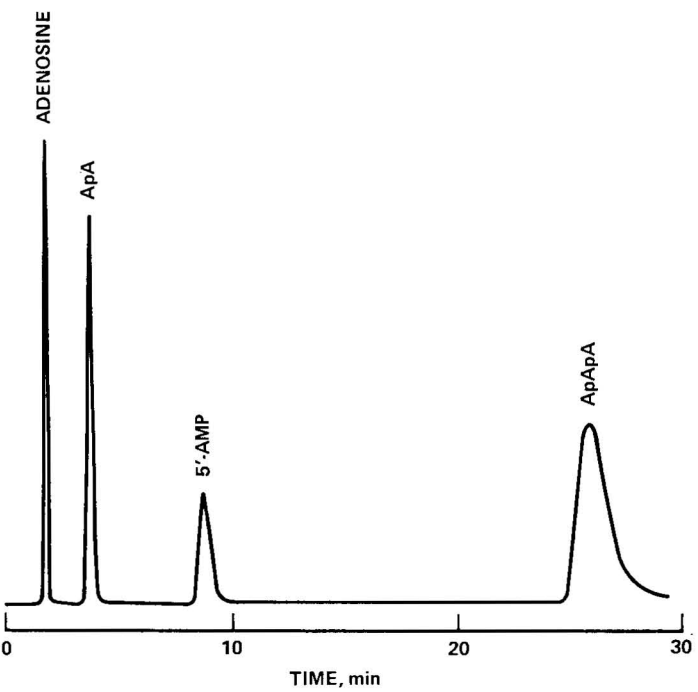


Fig. 2. HPLC separation of adenosine, 5′-AMP, ApA and ApApA by isocratic elution with 0.01 *M* KH_2PO_4 (pH 2.85). Flow-rate, 80 ml/h; detection, 259 nm, 0.0025 AUFS.

of ion-exchange separations. It previously had been difficult to achieve conditions for isocratic ion-exchange separation of nucleotides under which nucleosides are also resolved; using aqueous buffers, nucleosides are generally poorly retained. With a silica-based ion exchanger, addition of an organic modifier to the mobile phase decreases the absolute solubility of solutes in the mobile phase and thus increases retention times of nucleosides and bases. An organic modifier also serves to increase the relative affinity of deoxynucleosides for the stationary phase compared to ribo compounds, enhancing the resolution of the two classes. Gradient programming may then be used to elute the more highly charged nucleotides by increasing the ionic strength and pH of the mobile phase. Complete isocratic separation of a mixture of four bases and four nucleosides was accomplished using a mixture of 20% 0.01 *M* KH_2PO_4 (pH 2.85) and 80% acetonitrile (Fig. 3). By increasing both the molarity and pH of the aqueous mobile phase, it was possible to resolve a complex mixture of nucleoside mono-, di-, and triphosphates (Fig. 4).

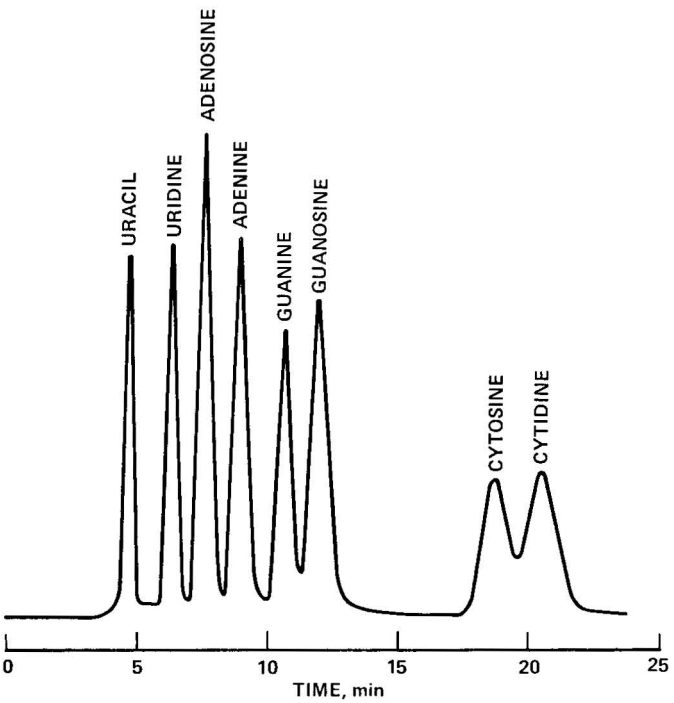


Fig. 3. HPLC separation of nucleosides and bases by isocratic elution with 80% acetonitrile–20% 0.01 *M* KH_2PO_4 (pH 2.85). Flow-rate, 48 ml/h; detection, 259 nm, 0.01 AUFS.

The separation of ribo- from deoxyribonucleotides has been successfully carried out on anion-exchange resins using gradient elution[6,15]; however, complete elution of all nucleotides required 1–3 h, and some pairs of ribo- and deoxyribonucleoside monophosphates were poorly resolved. Separation of ribo- and deoxyribonucleoside mono-, di-, and triphosphates has been attempted using a silica-based, strong anion exchanger, but it was not possible to obtain simultaneously good resolution and a satisfactory analysis time[16,17].

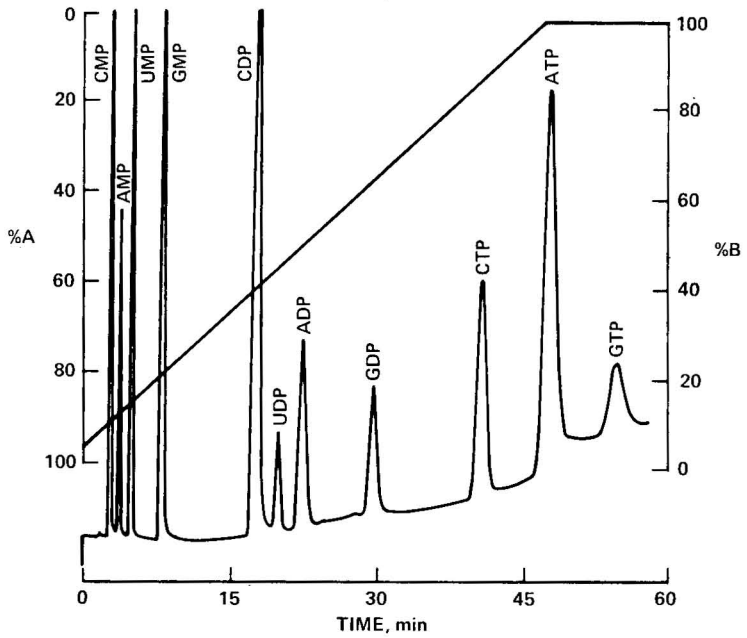


Fig. 4. HPLC separation of nucleoside 5′-mono-, di-, and triphosphates by gradient elution. A = 0.01 *M* KH_2PO_4, pH 2.85; B = 0.75 *M* KH_2PO_4, pH 4.4. Gradient program: 5–100% B; step 1, +2% B/min for 47.5 min; step 2, isocratic, 100% B for 12.5 min. Flow-rate, 120 ml/h; detection, 260 nm, 0.5 AUFS (0–15 min), 0.2 AUFS (15–60 min).

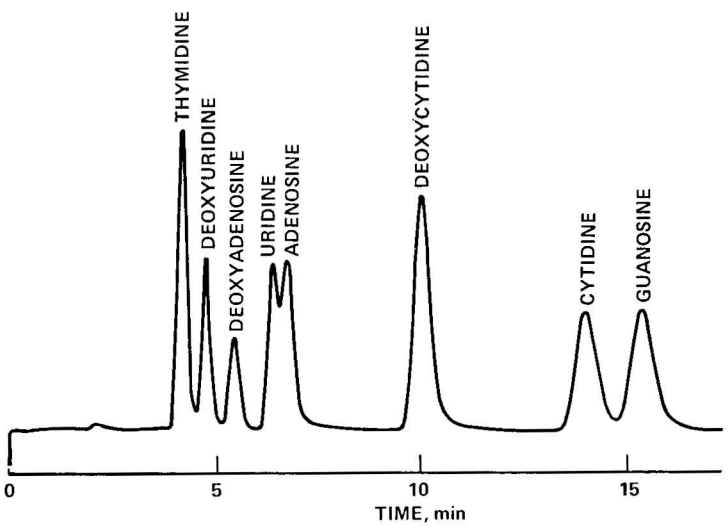


Fig. 5. HPLC separation of nucleosides and deoxynucleosides by isocratic elution with 82% acetonitrile–18% 0.0125 *M* KH_2PO_4. Flow-rate, 120 ml/h; detection, 270 nm, 0.2 AUFS.

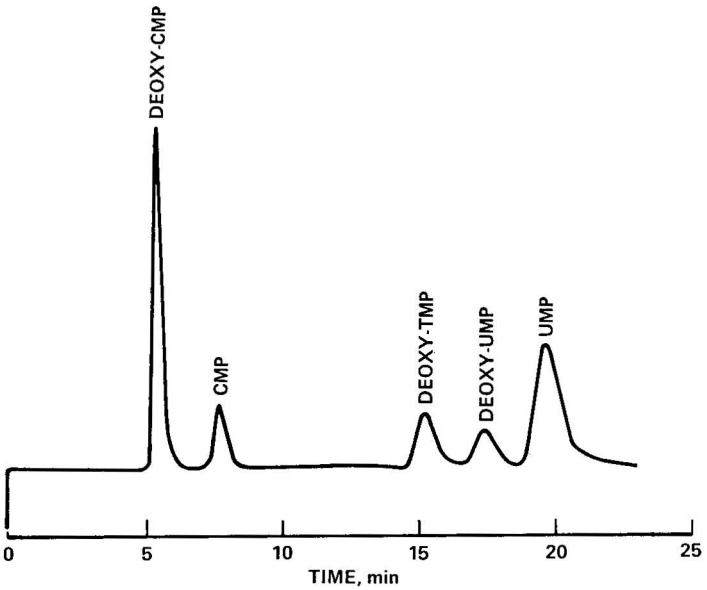


Fig. 6. HPLC separation of 5′-nucleotides and deoxynucleotides by isocratic elution with 40% acetonitrile–60% 0.01 *M* KH_2PO_4 (pH 2.85). Flow-rate, 120 ml/h; detection, 270 nm, 0.2 AUFS.

The separation of mixtures of nucleosides and deoxynucleosides or nucleotides and deoxynucleotides was readily obtained isocratically. The separation of a mixture of nucleosides and deoxynucleosides (Fig. 5) was achieved by elution with

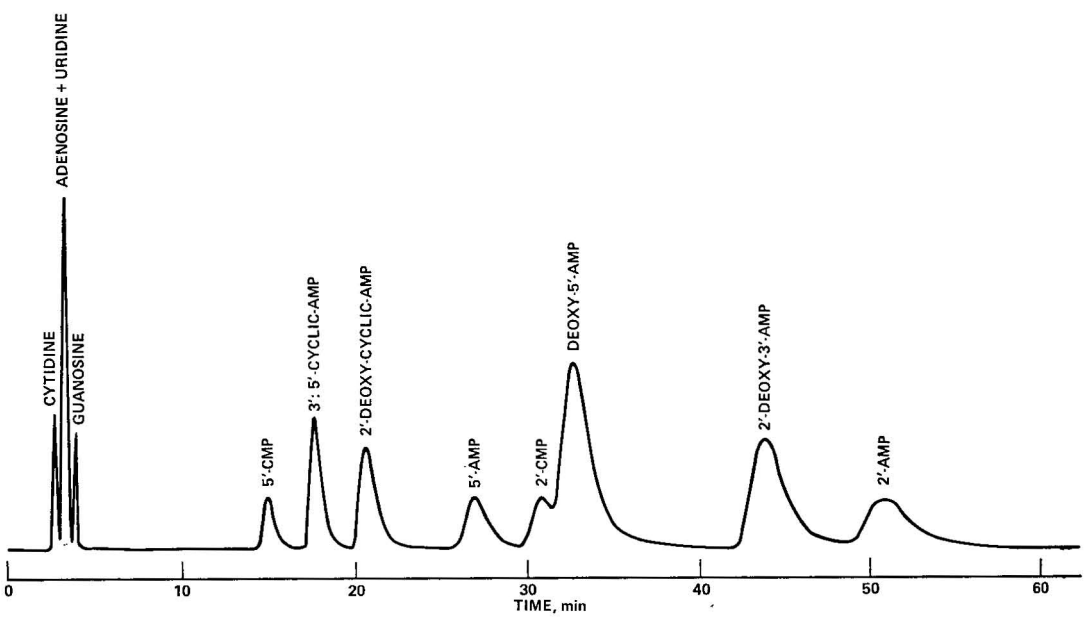


Fig. 7. HPLC separation of nucleosides, nucleotides, cyclic nucleotides, and deoxynucleotides by isocratic elution with 0.01 *M* KH_2PO_4 (pH 3.52). Flow-rate, 48 ml/h; detection, 259 nm, 0.01 AUFS.

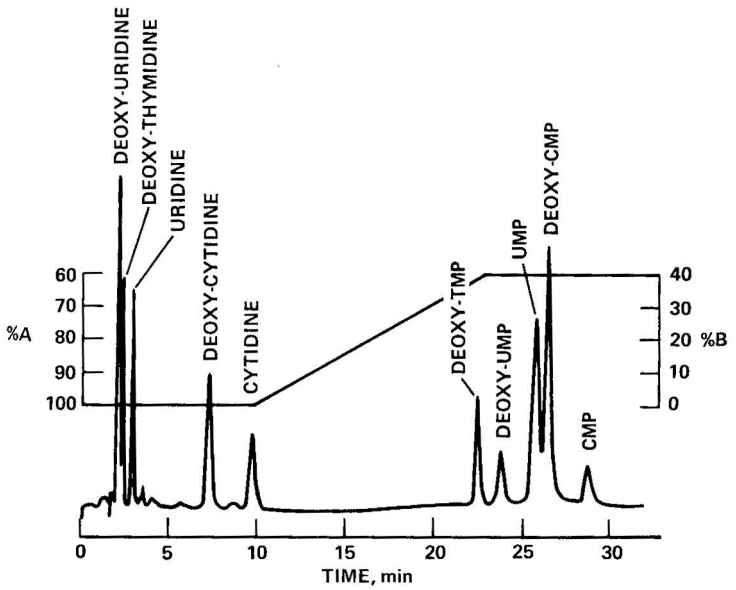


Fig. 8. HPLC separation of nucleosides, deoxynucleosides, and 5′-nucleotides and deoxynucleotides by gradient elution. A = 80% acetonitrile–20% 0.01 *M* KH_2PO_4 (pH 2.85); B = 0.01 *M* KH_2PO_4, pH 2.85. Gradient program: 0–40% B; step 1, isocratic, 0% B for 10 min; step 2, +3% B/min for 13.33 min; step 3, isocratic, 40% B for 6.67 min. Flow-rate, 120 ml/h; detection, 260 nm, 0.05 AUFS.

82% acetonitrile–18% 0.0125 *M* KH_2PO_4, whereas the separation of a mixture of nucleotides and deoxynucleotides (Fig. 6) was accomplished by elution with 40% acetonitrile–60% 0.01 *M* KH_2PO_4 (pH 2.85). (Note that deoxy-CMP is completely resolved from CMP and deoxy-TMP from UMP; in a reported analysis by gradient

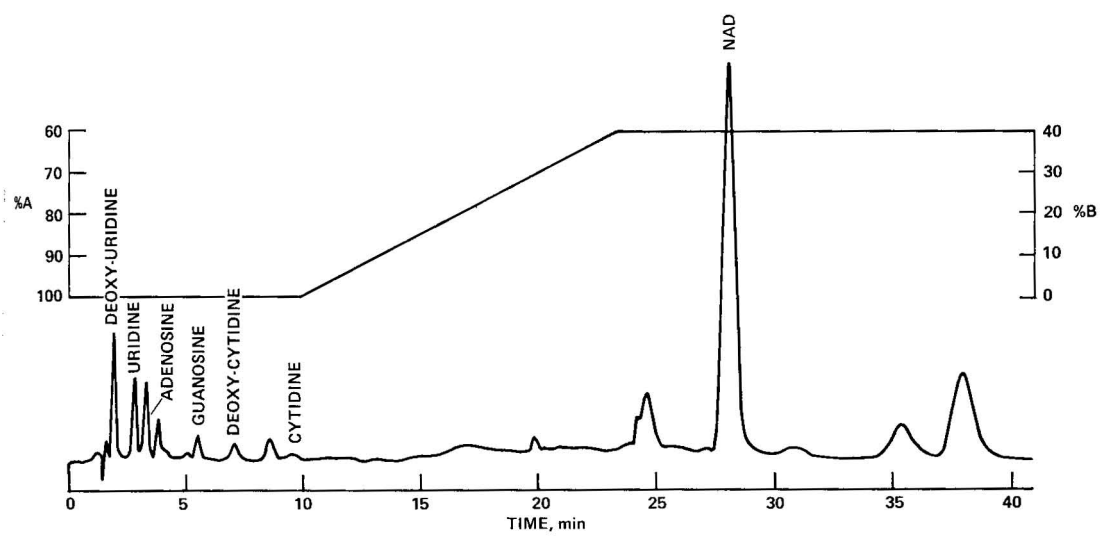


Fig. 9. HPLC separation of perchloric acid extract of Balb-C mouse liver. 10 μl was injected, equivalent to 2 mg of liver. Conditions same as those in Fig. 8.

elution on an anion-exchange resin, these pairs were poorly resolved[15].) A mixture of nucleosides, nucleotides, and deoxynucleotides was partially resolved isocratically using an aqueous mobile phase (Fig. 7).

The effect of mobile phase pH and addition of organic modifiers on retention of a wide range of nucleic acid constituents was investigated (Table I). In some cases (*e.g.*, 2′- and 3′-CMP) changing the pH provided partial resolution of coeluting compounds, while addition of acetonitrile allowed separation of the less polar bases and nucleosides. An excellent separation of nucleosides, nucleotides, deoxynucleosides, and deoxynucleotides was achieved using a gradient elution of decreasing acetonitrile concentration (Fig. 8). This technique was used to analyze the nucleic acid constituents in an extract of Balb-C mouse liver (Fig. 9).

CONCLUSION

The separations described demonstrate that a large variety of nucleic acid components can be chromatographically separated in a minimum of time using relatively simple experimental techniques. Many of the separations can be accomplished without the use of gradient elution by optimizing the buffer concentration and pH or, in the case of nucleosides and bases, by the addition of an organic modifier to the buffer. Using these techniques, it has been possible to readily accomplish relatively difficult separations, such as the separation of 2′-, 3′-, and 5′-AMP or the separation of deoxynucleotides and nucleosides.

LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CMP	Cytidine monophosphate
UMP	Uridine monophosphate
UDP	Uridine diphosphate
UTP	Uridine triphosphate
GMP	Guanosine monophosphate
ApA	Adenylyl-(3′→5′)-adenosine
ApApA	[Adenylyl-(3′→5′)]$_2$-adenosine
NAD	Nicotinamide-adenine dinucleotide

Note: All nucleotides are the 5′-isomer unless otherwise specified.

ACKNOWLEDGEMENT

This work was performed, in part, under a National Research Council Associateship granted to E. Edelson.

REFERENCES

1 P. R. Brown, *High Pressure Liquid Chromatography*, Academic Press, New York, 1973.
2 C. Horváth, *Methods of Biochem. Anal.*, 21 (1973) 79–154.

3 J. X. Khym, *Analytical Ion-exchange Procedures in Chemistry and Biology*, Prentice-Hall, Englewood Cliffs, N.J., 1974, pp. 168–182.
4 C. Ponnamperuma and R. Mack, *Science*, 148 (1965) 1221.
5 G. H. Handschuh, R. Lohrmann and L. E. Orgel, *J. Mol. Evol.*, 2 (1973) 251.
6 J. X. Khym, *Clin. Chem.*, 21 (1975), 1245.
7 J. Arendes, R. K. Zahn and W. E. G. Müller, *J. Chromatogr.*, 140 (1973) 118.
8 *Analysis of Nucleic Acid Constituents by High Performance Liquid Chromatography*, Bulletin 116, Whatman, Inc., Clifton, N.J., 1976.
9 M. Kratovich and B. A. Roe, *J. Chromatogr.*, 155 (1978) 407.
10 W. E. Cohn, in E. Heftmann (Editor), *Chromatography*, Reinhold, New York, 1961, pp. 560–563.
11 T. Uematsu and R. J. Suhadolnik, *J. Chromatogr.*, 123 (1976) 347.
12 C. W. Gehrke, K. C. Kuo, G. E. Davis, R. D. Suits, T. P. Waalkes and E. Borek, *J, Chromatogr.*. 150 (1978) 455.
13 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 126 (1976) 679.
14 A. M. Krstulovic, P. R. Brown and D. M. Rosie, *Anal. Chem.*, 49 (1977) 2237.
15 J. X. Khym, *J. Chromatogr.*, 124 (1976) 415.
16 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651.
17 M. McKeag and P. R. Brown, *J. Chromatogr.*, 152 (1978) 253.

Journal of Chromatography, 174 (1979) 421–433

CHROM. 11,769

DOSAGE DES MÉDICAMENTS PAR SPECTROPHOTOMÉTRIE *IN SITU* DES CHROMATOGRAMMES EN VUE DE LEUR ÉTUDE PHARMACOCINÉTIQUE

I. SULPIRIDE ET AUTRES BENZAMIDES, VINCAMINE, NAFTAZONE

F. BRESSOLLE, J. BRES et S. BRUN

Laboratoire de Chimie Analytique, Faculté de Pharmacie, Avenue Charles-Flahault, 34060 Montpellier (France)

et

E. RECHENCQ

Laboratoire de Pharmacodynamie, Faculté de Pharmacie, Avenue Charles-Flahault, 34060 Montpellier (France)

(Reçu le 25 juillet 1978; manuscrit modifié reçu le 22 janvier 1979)

SUMMARY

Quantitative determination of drugs by in situ spectrophotometry of chromatograms for pharmacokinetic studies. I. Sulpiride and other benzamides, vincamine, naftazone.

Assays are proposed for sulpiride and other benzamides, vincamine and naftazone in plasma (or blood) and urine with direct UV reflectance spectrophotometry on thin-layer chromatography (TLC) at 293, 280 and 270 nm respectively. Urine samples are applied directly on TLC along with a calibration curve on each plate. Plasma (or total blood) samples are extracted, and an internal standard is added before application; slopes of the obtained calibration curves do not change significantly from plate to plate, thus allowing several determinations on the same plate.

The sensitivity is 2 μg in a 1-ml sample (amount applied 30 ng) for sulpiride and related compounds and about the same for vincamine. Naftazone is determined in plasma with simultaneous reflectance and transmittance spectrophotometric measurements at 520 nm on chromatoplates sprayed with lead acetate, the sensitivity reached is 10 ng in a 1-ml sample (amount applied 0.5 ng).

For all drugs studied, the proposed techniques are specific, reliable and sensitive enough and can be used to perform pharmacokinetic studies in human or in animal after administration of doses in the therapeutic range.

INTRODUCTION

L'interprétation des données expérimentales acquises au cours d'une étude pharmacocinétique: variations de la concentration en médicament dans le plasma en fonction du temps et du pourcentage de la dose administrée, retrouvé dans les urines soit sous forme inchangée, soit sous forme de métabolites, n'est en toute rigueur

possible que si l'on peut disposer d'une technique analytique permettant de doser sélectivement le médicament sous forme inchangée. Les propriétés physio-chimiques des métabolites formés étant souvent proches de celles du médicament administré, la technique utilisée pour le dosage devra comporter une phase de séparation avec un haut pouvoir de résolution. Cette séparation préalable permet en outre d'éliminer des produits se trouvant naturellement dans les milieux biologiques, qui peuvent soit interférer avec les produits à doser, soit donner des blancs élevés.

Les moyens de séparation actuellement utilisés sont pour la pluspart des procédés chromatographiques: chromatographie sur colonne de type gaz–liquide (GLC), ou de type liquide–liquide (LC ou HPLC) et chromatographie sur couche mince (TLC).

En GLC ou en HPLC les séparations sont suivies d'une détermination quantitative, la sensibilité de la méthode, dépendant principalement du système de détection utilisé, en TLC ces déterminations quantitatives peuvent être faites soit après élution, soit par spectrophotométrie directe des chromatogrammes.

Cette dernière méthode se prête bien au dosage des médicaments en milieu biologique[1–14], elle a l'avantage de permettre de doser certains métabolites et le médicament inchangé sur une même plaque chromatographique[2,3,6,11,13,14] en utilisant soit un système de détection différent[14] soit pour la migration des mélanges de solvants de polarités différentes lorsque les métabolites formés sont pas trop différents du produit administré[11].

La reproductibilité des dosages est nettement améliorée par l'utilisation d'un étalon interne[5,6,11,12,15] qui n'est cependant pas encore aussi sytématique qu'en GLC.

Nous avons étudié les possibilités apportées par la spectrophotométrie *in situ* des chromatogrammes au dosage du sulpiride et de molécules appartenant également à la série des benzamides, de la vincamine et de la naftazone, et éventuellement de leurs métabolites, en milieu biologique. La séparation par chromatographie sur couche mince semblait particulièrement bien adaptée à ces molécules[10,16] dont nous devions réaliser l'étude pharmacocinétique et la détermination de la biodisponibilité après leur administration au chien ou à l'homme à des doses aussi proches que possible de celles utilisées en thérapeutique.

Le dosage du sulpiride en milieu biologique a été fait jusqu'ici soit par colorimétrie[16], soit par spectrophotométrie[16], soit par spectrofluorimétrie[17]; toutes ces méthodes présentent le risque de doser simultanément le sulpiride et des métabolites du sulpiride, la méthode spectrofluorimétrique donne quant à elle des blancs élevés[17]; une étude récente a montré que la spectrophotométrie par réflexion en inhibition de fluorescence après séparation chromatographique sur couche mince permettait d'atteindre la sensibilité requise pour la réalisation d'une étude pharmacocinétique[10]. Des essais préliminaires nous ayant montré d'une part que la spectrophotométrie par réflexion dans l'ultraviolet (UV) conduit à une meilleure sensibilité que la technique par inhibition de fluorescence, et d'autre part que l'interférence des substances normalement présentes dans les milieux biologiques est moindre dans ce cas, nous avons retenu cette méthode pour le dosage du sulpiride et des molécules appartenant également à la série des benzamides.

La vincamine est dosée dans le plasma par chromatographie en phase gazeuse après extraction et dérivatisation[18–20] avec une très bonne sensibilité, bien que moins

sensible nous avons pu employer la spectrophotométrie par réflexion dans l'UV pour le dosage de la vincamine dans le sang total.

Pour le dosage de la naftazone nous aurions pu utiliser comme précédemment pour le sulpiride et la vincamine, la spectrophotométrie par réflexion dans l'UV, mais en raison de la présence dans l'extrait obtenu à partir du plasma d'une substance migrant avec le même R_F dans tous les mélanges solvants étudiés[11] et absorbant à la même longueur d'onde, nous avons été amené à préconiser une technique par spectrophotométrie des chromatogrammes dans le visible après révélation par l'acétate neutre de plomb et formation d'un complexe coloré de type chélate.

Dans ce travail nous décrivons les méthodes employées pour le dosage de chacune des molécules étudiées et nous montrons qu'elles peuvent être appliquées à des études pharmacocinétiques par leur spécificité, leur reproductibilité et leur sensibilité.

MATÉRIEL ET MÉTHODES

Réactifs et produits de référence

Le sulpiride ou N-[(éthyl-1-pyrrolidinyl-2) méthyl]-méthoxy-2-sulfamoyl-5-benzamide, et les produits apparentés: TER 1546 ou N-[(méthyl-1-pyrrolidinyl-2)-méthyl]-méthoxy-2-sulfamoyl-5-benzamide et GRI 1665 ou N-[(propyl-1 pyrrolidinyl-2)méthyl]-méthoxy-2-sulfamoyl-5-benzamide, nous ont été gracieusement fournis par le laboratoire Delagrange (Paris, France).

La naftazone, ou semicarbazone de la naphtoquinone-1,2, nous a été gracieusement fournie par la Société S.E.R.E.S.C.I. (Bruxelles, Belgique).

La théophylline et la strychnine qui seront utilisées comme étalons internes, ainsi que tous les produits chimiques utilisés, sont des produits purs pour analyse. Tous les solvants utilisés ont été redistillés avant leur emploi.

Ne disposant pas de matière première, la mise au point du dosage de la vincamine ou 14,15-dihydro-14-β-hydroxy-14-β-acétoxy(3α, 16α)éburnaménine, a été effectué en utilisant comme produit de référence le soluté injectable de Pervincamine®, titrant 15 mg pour 3 ml, des laboratoires Dausse (Paris, France).

Matériel divers

Spectrophotomètre chromatographique Zeiss PMQ II, les conditions d'enregistrement des spots pour chacune des molécules étudiées sont les suivantes: sensibilité 10 pour les mesures en réflexion diffuse, 1 pour les mesures en réflexion transmission simultanées; atténuation II; stabilité III; vitesse de la plaque 50 mm/min; vitesse de déroulement du papier de l'enregitreur: 60 mm/min. Les longueurs d'onde de mesure sont données sous les tableaux de résultats correspondants à chacune des molécules étudiées.

Intégrateur de pic, Minigrator, Intersmat; l'intégrateur déclenché au même moment que l'enregistreur nous donne directement la surface intégrée de chacun des pics enregistrés.

Plaques Merck (20 × 20 cm) (Réf. 5721) recouvertes de gel de silice G de 0.25 mm d'épaisseur sur support de verre, sans indicateur de fluorescence.

Microseringues Hamilton de 10 μl gradués par 0.1 μl.

Dosage du sulpiride et du produit TER 1546 das le plasma

Dans des tubes à centrifuger en verre,introduire 2, 3 ou 4 ml de plasma à doser, de l'eau distillée q.s.p. 4 ml, 2 g de sulfate d'ammonium et 0.8 ml d'un tampon pH 10 (Tampon Jensen: 3.754 g glycocolle, 37 ml 1 *N* NaOH, eau q.s.p. 1 l). Agiter. Laisser en contact 3 min, extraire par 20 ml de chloroforme. Centrifuger. Prélever 15 ml de phase organique, porter dans une "capsule antigrimpante" contenant 0.5 ml d'une solution de produit GRI 1665, étalon interne, à 0.02 mg/ml préparée extemporanément à partir d'une solution mère à 1 mg/ml dans l'éthanol absolu.

Abandonner à la température ambiante jusqu'à réduction du volume à 2 ml environ. Transvaser dans des tubes à hémolyse en verre et laisser le solvant s'évaporer à sec. Reprendre le résidu par environ 0.3 ml de chloroforme et déposer sur la plaque Merck 10 μl de cette solution.

Introduire la plaque dans une cuve à chromatographie saturée (2 h) par le mélange acétone–*n*-butanol–eau distillée–ammoniaque (66:30:3:1).

Après développement du chromatogramme, sécher la plaque et procéder à l'enregistrement photodensitométrique, en réflexion diffuse à 293 nm, des spots correspondant au médicament et à l'étalon interne.

Établir le rapport *R* des surfaces intégrées relatives à ces spots.

Droite d'étalonnage. À quatre échantillons de 2 ml d'un plasma témoin, ajouter respectivement 0.1, 0.2, 0.4 et 0.6 ml d'une solution de sulpiride (ou de produit TER 1546) à 0.1 mg/ml préparée extemporanément à partir d'une solution mère à 1 mg/ml dans l'eau distillée, et suivre le mode opératoire adopté pour le plasma à doser. Les éléments de la gamme d'étalonnage sont déposés simultanément sur la plaque avec les échantillons de plasma à doser.

Dosage de la vincamine dans le sang total

Dans des tubes à centrifuger en verre, introduire 1, 2, 3, 4 ou 5 ml de sang à doser, 2 ml d'acide trichloracétique à 50% (v/v); laisser en contact 5 min. Ajouter 2 ml de 4 *N* NaOH. Extraire par 20 ml de chloroforme. Centrifuger. Prélever 15 ml de phase organique, porter dans une "capsule antigrimpante" contenant 0.2 ml de solution de strychnine, étalon interne, à 0.1 g/l dans le chloroforme. Abandonner à la température ambiante jusqu'à réduction du volume à 2 ml environ. Transvaser dans des tubes à hémolyse en verre et laisser le solvant s'évaporer à sec. Reprendre le résidu par environ 0.3 ml de chloroforme et déposer sur plaque Merck 10 μl de solution chloroformique. Introduire la plaque dans une cuve à chromatographie saturée (2 h) par le mélange chloroforme–méthanol (90:10). Après développement du chromatogramme, sécher la plaque et procéder à l'enregistrement photodensitométrique en réflexion diffuse à 280 nm, des spots correspondant à la vincamine et à l'étalon interne.

Établir le rapport *R* des surfaces intégrées relatives à ces spots.

Droite d'étalonnage. À trois échantillons de 2 ml d'un sang témoin, ajouter respectivement 0.05, 0.1 et 0.15 ml de solution de vincamine à 0.1 g/l préparée extemporanément à partir d'une solution mère à 5 mg/ml dans l'eau distillée et suivre le mode opératoire adopté pour le sang à doser. Ces trois échantillons étalons sont déposés sur la plaque en même temps que les échantillons à doser.

Dosage de la naftazone dans le plasma

Dans des tubes à centrifuger en verre, introduire 2, 4, 5 ou 7 ml de plasma

à doser, de l'eau distillée q.s.p. 7 ml et 5 g de sulfate d'ammonium. Laisser en contact 5 min. Extraire en agitant durant 5 min par 20 ml d'acétate d'éthyle. Centrifuger. Prélever 15 ml de phase organique, porter dans une "capsule antigrimpante" contenant 0.1 ml d'une solution de théophylline, étalon interne, à 4 μg/ml dans l'éthanol à 95%. Évaporer l'acétate d'éthyle au bain d'eau thermostaté à 80°. Reprendre le résidu par l'éthanol à 95%, transvaser dans des tubes à hémolyse en verre. Évaporer la solution alcoolique. Reprendre le résidu par environ 0.2 ml d'éthanol à 95% et déposer sur plaque Merck 10 μl de cette solution.

Introduire la plaque dans une cuve à chromatographie saturée (2 h) par le mélange chloroforme–méthanol (90:10). Après développement du chromatogramme, sécher la plaque et procéder à l'enregistrement photodensitométrique en réflexion diffuse à la longueur d'onde 270 nm du spot correspondant à la théophylline. Révéler par une solution aqueuse saturée d'acétate neutre de plomb et procéder à l'enregistrement photodensitométrique à 520 nm par réflexion et transmission simultanées du spot correspondant à la naftazone.

Établir le rapport R des surfaces* relatives au spot de la naftazone et à celui de l'étalon interne.

Droite d'étalonnage. À trois échantillons de 2 ml d'un plasma témoin, ajouter respectivement 0.1, 0.2 et 0.4 ml d'une solution de naftazone à 1 μg/ml dans l'éthanol à 95% et suivre le mode opératoire adopté pour le plasma à doser. Les éléments de la gamme d'étalonnage sont déposés simultanément sur la plaque avec les échantillons de plasma à doser.

Dosage dans les urines

Les différents échantillons d'urine recueillis sont directement déposés sur la plaque chromatographique. On dépose sur chaque plaque 2 μl de solutions étalons du médicament étudié préparées dans une urine témoin et titrant: (i) pour le sulpiride et le produit TER 1546: 250, 500 et 750 μg/ml; (ii) pour la vincamine: 125, 250 et 500 μg/ml; (iii) pour la naftazone: 2, 4, 8 et 10 μg/ml.

Les différents échantillons d'urine à analyser sont dilués ou concentrés** sous vide à 30° pour avoir une concentration finale comprise dans la gamme étalon.

Après migration, les plaques sont séchées et les spots correspondant au médicament étudié sont enregistrés au spectrophotomètre chromatographique dans les mêmes conditions que pour les échantillons de plasma.

RÉSULTATS ET DISCUSSION

Interprétation quantitative des enregistrements

Les méthodes les plus fréquemment utilisées pour un traitement mathématique rigoureux des données obtenues en photodensitométrie font appel aux théories classiques de la spectrophotométrie et plus spécialement à la loi de Lambert–Beer (milieux infiniment transparents) ainsi qu'à un cas particulier de la théorie de

* Ne disposant pas encore d'un intégrateur de pic au moment de la réalisation de ces dosages, les surfaces ont été calculées par le produit de la hauteur du pic par sa base à mi-hauteur.

** Des essais préliminaires ont montré que la teneur en médicament de l'urine n'est pas modifiée par évaporation.

Kubelka et Munk (milieux très réfléchissants et d'une épaisseur pouvant être considérée comme infinie[7,21–23]). Le domaine de linéarité des équations obtenues en fonction de la concentration des solutions déposées est meilleur pour les faibles concentrations dans le premier cas et pour les fortes concentrations dans le second cas. Treiber[7,22] a développé des équations tenant compte de ces deux théories classiques et qui présentent un domaine de linéarité, en fonction de la quantité déposée, plus important.

Le maniement de ces différents types d'équations est peu aisé et nécessite de nombreux calculs, aussi recherche-t-on en pratique d'autres paramètres plus facilement mesurables et dont les variations en fonction de la concentration de la solution déposée puissent être considérées comme linéaires dans un domaine de concentration aussi grand que possible.

Ces paramètres sont le plus souvent reliés au pic correspondant à l'enregistrement d'un spot à son maximum d'absorption, c'est-à-dire à l'absorbance maximale du spot; ce sont soit la hauteur de ce pic[3,4,6,9], soit sa surface[1,2,5,8,11,14], soit encore sa surface au carré[5].

Nous nous sommes toujours placés dans un domaine de concentration pour lequel la surface intégrée du pic correspondant au médicament étudié varie linéairement en fonction de la concentration de la solution déposée (plus exactement de la quantité déposée, les volumes déposés étant toujours identiques).

Dosage direct dans les urines

La surface intégrée du pic correspondant au sulpiride est proportionnelle à sa concentration dans les échantillons d'urine, dans le domaine de concentrations allant de 100 à 750 μg/ml d'urine (soit des quantités allant de 200 à 1500 ng dans le dépôt) comme l'indique la valeur du coefficient de corrélation obtenu par ajustement linéaire des données (méthode des moindres carrés) (Tableau I). La grande variabilité de la pente m et de l'ordonnée à l'origine y_0 souligne la nécessité de déposer une gamme d'étalonnage sur chaque plaque. Il en est de même pour le produit TER 1546.

Après administration à l'homme par voie orale de sulpiride à la dose de 6 mg/kg, nous avons observé, chez certains sujets la présence d'un métabolite qui n'a pas encore pu être identifié et qui peut être dosé par la technique employée pour le sulpiride (Fig. 1).

Pour la vincamine, le domaine de linéarité s'étend de 75 à 500 μg/ml d'urine (soit des quantités allant de 150 à 1000 ng dans le dépôt).

En ce qui concerne la naftazone, le produit inchangé n'est pas détecté dans les urines après administration de 1 ou 2 mg/kg au chien par voie intraveineuse ou orale. On le retrouve après hydrolyse chlorhydrique ou enzymatique[11].

Dans chacun des cas étudiés, nous n'avons pas observé de pic interférant avec celui du médicament.

Dosage dans le plasma ou le sang après extraction, utilisation d'un étalon interne

Les différents produits étudiés sont dosés dans le plasma après extraction à l'aide d'un solvant organique approprié. Malgré l'utilisation de microseringues de précision, l'erreur sur les volumes déposés, lorsqu'on dépose des solutions alcooliques ou chloroformiques de tension de vapeur élevée, est loin d'être négligeable. Afin de compenser cette erreur, nous avons eu recours à l'addition d'un étalon interne, procédé

TABLEAU I

ÉQUATION DE LA DROITE DE RÉGRESSION LINÉAIRE $y = mx + y_0$ OBTENUE EN UTILISANT LA MÉTHODE DES MOINDRES CARRÉS SUR LES DONNÉES (x_i, y_i)

x_i = Concentration en sulpiride ou en produit TER 1546 dans les urines en µg/ml (250, 500 et 750); y_i = surface intégrée des pics correspondant aux différentes concentrations (valeurs extrêmes 1500 à 6000 unités d'intégration). Mesures en réflexion diffuse à 293 nm.

Sulpiride					*Produit TER 1546*				
Urines	*Plaques*	*Coefficient de corrélation*	*Pente m*	*Ordonnée à l'origine, y_0*	*Urines*	*Plaques*	*Coefficient de corrélation*	*Pente m*	*Ordonnée à l'origine, y_0*
Sujet A	1	0.9957	9.24	−361	Sujet A	1	0.9999	6.94	+ 11
	2	0.9996	7.69	+267		2	0.9995	7.60	+ 99
	3	0.9999	8.72	−235	Sujet B	1	0.9996	6.40	− 25
Sujet B	1	0.9995	9.92	−188	Sujet C	1	0.9999	6.88	+ 24
	2	0.9991	6.68	+406	Sujet D	1	0.9982	7.09	+241
	3	0.9997	6.97	+587		2	0.9999	7.16	−161
	4	0.9997	9.55	−382	Sujet E	1	0.9976	6.73	+ 75
Sujet C	1	0.9878	5.75	+348		2	0.9994	6.03	+202
	2	0.9999	8.71	+758	Sujet F	1	0.9995	6.97	+309
	3	0.9997	6.05	+ 26		2	0.9996	7.13	+516
	4	0.9962	8.43	−305	Sujet G	1	0.9998	6.08	+ 63
						2	0.9999	6.71	+368
					Sujet H	1	0.9999	7.79	+242
					Sujet I	1	0.9996	6.97	+222
						2	0.9999	7.43	+224
Chien A	1	0.9997	7.10	+404	Chien A	1	0.9996	7.04	−133
	2	0.9999	7.68	+281		2	0.9999	7.04	+136
	3	0.9999	8.02	+274		3	0.9998	6.67	+147
	4	0.9999	8.22	+484		4	0.9998	6.44	+126
	5	0.9924	5.91	+493	Chien B	1	0.9998	6.99	−100
	6	0.9997	8.12	+229		2	0.9999	7.53	+266
	7	0.9978	6.66	+130		3	0.9992	6.26	+161
Chien B	1	0.9999	7.03	+ 54		4	0.9999	7.32	− 81
	2	0.9976	7.42	−146	Chien C	1	0.9999	6.79	+354
	3	0.9995	7.49	− 38		2	0.9999	6.91	+262
Chien C	1	0.9996	7.72	+147	Chien D	1	0.9999	6.70	+373
	2	0.9999	7.94	+102		2	0.9999	6.77	+261
	3	0.9998	7.96	+512		3	0.9999	5.73	+452
	4	0.9999	8.33	+ 54	Chien E	1	0.9983	7.99	+ 27
	5	0.9999	7.39	+219		2	0.9992	7.82	+ 55
						3	0.9999	7.65	+182

qui a également l'avantage de compenser les pertes intervenant au cours des transvasements et qui, de plus, permet de pallier le manque de reproductibilité d'une plaque à l'autre, comme nous allons le souligner à l'aide des résultats que nous avons obtenus.

Choix de l'étalon interne. Le produit servant d'étalon doit avoir un R_F nettement distinct de celui de la substance à doser (et éventuellement de ses métabolites), il doit présenter si possible un coefficient d'extinction spécifique élevé à la longueur d'onde d'absorbance maximale du médicament à doser. Si ce n'est pas le cas, la plaque peut être enregistrée à deux longueurs d'onde différentes.

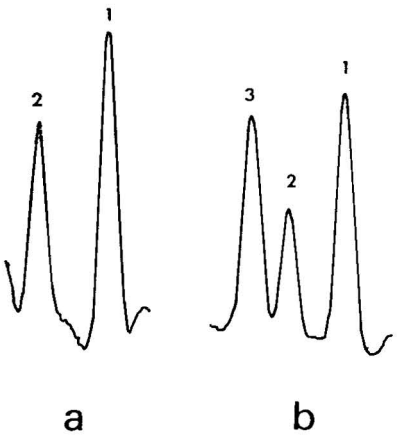


Fig. 1. Enregistrements au spectrophotomètre Zeiss PMQ II, en réflexion à 293 nm d'un échantillon d'urine (a) et d'un échantillon de plasma extrait selon le protocole adopté (b) prévelés chez l'homme après administration orale unique de deux comprimés de sulpiride dosés à 200 mg (a) ou repetées pendant plusieurs jours de quatre comprimés par jour (b). 1 = Sulpiride; 2 = métabolite du sulpiride; 3 = étalon interne. Amplification ×4. Quantités déposées: 2 μl pour l'échantillon d'urine, 10 μl pour l'échantillon de plasma.

(1). Pour le dosage du sulpiride et du produit TER 1546, nous avons choisi un dérivé de la même série chimique, le produit GRI 1665. Ces trois composés ont une absorbance maximale vers 293 nm[16] et des R_F nettement distincts avec la phase mobile utilisée (acétone–*n*-butanol–ammoniaque–eau, 66:30:1:3) (Fig. 1 et 2). R_F produit GRI 1665: 0.65; R_F métabolite du sulpiride (non identifié): 0.55; R_F sulpiride: 0.45; R_F produit TER 1546: 0.30.

(2). Pour le dosage de la vincamine, nous avons choisi un autre alcaloide, la strychnine qui a un coefficient d'extinction très élevé à 280 nm, longueur d'onde

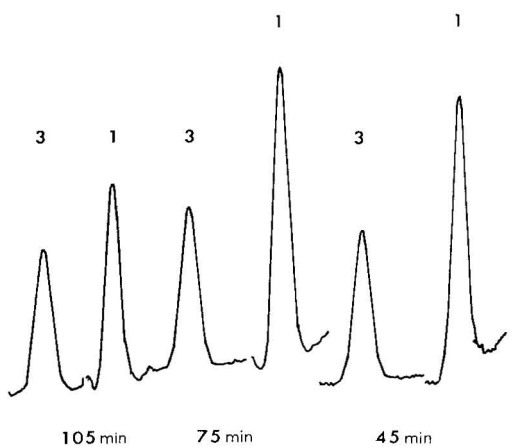


Fig. 2. Enregistrements au spectrophotomètre chromatographique Zeiss PMQ II, en réflexion à 293 nm, de trois échantillons de plasma humain extraits selon le protocole adopté, et prélevés 45 min, 75 min et 105 min après administration orale de deux comprimés de sulpiride dosés à 200 mg. 1 = Sulpiride, amplification ×10; 3 = étalon interne, amplification ×4. Quantités déposées: 10 μl.

d'absorbance maximale de la vincamine. Les R_F de ces deux composés sont distincts avec la phase mobile utilisée (chloroforme–méthanol, 90:10). R_F vincamine: 0.53, R_F strychnine: 0.25.

Nous n'avons pas observé de pic interférant avec celui du sulpiride, du produit TER 1546 dans les extraits obtenus à partir des échantillons de plasma prélevés avant leur administration; de plus, dans ces derniers, nous n'avons pas observé de pic interférant avec l'étalon interne. Il en est de même pour la vincamine.

(3). Pour le dosage de la naftazone, nous avons choisi comme étalon interne la théophylline qui présente une absorbance maximale à 270 nm, longueur d'onde identique à celle du maximum d'absorption de la naftazone dans l'UV[5,11,12]. Les R_F de ces deux composés sont distincts avec la phase mobile utilisée (chloroforme–méthanol, 90:10) (Fig. 3). R_F naftazone: 0.63, R_F théophylline: 0.29.

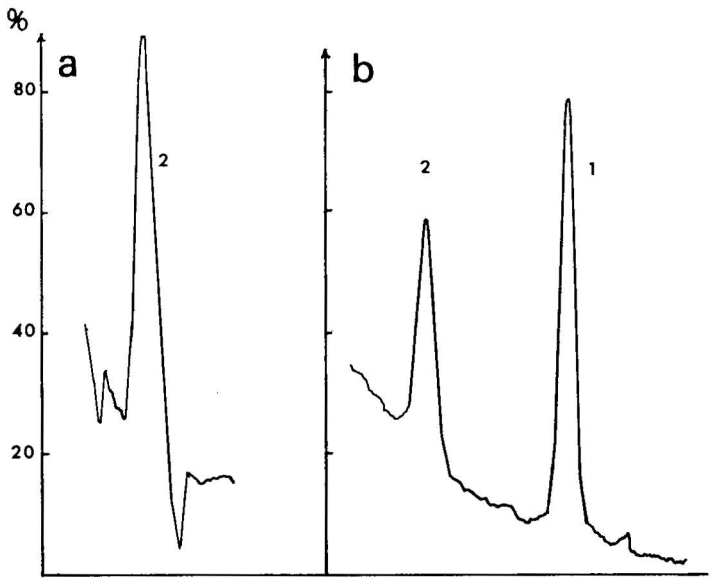


Fig. 3. Enregistrements au spectrophotomètre chromatographique Zeiss PMQ II en réflexion et transmission simultanées à 520 nm (a) et en réflexion diffuse à 270 nm (b) d'une solution alcoolique titrant 7.5 ng par μl en naftazone (2) et 20 ng par μl en théophylline (1). Dépôt 5 μl, amplification $\times 10$.

Le dosage de la naftazone dans le plasma par absorption dans l'UV s'étant avéré inapplicable par suite de la présence d'une substance interférante de même R_F dans tous les mélanges solvants étudiés[11], nous avons néanmoins gardé comme étalon interne la théophylline ce qui nous a obligé à travailler successivement à 270 nm puis à 520 nm après révélation par l'acétate neutre de plomb.

Dosage du sulpiride et du produit TER 1546 dans le plasma. Afin d'évaluer les possibilités de la méthode envisagée différents échantillons de plasma surchargés en sulpiride ou en produit TER 1546 ont été extraits selon le protocole adopté et déposés sur différentes plaques.

TABLEAU II

ÉQUATION DE LA DROITE DE RÉGRESSION LINÉAIRE $y = mx + y_0$ OBTENUE EN UTILISANT LA MÉTHODE DES MOINDRES CARRÉS SUR LES DONNÉES (x_i, y_i)

x_i = Concentration en sulpiride ou en produit TER 1546 dans le plasma en μg/ml (5, 10, 20 et 30). $y_i = R$ = SM/SE, rapport des surfaces intégrées des pics correspondant au sulpiride ou au produit TER 1546, SM, et à l'étalon interne, SE. Mesures en réflexion diffuse à 293 nm.

Sulpiride					*Produit TER 1546*				
Plasmas	*Plaques*	*Coefficient de corrélation*	*Pente m*	*Ordonnée à l'origine, y_0*	*Plasmas*	*Plaques*	*Coefficient de corrélation*	*Pente m*	*Ordonnée à l'origine, y_0*
Sujet A	3	0.9999	0.0899	+0.062	Sujet A	1	0.9999	0.0547	−0.034
Sujet B	5	0.9999	0.0899	+0.056	Sujet D	5	0.9987	0.0541	−0.016
Sujet C	7	0.9999	0.0893	+0.064	Sujet F	7	0.9990	0.0547	−0.024
Sujet D	8	0.9999	0.0895	+0.066					
Sujet E	10	0.9997	0.0895	+0.069					
	11	0.9999	0.0895	+0.063					
Chien A	1	0.9985	0.0958	+0.015	Chien A	1	0.9987	0.0619	+0.022
	2	0.9994	0.0965	+0.024	Chien E	9	0.9987	0.0621	+0.024
Chien B	3	0.9990	0.0970	+0.062	Chien F	11	0.9999	0.0664	−0.044
	4	0.9988	0.0969	+0.018	Chien H	15	0.9994	0.0622	+0.002
Chien C	5	0.9986	0.0970	+0.055					
Chien D	6	0.9999	0.0978	+0.025					
Chien E	9	0.9988	0.0985	−0.003					
Chien F	12	0.9996	0.0989	+0.024					
	13	0.9997	0.0986	+0.012					
Chien G	15	1.0000	0.0990	+0.015					
	16	0.9999	0.0984	+0.013					

Le rapport R de la surface intégrée du pic correspondant au sulpiride (ou au produit TER 1546), SM, à celle du pic correspondant à l'étalon interne, SE, varie linéairement en fonction de la concentration en sulpiride ou en produit TER 1546 pour un domaine de concentrations allant de 5 à 30 μg/ml (soit des quantités déposées allant de 125 à 750 ng) comme le montrent les valeurs des coefficients de corrélation obtenus par ajustement linéaire des données (méthode des moindres carrés) (Tableau II). La sensibilité de la méthode est de 2 μg pour une prise d'essai de 1 ml.

La très bonne reproductibilité des valeurs obtenues pour la pente m des différentes droites d'étalonnage, l'ordonnée à l'origine y_0 étant dans tous les cas très proche de zéro, montre que l'emploi d'un étalon interne permet de pallier aux variations entre plaques et éventuellement d'éviter de déposer une gamme d'étalonnage sur chaque plaque. Les valeurs de m sont plus fortes pour le sulpiride que pour le produit TER 1546 ce qui est dû principalement à son coefficient d'extinction spécifique plus élevé ($\varepsilon_{\text{sulpiride}} = 8.58\ \text{g}^{-1}\,\text{l}\,\text{cm}^{-1}$; $\varepsilon_{\text{TER 1546}} = 6.63\ \text{g}^{-1}\,\text{l}\,\text{cm}^{-1}$). D'autre part, on peut remarquer que pour chacun de ces produits, les valeurs de m obtenues pour les plasmas de chiens sont plus élevées que celles obtenues pour les plasmas humains, en raison d'un coefficient d'extraction différent (99% et 85% pour le produit TER 1546 et le sulpiride respectivement à partir de plasma de chien, 79.2% et 80.4% pour ces deux produits à partir de plasma humain) ce qui pourrait être attribuée à un pourcentage de liaison aux protéines différent.

Après administrations orales répétées de sulpiride, (800 mg répartis en trois prises par jour), nous avons observé, chez certains sujets la présence dans l'extrait obtenu à partir du plasma d'un métabolite non identifié qui semble cependant être le même que celui que nous avons trouvé dans les urines (Fig. 1).

Dosage de la vincamine dans le sang total. Après administration intraveineuse de 20 mg de vincamine au chien, sa concentration dans le plasma devient rapidement très faible, inférieure à 300 ng/ml[18]; d'autre part nous avons constaté par des expériences de distribution réalisées *in vitro* et dont les résultats seront publiés par ailleurs que la vincamine présente un coefficient de partage très élevé en faveur des globules rouges; c'est pourquoi pour augmenter la sensibilité de notre méthode nous avons dosé la vincamine dans le sang total. La validité de la méthode proposée a été évaluée après extraction selon le protocole adopté de différents échantillons de sang surchargés en vincamine.

Pour une concentration donnée, le rapport R de la surface intégrée du pic correspondant à la vincamine, SM, à celle du pic correspondant à l'étalon interne, SE, est très reproductible. Ses variations en fonction de la concentration sont linéaires dans le domaine de concentrations allant de 2.5 à 7.5 μg/ml (soit des quantités allant de 63 à 188 ng dans le dépôt) (Tableau III).

TABLEAU III

ÉQUATION DE LA DROITE DE RÉGRESSION LINÉAIRE $y = mx + y_0$ OBTENUE EN UTILISANT LA MÉTHODE DES MOINDRES CARRÉS SUR LES DONNÉES (x_i, y_i)

x_i = Concentration en vincamine dans le sang en μg/ml (2.5, 5 et 7.5); $y_i = R$ = SM/SE, rapport des surfaces intégrées des pics correspondant à la vincamine, SM, et à l'étalon interne, SE. Mesures en reflexion diffuse à 280 nm.

Sangs (échantillons)	*Plaques*	*Coefficient de corrélation*	*Pente m*	*Ordonnée à l'origine, y_0*
Chien A	1	1.0000	0.160	0.000
	2	1.0000	0.150	+0.057
Chien B	3	0.9996	0.160	+0.033
	4	0.9999	0.156	+0.027
Chien C	5	0.9996	0.160	+0.027
	6	0.9998	0.158	0.000
Chien D	7	0.9999	0.160	+0.003
	8	1.0000	0.162	+0.017

La sensibilité de la méthode est de 1.5 μg pour une prise d'essai de 1 ml.

Dosage de la naftazone dans le plasma. La validité de la méthode envisagée à été évaluée après extraction, selon le protocole adopté de différents échantillons de plasma surchargés en naftazone. Le pourcentage d'extraction de la naftazone est de 97% et son coefficient d'extinction spécifique à 520 nm de 60 $g^{-1}\,l\,cm^{-1}$.

Les variations du rapport R en fonction de la concentration en naftazone sont linéaires dans le domaine de concentrations allant de 50 à 200 ng/ml (soit des quantités déposées allant de 1.88 à 7.5 ng) (Tableau IV). La reproductibilité est un peu moins bonne que lors des exemples précédents. Dans ce cas, en effet, les surfaces de pics ont été calculées et non intégrées, de plus l'étalon interne est enregistré dans

TABLEAU IV

ÉQUATION DE LA DROITE DE RÉGRESSION LINÉAIRE, $y = mx + y_0$ OBTENUE EN UTILISANT LA MÉTHODE DES MOINDRES CARRÉS SUR LES DONNÉES (x_i, y_i)

x_i = Concentration en naftazone dans le plasma en ng/ml (50, 100 et 200), $y_i = R$ = SM*/SE**, rapport des surfaces intégrées des pics correspondant à la naftazone, SM*, et à l'étalon interne, SE**.

Plasmas (échantillons)	*Plaques*	*Coefficient de corrélation*	*Pente m*	*Ordonnée à l'origine, y_0*
Chien A	1	0.9998	0.0066	−0.065
	2	0.9986	0.0052	+0.075
Chien B	3	0.9999	0.0054	+0.045
Chien C	4	0.9992	0.0061	−0.030
Chien E	6	0.9998	0.0060	−0.025

* Mesures en reflexion transmission simultanées à 520 nm.
** Mesures en reflexion diffuse à 270 nm.

l'UV à 270 nm alors que la naftazone est enregistrée dans le visible à 520 nm après révélation.

Étant donné le volume de la prise d'essai sur laquelle nous travaillons (qui peut aller jusqu'à 5 ml) nous voyons que cette méthode nous permet d'atteindre une sensibilité de l'ordre du nanogramme.

CONCLUSION

Dans tous les cas étudiés la sensibilité obtenue liée à une bonne reproductibilité et à une grande spécificité permet de retenir la spectrophotométrie par transmission après séparation chromatographiqe sur couche mince pour le dosage des médicaments en milieu biologique, en vue de leur étude pharmacocinétique.

Pour le sulpiride, nous obtenons une sensibilité équivalente à celle de la méthode fluorimétrique: 0.1 μg/ml, avec l'avantage de pouvoir séparer et doser l'un de ses métabolites, et également de diminuer l'influence de substances se trouvant naturellement dans les milieux biologiques et pouvant interférer avec le produit à doser.

La méthode que nous décrivons nous a permi de réaliser l'étude pharmacocinétique de cette molécule ainsi que celle du produit TER 1546 chez le chien après administration par voie intraveineuse, intramusculaire ou orale de doses comprises entre 7.5 et 60 mg/kg. Elle nous a permi également de déterminer la biodisponibilité de comprimés dosés à 200 mg en sulpiride ou en produit TER 1546 chez l'homme. Les résultats de ces études feront l'objet de publications ultérieures.

Pour la vincamine la sensibilité (15 μg/ml) est loin d'atteindre celle obtenue par chromatographie en phase gazeuse après extraction et dérivatisation (0.5 μg/ml), cependant la méthode que nous décrivons à l'avantage d'être rapide et de se prêter à des analyses de routine, elle nous a permi de déterminer la biodisponibilité de comprimés dosés à 20 mg chez le chien.

La très bonne sensibilité obtenue pour la naftazone de l'ordre du nanogramme nous a permi de réaliser son étude pharmacocinétique après administration au chien[11]. Dans ce cas, la séparation chromatographique sur couche mince ne nous a pas permi

d'éviter l'interférence d'un produit génant. Cette difficulté a pu être surmontée par l'utilisation d'une révélation spécifique.

RÉSUMÉ

Le dosage du sulpiride et des autres benzamides, de la vincamine et de la naftazone dans le plasma (ou le sang) et les urines peut être effectué par spectrophotométrie en réflexion diffuse des chromatogrammes à 293, 280 et 270 nm respectivement. Les échantillons d'urine sont déposés directement sur couche mince avec une gamme d'étalonnage sur chaque plaque. Les échantillons de plasma ou de sang sont extraits; par addition d'un étalon interne aux extraits avant leur dépôt, on obtient des droites d'étalonnage reproductibles d'une plaque à l'autre, ce qui permet de réaliser un plus grand nombre de dosages sur la même plaque. La sensibilité est de 2 μg pour une prise d'essai de 1 ml (30 ng déposés) pour le sulpiride, les molécules qui lui sont apparentées et la vincamine. La naftazone est dosée dans le plasma en réflexion et transmission simultanées à 520 nm après révélation à l'acétate de plomb, la sensibilité obtenue est de 10 ng pour une prise d'essai de 1 ml (0.5 ng déposés).

Pour toutes les molécules étudiées, les méthodes proposées, spécifiques, reproductibles et assez sensibles peuvent permettre la réalisation d'études pharmacocinétiques après administration de doses thérapeutiques à l'homme ou à l'animal.

BIBLIOGRAPHIE

1 D. B. Faber et W. A. Man in 't Veld, *J. Chromatogr.*, 93 (1974) 238.
2 J. A. F. de Silva, I. Bekersky et C. V. Puglisi, *J. Pharm. Sci.*, 63 (1974) 1837.
3 H. K. L. Hundt et E. C. Clark, *J. Chromatogr.*, 107 (1975) 149.
4 J. M. Steyn et H. K. L. Hundt, *J. Chromatogr.*, 111 (1975) 463.
5 J. Bres et F. Bressolle, *Trav. Soc. Pharm. Montpellier*, 35 (1975) 381.
6 J. M. Steyn, *J. Chromatogr.*, 120 (1976) 465.
7 L. R. Treiber, *J. Chromatogr.*, 123 (1976) 23.
8 J. Christiansen, *J. Chromatogr.*, 123 (1976) 57.
9 P. Haefelfinger, *J. Chromatogr.*, 124 (1976) 351.
10 J. Segura, L. Borja et O. M. Bakke, *Arch. Int. Pharmacodyn.*, 223 (1976) 88.
11 F. Bressolle, *Thèse Doct. Pharmacie*, Montpellier, 1976.
12 F. Bressolle et J. Bres, *Trav. Soc. Pharm. Montpellier*, 37 (1977) 113.
13 B. Wesley-Hadzija et A. M. Mattocks, *J. Chromatogr.*, 143 (1977) 307.
14 N. Strojny, K. Bratin, M. A. Brooks et J. A. F. de Silva, *J. Chromatogr.*, 143 (1977) 363.
15 S. Ebel et G. Herold, *Chromatographia*, 8 (1975) 569.
16 G. Pitel et Th. Luce, *Ann. Pharm. Fr.*, 28 (1970) 409.
17 T. Kleimola, O. Leppänen, J. Kanto, R. Mäntylä et E. Syvälahti, *Ann. Clin. Res.*, 8 (1976) 104.
18 V. H. Laufen, W. Juhran, W. Fleissig, R. Götz, F. Scharpf et G. Bartsch, *Arzneim.-Forsch.*, 27 (1977) 1255.
19 V. C. P. Siegers, H. Iven et O. Strubelt, *Arzneim-Forsch.*, 27 (1977) 1271.
20 V. C. P. Siegers, W. D. Heib et K. Kohlmeyer, *Arzneim-Forsch.*, 27 (1977) 1274.
21 S. Ebel et H. Kubmaul, *Chromatographia*, 7 (1974) 197.
22 L. R. Treiber, *J. Chromatogr.*, 100 (1974) 123.
23 S. Ebel et J. Hocke, *Z. Anal. Chem.*, 277 (1975) 105.

Journal of Chromatography, 174 (1979) 434–436

CHROM. 11,770

Note

Inner surface deterioration in glass-lined tubing

K. D. STEELE and J. A. ZABKIEWICZ
Forest Research Institute, Private Bag, Rotorua (New Zealand)
(Received February 6th, 1979)

Glass-lined tubing (GLT) has become widely used in gas chromatography for injectors, capillary connectors, and various manifolds since its introduction in the

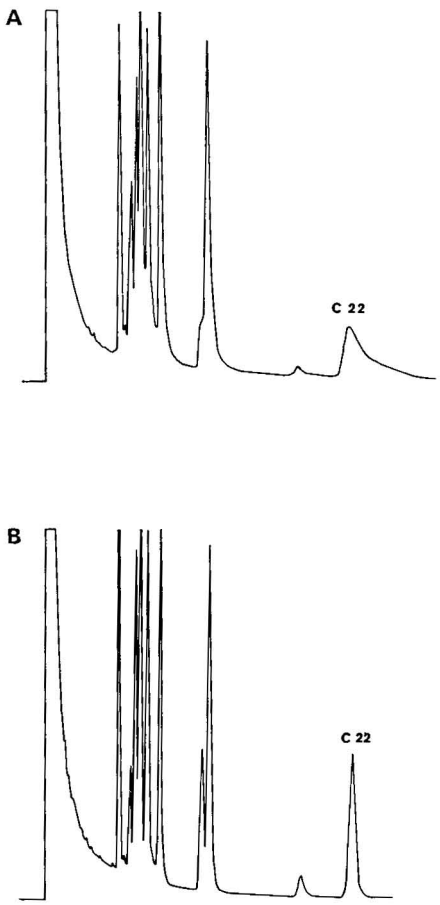


Fig. 1. Separation of monosaccharides and related compounds (as trimethylsilyl derivatives) on a 60 m × 0.5 mm I.D. glass SCOT SE-30 capillary column; temperature, 208°; helium flow, 7 ml/min. (A) Using the original injector; (B) using the same injector but with a replacement 0.7 mm I.D. inner GLT core. C_{22} indicates the *n*-alkane internal standard; mass injected, 150 ng in 0.3 μl.

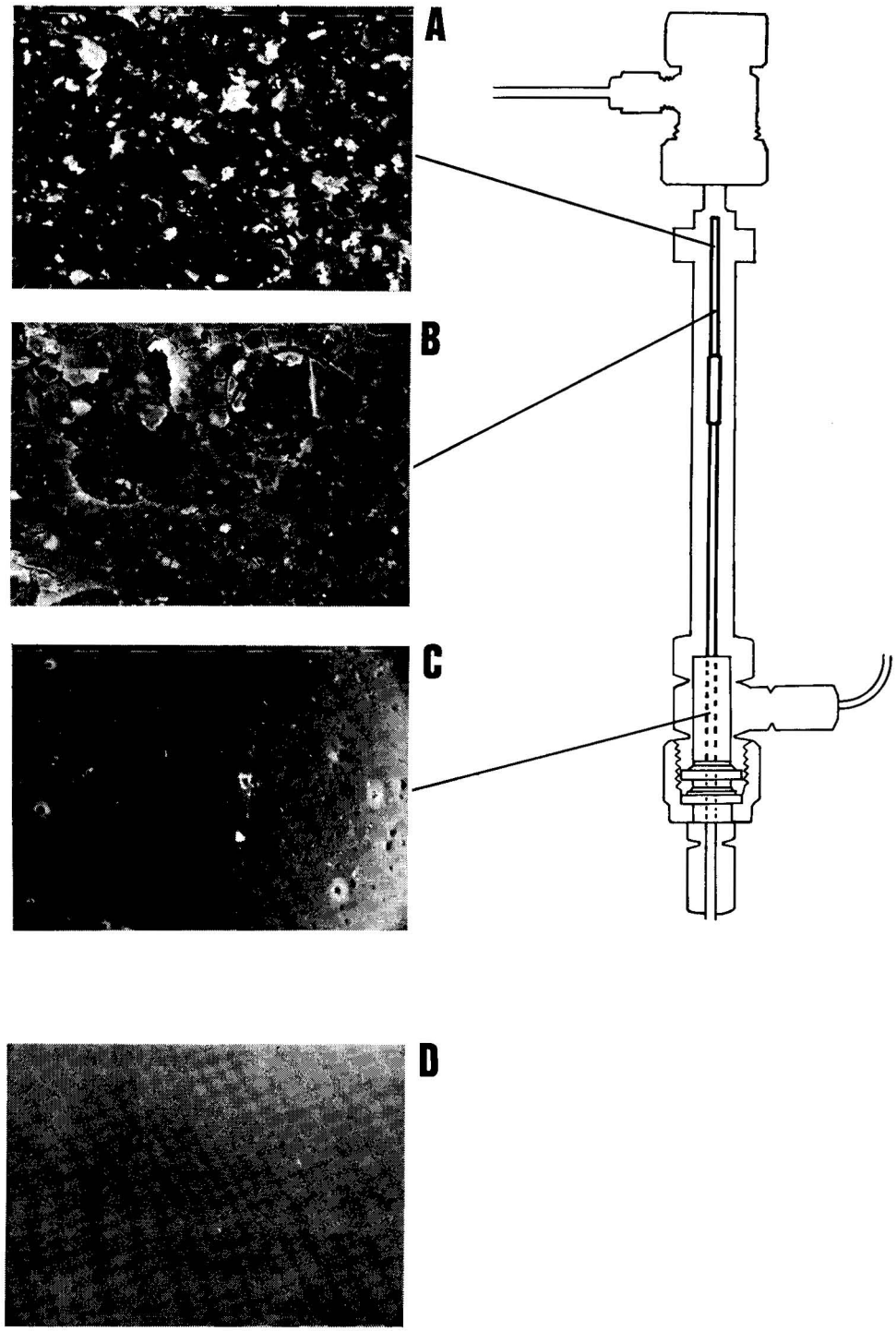


Fig. 2. Diagrammatic representation of the injector unit with the affected inner GLT core indicated in heavier outline. Arrows from each photograph indicate the point within the GLT at which the photographs were taken. Photograph D is of the inner surface of a new section of GLT for comparison. Magnifications: (A) ×900; (B) ×900; (C) ×1000; (D) ×1200. Each section of the bar scale on the photographs represents 10 μm.

early 1970s. Over the past four years we have been using a commercially available splitless injection unit with a glass support-coated open tubular (SCOT) capillary column assembly (Scientific Glass Engineering, North Melbourne, Australia) together with automatic sample injection. Lately persistent loss of overall column performance occurred, which forced us to seek the cause of this loss.

The severity of the problem is illustrated in Fig. 1A which shows both the loss of resolution with a mixture of carbohydrate derivatives and the very pronounced tailing of the n-C_{22} alkane used as internal standard. All parts of the chromatographic system were checked until it was finally determined that the cause lay within the injector. Involatile deposits or fractures of the glass layer were thought to be responsible. The manufacturers do specify a method for detecting cracks within the glass lining[1], but the method is hardly suitable for routine use on such assemblies. No lasting improvement was gained by various cleaning or injection techniques until finally the inner GLT core within the injector was replaced. The improvement in resolution and column efficiency is illustrated in Fig. 1B. Despite the fact that the replacement GLT used was 0.7 mm I.D. throughout (instead of 0.5 mm) column efficiency rose from N_{Eff} 14,400 to N_{Eff} 26,700 (for the C_{22} peak) under our routine operating conditions (SE-30 column, 208°, helium flow of 7.0 ml/min).

The original GLT injector core was cut open with a diamond abrasive disc for inspection and the sections sequentially cleaned by sonication in methanol, water, methanol and carbon tetrachloride to remove organic and particulate deposits. The glass lining was then examined with a scanning electron microscope at various points along its length after surface coating with gold in the normal way (Fig. 2). There is deep scarring and disruption of the surface in the region to which the needle tip penetrated, as well as deposition of irregular particles, possibly from displaced glass wall or septum or inorganic sample residues (Fig. 2A). Slightly lower down (Fig. 2B) there is further degradation of glass wall but of a different form. Here it seems as if there has been thermal shock, giving irregular "crazing", as well as regions where further chemical "etching" has removed more of the lining. Further "etching" is evident even near the base of the injector (Fig. 2C) where there is distinct pitting of the glass surface. This particular portion also includes what seems to be a linear fault in the deposition of the glass layer. The final photograph (Fig. 2D) is of the inner face of a new portion of GLT which shows a uniformly smooth surface.

It is obvious that regular checking and replacement of GLT will be essential in certain situations. It is difficult to forecast how soon performance may become unacceptable since our injection head has been in use over a four-year period, for both manual and automated injection. Sample throughput of fairly complex mixtures over that period (mainly as trimethylsilyl derivatives) is estimated at more than 20,000. It appears in retrospect that for continued good results the injector should have been refurbished much earlier or after a smaller number of samples.

ACKNOWLEDGEMENT

We wish to thank R. E. Gaskin for the SEM photographs.

REFERENCE

1 *Data sheet Reference GLT 4/75*, Scientific Glass Engineering Pty., North Melbourne, 1975.

Journal of Chromatography, 174 (1979) 437–439

CHROM. 11,797

Note

Sampling method in capillary column gas–liquid chromatography allowing injections of up to 250 μl

WOLFGANG VOGT, KARL JACOB and HANS WERNER OBWEXER

Institut für Klinische Chemie am Klinikum Grosshadern der Universität München, Postfach 701 260, D-8000 München 70 (G.F.R.)

(Received December 21st, 1978)

In gas chromatographic trace analysis, on the one hand we make great efforts to reduce detection limits to extreme levels, and on the other hand only a small amount of the isolated material is analysed, while most is usually wasted in capillary column gas–liquid chromatography. After splitting, only a fraction of a microlitre passes to the column and finally to the detector for quantification.

Various proposals have been made for overcoming this problem. The best known is the Grob and Grob-type splitless injection technique[1,2] and its modifications[3]. Another technique involves the use of a moving-needle system[4]. In general a maximal volume of 5 μl can be handled by these systems.

Because it is desirable to inject larger volumes, we have developed a split–splitless injector that allows the introduction of very large amounts of sample. Preliminary results of our studies are presented here.

EXPERIMENTAL

Injection system

The split–splitless injector was designed for a Varian 1440 gas chromatograph. The injection port together with the electronic control unit was removed and replaced by our injector, consisting of a thin-walled metal tube of very low thermal capacity, with a length similar to that of the original device. It can be heated rapidly by a heat-conducting coil (Philips Elektronik Industrie, Hamburg, G.F.R.) which is controlled by a thyristor circuit. For pre-heating, the carrier gas line was wound side by side with the heater coil. The glass insert was modified so as to obtain an optimal gas stream at the splitting point. Splitting was controlled by a vent-off valve. A 25-m SE-30 open-tubular glass capillary column (I.D. 0.27 mm) was used with nitrogen as the carrier gas. The inlet pressure was 1.0 bar and the flow-rate 1.2 ml/min.

Materials

The experiments were performed with test mixtures consisting of a homologous series of hydrocarbons (C_{18}–C_{30}) as proposed by Schomburg *et al.*[5], fatty acid methyl esters (C_{16}–C_{19}), and dimethylthiophosphinic esters of aromatic hydroxy acids[6].

RESULTS AND DISCUSSION

Like the moving-needle system, the proposed injector makes use of the different boiling points of the solvent and the sample constituents. However, its special construction enables us to apply far greater amounts of solutions.

The principles can be summarized as follows. Prior to the introduction of the sample, the injector is cooled to the appropriate temperature, which is preferably higher than the boiling point of the solvent used. Within approximately 40 sec up to 250 μl of the sample are slowly injected with the splitter valve fully open; the splitting ratio is 1:600. The solvent is thereby evaporated and flushed by the carrier gas through the vent-off valve. The sample constituents remain in the glass insert. After a few seconds, the splitter valve is closed and the interior of the injector heated immediately to the volatilization temperature of the sample (*e.g.*, 340°). The column is held at the starting temperature of the programme during this step. After a few minutes the temperature programme is run and the separation performed as usual.

The reproducibility of the technique was demonstrated by injecting different volumes of sample containing the same amounts of the compounds to be analysed. For example, a series of even-numbered alkanes (C_{18}–C_{30}) with alternating concentrations in carbon disulphide was analysed (Fig. 1). The peak areas from a 100-μl

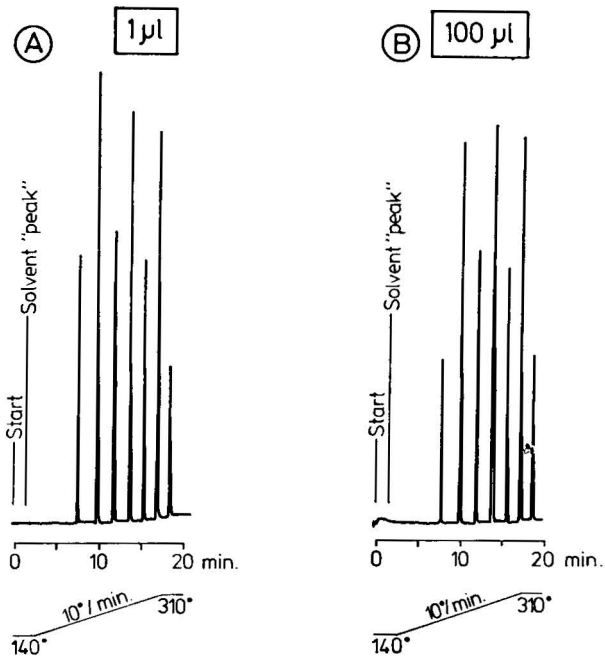


Fig. 1. Gas chromatograms from (A) a 1-μl and (B) a 100-μl injection of a series of even-numbered alkanes dissolved in carbon disulphide. The total amounts of the hydrocarbons were the same in each instance. The concentrations in solution A were C_{18}, C_{22}, C_{26} and C_{30} 250 ng/μl and C_{20}, C_{24} and C_{28} 500 ng/μl, and in solution B 2.5 and 5.0 ng/μl, respectively. The injector starting temperature was 50° and the vaporization temperature 320°. The column was 25 m × 0.27 mm I.D. SE-30, with nitrogen as carrier gas at a flow-rate of 1.2 ml/min, temperature programmed from 140° (4 min) to 310° at 10°/min. A flame-ionization detector was used, temperature 290°.

injection (Fig. 1A) agree well with those from a 1-μl injection (Fig. 1B), with the exception of the first (C_{18}). Similar results were achieved with other compounds, such as methyl esters of fatty acids or dimethylthiophosphinates. In addition, the retention times correspond well with each other. The peaks were sharp and showed no tailing. With carbon disulphide no solvent peak was observed at the expected retention time (Fig. 1), whereas only a small peak appeared when using hydrocarbons such as *n*-hexane. This solvent-free chromatography is an advantage over the common splitless injection techniques; the lifetime of the capillary column is prolonged and the chromatographic properties are enhanced.

A disadvantage of all large-volume injection systems is the relatively rapid contamination of the insert[7]. In some instances the glass insert has to be changed after each analysis. This effect has been considered in the mechanical construction of our device, and the replacement of the insert is rapid and simple.

In our opinion the proposed sampling technique increases decisively the overall sensitivity in gas chromatographic trace analysis.

REFERENCES

1 K. Grob and G. Grob, *J. Chromatogr. Sci.*, 7 (1969) 584.
2 K. Grob and G. Grob, *J. Chromatogr. Sci.*, 7 (1969) 587.
3 M. Novotny and A. Zlatkis, *J. Chromatogr. Sci.*, 8 (1970) 346.
4 P. M. J. van den Berg and Th. P. H. Cox, *Chromatographia*, 5 (1972) 301.
5 G. Schomburg, H. Behlau, R. Dielmann, F. Weeke and H. Husmann, *J. Chromatogr.*, 142 (1977) 87.
6 K. Jacob, E. Maier, G. Schwertfeger, W. Vogt and M. Knedel, *Biomed. Mass Spectrom.*, 5 (1978) 302.
7 F. J. Yang, A. C. Brown, III and S. P. Cram, *J. Chromatogr.*, 158 (1978) 91.

Journal of Chromatography, 174 (1979) 440–442

CHROM. 11,821

Note

Studies on steroids

CXLVI. Chromatographic behaviour of organic sulphates on Sephadex LH-20

KAZUTAKE SHIMADA and TOSHIO NAMBARA*

Pharmaceutical Institute, Tohoku University, Aobayama, Sendai (Japan)

(First received February 6th, 1979; revised manuscript received February 27th, 1979)

The occurrence of numerous kinds of organic sulphates as natural products[1,2], metabolites of drugs and steroid hormones[3] is well documented and a variety of chromatographic methods have been applied to the separation of these highly polar compounds. These methods involve the separation of molecular species of different anionic moieties because they are incapable of differentiating cationic moieties from one another. This paper deals with the chromatographic behaviour of organic sulphate salts consisting of various cations on Sephadex LH-20.

EXPERIMENTAL

Instruments

A Hitachi Model 124 ultraviolet spectrophotometer and a Hitachi Model 208 atomic-absorption spectrophotometer were used.

Reagents and materials

Sephadex LH-20 (particle size 25–100 μm) was supplied by Pharmacia (Uppsala, Sweden). All reagents and chemicals used were of analytical-reagent grade. Distilled water was deionized by percolation through a mixed-bed column of ion exchangers. Dowex 50W-X8 (100–200 mesh) ion-exchange resin was conditioned in the usual manner and then converted into various cationic forms by washing with 2 *N* NaOH, KOH, Li_2SO_4, RbCl, CsCl, $AgNO_3$ and $BaCl_2$ solutions. Marinobufagin 3-sulphate sodium salt was obtained from toad venom in these laboratories. Other salts were prepared by passing an aqueous solution of the sodium salt through a column packed with Dowex 50W-X8 resin in the corresponding cationic form. *p*-Toluenesulphonic acid and benzoic acid were similarly converted into the desired salts as described above.

Chromatography on Sephadex LH-20

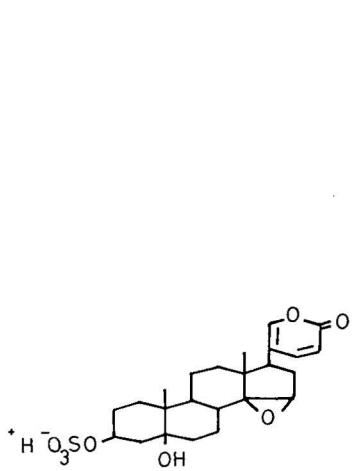
Sephadex LH-20 was swollen in each solvent overnight and then packed in a column (35 × 1.5 cm I.D.). The void volume was estimated to be 18 ml when

* To whom correspondence should be addressed.

methylated Blue Dextran 2000 (methanol) and Blue Dextran 2000 (water) were used. A synthetic mixture of sulphate salts (each *ca.* 1 mg) dissolved in solvent (1–2 ml) was applied to the column and then eluted with each solvent at a flow-rate of 0.6ml/min.

RESULTS AND DISCUSSION

When submitted to gel chromatography on Sephadex LH-20 using methanol as solvent, a mixture of marinobufagin 3-sulphate (Fig. 1), lithium, sodium and potassium salts were distinctly separated, as illustrated in Fig. 2. A mixture of *p*-toluenesulphonic acid lithium, sodium, potassium and silver salts showed a similar chromatogram under the same conditions. In addition, *p*-toluenesulphonic acid lithium salt and marinobufagin 3-sulphate potassium salt were resolved to give the corresponding peaks (Fig. 3).



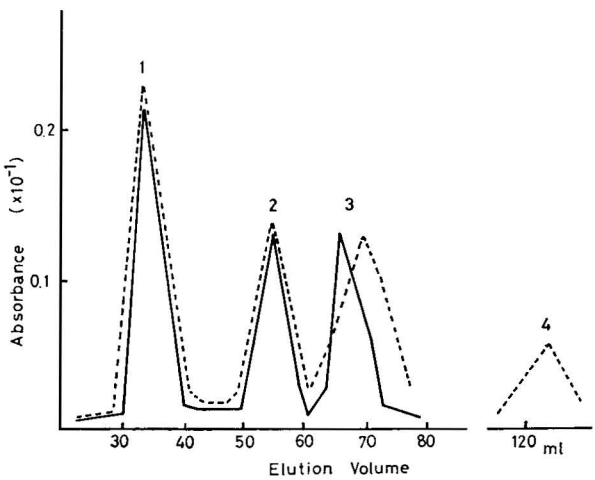
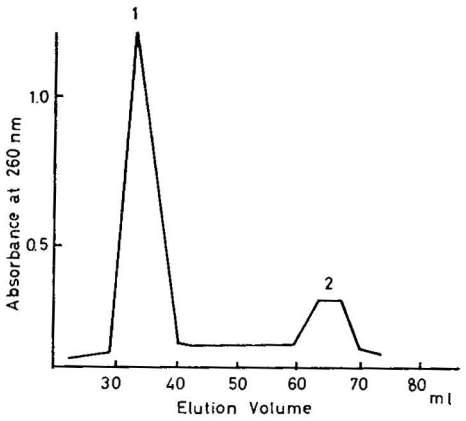


Fig. 1. Structure of marinobufagin 3-sulphate.

Fig. 2. Separation of various salts of marinobufagin 3-sulphate (——) and *p*-toluenesulphonic acid (– – – –) on Sephadex LH-20. Solvent: methanol. Detection: ——, 300 nm; – – – –, 260 nm. 1 = Li, 2 = Na, 3 = K and 4 = Ag salt.

The eluate in each fraction was unequivocally characterized by flame spectrophotometry or the silver chloride test and by converting the cationic moieties into one another by ion-exchange chromatography on Dowex 50W-X8 resin. These experiments revealed that no exchange of cationic moiety occurred among the sulphate salts during gel chromatography and the separation was solely dependent upon the cationic moiety of the sulphate. However, a mixture of benzoic acid lithium, sodium, potassium and silver salts provided no satisfactory resolution except for the lithium salt under the same conditions. Also, *p*-toluenesulphonic acid lithium, sodium and potassium salts were not separated, exhibiting a single peak when water or methanol containing 0.01 *M* sodium chloride was used as solvent. The divalent metal (barium) salts of marinobufagin 3-sulphate and *p*-toluenesulphonic acid showed a broad peak on the chromatogram.



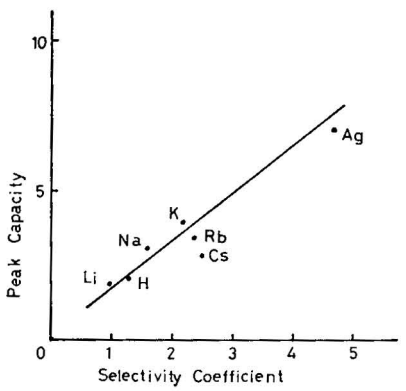


Fig. 3. Separation of *p*-toluenesulphonic acid lithium salt (1) and marinobufagin 3-sulphate potassium salt (2) on Sephadex LH-20. Solvent: methanol.

Fig. 4. Relationship between peak capacity of *p*-toluenesulphonic acid salt and selectivity coefficient of metal ion for the cation exchanger.

These results show that only univalent metal salts of an organic sulphate are distinctly separated by chromatography on Sephadex LH-20 with methanol. As illustrated in Fig. 4, there was a linear relationship between the peak capacity of *p*-toluenesulphonic acid salt and the selectivity coefficient of the metal ion for the cation exchanger[4], with the exception of the caesium salt. It is sufficiently substantiated that separation mechanisms other than molecular sieving, namely adsorption and ion exchange[5–7], are also operative in chromatography on Sephadex LH-20. The chromatographic behaviour of alkali metal ions that form ion pairs has previously been demonstrated[8]. This kind of ion-pair separation seems to be limited to alkali metals to methanol and ethanol–water mixtures.

Further studies on the chromatographic behaviour of biologically important steroid sulphates are being conducted in these laboratories.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan, which is gratefully acknowledged.

REFERENCES

1 K. Shimada, Y. Fujii, E. Yamashita, Y. Niizaki, Y. Sato and T. Nambara, *Chem. Pharm. Bull.*, 25 (1977) 714.
2 K. Shimada, M. Hasegawa, K. Hasebe, Y. Fujii and T. Nambara, *J. Chromatogr.*, 124 (1976) 79.
3 S. Bernstein and S. Solomon, *Chemical and Biological Aspects of Steroid Conjugation*, Springer-Verlag, Berlin, 1970, p. 182.
4 W. Rieman, III, and H. F. Walton, *Ion Exchange in Analytical Chemistry*, Pergamon Press, Oxford, 1970, p. 44.
5 M. Wilk, J. Rochlitz and H. Bende, *J. Chromatogr.*, 24 (1966) 414.
6 V. Di Gregorio and M. Sinibaldi, *J. Chromatogr.*, 129 (1976) 407.
7 S. C. Chattoraj, A. S. Fanous, D. Cecchini and E. W. Lowe, *Steroids*, 31 (1978) 375.
8 M. Lederer and C. Majani, *Chromatogr. Rev.*, 12 (1970) 239.

Journal of Chromatography, 174 (1979) 443–446

CHROM. 11,811

Note

Comparison of open-column and high-performance gel permeation chromatography in the separation and molecular-weight estimation of polysaccharides

T. W. DREHER, D. B. HAWTHORNE and B. R. GRANT*

Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052 (Australia)

(Received February 19th, 1979)

Gel permeation chromatography (GPC) on polydextran (Sephadex) or polyacrylamide (Bio-Gel) gels has been widely used in the fractionation and molecular-weight estimation of polysaccharides[1,2]; for polysaccharides of very high molecular weights, agarose gels have been used[3,4]. In this paper, we compare the soft gel columns of Ultrogel (LKB, Bromma, Sweden) with the μBondagel columns (Waters Assoc., Milford, Mass., U.S.A.) used in high-performance liquid chromatography (HPLC) as a means of separating polysaccharides and estimating their molecular weights. Ultrogels are co-polymers of agarose with polyacrylamide and allow high flow-rates at low pressures.

The HPLC system consisted of a Waters Assoc. Model 6000A solvent pump, Model U6K injector and a Model R401 differential refractometer. Two stainless-steel columns 300 × 3.9 mm) were used; one had a specified fractionation range of 2000 to 2,000,000 daltons (μBondage E-linear), and the other had a narrower range of 3000 to 100,000 daltons (μBondagel E-300). Separations in the HPLC system were carried out at ambient temperature (25°), with either methanol–water (2:3, v/v) or 0.1 *M* sodium acetate–acetic acid buffer solution of pH 5.5 (containing 0.02% of sodium azide) as mobile phase; flow-rates were maintained at 1 ml min^{-1} by pressures of 1200 and 900 p.s.i., respectively.

The GPC on Ultrogel packings was carried out at 20° with columns (17 mm × 450 mm) of AcA-22 (fractionation range 60,000 to 1,000,000 daltons) and AcA-34 (fractionation range 20,000 to 400,000 daltons), with bed volumes of 95 and 98 ml, respectively. The fractionation ranges quoted for both Ultrogel and Bondagel are those obtained with globular proteins. The eluent was 0.01 *M* acetate buffer solution of pH 5.5 containing 0.02% of sodium azide. By using a head of 75 cm, constant flow-rates of 25 ml h^{-1} could be obtained with the AcA-22 column and 30 ml h^{-1} with the AcA-34 column. The polysaccharide content of the fractions was measured by the phenol–sulphuric acid method[5].

The T-series of dextrans, T-10 to T-500, and blue dextran (Pharmacia, Uppsala, Sweden), Dextrans D4133 and D5251 (Sigma, St. Louis, Mo., U.S.A.), Pullulan (Calbiochem, Los Angeles, Calif., U.S.A.) and Dextran B and D-glucose (BDH,

* To whom correspondence should be addressed.

Poole, Great Britain) were used to compare the systems. The weight-average molecular weights, $\bar{M}_w$, for the T-dextran samples used as calibration standards are listed in Table I. All samples used in HPLC were prepared as 2% (w/v) solutions in the mobile phase, and 4 to 5 μl of solution were injected in each run. Samples for separation on Ultrogel were applied as 0.3% (w/v) solutions, 1 ml to each column. All samples were passed through filters (pore size 0.4 μm) (Nuclepore, Pleasanton, Calif., U.S.A.) immediately before application to the column.

TABLE I

MOLECULAR WEIGHTS ($\bar{M}_w$) OF T-DEXTRAN SERIES USED IN CALIBRATION OF COLUMNS

Dextran	$\bar{M}_w$
T-10	10,400
T-20	21,600
T-40	44,400
T-70	68,500
T-150	154,000
T-500	450,000
Blue dextran	>2,000,000

By using the T-series dextrans as standards, the relationship between $\bar{M}_w$ and elution volume (V_e) shown in Table II was obtained from the various columns, within their specified fractionation ranges. By using these relationships, estimates of the molecular weights of other dextrans and pullulan were compared in different chromatographic systems; the results are shown in Table III. The reproducibility of the values was 2% for the Ultrogels and 1% for HPLC. The E-linear and AcA-22 columns fractionated over a similar range, whereas the AcA-34 column fractionated over a narrower range than the E-300. The fractionation range of both E-linear and E-300 columns may extend below 10,000, as a sample of a 1,3-β-glucan[6] ($\bar{M}_w$ 5000) gave an estimated molecular weight of 5100 on both E-linear and E-300 columns. The limit of resolution of both μBondagel and Ultrogel columns was similar. Compounds with $\Delta\ln \bar{M}_w$ values of 2.0 or greater were separated on the two μBondagel

TABLE II

RELATIONSHIP BETWEEN MOLECULAR WEIGHT ($\bar{M}_w$) AND ELUTION VOLUME (V_e) FOR μBONDAGEL AND ULTROGEL COLUMNS FRACTIONATING DEXTRAN STANDARDS

Column	*Solvent*	*Fractionation range*	*Linear regression*	*Regression coefficient*
μBondagel E-linear	Methanol–water (2:3)	10,000–2,000,000*	$\ln \bar{M}_w = 29.325 - 7.576 V_e$**	0.970
	0.1 *M* Acetate (pH 5.5)		$\ln \bar{M}_w = 28{,}076 - 7.011 V_e$	0.980
μBondagel E-300	Methanol–water (2:3)	10,000– 500,000	$\ln \bar{M}_w = 20.665 - 4.017 V_e$	0.992
	0.1 *M* Acetate (pH 5.5)		$\ln \bar{M}_w = 20.102 - 3.790 V_e$	0.979
Ultrogel AcA-22	0.01 *M* Acetate (pH 5.5)	20,000–2,000,000*	$\ln \bar{M}_w = 18.517 - 0.100 V_e$	0.990
Ultrogel AcA-34	0.01 *M* Acetate (pH 5.5)	20,000– 150,000	$\ln \bar{M}_w = 14.322 - 0.048 V_e$	0.970

* Blue dextran was fractionated slightly on these columns.

** V_e is the elution volume (in ml).

TABLE III

MOLECULAR WEIGHTS OF SEVERAL DEXTRANS AND PULLULAN ESTIMATED BY CHROMATOGRAPHY ON μBONDAGEL AND ULTROGEL COLUMNS

Sample	*Column*	*Solvent*	*Estimated molecular weight*
Dextran B	μBondagel E-linear	Methanol–water (2:3)	126,600
		0.1 *M* Acetate	125,500
	Ultrogel AcA-34	0.1 *M* Acetate	133,000
Dextran D5251	μBondagel E-linear	0.1 *M* Acetate	430,400
	Ultrogel AcA-22	0.01 *M* Acetate	442,000
Dextran D4133	μBondagel E-linear	0.1 *M* Acetate	50,040
	Ultrogel AcA-34	0.01 *M* Acetate	49,000
Pullulan	μBondagel E-linear	0.1 *M* Acetate	1,060,000
	Ultrogel AcA-22	0.01 *M* Acetate	1,623,000

columns, but those which differed in molecular weight by less than this simply gave flat-topped single peaks (Fig. 1) or broad single peaks, intermediate in position between the peaks of individual dextrans. With the Ultrogel columns, the $\Delta \ln \bar{M}_w$ value had to exceed 2.5 before separation could be achieved. It was of interest that, although the μBondagel E-linear column separated over a wider range of molecular weights than the μBondagel E-300 column, both columns showed similar resolution characteristics.

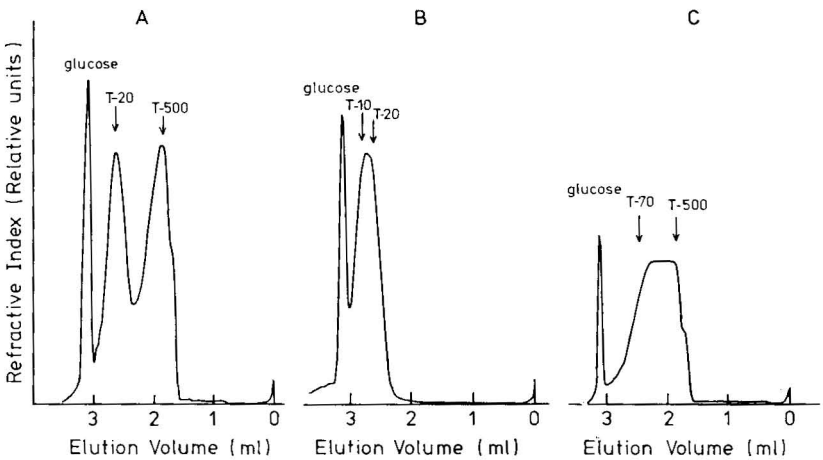


Fig. 1. Elution profiles of dextran mixtures on a μBondagel E-300 column, with methanol–water (2:3) as mobile phase. A, Mixture of D-glucose with Dextrans T-20 and T-500; B, mixture of glucose with Dextrans T-10 and T-20 (the single peak elutes between the positions of the two polymers); C, mixture of glucose, with Dextrans T-70 and T-500 showing a single, flat-topped peak extending over the individual elution positions.

The blue dextran normally used to locate the void volumes in GPC[7] fractionated on the μBondagel E-linear and the AcA-22 columns (this was also noted by Wu *et al.*[8], who used controlled-pore glass columns in an HPLC system. This leaves some uncertainty in the determination of the void volumes of these columns. When water is used as mobile phase, the blue dextran gives a sharper peak than

with acetate buffer solution; this is presumably due to hydrophobic absorptive interactions (which have previously been observed with μBondagel columns when separating proteins[9]). However, if charged polysaccharides such as dextran sulphates ($\bar{M}_w$ 500,000) were chromatographed with either water or buffer solution as mobile phase in either system, the apparent molecular weights obtained were abnormally large compared with those calculated from ultracentrifuge data.

The results obtained show that separations and estimations of the molecular weights of dextrans on the μBondagel and Ultrogel columns give similar values. The HPLC system has the advantage of speed (with complete separation in 5 min or less) and small sample size. Some 10 μg of carbohydrate could readily be detected with the refractive index detector. The Ultrogel open columns, although much slower (maximum elution rate 10 ml cm^{-2} h^{-1} for optimum separation[4]) are useful for preparative work with up to 20 mg of carbohydrate loaded in each run. We have used these systems to separate marine-algal polysaccharides with a wide range of molecular weights and linkage types.

REFERENCES

1 S. C. Charmo, *Advan. Carbohyd. Chem. Biochem.*, 25 (1970) 13.
2 A. Wasteson, *Biochem. Biophys. Acta*, 177 (1969) 152.
3 R. H. Pearce and B. J. Grimmer, *J. Chromatogr.*, 150 (1978) 548.
4 L. Hagel, *J. Chromatogr.*, 160 (1978) 59.
5 M. Dubois, K. A. Gibs, J. K. Hamilton, D. A. Rebers and F. Smith, *Anal. Chem.*, 28 (1956) 350.
6 D. B. Hawthorne, W. H. Sawyer and B. R. Grant, *Carbohydr. Res.*, in press.
7 L. Fischer, in T. S. Work and E. Work (Editors), *Laboratory Techniques in Biochemistry and Molecular Biology, Vol. I, Part II, An Introduction to Gel Chromatography*, North-Holland, Amsterdam, London, 1969, p. 166.
8 A. C. M. Wu, W. A. Bough, E. C. Conrad and K. E. Alden, Jr., *J. Chromatogr.*, 128 (1976) 87.
9 R. V. Vivilecchia, B. C. Lightbody, N. Z. Thimot and H. M. Quinn, *J. Chromatogr. Sci.*, 15 (1977) 424.

Journal of Chromatography, 174 (1979) 447–450

CHROM. 11,820

Note

Structure–R_M investigation of 3-acyloxy-1,4-benzodiazepines

GÁBOR MAKSAY, ZSUZSANNA TEGYEY and LÁSZLÓ ÖTVÖS

Central Research Institute for Chemistry of the Hungarian Academy of Sciences, Pusztaszeri ut 59-67, H-1025 Budapest (Hungary)

(First received January 5th, 1979; revised manuscript received February 28th, 1979)

The study of quantitative structure–activity relationships (QSAR) of 1,4-benzodiazepines is based on different hydrophobic parameters. Partition coefficients (P) were the first to be considered[1–3]. After Tomlinson[4] had pointed out the advantages of thin-layer chromatographic (TLC)R_M values in QSAR studies, Hulshoff and Perrin[5] developed a method for the determination of the R_M values of very lipophilic compounds. This method was applied to 1,4-benzodiazepines and compared with a high-performance liquid chromatographic method[6].

R_M values can also be used in QSAR studies of prodrugs. This is supported by a structure–pharmacokinetic study of 1,4-benzodiazepine prodrugs[7], which indicated a correlation between the brain penetration of oxazepam and the R_M values of its esters. In this work, the R_M values of 33 closely related potential prodrugs have been determined. The compounds investigated were all 3-acyloxy-1,4-benzodiazepines differing either in the acyl moiety or in ring substitution.

EXPERIMENTAL

Materials

The acetoxy-1,4-benzodiazepines were prepared by rearrangement of the corresponding N^4-oxides[8]. Other esters were synthesized by pyridine-catalysed acylation using alcohols and acyl chlorides[9].

Light petroleum (b.p. 40–70°) was supplied by Carlo Erba (Milan, Italy) and Kieselgel HF_{254} by Merck (Darmstadt, G.F.R.). Distilled water was used throughout.

TLC experiments

Glass plates (20 × 20 cm) were coated with an aqueous slurry of Kieselgel HF_{254} to a thickness of 0.25 mm, using standard equipment. Water was allowed to evaporate at room temperature for at least 1 day, then the plates were treated with light petroleum containing 5% of liquid paraffin. The substrates (in 10 μl of dimethyl sulphoxide) were spotted on a line 2 cm from the lower edge of the plate. The plates were equilibrated overnight in a saturated chamber[10] and then developed in aqueous methanol. The spots were detected under ultraviolet light at 254 nm.

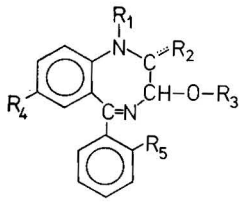


Fig. 1. Chemical structure of 1,4-benzodiazepines.

RESULTS AND DISCUSSION

R_M values of 33 3-acyloxy-1,4-benzodiazepines were determined. The effects of the following structural modifications were examined:

(1) different esters of oxazepam and lorazepam with varying alkyl chain length and branching, and ω-phenyl and/or α-hlaogen substituents in the acyl moiety;

(2) 3-acetoxy-1,4-benzodiazepines with different ring substituents in positions 1-, 2-, 7- and 5-(2'). The effect of methanol concentration on R_F values was examined for 17 oxazepam esters (Table I). For the determination of the corresponding R_M data a methanol concentration of 65% was used, which resulted in a suitable R_F range for the whole series.

TABLE I

R_F AND R_M VALUES OF OXAZEPAM ESTERS IN DIFFERENT METHANOL–WATER MIXTURES

R_1=R_5=H, R_2=O, R_4=Cl. Results are averages of six determinations. R_M values were calculated from a linear fit of the R_M–concentration function.

Compound No.	R_3	R_F				R_M*
		60% MeOH	*65% MeOH*	*70% MeOH*	*75% MeOH*	
1	H	0.52	0.66	0.71	0.83	−0.25
2	$COCH_3$	0.44	0.60	0.70	0.81	−0.15
3	$COCH_2CH_3$	0.36	0.53	0.67	0.81	−0.03
4	$COCH(CH_3)_2$		0.43	0.61	0.76	0.12
5	$CO(CH_2)_2CH_3$	0.23	0.40	0.43	0.77	0.17
6	$CO(CH_2)_2COOCH_3$	0.27	0.58	0.68	0.80	−0.03
7	$COCH_2CH(CH_3)_2$		0.32	0.50	0.71	0.34
8	$COC(CH_3)_3$		0.33	0.52	0.72	0.30
9	$COCH(CH_2CH_3)_2$		0.26	0.45	0.69	0.46
10	$COCH(CH_2)_3CH_3$ | CH_2CH_3		0.12**	0.28**	0.55	0.88
11	$COCH_2Ph$		0.33	0.54	0.76	0.32
12	$COCH_2CH_2Ph$	0.11	0.24	0.42	0.69	0.50
13	$COCH(CH_3)CH_2Ph$		0.19	0.39	0.63	0.64
14	$COCHClCH_2Ph$		0.20	0.41	0.65	0.60
15	$COCHBrCH_2Ph$***		0.18	0.35	0.63	0.65
16	$COC(CH_3)_2CH_2Ph$		0.17	0.34	0.58	0.69
17	$CO(CH_2)_3Ph$		0.19	0.36	0.64	0.65

* 65% methanol.

** Tailing of the spots occurred.

*** Partial decomposition during chromatography.

TABLE II

R_F AND R_M VALUES OF 3-ACYLOXY-1,4-BENZODIAZEPINES

Results are average values of six determinations in 65% methanol.

Substituent					R_F	R_M
R_1	R_2	R_3	R_4	R_5		
H	O	H	Cl	Cl	0.62	−0.21
H	O	$COCH_3$	Cl	Cl	0.61	−0.19
H	O	$COCH(CH_3)_2$	Cl	Cl	0.48	0.04
H	O	$COCH_2CH(CH_3)_2$	Cl	Cl	0.39	0.20
H	O	$COC(CH_3)_3$	Cl	Cl	0.39	0.20
H	O	$COCH(CH_2CH_3)_2$	Cl	Cl	0.30	0.36
H	O	$COCH_2CH_2Ph$	Cl	Cl	0.29	0.39
H	O	$COCH(CH_3)CH_2Ph$	Cl	Cl	0.24	0.49
H	O	$COCH_2Cl$	Cl	H	0.59*	−0.16
H	O	$COCHCl_2$	Cl	H	0.58*	−0.14
CH_3	O	$COCH_3$	Cl	H	0.49	0.01
H	O	$COCH_3$	NO_2	H	0.61	−0.20
—	$NHCH_3$	$COCH_3$	Cl	H	0.34	0.29
$(CH_2)_2COOEt$	O	$COCH_3$	Cl	H	0.45	0.08
CH_2COOEt	O	$COCH_3$	Cl	H	0.49	0.01
$(CH_2)_2COOH$	O	$COCH_3$	Cl	H	0.67	−0.31

* Partial hydrolysis during chromatography.

Table II gives the R_F values of the other 3-acyloxy-1,4-benzodiazepines measured with a methanol concentration of 65%.

A regular increase in the carbon number of the acyl moiety resulted in a linear increase in R_M, as can be seen in Fig. 2. Compounds with an ω-phenyl substituent in the acyl moiety give a separate line. Early investigations[11] indicated a possible interaction between two aromatic systems united by a methylene chain, when varying π-increments of the successive methylene units are found. This is not the

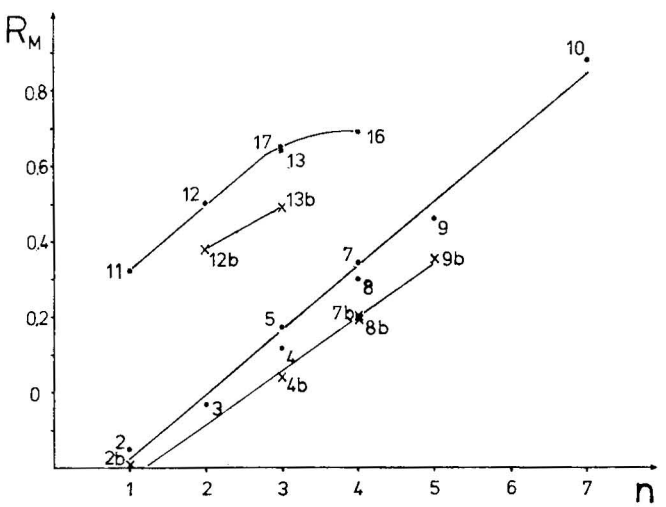


Fig. 2. Relationship between carbon number of acyl moiety (n) and R_M for oxazepam esters (compounds 2–17) and lorazepam esters (compounds 2b–13b).

case with ω-phenyl-substituted esters, because these compounds have similar ΔR_M increments per methylene unit.

Linearity of R_M values with increasing carbon number is similar to that found for aliphatic amines[12] and for the log P values of barbiturates[13]. Branching results in a decrease in R_M[4,12] which is characteristic of α,α-dimethyl-β-phenylpropionyl oxazepam (compound 16 in Fig. 2). The slopes in Fig. 2 are similar, indicating that ω-phenyl or 5-(2′-chloro) substitution does not affect the dependence on n, but causes a constant change in hydrophobicity manifested by a vertical shift of the lines.

Halogen substitution in the α-position (compounds 14 and 15) causes a similar change to that of methyl substitution, as ΔR_M is proportional to the partial molar volume of the groups[14].

The effect of ring substituents was also investigated, maintaining the acyl moiety constant (acetyl esters). Table III gives the R_M values of 3-acetoxy-1,4-benzodiazepines bearing some pharmacologically potent substituents. Lipophilicity is increased by N^1-alkyl substitution and substitution of the 2-oxo group for the methylamino group and is decreased by 5-(2′-chloro) substitution.

TABLE III

R_M VALUES OF 3-ACETOXY-1,4-BENZODIAZEPINES

R_2=$COCH_3$, R_5=H. Results are average values of six determinations in 65% methanol.

Substituent			R_M
R_1	R_2	R_4	
CH_3	O	Cl	0.01
H	O	NO_2	—0.20
—	$NHCH_3$	Cl	0.29
$(CH_2)_2COOEt$	O	Cl	0.08
CH_2COOEt	O	Cl	0.01
$(CH_2)_2COOH$	O	Cl	—0.31

ACKNOWLEDGEMENTS

The authors thank Mrs. E. Simon for supplying the lorazepam esters.

REFERENCES

1 W. Müller and U. Wollert, *Naunyn-Schmiedebergs Arch. Pharmacol.*, 278 (1973) 301.
2 R. W. Lucek and C. B. Coutinho, *Mol. Pharmacol.*, 12 (1976) 612.
3 T. Miyadera, A. Terada, C. Tamura, M. Yoshimoto and R. Tachikawa, *An. Rep. Sankyo Res. Lab.*, 28 (1976) 1.
4 E. Tomlinson, *J. Chromatogr.*, 113 (1975) 1.
5 A. Hulshoff and J. H. Perrin, *J. Chromatogr.*, 120 (1976) 65.
6 A. Hulshoff and J. H. Perrin, *J. Chromatogr.*, 129 (1976) 263.
7 G. Maksay, Zs. Tegyey, V. Kemény, I. Lukovits and L. Ötvös, *J. Med. Chem.*, submitted for publication.
8 S. T. Bell and S. J. Childress, *J. Org. Chem.*, 27 (1962) 1691.
9 G. Maksay, Zs. Tegyey and L. Ötvös, *Hoppe-Seylers Z. Physiol. Chem.*, 359 (1978) 879.
10 M. S. J. Dallas, *J. Chromatogr.*, 17 (1965) 267.
11 J. Iwasa, T. Fujita and C. Hansch, *J. Med. Chem.*, 8 (1965) 150.
12 C. Prandi, *J. Chromatogr.*, 155 (1978) 149.
12 C. Prandi, *J. Chromatogr.*, 155 (1978) 149.
13 K. Kakemi, T. Arita, R. Hori and R. Konishi, *Chem. Pharm. Bull.*, 15 (1967) 1705.
14 E. Bush, *Methods Biochem. Anal.*, 13 (1975) 357.

Journal of Chromatography, 174 (1979) 451–453

CHROM. 11,796

Note

Determination of hecogenin in *Agave sisalana* by gas–liquid chromatography

YASUHIKO MATSUKI, KATSUHARU FUKUHARA and TOHRU YUI
Hatano Research Institute, Food and Drug Safety Centre, Hatano, Kanagawa (Japan)
and
TOSHIO NAMBARA
Pharmaceutical Institute, Tohoku University, Aobayama, Sendai (Japan)
(Received January 30th, 1979)

The occurrence of steroidal saponins in *Agave sisalana* has previously been reported. The plant is harvested all the year round in tropical areas and used as a fibre source[1]. A major saponin in this plant, hecogenin [3β-hydroxy-(25*R*)-5α-spirostan-12-one], is a useful starting material for the manufacture of steroid hormones. The methods for the determination of hecogenin so far devised involve densitometry on a thin-layer chromatogram[2] and gas–liquid chromatography of the acetate[3] or trimethylsilyl derivative[4]. These methods, however, are not satisfactory with respect to feasibility and sensitivity. An urgent need to clarify the hecogenin contents of the crude sapogenin concentrates known as "coffee ground" prompted us to develop a reliable method for the quantitation of hecogenin.

EXPERIMENTAL

Materials

Authentic hecogenin was purchased from Steraloids (Pawling, N.Y., U.S.A.) and its purity was checked by thin-layer chromatography prior to use. Cholest-4-en-3-one (internal standard) was prepared in these laboratories. Trifluoroacetic anhydride and other chemicals were of analytical-reagent grade.

Gas chromatography

A Shimadzu Model 4BM gas chromatograph equipped with a hydrogen flame-ionization detector and a coiled glass column (1 m × 2 mm I.D.) was used. The column was packed with 1% XE-60 on Gas-Chrom Q (80–100 mesh). The detector and flash heater were kept at 230° and the column temperature was 215°. Nitrogen was used as the carrier gas at a flow-rate of 40 ml/min.

Gas chromatography–mass spectrometry

A Shimadzu Model 9000B gas chromatograph–mass spectrometer was used. A coiled glass column (2 m × 2 mm I.D.) was packed with 1% OV-1 on Gas-Chrom Q (80–100 mesh), and the flow-rate of carrier gas (helium) was 30 ml/min. The column temperature was 250° and the injection port and ion source were kept at 270°.

The accelerating voltage, ionization voltage and trap current were 3.5 kV, 70 eV and 60 μA, respectively.

Preparation of the trifluoroacetate

A sample (0.9–7.2 μg) was treated with trifluoroacetic anhydride (0.2 ml) and allowed to stand at room temperature for 10 min. After removal of the excess of reagent by means of a stream of nitrogen gas the residue was dissolved in chloroform (0.5 ml) containing a known amount of cholest-4-en-3-one (*ca.* 200 μg). A 1–2-μl volume of this solution was injected into the gas chromatograph. The retention times of hecogenin and cholest-4-en-3-one were 8.7 and 3.9 min, respectively.

Determination of hecogenin in crude sapogenin concentrates

A ground sample (100–339 mg) was extracted twice with 20 ml of methanol at 50°. The solid was removed by filtration and washed twice with 10 ml of cold methanol. The filtrate and washings were combined and concentrated under reduced pressure, and the whole volume was brought to 30 ml with methanol. An 1-ml aliquot of this solution was transferred into a tube, evaporated to dryness and submitted to gas chromatographic determination. Another 1-ml aliquot was similarly evaporated, and the residue was treated with 2 *N* hydrochloric acid at 90° for 1–3 h. The hydrolysate was extracted twice with 10 ml and once with 5 ml of ethyl acetate and the organic layer was dried over anhydrous sodium sulphate and evaporated to dryness. After addition of the internal standard to the extract the solution was subjected to gas chromatographic determination.

RESULTS AND DISCUSSION

Previous papers have described the gas–liquid chromatography of hecogenin in the form of the acetate[3] or trimethylsilyl ether[4]. These derivatives, however, are unsatisfactory with respect to volatility and sensitivity. Therefore, trifluoroacetylation was used as the derivatization method prior to gas chromatography.

Treatment of hecogenin with trifluoroacetic anhydride in the usual manner gave a single peak of the correct theoretical shape on the gas chromatogram. The structure of the resulting trifluoroacetate was characterized by gas chromatography–mass spectrometry. The mass spectrum (Fig. 1), exhibiting characteristic fragment ions (*m/e* 467, 454, 139, 115), provided unequivocal support for the assignment of the structure as hecogenin 3-trifluoroacetate[5].

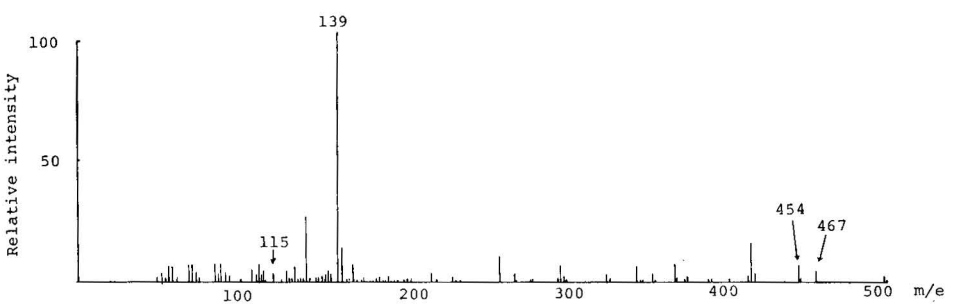


Fig. 1. Mass spectrum of trifluoroacetyl derivative of hecogenin in crude sapogenin concentrates.

A calibration graph was constructed by plotting the ratio of the peak height of hecogenin to that of the internal standard (cholest-4-en-3-one) against the weight ratio of the two; satisfactory linearity was observed in the range 0.9–7.2 μg of hecogenin. Quantitation of hecogenin in the crude preparation was then carried out. Initially, the content of conjugated hecogenin was measured by hydrolytic cleavage with hydrochloric acid followed by determination of the hydrolysate by gas chromagraphy. No substantial difference was found between the values obtained with and without prior hydrolysis. Hecogenin contents of crude sapogenin concentrates determined by the present method are given in Table I. The results are comparable with those previously reported by Cripps and Blunden[4]. It is of interest that the hecogenin content is dependent on the season when the plant is harvested.

TABLE I

HECOGENIN CONTENTS OF CRUDE SAPOGENIN CONCENTRATES DETERMINED BY THE PRESENT METHOD

*Sample**		*Hecogenin content***	
No.	*mg*	*mg*	*%*
1	100	32.2	32.2
2	152	46.5	30.6
3	120	23.5	19.6

* The three samples were obtained in different seasons.
** Mean value of three determinations.

It is hoped that the availability of the convenient and reliable method for the determination of hecogenin will provide a more precise knowledge of its content in *Agave sisalana* and related plants.

REFERENCES

1 G. Blunden, Y. Yi and K. Jewers, *Lloydia*, 37 (1974) 10.
2 G. Blunden and R. Hardman, *J. Chromatogr.*, 34 (1968) 507.
3 B. A. Vela and H. F. Acevedo, *Steroids*, 14 (1969) 499.
4 A. L. Cripps and G. Blunden, *Steroids*, 31 (1978) 661.
5 H. Budzikiewicz, C. Djerassi and D. H. Williams, *Structure Elucidation of Natural Products by Mass Spectrometery*, Vol. II, Holden-Day, San Francisco, Calif., 1964, pp. 110–120.

Journal of Chromatography, 174 (1979) 454–460

CHROM. 11,798

Note

Glass capillary column gas chromatography of narcotic drugs after flash-heater trimethylsilylation

ASBJØRG S. CHRISTOPHERSEN and KNUT E. RASMUSSEN

Department of Pharmaceutical Chemistry, Institute of Pharmacy, P.O. Box 1068, Blindern, Oslo 3 (Norway)

(Received January 23rd, 1979)

Glass capillary column gas chromatography is widely used for the analysis of biologically important substances such as steroid hormones[1] and fatty acids[2]. However only a few papers have been published on drug analysis[3–5] in spite of the fact that improved specificity and sensitivity are obtained with capillary columns in comparison with packed columns. It is often necessary to derivatize the drugs before gas chromatography. Even good glass capillary columns often show undesirable adsorption characteristics and the problem becomes more serious with decreasing amounts of sample. One means of overcoming this problem is to convert polar drugs into non-polar derivatives. Derivatization may also increase the resolution and the difference in the retention times of a drug, and the drug derivative may give extra information during the identification of unknown substances. This is of importance in the analysis of narcotic drugs.

In earlier papers we have described the technique of on-column derivatization[6–9]. Packed columns were used in these studies and the silyl and acyl derivatives of the drugs were formed by simultaneous injection of the sample and the reagent into the column. The method was used successfully for qualitative and quantitative drug analyses using flame-ionization and electron-capture detection. It was the purpose of this investigation to show that derivatization of drugs in a heated capillary injector (flash-heater derivatization) can be used for resolving complex drug mixtures and also for quantitative drug analysis in the nanogram range.

Narcotic drugs were used as model substances and reagents suitable for forming trimethylsilyl derivatives are studied. The reproducibility of quantitative analysis and the minimal detectable amounts of codeine and morphine were evaluated and the results are compared with earlier findings.

MATERIALS AND METHODS

Reagents

Table I lists the compounds used. Methyl phenidate and phenmetrazine were supplied by Ciba-Geigy (Basle, Switzerland). Hexadecane and eicosane were purchased from Koch-Light (Colnbrook, Great Britain). Heroin was synthesized from morphine. The other drugs were of pharmacopoeial grade and supplied by Norsk

Medisinaldepot (Oslo, Norway). Ampoules of 1 ml of N,O-bis(trimethylsilyl)-acetamide (BSA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-trimethylsilylimidazole (TMSIM), used as derivatization reagents, were purchased from Supelco (Bellefonte, Pa., U.S.A.). After opening the ampoules were kept as described earlier[9].

Analytical-reagent grade ethyl acetate and chloroform were obtained from E. Merck (Darmstadt, G.F.R.). Stock standard solutions of the compounds were prepared in ethyl acetate at concentrations of 5 mg/ml, except for morphine, the concentration of which in ethyl acetate was 1 mg/ml. These solutions were diluted to give a concentration of 250 μg/ml for gas chromatography.

Gas chromatography

A Fractovap 2300 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame-ionization detector (FID) and a capillary column splitless injector was used. The glass capillary column (20 m $\times$ 0.35 mm I.D.) (H. and J. Jaeggi, Trogen, Switzerland) was wall-coated with SE-30. The injection port temperature was 250° and the samples were injected at an oven temperature of 50° or 70°. The temperature was programmed at 5°/min up to 250°. Nitrogen was used at the carrier gas at an inlet pressure of 0.4 kp/cm^2, which gave a flow-rate of 1.4 ml/min through the column. The splitting ratio of the injector was 1:40 for the identification test and the sensitivity setting was 10 $\times$ 8.

Samples for the calibration graph were injected without splitting. The splitter was closed before the injection and re-opened to a splitting ratio of 1:40 30 sec after the injection. The sensitivity setting was 1 $\times$ 8 and a Spectra Physics Autolab Minigrator was connected to the gas chromatograph for peak area measurements.

Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry (GC–MS) was carried out using a Varian Model 112 mass spectrometer (Varian-MAT, Bremen, G.F.R.) combined with a Varian Model 1400 gas chromatograph (Varian, Walnut Creek, Calif., U.S.A.). The glass capillary column (50 m $\times$ 0.28 mm I.D.) (LKB, Stockholm, Sweden) was wall-coated with SE-30.

Identification of narcotic drugs

A mixture of the compounds listed in Table I was prepared to give a concentration of 250 μg/ml in ethyl acetate for each. This solution was also diluted 1:10 and 1:100. A 1-μl volume of derivatization reagent was drawn into the syringe (Hamilton 701 N) followed by 1 μl of the test solution, and the mixture was injected into the gas chromatograph. About 50 mg of the illicit heroin samples were dissolved in 50 ml of chloroform, the solutions were centrifuged and 1 μl was injected into the gas chromatograph together with 1 μl of derivatization reagent.

Calibration graph

Calibration graphs for the concentration range 1–10 μg/ml in ethyl acetate were constructed for codeine and morphine using ethylmorphine as internal standard. The concentration of the internal standard was 10 μg/ml. A 1-μl volume of BSA was injected into the gas chromatograph together with 1 μl of the test solution. The

peak-area ratios (drug derivative to internal standard derivative) were plotted against the drug concentration. Five assays of each solution were carried out and the regression lines and the correlation coefficients were calculated.

Reproducibility of quantitative analysis after derivatization

A test solution containing 5 μg/ml of codeine and morphine and 10 μg/ml of internal standard was analysed as described above and the peak-area ratios were calculated. The mean and the relative standard deviations (RSDs) for ten analyses were calculated.

RESULTS AND DISCUSSION

Information relating to some of the drugs that form trimethylsilyl derivatives under the above conditions are given in Table I. The results obtained by injecting a mixture of fourteen different compounds together with BSA is shown in Fig. 1. The mixture diluted 1:10 to give a concentration of 25 μg/ml also gave peaks that permitted easy identification. When the mixture was diluted 1:100 to give a concentration of 2.5 μg/ml, the attenuation had to be set to a higher value, and impurities from BSA interfered with the early eluted drugs. With more purified reagents the compounds can also be easily identified in this concentration range.

TABLE I

COMPOUNDS EXAMINED AND THEIR ELUTION CHARACTERISTICS

Compound	*Functional group for derivatization*	*Compound derivatized*	*Temperature when compound was eluted (°C)*	*Temperature when derivative was eluted (°C)*
Amphetamine	Primary amine	Yes	91 (small, broad)	96
Ephedrine	Secondary amine	Yes	105	108
	Hydroxyl	Yes		
Phenmetrazine	Secondary amine	No	113	
Hexadecane	None	No	137	
Methyl phenidate	Secondary amine	No	148	
Pethidine	None	No	150	
Caffeine	None	No	163	
Eicosane	None	No	180	
Methadone	None	No	192	
Cocaine	None	No	198	
Codeine	Hydroxyl	Yes	216 (broad)	220
Ethylmorphine	Hydroxyl	Yes	219 (broad)	223
Morphine	Hydroxyl	Yes	—	227
Heroin	None	No	236	

The identity of the drug derivatives was checked by GC–MS. This investigation showed that N,N-bis(trimethylsilyl)amphetamine, N,O-bis(trimethylsilyl)ephedrine, O-trimethylsilylcodeine, O-trimethylsilylethylmorphine and O,O-bis(trimethylsilyl)morphine were formed. No underivatized drug could be detected, and it was concluded that the reaction was complete. The secondary cyclic amines in methyl phenidate and phenmetrazine were not derivatized, and no derivative formation was

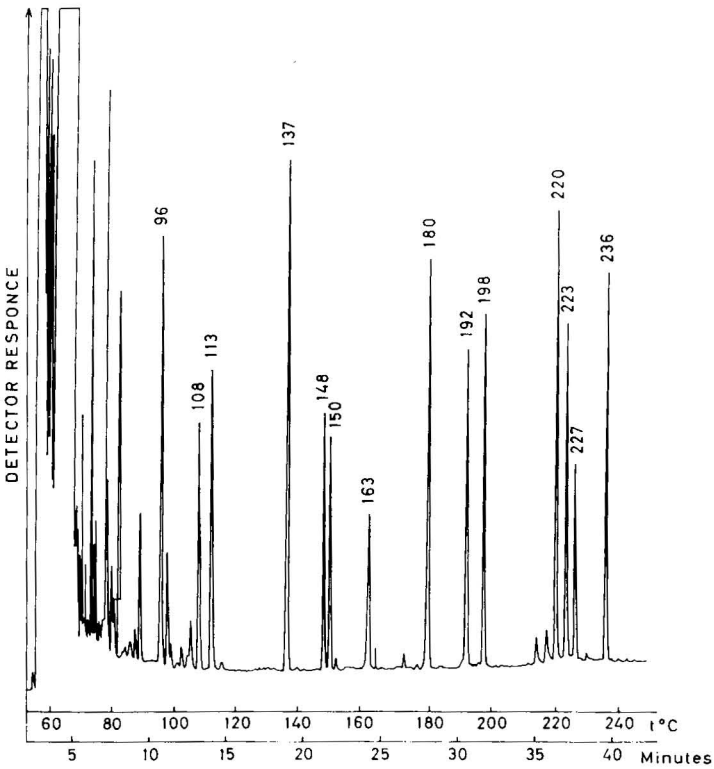


Fig. 1. Chromatogram obtained after flash-heater derivatization by injecting 1 μl of BSA together with 1 μl of an ethyl acetate solution containing 250 ng of each of the following components: 96 = amphetamine; 108 = ephedrine; 113 = phenmetrazine; 137 = hexadecane; 148 = methyl phenidate; 150 = pethidine; 163 = caffeine; 180 = eicosane; 192 = methadone; 198 = cocaine; 220 = codeine; 223 = ethylmorphine; 227 = morphine; 236 = heroin. Column temperature: 50° at start, programmed at 5°/min.

observed when the volume of BSA injected was increased. The lower reactivity of secondary amines has been described for several derivatization reagents[10].

The drugs were also dissolved in solvents such as chloroform, benzene and carbon disulphide and the reaction was found to be independent of the solvent. It is well known that silylation reactions with amine groups are markedly dependent on the solvent when performed off-column[10]. The same derivatives were also formed with the drug salts dissolved in chloroform.

BSTFA and TMSIM were also tried as derivatization reagents but only with BSTFA was the reaction complete. TMSIM, which is the preferred reagent for the silylation of all OH groups, will not derivatize primary and secondary aliphatic amines[10]. This reagent therefore cannot be used for the identification of amphetamine and ephedrine.

Fig. 2 shows a chromatogram relating to an illicit heroin sample that was extracted with chloroform and derivatized with BSA. From this chromatogram caffeine, morphine, monoacetylmorphine and heroin could be detected. The peak eluted at 275° was identified as strychnine. A GC–MS investigation was also carried

out to verify the results. The experimental aspects of the splitless sampling technique have recently been evaluated[11]. To avoid overloading of the column, it was necessary to inject the codeine–morphine samples without splitting at an oven temperature of 70° and to re-open the splitter 30 sec after the injection.

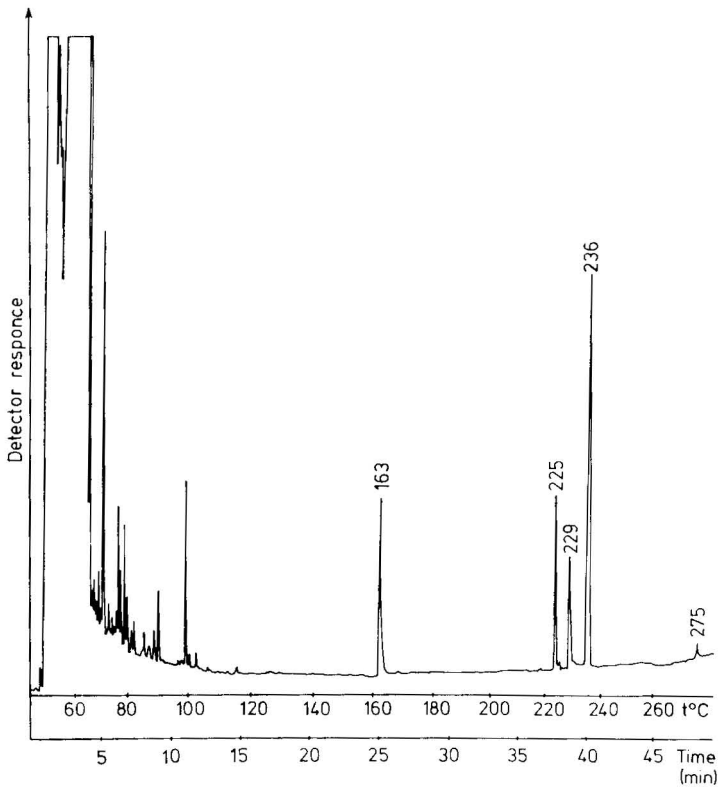


Fig. 2. Chromatogram obtained after flash-heater derivatization of a chloroform extract of an illicit heroin sample. A 1-μl volume of BSA was injected together with 1 μl of the sample. 163 = Caffeine; 225 = monoacetylmorphine; 229 = morphine; 236 = heroin; 275 = strychnine. Column temperature: 50° at start, programmed at 5°/min.

In order to check the linearity of the derivatization method in the temperature-programmed mode, calibration graphs were constructed for codeine and morphine. The calibration graphs were calculated according to the method of least squares, relating y (the peak-area ratio of the drug derivative to the internal standard derivative) to x (the concentration of the drug solution in micrograms per millilitre). The calibration graph for the concentration range 1–10 μg/ml was $y = 0.100x - 0.033$ with a correlation coefficient of 0.9991 for codeine and $y = 0.083x - 0.063$ with a correlation coefficient of 0.9963 for morphine.

The data obtained from the reproducibility test showed that at 5.0 μg/ml the RSD was 1.5% for codeine and 4.3% for morphine. These values are similar to those found earlier using packed columns for the analysis of codeine after on-column

derivatization with heptafluorobutyrylimidazole, heart-cutting and electron-capture detection[9].

Fig. 3 shows a chromatogram after flash-heater silylation of 10 ng of codeine, 10 ng of ethylmorphine and 10 ng of morphine. A blank run showed no interfering peaks with the codeine, ethylmorphine or morphine derivative. With this method the minimal detectable amount was about 500 pg for codeine and morphine. However, with the earlier described method with electron-capture detection smaller amounts could be detected.

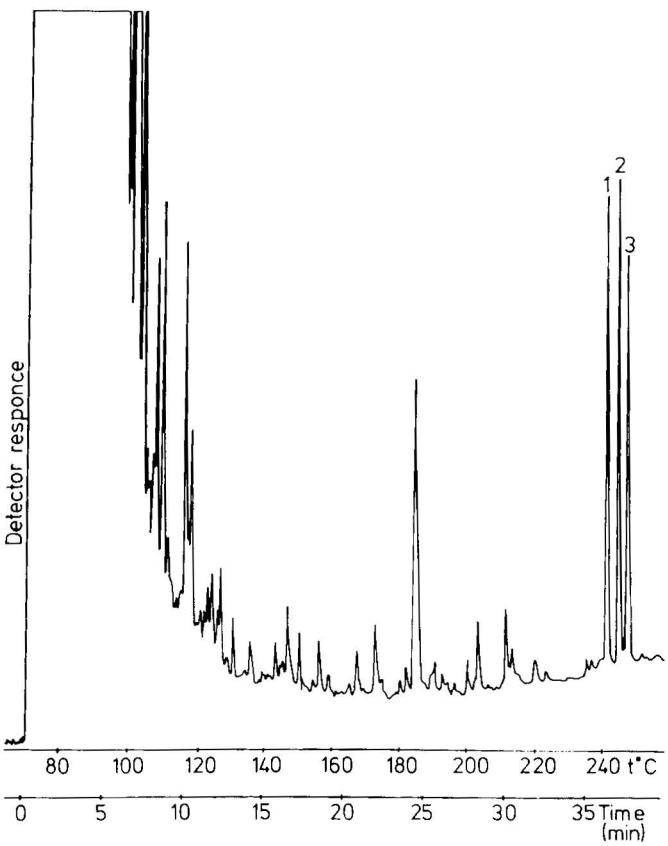


Fig. 3. Chromatogram of a sample containing 10 ng of codeine (peak 1), 10 ng of ethylmorphine (peak 2) and 10 ng of morphine (peak 3) after flash-heater silylation. Column temperature: 70° at start, programmed at 5°/min.

On the basis of these results the derivatization reagent and the sample can be injected directly into a gas chromatograph equipped with a capillary column. Less time is required for forming derivatives, and also there is no chance of the hydrolysis which sometimes occurs with certain derivatives in damp atmospheres. The method can be used successfully for the identification of complex mixtures of narcotic drugs and also for the quantitative analysis of drugs in the nanogram range.

REFERENCES

1 W. J. J. Leunissen and J. H. H. Thijssen, *J. Chromatogr.*, 146 (1978) 365.
2 H. J. Jaeger, H.-U. Klør, G. Blos and H. Ditschuneit, *Chromatographia*, 8 (1975) 507.
3 E. Bailey, M. Fenoughty and L. Richardson, *J. Chromatogr.*, 131 (1977) 347.
4 A. G. de Boer, J. Röst-Kaiser, H. Bracht and D. D. Breimer, *J. Chromatogr.*, 145 (1978) 105.
5 M. T. Rosseel, M. G. Bogaert and M. Claeys, *J. Pharm. Sci.*, 67 (1978) 802.
6 K. E. Rasmussen, *J. Chromatogr.*, 114 (1975) 250.
7 K. E. Rasmussen, *J. Chromatogr.*, 120 (1976) 491.
8 G. Brugaard and K. E. Rasmussen, *J. Chromatogr.*, 147 (1978) 476.
9 A. S. Christophersen and K. E. Rasmussen, *J. Chromatogr.*, 168 (1979) 216.
10 K. Blau and G. S. King, *Handbook of Derivatives for Chromatography*, Heyden & Son, London, 1977, p. 179.
11 F. J. Yang, A. C. Brown, III and S. P. Cram, *J. Chromatogr.*, 158 (1978) 91.

Journal of Chromatography, 174 (1979) 461–464

CHROM. 11,824

Note

Analysis of biosynthesised terpene alcohols facilitated by C_{18} phase-bonded silica

THOMAS G. McCLOUD and PETER HEINSTEIN

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Ind. 47907 (U.S.A.)

(First received September 18th, 1978; revised manuscript received February 28th, 1979)

During recent studies on the biosynthesis of terpene alcohols by a partially purified prenyl transferase (EC 2.5.1.1), we considered it desirable to combine analytical with preparative chromatography of the products. The currently available methods presented several disadvantages. Thin-layer chromatography (TLC) on silica gel containing silver nitrate will easily separate the isomers of terpene alcohols, but the relative mobilities of several C_5, C_{10} and C_{15} terpenes are so close[1–3] that use of this method for identifying a single product in a mixture containing several mono- through sesqui-terpenes would not be reliable. Consequently, preliminary classification of alcohols by chain length is necessary. Reversed-phase TLC using a silicone or mineral oil has been applied to this purpose[2,4,5], but this is both messy and time-consuming, and for further analysis, elution and removal of the silicone or mineral oil from the sample is necessary. The use of gas–liquid chromatography (GLC) proved to be unsatisfactory because recovery of injected standard [^{14}C]farnesol was consistently low (12%) and there was isomerisation on the column, as shown in Fig. 1.

High-performance liquid chromatography (HPLC) has recently been used to achieve some excellent separations of natural products[6,7]. Ross has been able to identify components of cinnamon oil by HPLC on silica[8], and of ylang-ylang and spearmint oil volatiles by reversed-phase HPLC on phase-bonded C_{18} silica[9]. Some success has been reported in separating geometric isomers by this reversed-phase technique[10]. In all these instances, the instrumentation was of the rather sophisticated and expensive type commercially available. However, many of the advantages of HPLC can be retained by performing low-pressure chromatography on phase-bonded silica in a glass column and using pumps, detectors and accessory equipment more commonly available in the laboratory.

A chromatographic column (22 × 1.2 cm) was constructed from Ace Glass No. 7 threated fittings*. The PTFE end-pieces and injection port were machined to our specifications to seal the ends of the glass column without use of additional O-rings and to be compatible with the Altex brand of fittings. During packing, a reservoir was attached to the column so that a slurry of C_{18} phase-bonded silica of

* Columns and PTFE end-fittings similar to those described are now being made available by the Ace Glass Company, Vineland, N.J., U.S.A.

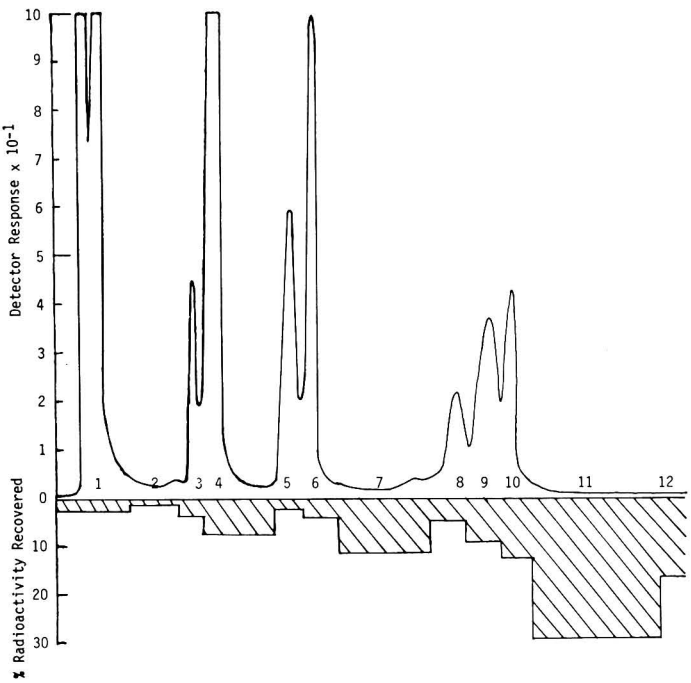


Fig. 1. Analysis of *trans,trans*-farnesol by GLC on a column packed with 10% of Carbowax on Chromosorb W (60–80 mesh) and temperature-programmed from 145° to 230° at 4°/min, with helium (0.8 ml/sec) as carrier gas and a thermal-conductivity detector. The area beneath peaks 8, 9 and 10 accounts for 26% of the total counts recovered as one of the four isomers of farnesol, whereas only 12% lies beneath the peak for *trans,trans*-farnesol; 46% of the counts recovered were eluted at times exceeding the retention time of farnesol.

the type manufactured by E. Merck (LiChroprep RP-18) could be poured in a single step and solvent under pressure could be passed through the assembly. A constant flow of solvent at 20 p.s.i. was maintained during packing, injection and elution of sample by means of a Milton-Roy high-pressure piston pump. The solvent used throughout was methanol–water–acetonitrile (7:2:1) and, during analytical runs, the flow-rate was 1 ml/min. An ISCO UA-4 ultraviolet monitor set at 254 nm was used to detect compounds as they were eluted from the column.

Samples were prepared, following incubation of various combinations of C_5 and C_{10} terpene substrates with a prenyl transferase, by hydrolysing with alkaline phosphatase, extracting with light petroleum (b.p. 30–60°), drying and concentrating[11]. To each sample was added 5 μl of isopentenol, 5 μl of nerol and 10 μl of farnesol, all from commercial sources, and the mixture was further concentrated to 50 μl, of which 20 μl were injected directly on to the column. Various standards obtained from commercial sources were also analysed in this way.

In Fig. 2 is shown a typical elution profile for an enzyme-product analysis run; the positions at which several standard compounds are eluted are indicated by arrows. As can be seen, there is excellent separation of these compounds according to the number of carbon atoms, with each group being eluted almost precisely at

the same time, regardless of their individual geometries or substituents. After elution of the last desired component, the next sample may be injected immediately, without any reconditioning of the column. Terpenoids of higher molecular weight (such as phytol and squalene) are retained on the column for extended periods, though they also may be eluted if the solvent system is made less polar.

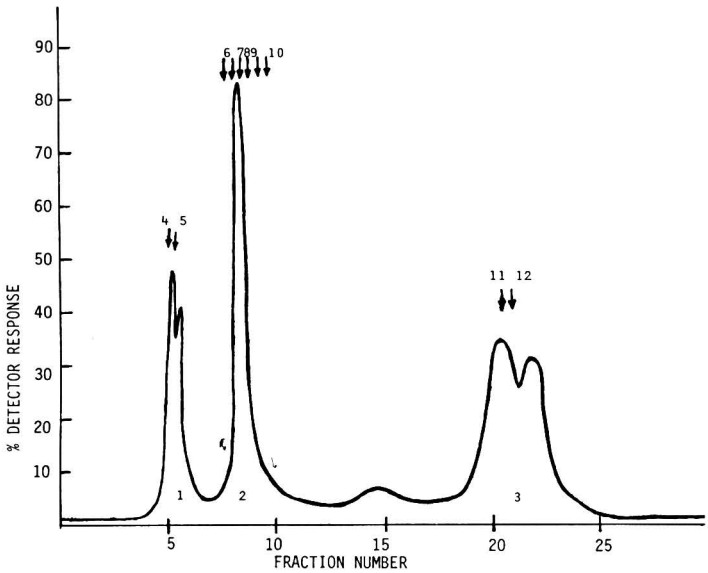


Fig. 2. Elution pattern of terpenes from phase-bonded silica. Peaks: 1 = isopentenol; 2 = nerol; 3 = farnesol (mixture of standards used in enzyme-product analysis); 4 = dimethylallylalcohol; 5 = mevalonic acid; 6 = carvone; 7 = carvacrol; 8 = geraniol; 9 = linalool; 10 = citral; 11 = bisabolol; 12 = cadinene. Fraction volume, 2.5 ml.

Fractions collected from the column were scanned for radioactivity or spotted on to a silica gel plate and sprayed with anisaldehyde reagent[12]. The peak fractions were concentrated and applied to a Merck silica gel 60 plate that had been dipped into a solution of 20% silver nitrate in acetonitrile, then briefly dried. Development was with cyclohexane–ethyl acetate (1:1), the plates were sprayed with anisaldehyde reagent, and the appropriate zones were scraped into scintillation vials for counting. Separation of the terpene alcohols by geometry has thus been obtained, and the quantitation of each individual isomer, even of such closely matched pairs as *cis,trans*- and *trans,cis*-farnesols is possible.

In addition to its uses in research, the technique presented here would seem to have great potential in industry, as it would allow for preliminary fractionation of complex mixtures, such as essential oils or fermentation products, by carbon number, thus facilitating the clean-up procedure.

ACKNOWLEDGEMENT

This investigation was supported by grant number GM 23249, awarded by the National Institute of General Medical Sciences, DHEW.

REFERENCES

1 E. Cardemil, J. R. Vicuna, A. M. Jabalquinto, and O. Cori, *Anal. Biochem.*, 59 (1974) 636.
2 E. Stahl and H. Vollman, *Talanta*, 12 (1965) 525.
3 E. Stahl and J. Jork, in E. Stahl (Editor), *Thin-Layer Chromatography*, 2nd ed., Springer, Berlin, 1969, p. 227.
4 G. P. McSweeney, *J. Chromatogr.*, 17 (1965) 183.
5 M. O. Oster and C. A. West, *Arch. Biochem. Biophys.*, 127 (1968) 112.
6 *Application Highlights No. H93 (Natural Products Isolation)*, Waters Assoc., Milford, Mass., 1978.
7 *Application Highlights No. H313 (Essential Oils)*, Waters Assoc., Milford, Mass., 1974.
8 M. S. F. Ross, *J. Chromatogr.*, 118 (1976) 273.
9 M. S. F. Ross, *J. Chromatogr.*, 160 (1978) 199.
10 J. D. Warthen, Jr., *J. Amer. Oil Chem. Soc.*, 52 (1975) 151.
11 R. Widmaier, J. Howe and P. Heinstein, *Arch. Biochem. Biophys.*, in press.
12 E. Stahl, in E. Stahl (Editor), *Thin-Layer Chromatography*, 2nd ed., Springer, Berlin, 1969, p. 857.

Journal of Chromatography, 174 (1979) 465–468

CHROM. 11,807

Note

High-performance liquid chromatographic method for the determination of 5-vinyl-2-oxazolidinethione in milk

EGON JOSEFSSON and LARS ÅKERSTRÖM

The Swedish National Food Administration, Food Laboratory, S-751 26 Uppsala (Sweden)

(First received January 17th, 1979; revised manuscript received February 19th, 1979)

Rapeseed meal is a valuable source of protein for dairy cows. However, it contains glucosinolates, of which 2-hydroxy-3-butenylglucosinolate is the most important[1]. The glucosinolates are hydrolysed by an enzyme system, myrosinase, which always yields glucose and sulphate. Further, thiocyanates, isothiocyanates, cyano compounds or oxazolidinethiones are formed[2]. Although the myrosinase is inactivated in commercial rapeseed meal, some of the glucosinolates may be hydrolysed *in vivo*[3], in which case 5-vinyl-2-oxazolidinethione (I) may be formed from 2-hydroxy-3-butenylglucosinolate. The oxazolidinethione causes goitre in animals[4]. Virtanen *et al.*[5] found that cows given feed containing 2-hydroxy-3-butenylglucosinolate excreted about 0.05% of the amount of I found in the minced feed. The content of I in the milk did not exceed 100 μg/l. After studying the influence of I on the uptake of radioactive iodide in the thyroid gland, Vilkki *et al.*[6] concluded that the content of I in milk from cows fed large amounts of green *Brassica* plants was not high enough to cause goitre in man. However, Peltola and Krusius[7] found evidence that a daily dose of 100 μg of I given to man decreased the amount of thyroxine in the blood. In view of this, it is of interest to analyse I in milk.

Kreula and Kiesvaara[8] developed a method for the determination of I in milk. After extraction and clean-up, the sample was chromatographed with two-dimensional paper chromatography. The spot of I was eluted and the I content determined spectrophotometrically.

McLeod *et al.*[9] showed that in milk I may be detected as its heptafluorobutyl derivative by gas–liquid chromatography using an electron-capture detector, after clean-up by high-performance liquid chromatography (HPLC). However, this seems to be a time-consuming method and experiments with milk were performed only at the very high level of 2 mg/kg of I.

Since Kreula and Kiesvaara[8] published their method, the technique of HPLC has developed rapidly. This technique is regarded as being more precise and less time consuming than the one based on paper chromatography and spectrophotometry and was utilized for the development of the present method.

EXPERIMENTAL

Materials

Milk from cows that had not been fed on rapeseed meal or *Brassica* plants was obtained from the Department of Animal Husbandry, Swedish University of Agricultural Sciences, Öjebyn, Sweden.

5-Vinyl-2-oxazolidinethione was prepared from rapeseed meal. The purity was 99% as determined by spectrophotometry. The compound was dissolved in chloroform to give a concentration of 10 μg/ml.

Extraction and clean-up

The extraction and clean-up were carried out essentially according to the method of Kreula and Kiesvaara[8], with the exception that dichloromethane was used instead of ethyl acetate for the extraction step. Milk (300 ml) was heated at 85–90° for *ca.* 5 min and then cooled rapidly. The sample was extracted twice with 500 ml of dichloromethane in a separating funnel and the dichloromethane solutions were evaporated to dryness. The residue was extracted with 5 ml of 1% ammonia solution by gentle shaking whilst warming in a water-bath. This procedure was repeated three times. The combined ammonia extracts were adjusted to pH 6.5–7.5 with 18% acetic acid. Fat was removed by extraction with two 5-ml volumes of *n*-hexane in a separating funnel. The aqueous phase was extracted with four 30-ml volumes of dichloromethane. After evaporation to dryness of the combined dichloromethane extracts, the residue was dissolved in 1.0 ml of dichloromethane.

Liquid chromatography

The liquid chromatograph used was a Spectra-Physics Model 3500B equipped with a 250 × 3 mm I.D. stainless-steel column packed with Spherisorb 5-μm silica (Spectra-Physics, Santa Clara, Calif., U.S.A.). I was detected by its UV absorption using a Schoeffel Model SF 770 spectroflow monitor (Schoeffel, Westwood, N.J., U.S.A.) or an SP 8200 (Spectra-Physics). Chloroform–*n*-hexane (2:1) (analytical-reagent grade) was used as the mobile phase with a flow-rate of 1.2 ml/min. The UV detector was set at a wavelength of 249 nm (SF 770) or 254 nm (SP 8200). Usually 10 μl of sample were injected into the chromatograph. The injections were made with a loop valve injector, which provides reproducible injections. Peak areas were measured by multiplying the peak height by the width at half-height. The content of I was calculated by comparing the peak area obtained with the sample with that obtained with standard solutions. The detector response was linear for the range tested (3-100 ng).

RESULTS AND DISCUSSION

Recoveries were studied by adding I dissolved in 100–200 μl of chloroform, which was evaporated before the heat treatment of the milk.

Kreula and Kiesvaara[8] found that I disappeared very rapidly from milk if it was not heated. Even when the milk was analysed immediately after the addition of I, heating to 85° increased the recovery.

TABLE I

RECOVERY OF 5-VINYL-2-OXAZOLIDINETHIONE ADDED TO MILK CONTAINING 3% OF FAT

Amount added (μg/l)	*Recovery (%)*
143	82
100	70
67	71
67	70
67	70
67	90
33	70
33	69
	Mean: 74

Kreula and Kiesvaara[8] reported a recovery of about 75%. Using ethyl acetate extraction and the clean-up according to their method, we obtained recoveries of only about 50%. A better recovery was obtained when dichloromethane was utilized for the extraction.

As shown in Table I, the average recovery of I added to milk was 74%. The limit of detection of the method is 1 μg/l. A typical chromatogram of a spiked sample is shown in Fig. 1.

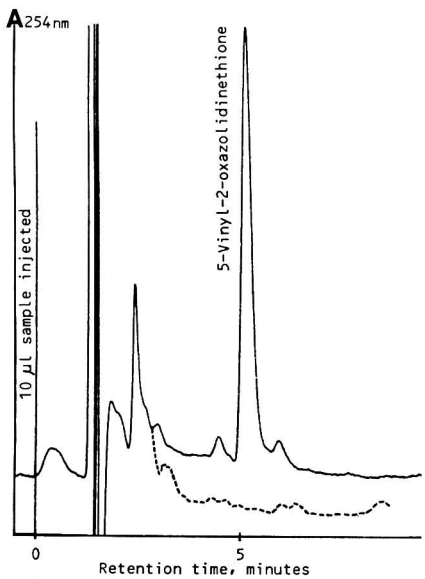


Fig. 1. High-performance liquid chromatograms of milk samples without 5-vinyl-2-oxazolidinethione (broken line) and after the addition of 67 μg/l of 5-vinyl-2-oxazolidinethione (solid line). Column, Spherisorb 5-μm silica; mobile phase, chloroform–*n*-hexane (2:1); flow-rate, 1.2 ml/min; Spectra-Physics SP 8200 UV detector, 0.04 a.u.f.s.

REFERENCES

1 E. Josefsson and L.-Å. Appelqvist, *J. Sci. Food Agr.*, 19 (1968) 564.
2 C. H. VanEtten and I. A. Wolff, in Committee on Food Protection, NRC, *Toxicants Occurring Naturally in Foods*, National Academy of Sciences, Washington, D.C., 1973, p. 210.
3 M. A. Greer and J. M. Deeney, *J. Clin. Invest.*, 38 (1959) 1465.
4 E. B. Astwood, M. A. Greer and M. G. Ettlinger, *J. Biol. Chem.*, 181 (1949) 121.
5 A. I. Virtanen, M. Kreula and M. Kiesvaara, *Acta Chem. Scand.*, 13 (1959) 1043.
6 P. Vilkki, M. Kreula and E. Piironen, *Ann. Acad. Sci. Fenn. Ser. A2 Chem.*, No. 110 (1962).
7 P. Peltola and F.-E. Krusius, in K. Fellinger and R. Höfer (Editors), *Further Advances in Thyroid Research*, Verlag der Wiener Medizinischen Akademie, Vienna, 1971, p. 149.
8 M. Kreula and M. Kiesvaara, *Acta Chem. Scand.*, 13 (1959) 1375.
9 H. A. McLeod, G. Benns, D. Lewis and J. F. Lawrence, *J. Chromatogr.*, 157 (1978) 285.

Journal of Chromatography, 174 (1979) 469–473

CHROM. 11,779

Note

Measurement of furosemide by high-performance liquid chromatography

SARAH E. SWEZEY, PETER J. MEFFIN* and TERRENCE F. BLASCHKE

Divisions of Clinical Pharmacology and Cardiology, Stanford University Medical Center, Stanford, Calif. 94305 (U.S.A.)

(Received February 7th, 1979)

Furosemide is a potent diuretic agent widely used in the treatment of disease states complicated by sodium retention. A variety of different assay methods are available for measuring furosemide[1–6], but most have problems related to specificity or sensitivity, or are too complicated for use in detailed pharmacokinetic studies. Recently, Carr *et al.*[7] described a method for measurement of furosemide which has many advantages over the previous methods. Using high-performance liquid chromatography (HPLC) and an internal standard, they have developed a sensitive and selective method which has improved accuracy and precision. We have concurrently developed an HPLC method using a different internal standard which has five times the sensitivity and significantly reduces the time required for chromatography when compared with the method of Carr *et al.*[7]

EXPERIMENTAL

Standards

Standard solutions of both furosemide and the internal standard, N-benzyl-4-chloro-5-sulfamoylanthranilic acid (both gifts from Hoechst, Frankfurt/M, G.F.R.) are made in pH 10.2 0.1 *M* carbonate–bicarbonate buffer. Standard furosemide concentrations ranged from 0.02 μg to 50 μg per 100 μl buffer. Concentration of the internal standard was 0.5 μg per 100 μl buffer or 10.0 μg per 100 μl buffer, depending on the range of concentrations expected in unknown samples. New solutions of both compounds were made every 2 weeks and stored at 4°.

Extraction

The extraction procedure shown in Fig. 1 is a simple two-step process involving an acid extraction into diethyl ether and back extraction into aqueous sodium hydroxide. A 0.5 μg/100 μl aliquot of internal standard is pipetted into a culture tube (13 × 100 mm) with a PTFE-lined screw cap to which are added 0.1–1.0 ml plasma or urine and 2.0 ml water. Next, the plasma or urine is acidified with 150 μl of 4 *M* HCl. Five ml of diethyl ether are then added to the tube and the

* Present address: Department of Clinical Pharmacology, Flinders Medical Centre, Bedford Park 5A5042, Australia.

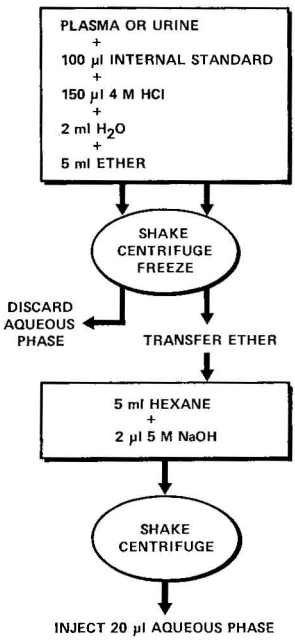


Fig. 1. Flow diagram showing the extraction procedure.

mixture is allowed to shake on a Labquake shaker for 10 min followed by centrifugation at relative centrifugal force of 1100 *g* for 5 min. The tube is immersed in a dry ice–acetone bath and the aqueous phase frozen. The ether phase is then poured into a tube with an elongated cone at its base which contains 2 µl 5 *M* NaOH (measured with a 10-µl Hamilton syringe) and 5 ml *n*-hexane. This mixture is allowed to shake 10 min and centrifuged for 5 min. The entire aqueous phase (now consisting of *ca.* 20 µl) is then sampled from the tube with a 25-µl Hamilton syringe. One µl of 5 *M* acetic acid is also drawn into the syringe, and the total of 21 µl is injected into the chromatograph.

Chromatographic conditions

A Varian dual-pump high pressure liquid chromatograph fitted with a Rehodyne loop injector and a Varian CH-10 reversed-phase column is used for the separation. The solvent mixture is 0.5% acetic acid in acetonitrile–water (30:70). The flow-rate is 60 ml/h which results in a column input pressure of 170 atm.

Detector

A Schoeffel FS970 fluorescence monitor with a quartz cell and a deuterium lamp is used. The excitation monochromator is set at 350 nm and a 389-nm cut-off emission filter is used. The output from the detector is recorded on a Varian Model 9176 recorder.

Calibration

The assay is calibrated by analyzing 1-ml aliquots of control plasma to which

has been added 0.02–5.0 μg of furosemide and 0.5 μg of internal standard or 1.0–50.0 μg of furosemide and 10 μg of internal standard. Control urine samples of 0.1 ml containing 0.5–5.0 μg of furosemide and 0.5 μg internal standard are used for the urine calibration graph.

For each sample, the ratio of furosemide peak height to internal standard peak height is determined. Each ratio is divided by the amount of furosemide in that sample to give a normalized peak-height ratio. The peak-height ratios are plotted against the concentrations to determine the linearity of the extraction and detector response. The normalized peak-height ratios are averaged and the mean value is used to determine the amounts of furosemide in unknown samples. Precision of the assay is estimated by determining the coefficient of variation of the normalized peak-height ratios.

RESULTS AND DISCUSSION

Retention times under the conditions described are 3.0 min for furosemide and 6.5 min for the internal standard. One sample can be chromatographed in 8–9 min. Fig. 2 shows chromatograms of 1.0 ml control plasma (A) and 0.1 ml of control urine (C) as well as 1.0 ml plasma containing 0.2 μg furosemide and 0.5 μg internal standard (B) and 0.1 ml urine containing 0.2 μg furosemide and 0.5 μg internal standard (D). The two compounds are well separated, and there were no significant peaks which interfere with the peaks of interest.

Reproducibility

The reproducibility of the assay was measured by determining the coefficients of variation of five replicate samples of 0.02, 0.05 and 0.50 μg furosemide in 1 ml plasma or 0.1 ml urine. Table I shows coefficient of variation for the calibration graphs and the reproducibility studies. These results indicate that the overall variability of the assay is quite acceptable. The calibration graphs are linear and pass through the origin. The lower limit of sensitivity for the assay is 20 ng extracted from either 1 ml plasma or 0.1 ml urine. This is sufficient sensitivity for use in pharmacokinetic studies of the drug. The reproducibility studies at different concentrations showed low variability for both plasma and urine, even at 20 ng/ml. Reproducibility with varying amounts of plasma or urine was determined by extracting 0.5 μg furosemide from 0.1, 0.2, 0.5 and 1.0 ml of plasma or urine. Reproducibility with different volumes of plasma or urine was essentially the same as the reproducibility with equal volumes and there was no consistent trend in the data.

Efficiency

The efficiency of the extraction procedure was determined by extracting 110 ng (1350 dpm) of generally labelled [^{3}H]furosemide (prepared by New England Nuclear, Boston, Mass., U.S.A., and purified at Syntex Labs., Palo Alto, Calif., U.S.A.) and counting aliquots of each phase in the extraction procedure with a Beckman LS230 liquid scintillation counter. Recovery studies with [^{3}H]furosemide showed that an average of 93% of the drug was extracted into the ether during the first extraction, and 66% of the total was recovered in the aqueous phase after the back extraction.

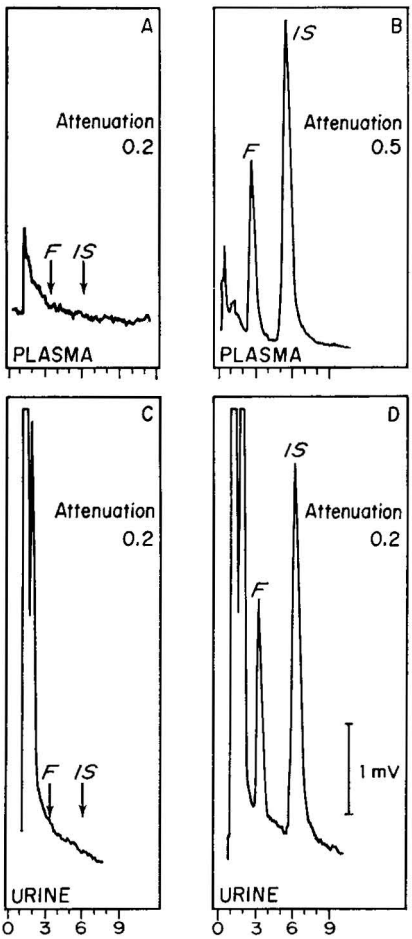


Fig. 2. Chromatograms of plasma and urine extracts. Arrows show where furosemide (arrow farthest to the left) and the internal standard would elute if they were present. (A) 1.0 ml of control plasma; (B) 1.0 ml of plasma containing 0.02 μg of furosemide and 0.5 μg internal standard; (C) 0.1 ml of control urine; (D) 0.1 ml of urine containing 0.2 μg furosemide and 0.5 μg internal standard.

Interference by other drugs

Several blank plasma samples spiked with various drugs were taken through the analysis to see whether or not they caused interference. These drugs, which seemed likely to be given coincidentally with furosemide, were propranolol, digoxin, hydralazine, methyldopa, quinidine, NAPA, prazosin, mixiletene and disopyramide.

Of the drugs which were checked for interference in the assay, prazosin, NAPA and quinidine were found to have similar retention times and to fluoresce under these conditions. However, at normal therapeutic concentrations they are not extracted from plasma using the procedure described above.

TABLE I

SUMMARY OF DATA FOR CALIBRATION GRAPHS AND REPRODUCIBILITY STUDIES

	Furosemide (μg)	*Internal standard (μg)*	*Sample size (ml)*	*n**	*Coefficient of variation (%)*
Plasma					
Calibration graphs	0.02– 5.0	0.5	1.0	7	4.2
	1.0 –50.0	10.0	1.0	5	4.3
Reproducibility studies	0.02	0.5	1.0	5	8.0
	0.05	0.5	1.0	5	7.5
	0.5	0.5	1.0	5	1.3
	0.5**	0.5	0.1–1.0	4	3.8
Urine					
Calibration graphs	0.02– 5.0	0.5	0.1	7	7.4
	1.0 –50.0	10.0	0.1	5	4.4
Reproducibility studies	0.02	0.5	0.1	5	7.7
	0.05	0.5	0.1	5	4.6
	0.5	0.5	0.1	5	0.8
	0.5**	0.5	0.1–1.0	4	3.2

* Number of replicates in each study.
** Reproducibility with varying volumes.

ACKNOWLEDGEMENTS

This study was supported by National Institutes of Health Grant GM22209. Dr. Blaschke is a Burroughs Wellcome Scholar in Clinical Pharmacology, and recipient of a Research Career Development Award (GM00407) from the National Institutes of Health.

We gratefully acknowledge the help of Dr. Howard Parneff of Syntex Laboratories in purifying the labelled furosemide and Linda Halloran for preparation of the manuscript.

REFERENCES

1 F. Andreasen and P. Jakobsen, *Acta Pharm. Toxicol.*, 35 (1974) 49.
2 A. D. Blair, A. W. Forrey, B. T. Meijsen and R. E. Cutler, *J. Pharm. Sci.*, 64 (1975) 1334.
3 B. Lindström, *J. Chromatogr.*, 100 (1974) 189.
4 B. Lindström and M. Molander, *J. Chromatogr.*, 101 (1974) 219.
5 M. L. MacDougall, D. W. Shoeman and D. L. Azarnoff, *Res. Commun. Chem. Pathol. Pharmacol.*, 10 (1975) 285.
6 E. Mikkelson and F. Andreason, *Acta Pharm. Toxicol.*, 41 (1977) 254.
7 K. Carr, A. Rane and J. C. Frölich, *J. Chromatogr.*, 145 (1978) 421.

Journal of Chromatography, 174 (1979) 474–477

CHROM. 11,823

Note

Enzymic hydrolysis of peptides and their analysis on a gradient-operated Chromaspek amino acid analyser*

A. CASTEL, J. M. DE GRAAF and B. KRAAL

Department of Biochemistry, State University of Leiden, P.O. Box 9505, 2300 RA Leiden (The Netherlands)

(First received December 27th, 1978; revised manuscript received February 26th, 1979)

In the course of investigations on the elucidation of the primary structure of the coat protein of alfalfa mosaic virus strain VRU we used enzymic hydrolysis with aminopeptidase M and carboxypeptidases A and B. The digestion was performed by standard methods[1,2] in 0.2 *M* sodium hydrogen carbonate buffer and was stopped by the addition of an equal volume of 14% (w/v) trichloroacetic acid containing 0.2 *M* sodium acetate[3]. After centrifugation the supernatant used to be directly applied to a Unichrom amino acid analyser (Beckman, Munich, G.F.R.) based on a stepwise elution buffer system. More recently, when using a gradient-operated Chromaspek amino acid analyser (Rank Hilger, Margate, Great Britain), we faced the problem of very bad resolution of peaks, especially at the beginning of the run (Fig. 1A). This was caused by a serious disturbance of the programmed pH gradient in the elution buffer, owing to the strongly acidic character and high salt concentration of the sample applied. The pH gradient on the column is produced by mixing two buffers of pH 2.2 and 11.5, controlled by a profile on a programme drum[4].

At first we tried to solve this problem by titrating the samples with 1 *N* sodium hydroxide solution, but the results were unsatisfactory. The high salt concentration caused serious broadening of the peaks, especially for Asp, Thr, Ser, Glu, Gly and Ala, which resulted in a bad separation. Although we succeeded in designing a programme that gave a better separation of amino acids at the beginning of the run, it was not possible to separate clearly all of the amino acids within the same total run time.

Therefore, we decided to develop an alternative procedure, in which the enzymic digestion takes place in 0.1 *M* ammonium hydrogen carbonate solution. To avoid disturbances to the analysis by trichloroacetic acid or sodium acetate in the sample, the incubation with the enzyme was stopped by deproteinization with acetone. Experimental details of this method are as follows.

In digestion with the carboxypeptidases, the reaction mixtures contained 10–20 nmoles of peptide and in all instances 20 nmoles of norleucine were added as an internal standard. Carboxypeptidase A (E.C. 3.4.12.1) was an aqueous suspension

* Dedicated to Prof. E. Havinga on the occasion of his 70th birthday and his retirement from the chair of Organic Chemistry at Leiden University.

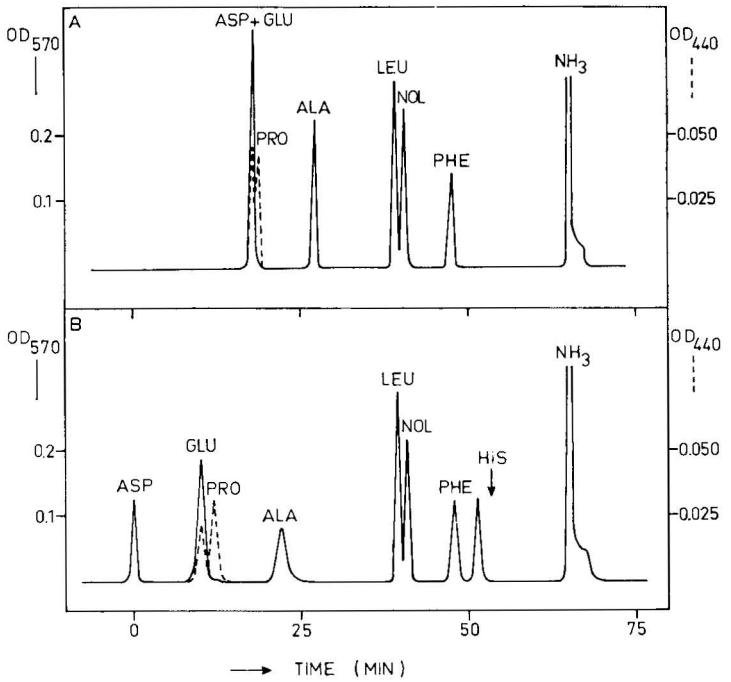


Fig. 1. Results of an aminopeptidase M digestion of the peptide Phe–Asp–Ala–Leu–Pro–Glu on a Chromaspek amino acid analyser. (A) Result with conventional procedure; (B) result with the proposed method. Experimental details are given in the text. The extra peak in B between phenylalanine and histidine is a result of the triethylamine treatment to remove excess of ammonia from the sample.

(21 mg/ml) and obtained from Sigma (St. Louis, Mo., U.S.A.). Carboxypeptidase B (E.C. 3.4.12.3) was a suspension (2 mg/ml) in 0.1 *M* sodium chloride solution, also obtained from Sigma. All other reagents, except for triethylamine [Pierce, (Rockford, Ill., U.S.A.), "sequenal grade"], were of analytical-reagent grade and were obtained from Merck (Darmstadt, G.F.R.). Digestion was carried out as follows: 2 μl of a suspension of carboxypeptidase A and 10 μl of a suspension of carboxypeptidase B were added to a solution of 10–20 nmoles of peptide in 0.25 ml of 0.2 *M* ammonium hydrogen carbonate buffer (pH 8.5). The mixtures were incubated at 37° for 2–24 h, after which they were placed in ice and 0.75 ml of acetone was added. They were thoroughly mixed and left in ice for at least 1 h. Centrifugation of the samples was carried out for 10 min at 9000 *g* in the cold and a clear supernatant appeared. The supernatant was freed of acetone in a stream of nitrogen and dried *in vacuo*, after which 4% (v/v) triethylamine was added and the sample was dried again. This procedure was repeated once more with pure triethylamine to remove all of the ammonia. Finally, the sample was taken up in 200 μl of 0.025 *M* hydrochloric acid and applied to the Chromaspek instrument for amino acid analysis by means of the citrate–borate buffer gradient system[5].

Aminopeptidase M (E.C. 3.4.11.2) was a crystalline suspension in a solution containing 3.2 *M* ammonium sulphate and 10 m*M* magnesium chloride (pH 6.0) and was obtained from Boehringer (Mannheim, G.F.R.). Digestion was carried out by

adding 20 μl of the enzyme suspension to a solution of 10–20 nmoles of peptide in 0.2 ml of 0.1 *M* ammonium hydrogen carbonate buffer (pH 8.5). Incubation took place at 37° for 24 h, after which the samples were prepared for amino acid analysis as described for the carboxypeptidase digestions.

In control experiments using a standard calibration mixture the recoveries of different amino acids in this deproteinization procedure were tested. From Table II, it is clear that the recoveries are very good: amino acids seem not to be trapped in the precipitate. In another control experiment it was established that 42 μg of carboxypeptidase A and 20 μg of carboxypeptidase B could be precipitated in the final volume (1.0 ml) of 75% (v/v) acetone solution to the extent of more than 99%. To avoid a disturbance of the chromatogram in the region of arginine, the excess of ammonia in the samples was removed by repeated addition of triethylamine and drying *in vacuo*.

TABLE I

SOME EXAMPLES OF ENZYMIC DIGESTIONS WITH CARBOXYPEPTIDASE A AND B OR AMINOPEPTIDASE M

Enzyme	*Substrate*	*Amino acids released (residues/molecule)*
Carboxypeptidase A and B	N-Acetyl-Ser–Ser–Ser–Gln–Lys	Lys (0.7)
	Lys–Ala–Gln–Leu–Pro–Lys–Pro–Pro–Ala–Leu–Lys	Lys (1.0), Leu (1.0), Ala (<0.1)
Aminopeptidase M	Met–Ala–Ser	Met (1.0), Ala (1.0), Ser (1.0)
	Phe–Asp–Ala–Leu–Pro–Glu	Phe (0.9), Asp (0.7), Ala (0.9) Leu (1.2), Pro (1.0), Glu (1.1)

TABLE II

RECOVERY OF AMINO ACIDS AFTER ACETONE PRECIPITATION

A standard calibration mixture, containing 10 nmoles of each amino acid, was taken up in 0.2 ml of 0.1 *M* ammonium hydrogen carbonate buffer (pH 8.5) containing 20 μl of enzyme suspension. The mixture was placed in ice and 0.6 ml of acetone was added. Amino acid analysis of the supernatant was performed as described in the text.

Amino acid	*Recovery (%)**	*Amino acid*	*Recovery (%)**
Aspartic acid	97	Methionine	100
Threonine	100	Isoleucine	99
Serine	104	Leucine	95
Glutamic acid	105	Norleucine	100
Proline	98	Tyrosine	103
Glycine	100	Phenylalanine	95
Alanine	103	Histidine	100
Cystine (half)	102	Lysine	98
Valine	99	Arginine	97

* Mean values of triplicate experiments.

As a result of the triethylamine treatment an extra peak was seen in the chromatogram in a position between phenylalanine and histidine; this was not investigated further, however, because it did not interfere with the analysis.

Some examples of enzymic digestions carried out according to this new method are listed in Table I. Although digestion with aminopeptidase M in an

ammonium hydrogen carbonate buffer has been reported before[6], this procedure is not very commonly used.

We found that in this buffer the enzyme can hydrolyse peptides very efficiently (Table I). In the fragment Phe–Asp–Ala–Leu–Pro–Glu even proline is split off quantitatively. A typical example of a Chromaspek run of a peptide hydrolysed by aminopeptidase M by means of the new procedure is shown in Fig. 1B. Throughout the chromatogram the separation of amino acids is satisfactory and allows a straightforward determination of the result. In a number of duplicate experiments the results were essentially the same.

In conclusion, the proposed micro-scale method of analysis of an enzymic hydrolysate is convenient and reproducible. Digestion in a volatile buffer and deproteinization of the samples by acetone precipitation allows an easy interpretation of the results obtained on a gradient-operated high-sensitivity instrument such as the Chromaspek amino acid analyser.

REFERENCES

1 R. P. Ambler, *Methods Enzymol.*, 25 (1972) 262.
2 A. Light, *Methods Enzymol.*, 25 (1972) 253.
3 B. Kraal, J. M. de Graaf, T. A. Bakker, G. M. A. van Beynum, M. Goedhart and L. Bosch, *Eur. J. Biochem.*, 28 (1972) 20.
4 A. J. Thomas, in A. Baillie and R. J. Gilbert (Editors), *Microbiology of Applied Bacteriology*, Vol. 4, Academic Press, London, 1970, p. 107.
5 C. Murren, D. Stelling and G. Felstead, *J. Chromatogr.*, 115 (1975) 236.
6 D. Wouters-Tyrou, P. Sautière and G. Biserte, *FEBS Lett.*, 65 (1976) 225.

Journal of Chromatography, 174 (1979) 478–482

CHROM. 11,818

Note

Auftrennung und Remissionsmessung *in situ* von Flavonoid-Aglykonen auf Hochleistungsdünnschichtchromatographie-Fertigplatten RP-18 $F_{254}S$

A. HIERMANN

Institut für Pharmakognosie der Universität Graz, Universitätsplatz 4/I, A-8010 Graz (Österreich)

(Eingegangen am 27. Februar 1979)

Die Auftrennung von Flavonoiden mittels Dünnschichtchromatographie (TLC) erfolgt im allgemeinen auf Cellulose-, Polyamid- oder Kieselgelschichten. In diesem Zusammenhang ist es oft von Interesse eine qualitative und quantitative Analyse der aufgetrennten Flavonoide unmittelbar auf der Sorptionsschicht durchzuführen. Zu diesem Zweck werden die Absorptions- oder (nach entsprechender Detektion) die Fluoreszenzspektren *in situ* aufgenommen[1,2]. Als Sorbentien für die Absorptionsmessung eignen sich vor allem Cellulose und Polyamid. Die auf normalen Kieselgelgeschichten aufgetrennten Flavonoide zeigen sehr abgeflachte und bathochrom verschobene Absorptionsspektren. Dies dürfte auf eine Chelatbildung der Flavonoide mit anwesenden Metallsalzen zurückzuführen sein. Nur bei Verwendung der Hochleistungsdünnschichtchromatographie (HPTLC)-Fertigplatten Kieselgel 60 konnten einwandfreie, deutlicht ausgeprägte Absorptionsbanden erhalten werden[3].

In der vorliegenden Arbeit wird die Auftrennung sowie die qualitative und quantitative Analyse mittels Remissionsmessung auf HPTLC-Fertigplatten RP-18 $F_{254}S$ beschrieben.

EXPERIMENTELLES

TLC-Auftrennung

Sorptionsschicht: HPTLC-Fertigplatten RP-18 $F_{254}S$ (10 × 10 cm; Merck, Darmstadt, B.R.D.). Auftragen: Mikrokapillare bis max. 1 μl (1 cm vom unteren Plattenrand, seitlich 0.5 cm). Fliessmittel: FG 1 (für wenig methylierte und nicht methylierte Flavonoid-Aglykone); Methanol–Wasser–Ameisensäure–(28:10:5); Laufstrecke 8 cm. FG 2 (für höher methylierte Flavonoid-Aglykone); Methanol–Wasser–Ameisensäure (28:4:5). Detektion; UV-254 und Naturstoffreagens (1 % in Methanol) Betrachten im UV-360.

In Tabelle I sind die R_F-Werte der untersuchten Flavonoid-Aglykone aufgezeigt.

Remissionsmessung in situ

Qualitative Auswertung. Für die qualitative Analyse der aufgetrennten Flavonoid-Aglykone werden nach Vertreiben des Fliessmittels die Absorptionsspektren mit einem Spektralphotometer (Zeiss PMQ 3) im Bereich von 500–200 nm aufgenommen (Parameter siehe *Quantitative Auswertung*). Die untere Nachweisgrenze liegt bei etwa

TABELLE I

R_F-WERTE UND ABSORPTIONSMAXIMA VON FLAVONOID-AGLYKONEN AUF HPTLC-FERTIGPLATTEN RP-18 $F_{254}S$

FG 1 = Methanol–Wasser–Ameisensäure (28:10:5), FG 2 = Methanol–Wasser Ameisensäure (28:4:5).

Flavonoid-Aglykone	*R_F-Werte*		*Absorption in Methanol*		*Absorption in situ*	
	FG 1	*FG 2*	*Bande I*	*Bande II*	*Bande I*	*Bande II*
Flavone						
Luteolin	0.66	0.77	349	253	351	266
Apigenin	0.59	0.70	336	268	340	271
Nepetin	0.52	0.80	346	273	350	270
Chrysoeriol	0.52	0.81	347	269	348	271
Dinatin	0.49	0.71	335	274	340	276
Diosmetin	0.48	0.70	344	267	346	270
Jaceosidin	0.47	0.71	342	275	349	276
Digicitrin	0.37	0.63	336	281	345	285
Chrysin	0.35	0.54	313	268	327	270
Acacetin	0.26	0.49	327	269	342	265
Pectolinaringenin	0.23	0.49	332	276	340	271
Flavon	0.22	0.44	294	250	315	255
Apigenin 5,7,4′-OCH_3	0.08	0.33	333	270	344	270
Flavonole						
Quercetagetin	0.99	0.99	375	264	372	270
Myricetin	0.84	0.92	374	254	372	270
Fisetin	0.73	0.88	363	248	365	265
Morin	0.73	0.91	370	264	372	271
Quercetin	0.71	0.82	370	256	376	261
Kämpferol-7,4′-OCH_3	0.56	0.25	367	270	380	270
Kämpferol-3-OCH_3	0.54	0.73	351	270	358	269
Kämpferol	0.52	0.91	367	266	369	276
Quercetin-3,3′-OCH_3	0.51	0.72	355	256	361	260
Rhamnetin	0.42	0.68	371	256	377	265
Galangin	0.30	0.59	359	267	367	276
Kämpferol-7-OCH_3	0.28	0.53	368	269	375	271
Myricetin-7,3′,4′-OCH_3	0.26	0.53	371	264	372	270
Kämpferol-4′-OCH_3	0.25	0.51	367	267	375	269
3-Hydroxyflavon	0.19	0.40	344	239	353	246
Kämpferol-3,7,4′-OCH_3	0.11	0.33	350	270	362	260
Quercetin-7,3′,4′-OCH_3	0.09	0.31	370	256	376	260
Flavanone						
Naringenin	0.76	0.85	326	289	330	295
Hesperetin	0.71	0.84	326	282	—	302
Dihydroflavonole						
Dihydroquercetin	0.94	0.96	340	293	—	296
Dihydrofisetin	0.94	0.96	310	278	317	286

50 ng. In Fig. 1 sind die Absorptionsspektren von verschiedenen Flavonoidtypen (Flavon, Flavonol, Flavanon und Dihydroflavonol) aufgezeigt.

Quantitative Auswertung. Für die quantitative Analyse wird die Flavonoidlösung mittels einer Mikrokapillare mit Festvolumen 0.1 µl aufgetragen (siehe *TLC-Auftren-*

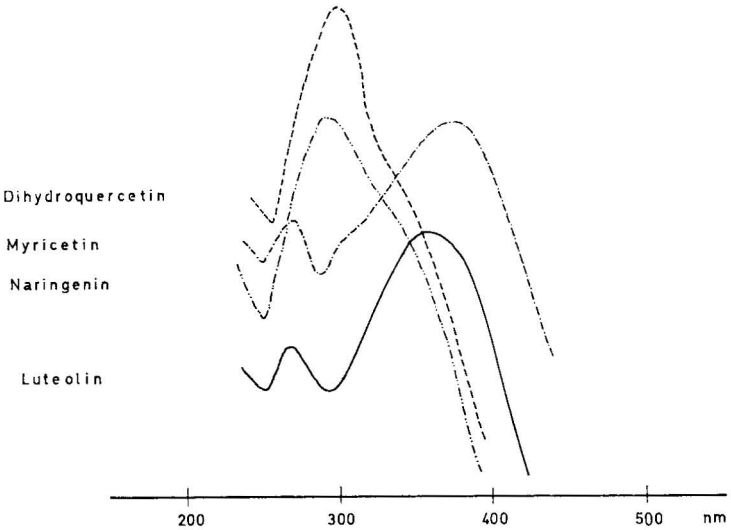


Fig. 1. Remissionsspektren von Flavonoid-Aglykonen auf RP-18-Schicht.

nung). Nach dem Entwickeln des Chromatogrammes wird das Fliessmittelgemisch im Luftstrom vertrieben und die Absorption der zu bestimmenden Flavonoid-Aglykone bei der entsprechenden Wellenlänge (siehe Absorptionsmaxima in Tabelle I) in Remission gemessen. Werden mehrere Flavonoid-Aglykone auf einer Laufbahn in einem Scan quantitativ erfasst, so wird die Absorption bei einer mittleren Wellenlänge von 360 nm (Flavone und Flavonole und 290 nm (Flavanone und Dihydroflavonole) gemessen. Die untere Nachweisgrenze liegt bei etwa 5 ng. Der Durchschnitt der mittleren Standardabweichung beträgt $\pm$ 2.6%.

Parameter: Spektralphotometer Zeiss PMQ 3; Spaltbreite 6 mm, Monochromatorspalt 0.5 mm, Dämpfung 3, Servogor S-Schreiber, Spreizung 20 mV, qualitativ 50 mV.

ERGEBNISSE UND DISKUSSION

Zur Auftrennung von Flavonoiden auf Reversed-Phase (RP) HPTLC-Schichten haben sich als Fliessmittel vor allem Methanol–Wasser-Gemische geeignet. Wie aus Fig. 2 zu ersehen ist, können die Trennbedingungen durch Erhöhung bzw. Erniedrigung des Wasseranteiles beim Fliessmittelsystem Methanol–Wasser–Ameisensäure wesentlich beeinflusst werden. Um eine Schwanzbildung bei der TLC-Auftrennung weitestgehend zu vermeiden, ist der Zusatz von Ameisensäure erforderlich. Es ist dabei zweckmässig, höher methylierte Aglykone mit dem Fliessmittelgemisch FG 1 Methanol–Wasser–Ameisensäure (28:4:5) und wenig methylierte sowie nicht methylierte Aglykone mit dem Fliessmittelgemisch FG 2 Methanol–Wasser–Ameisensäre (28:10:5) aufzutrennen. Das TLC Verhalten der Flavonoid-Aglykone entspricht dabei im wesentlichen den allgemeinen Kriterien der Reversed-Phase Chromatographie.

Für eine generelle TLC-Auftrennung von Flavonoid-Glykosiden scheint uns dagegen dieses lipophile Sorptionsmittel nicht besonders geeignet, da der Wasseranteil des Fliessmittels so weit erhöht werden müsste, dass die Benetzbarkeit der hydropho-

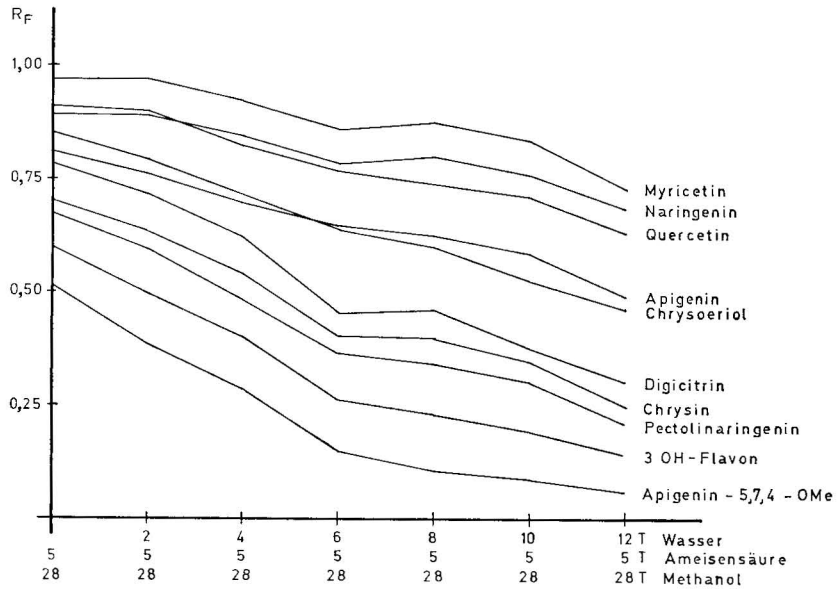


Fig. 2. Einfluss des Wasseranteiles auf die Trennung von Flavonoid-Aglykonen auf RP-18-Schicht.

ben RP-Schicht überschritten wird. Für die Identifizierung bzw. qualitativen Analyse wurden die Absorptionsspektren in Remission aufgenommen.

Die Absorptionsmaxima der von uns untersuchten Flavonoid-Aglykone sind in Tabelle I aufgezeigt. Interessant erscheint dabei, dass die Lage der Absorptionsbanden strukturbedingten Gesetzmässigkeiten unterliegt, wie sie von Mabry *et al.*[4] bei der UV-spektroskopischen Analyse der in Methanol aufgenommenen Spektren beschrieben sind. Bei den in Remission auf RP-Schichten aufgenommenen Absorptionsspektren sind die Maxima gegenüber den in Methanol aufgenommenen nur geringfügig bathochrom verschoben. Die Absorptionsmaxima der uns zur Verfügung stehenden Flavonoid-Aglykone lagen bei *Flavonen*, Bande I, bei 315–351 nm (in Methanol bei 304–350 nm), bei *Flavonolen*, Bande I, bei 353–385 nm (in Methanol bei 352–384 nm) (bei nicht substituierter 3-OH-Gruppe) und bei *Flavanonen* und *Dihydroflavonolen*, Bande II, bei 287–296 nm (in Methanol bei 270–295 nm).

Aus den Ergebnissen der vorliegenden Arbeit ist ersichtlich, dass auch lipophile Sorptionsschichten für die Auftrennung von Flavonoid-Aglykonen geeignet sind. Die TLC-Untersuchungen wurden sowohl an Gemischen von Reinsubstanzen als auch an Flavonoid-führenden Pflanzenextrakten durchgeführt (*Crataegus*, *Digitalis*, *Aesculus* und *Convallaria*). Lipophile Begleitstoffe wie Chlorophylle, Lipide, Carotinoide etc. verbleiben grösstenteils an der Startlinie. Hydrophile Begleitstoffe wie Gerbstoffe, Pflanzensäuren etc. wandern an der Front. Die aufgetrennten Flavonoid-Aglykone konnten durch Remissionsmessung *in situ* qualitativ und quantitativ analysiert werden.

DANK

Für die Überlassung von Kämpferol-7-OCH_3, -7,4'-OCH_3 und -3,7,4'-OCH_3,

Quercetin-3,3′-OCH_3 und -7,3′,4′-OCH_3, und Myricetin-7,3′,4′-OCH_3 danke ich Herrn Dozent E. Wollenweber, Botanisches Institut der Technischen Hochschule Darmstadt herzlich.

LITERATUR

1 P. Spiegel, Ch. Dittrich und K. Jentzsch, *Sci. Pharm.*, 44 (1976) 126.
2 P. P. S. Schmid, *J. Chromatogr.*, 157 (1978) 217.
3 A. Hiermann und Th. Kartnig, *J. Chromatogr.*, 140 (1977) 322.
4 T. J. Mabry, K. R. Markham und M. B. Thomas, *The Systematic Identification of Flavonoids*, Springer, New York, Heidelberg, Berlin, 1970, S. 33–249.

Journal of Chromatography, 174 (1979) 483–487

CHROM. 11,817

Note

Analytical utility of 2-halopyridinium salts

I. Paper electrophoretic characterization of thiols as 2-alkyl(aryl)thio-1-methylpyridinium *p*-toluenesulphonates

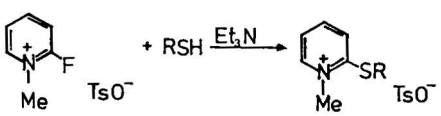
EDWARD BALD

Department of Chemical Technology, Lodz University, 18 Nowotki Street, 91-416 Lodz (Poland)

(Received March 1st, 1979)

In recent years, a new type of dehydration condensation reaction utilizing 2-halopyridinium salts has been developed by Mukaiyama's group (*e.g.*, refs. 1–3). This reaction has a wide range of applications; it is suitable, for example, for the synthesis of esters[1], amides[2] and thiol esters[3]. It appeared to the author that 2-fluoro-1-methylpyridinium *p*-toluenesulphonate could be a promising reagent for the derivatization of thiols subjected to electrophoretic separation, and results are reported here that support this suggestion.

2-Alkyl(aryl)thio-1-methylpyridinium *p*-toluenesulphonates (3) (Fig. 1) were produced easily and rapidly by a nucleophilic attack of thiols (2) on 2-fluoro-1-methylpyridinium *p*-toluenesulphate (1) in the presence of triethylamine. The reaction was carried out in various solvents such as acetonitrile, chloroform, benzene, ethanol and water and no substantial solvent effect was observed. Based on the above considerations, several reactions of thiols and pyridinium salt (1) were carried out, and corresponding derivatives (3) were obtained. The derivatives (3) were separated by high-voltage paper electrophoresis followed by detection with iodine vapour.



1 2 3

Fig. 1. General reaction of thiols and 2-fluoro-1-methylpyridinium *p*-toluenesulphonate.

Few electrophoretic separations of thiols have been described, apart from mostly thiols that have other ionizable functions in the same molecule. Schrauwen[4] referred to the separation of thiols by paper electrophoresis at pH 6, using acetate buffer, at 220 V. His work was concerned primarily with the detection procedure, which he performed with 1-(4-acetoxymercuriphenylazo)-2-naphthol. Wroński[5] described the high-voltage paper electrophoretic separation of thiols, disulphides and

other sulphur-containing compounds. He used *o*-hydroxymercuribenzoic acid and dithiofluorescein as detection reagents.

The electrophoretic separation of simple thiols does not appear to have been described previously. The procedure reported here provides a useful complementary method to chromatographic techniques (for a comprehensive survey, see ref. 6), which normally require some preliminary separation. The derivatization procedure is simple and the equipment involved is simple and inexpensive.

EXPERIMENTAL

Chemicals

2-Fluoro-1-methylpyridinium *p*-toluenesulphonate was prepared in this laboratory. To 2-fluoropyridine (4.85 g, 50 mmole) was added methyl *p*-toluenesulphonate (9.4 g, 50 mmole) and the mixture was stirred for 3 h followed by heating (oil-bath, 80°) for a further 3 h. A precipitate appeared, which was filtered off and washed with dry diethyl ether (25 ml). After drying under reduced pressure, 2-fluoro-1-methylpyridinium *p*-toluenesulphonate (13.9 g, 92%) was obtained and used for the derivatization of thiols without recrystallization. The 2-fluoropyridine and methyl *p*-toluenesulphonate used (Fluka, Buchs, Switzerland) were of synthetic grade.

n-Hexanethiol, *n*-nonanethiol and benzylmercaptan were synthesized in this laboratory from the corresponding iodides according to the literature[7] and purified by double distillation. All other thiols were of analytical-reagent grade from Fluka, Merck (Darmstadt, G.F.R.) or Aldrich Europe (Beerse, Belgium).

Triethylamine (Loba Chemie, Vienna, Austria) was redistilled before use.

AnalaR-grade solvents were used without drying.

Solutions

Buffer solution of pH 7.6 consisted of an aqueous solution of 0.5 mole/l boric acid 0.1 mole/l diethanolamine and 0.025 mole/l disodium salt of ethylene-diamine-tetraacetic acid (EDTA).

Other solutions were a 0.1 *M* solution of 2-fluoro-1-methylpyridinium *p*-toluenesulphonate in acetonitrile and a 0.1 *M* solution of triethylamine in acetonitrile.

Sample derivatization procedure

A 0.1-ml volume of sample solution in the appropriate solvent (or neat) containing not less then 50 nmole of the thiol was pipetted into a small glass test-tube, then 0.1 ml of 2-fluoro-1-methylpyridinium *p*-toluenesulphonate solution and 0.1 ml of triethylamine solution were added*. After shaking the tube and diluting with buffer, the sample was ready for electrophoretic separation.

Electrophoresis procedure

The apparatus for high-voltage electrophoresis (type AEA; WSR-Olsztyn, Poland) was set up in accordance with the manufacturer's instruction. Whatman No. 1 chromatography paper (12 × 37 cm) was impregnated with buffer solution by immersion

* If the sensitivity limit was to be determined, 5 μl of each solution were applied with a Hamilton microdispenser.

in a trough containing the boric acid–diethanolamine–EDTA buffer and excess of buffer solution was removed by light blotting with filter-paper. Sample solutions were applied across the moist paper, 5 cm from the edge, as 5-μl spots using a microsyringe. The safety case of the electrophoresis unit was then closed and a potential of 3000 V applied for 30 min. The paper was removed and the compounds were made visible with iodine vapour as violet-brown compact spots on a white background. All spots were 0.9 $\pm$ 0.1 cm in diameter.

RESULTS

The results show that is possible to correlate migration distance with the carbon number of the *n*-alkyl group for series of C_1–C_{12} thiols.

Fig. 2 shows the electrophoretic migration pattern of the 2-alkylthio-1-methylpyridinium cations obtained for the series of *n*-alkylthiols when examined under the standard conditions described above. Standard mixtures of the thiol derivatives were applied on either side of the paper in order to show that uniform migration occurred across the paper.

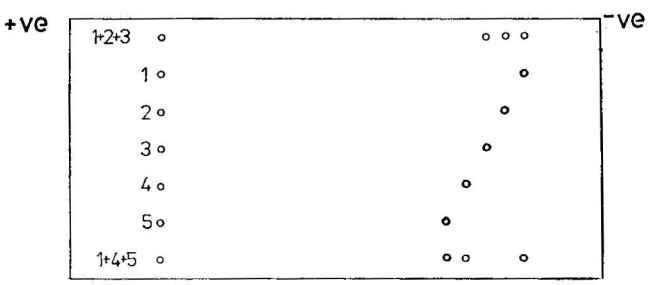


Fig. 2. Migration patterns of thiols separated as 2-alkylthio-1-methylpyridinium *p*-toluenesulphonates. 1 = Methyl; 2 = ethyl; 3 = *n*-propyl; 4 = *n*-butyl; 5 = *n*-pentyl.

Fig. 3 shows the linear relationship between migration distance and carbon number of the *n*-alkyl group for the series of C_1–C_{12} thiols. The aryl series also shows a linear relationship although the migration distances of the cations were smaller.

The relative mobilities and the sensitivity limits of 2-alkyl (aryl)thio-1-methylpyridinium cations are listed in Table I. The relative mobility data are average values of duplicate measurements, and in no instance was the variation between duplicates greater than 4.

Stability of thiol derivatives

The stability of 2-alkyl(aryl)thio-1-methylpyridinium *p*-toluenesulphonates diluted with buffer was followed for 2 weeks and no changes were noticed.

CONCLUSIONS

The method described appears to be a general procedure for the rapid separation of thiols. The method has several advantages: (a) it can be used for

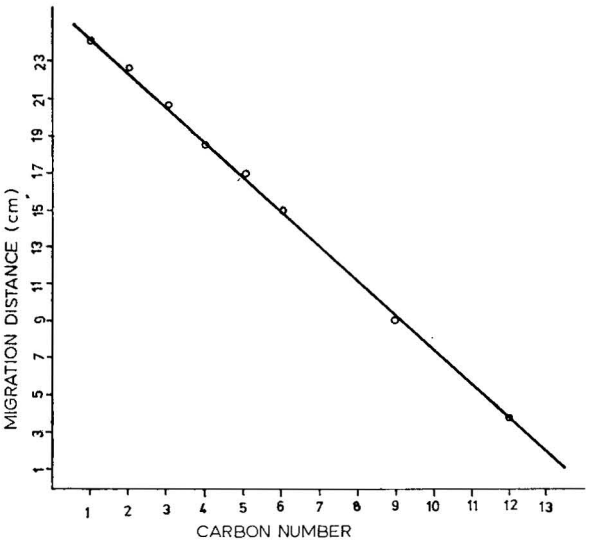


Fig. 3. Relationship between migration distance and carbon number.

TABLE I

CHARACTERIZATION OF THIOLS AS 2-ALKYL(ARYL)THIO-1-METHYLPYRIDINIUM *p*-TOLUENESULPHONATES

Run	*R in thiol*	*Derived cation (Mm × 100)* *	*Sensitivity limit (pmole)* **
1	Methyl	100	1000
2	Ethyl	93	750
3	*n*-Propyl	87	500
4	Isopropyl	88	500
5	*n*-Butyl	76	500
6	Isobutyl	76	500
7	*tert.*-Butyl	79	500
8	*n*-Amyl	71	500
9	Isoamyl	70	500
10	*n*-Hexyl	62	500
11	Cyclohexyl	94	1000
12	*n*-Nonyl	37	500
13	*n*-Dodecyl	16	500
14	Benzyl	64	250
15	Phenyl	71	500
16	2-Pyridyl	75	1000
17	*p*-Cresyl	63	750
18	2-Naphthyl	45	750
19	Allyl	87	250
20	Diethyl dithiophosphate	19	500

* *Mm* values express mobilities relative to the 2-methylthio-1-methylpyridinium ion, which moved approximately 23 cm.

** Sensitivity limit data are, in fact, the combined effect of two procedures, formation of the cation and detection.

the rapid characterization of the thiol compositions in wide range of solvents; (b) it works also with moist samples; (c) it involves readily available reagents and simple and inexpensive equipment; (d) it has a high sensitivity.

Electrophoresis, however, possesses certain disadvantages: (a) it is possible for mixtures containing alkyl, aryl and other thiols to have similar electrophoretic mobilities and thus be indistinguishable; (b) difficulty is experienced with mixtures of isomeric alkyl derivatives, except *tert.*-butyl (see Table I).

Further analytically useful applications of 2-halopyridinium salts are currently being explored.

REFERENCES

1 T. Mukaiyama, M. Usui, E. Shimada and K. Saigo, *Chem. Lett.*, (1975) 1045.
2 E. Bald, K. Saigo and T. Mukaiyama, *Chem. Lett.*, (1975) 1163.
3 Y. Watanabe, S. Shoda and T. Mukaiyama, *Chem. Lett.*, (1976) 741.
4 J. A. M. Schrauwen, *J. Chromatogr.*, 10 (1963) 113.
5 M. Wroński, *Z. Anal. Chem.*, 264 (1973) 406.
6 M. R. F. Ashworth, *The Determination of Sylphur-Containing Groups, Vol. II, Analytical Methods for Thiol Groups*, Academic Press, London, 1976.
7 A. I. Vogel, *A Text-Book of Practical Organic Chemistry*, Longmans, London, 1961.

Journal of Chromatography, 174 (1979) 488–489

CHROM. 11,819

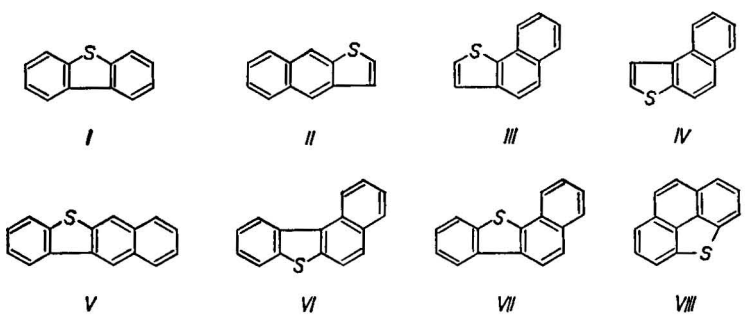
Letter to the Editor

Contamination of some polynuclear aromatic standards

Sir,

In a recent letter[1] there was noted, in connection with the testing of a flame photometric detector (FPD)[2], an unpleasant contamination of some polynuclear aromatic standards, particularly of phenanthrene, with unknown minor compounds which were eluted with, or close to, the major components and responded strongly in the FPD. The impurities were assumed to be sulphur-containing polynuclear aromatics of appropriate volatility. Being engaged in studies on the composition and analysis of coal-tar products, we can support this assumption and describe some of the contaminants.

On the basis of a survey of coal-tar compounds arranged according to their boiling points[3] and taking into account further data[4–6], it is possible, by analogy with the known contamination of benzene with thiophene and of naphthalene with benzo[*b*]thiophene, to postulate the contamination of phenanthrene (b.p. 338°) and anthracene (b.p. 340°) with dibenzo[*b*,*d*]thiophene (I, b.p. 331°), naphtho[2,3-*b*]-thiophene (II, b.p. 335°), naphtho[1,2-*b*]thiophene (III, b.p. 330°) and naphtho-[2,1-*b*]thiophene (IV, b.p. 330°). The sulphur impurities in benz[*a*]anthracene (b.p. 438°) should be benzo[*b*]naphtho[2,3-*d*]thiophene (V, b.p. 440°), benzo[*b*]baphtho-[1,2-*d*]thiophene (VI, b.p. 440°) and benzo[*b*]naphtho[2,1-*d*]thiophene (VII, b.p. 430°). Pyrene should be contaminated with phenanthro[4,5-*bcd*]thiophene (VIII).



In this Institute we have carried out gas chromatographic (GC) analyses of phenanthrene and anthracene, and in both hydrocarbons we found compounds I and II; compound I dominated in phenanthrene and compound II in anthracene. The full data will be given in a paper dealing with GC analyses of coal-tar products on selective packed columns, which is in preparation.

NOTE ADDED IN PROOF

On the basis of a recent paper on the separation and identification of polynuclear aromatics in coal tar[7] it is also possible to suppose that the dibenzophenanthrene and dibenzanthracene standards are contaminated with dinaphthothiophenes.

Research Institute for Coal Tar Chemistry,
Urxovy závody, n.p.,
757 27 Valašské Meziříčí
(Czechoslovakia)

JOSEF FRYČKA

1 W. A. Aue and C. G. Flinn, *J. Chromatogr.*, 153 (1978) 305.
2 W. A. Aue and C. G. Flinn, *J. Chromatogr.*, 142 (1977) 145.
3 K. F. Lang and I. Eigen, *Fortschr. Chem. Forsch.*, 8 (1967) 91.
4 G. Grimmer and A. Glaser, *Erdoel Kohle, Erdgas, Petrochem.*, 28 (1975) 570.
5 G. Collin and H. Köhler, *Erdoel Kohle, Erdgas, Petrochem.*, 30 (1977) 257.
6 H. Borwitzky, D. Henneberg, G. Schomburg, H. D. Sauerland and M. Zander, *Erdoel Kohle, Erdgas, Petrochem.*, 30 (1977) 370.
7 H. Borwitzky and G. Schomburg, *J. Chromatogr.*, 170 (1979) 99.

(Received February 26th, 1979)

Journal of Chromatography, 174 (1979) 490–491

CHROM. 11,804

Letter to the Editor

Determination of sulphapyridine and its N^4-acetyl metabolite in plasma using liquid chromatography

Sir,

Recently, a method for the determination of sulphapyridine using a system based on silica gel was published[1]. When the column packing used for the determination of sulphapyridine had to be replaced with a new batch of silica, it was not possible to obtain a chromatogram showing the same degree of separation as before (several batches of silica were tried). The conclusion must be drawn that the batch of silica used initially could not have been pure. To be able to continue the analysis of sulphapyridine it was necessary to change the eluent to chloroform–acetonitrile–methanol–35% ammonia (65.5:30:4:0.5) at a flow-rate of 1 ml/min. In other respects the chromatographic system and extraction method remained unchanged. This new solvent system made it possible to effect a simultaneous determination of sulphapyridine and its N^4-acetyl metabolite. Fig. 1 shows typical chromatograms.

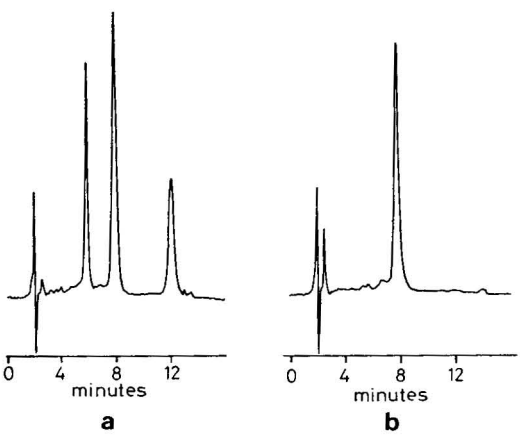


Fig. 1. Chromatograms of (a) plasma sample containing 10.5 μg/ml of sulphapyridine, 16.8 μg/ml of N^4-acetylsulphapyridine and internal standard (sulphamethazine) and (b) a blank plasma sample containing only internal standard. The retention times for sulphapyridine, sulphamethazine and N^4-acetylsulphapyridine were 5.6, 7.2 and 11.7 min, respectively.

When a plasma sample was analysed several times according to the method, the recovery of sulphapyridine sometimes varied considerably. The internal standard (sulphamethazine) behaved in the same manner, *i.e.*, the ratio between the two peaks resulting from sulphapyridine and sulphamethazine remained the same during the

repeated analyses. The metabolite, N^4-acetylsulphapyridine, did not show this variation. This difference may be due to varying adsorption losses caused by the primary amino groups in sulphapyridine and sulphamethazine and slight variations in the work-up procedure.

A calibration graph was constructed by analysing according to the above method samples to which had been added known amounts of sulphapyridine and plotting the peak-height or -area ratios of sulphapyridine to internal standard against concentration. To the samples had also been added different amounts of N^4-acetylsulphapyridine and the calibration graph for the determination of this substance was constructed by plotting the resulting peak heights or areas against concentration. The standard deviation obtained in the analysis of ten samples to which had been added 15 μg/ml of sulphapyridine was 2.7% and that for ten samples containing 15 μg/ml of N^4-acetylsulphapyridine was 2.5%.

National Board of Health and Welfare,
Department of Drugs, Box 607, 751 25 Uppsala (Sweden)

KERSTIN LANBECK
BJÖRN LINDSTRÖM

1 K. Lanbeck and B. Lindström, *J. Chromatogr.*, 154 (1978) 321.

(First received December 27th, 1978; revised manuscript received February 19th, 1979).

Author Index

Errata

J. Chromatogr., 170 (1979) 446–448

Page 448, legend to Fig. 2 should read: Fig. 2. The right-hand gel, containing bovine serum albumin, was covered with white complexes over the whole surface when it was incubated with tannic acid and subsequently with quinine after electrophoresis. The left-hand gel was stained with 1% Amido Black 10B in 7% acetic acid for 2 h and swollen about 10%. The amount of the protein applied to each gel was 44 μg.

J. Chromatogr., 172 (1979) 207–219

Page 209, *Interferons* section, 4th line: "1.4×10^5" should read "1.4×10^8".

J. Chromatogr., 173 (1979) 175–181

Page 179, legend to Fig. 2, 7th line: "flow-rate of 10 ml/min" should read "flow-rate of 1.0 ml/min".

Vol. 174, No. 2

journal of chromatography news section

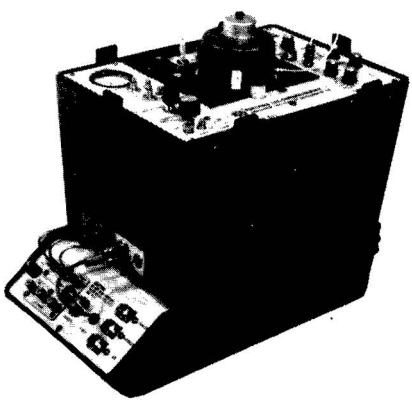
APPARATUS

N-1272

PORTABLE GAS CHROMATOGRAPH WITH PHOTOIONIZATION DETECTOR

Analytical Instrument Development has developed the Model 511-23 portable gas chromatograph system with a completely self-contained photoionization detector system. This system has an increased sensitivity for some materials of approximately 10 to 30 times that of the flame ionization detector, e.g. a sensitivity of 5 ppb for benzene.

The gas chromatograph has only one gas and the supporting electronics enclosed within the instrument; this makes the 511-23 very well suited for field measurements.

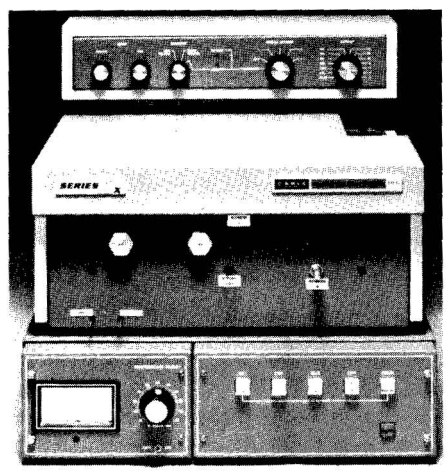


For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-1275

NEW GENERATION GAS CHROMATOGRAPHS

Carle Instruments has introduced the Series-Sx gas chromatographs. These instruments perform specific analyses automatically like the original Series-S instruments, but rather than employing a built-in valve programmer, the Series-Sx is controlled by the user's in-house computer or by a microprocessor-based integrator with external timed event capability.

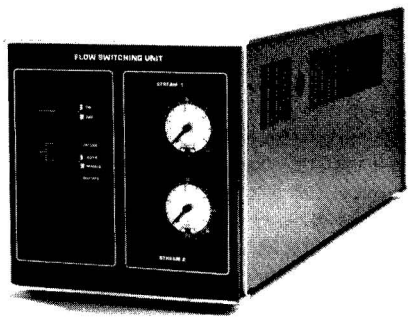


Both the new Series-Sx and the Series-S instruments will be offered in all 20 different application configurations presently available from Carle. The Series-Sx operation is based on automatic sample injection and column switching by motor-driven valves and is compatible with most of the currently available integrators and laboratory computers.

N-1283

NEW APPARATUS FOR GC

On the Model 204 gas chromatograph Pye Unicam, Cambridge, U.K., introduced the Flow Switching Unit. With this unit, which can be also used with other gas chromatographs, multi-dimensional gas chromatography is made easier.



The new unit enables analysis times to be cut by as much as 80% by back flushing unwanted components and recording only the required information. It prevents the accumulation of low volatility material which could otherwise affect column life and allows the heart cutting of trace components from major ones. The column switching technique makes time reductions possible by using the by-passing technique to switch groups of compounds from one column to another. With optional accessories, the multi-dimensional chromatograph can be operated automatically. Pye Unicam Ltd. also introduced a capillary version of the Model 204 gas chromatograph.

N-1281

INJECTION VALVES BROCHURE FROM SCHOEFFEL

In an 8-page brochure from the Schoeffel Instrument Division of Kratos the company's offering of high-pressure injection valves for liquid chromatography (products of Rheodyne) is detailed. A rotary valve for injection, column switching, recycling, etc. is also described. The injection valves include a high-pressure septumless syringe loading sample injector, with loops available from 10 to 100 µl. A full line of accessories is also described.

N-1259

CHROMATOGRAPHY DATA SYSTEM

An introduction to Perkin-Elmer's SIGMA 10 Chromatography Data Station is presented in a 20-page brochure now available from the manufacturer. Emphasized is the ease of programmability of the system using standard BASIC language, and its data handling features which allow it to control up to four chromatographs simultaneously. Several examples of the printer/plotter outputs are shown demonstrating the capabilities of the system. A large functional block diagram of the SIGMA 10 shows its capability of being interfaced with an auxiliary terminal, a larger computer system, or another gas or liquid chromatograph. Standard and special integrating features for baseline treatment are discussed. The brochure concludes with examples of user program procedures, illustrated with program listings and outputs.

MEETING

30th PITTSBURGH CONFERENCE ON ANALYTICAL CHEMISTRY AND APPLIED SPECTROSCOPY

The 30th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy was held this year from March 5 to March 10. This conference is organised annually by the Society for Analytical Chemistry and by the Spectroscopic Society of Pittsburgh.

At the 30th Pittsburgh Conference 739 papers were presented in 84 sessions, compared with 667 papers last year. Not only is the number of papers presented at the Conference increasing each year, but the exhibition area in the spacious Mall Exhibit Area of the Cleveland Convention Centre is also continually expanding.

The exhibition of modern laboratory equipment, which is traditionally organised during the Conference, required an increase of about 200 standard booths this year, and included some 390 instrument manufacturers and trading companies; 11% more than in 1978.

The theme of this 30th Pittsburgh Conference was "the continued growth in analytical chemistry and applied spectroscopy through expanded communication", which was underlined in the Conference program booklet by the statement made 200 years ago by Dr. Peter Tempelman, that "the surest method of improving science is by a generous intercourse of the learned in different countries and a free communication of knowledge".

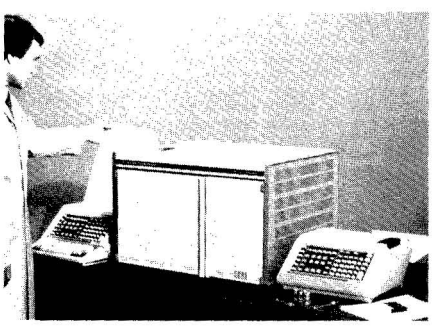
Nearly 15,000 conferees from all over the world attended the Pittsburgh Conference this year.

Once again, the Perkin-Elmer Corporation from Norwalk, Conn., occupied the largest booth on the exhibition floor (30 standard booth areas). In the field of chromatography Perkin–Elmer showed their new Series 3B liquid chromatograph, an improved version of the dual-pump, microprocessor-controlled Series 3 system.

New in the LC realm was also the Model LC-75 spectrophotometric detector. This complete new instrument with a sensitivity of 5×10^{-4} absorbance units replaces the Model 55 LC detector. The Model LC-75 Autocontrol Module combines electronic logic and positive linkage to lead the user step-by-step through difficult analyses. The results are presented digitally. Furthermore Perkin-Elmer offered a complete hardware and software gel permeation chromatography system, consisting of the Series 1 pump, the LC-100 oven, a selection of GPC columns and the SIGMA-10 data system.

The most striking news in the gas chromatography realm was the new HP 5880 series of gas chromatographic instrumentation from Hewlett-Packard's Avondale, Pa. Division. The 5880 Series is a complete program of gas chromatographs, ranging from a simple, small and relatively inexpensive routine instrument to a sophisticated GC system complete with a BASIC programmable data system. Also the Packard Instruments Corporation, Downers Grove, Ill., showed a new gas chromatograph, the top-of-the-line member of the Packard-Becker range of instruments. This Model 433 is, in fact, a Model 429 completed with a built-in digital integrator and a reporting printer/plotter.

Shimadzu Scientific Instruments, Inc., Columbia, Md., the local representatives of the Tokyo based Japanese company, showed their new microprocessor gas chromatograph GC-RIA, the new low-cost GC-7AG, and improved versions of GC detectors. The AEI division of Kratos Instruments showed the Model MS 25 GC–MS combination, equipped with a Pye Unicam Model 204 gas chromatograph. Du Pont Scientific Instruments, Wilmington, Del., presented the Model DP-102, a com-



The Perkin-Elmer Series 3B liquid chromatograph is a dual-pump microprocessor-controlled solvent delivery module (left).

Hewlett-Packard 5880 Series gas chromatographs feature flexibility in data handling and easy expansion of chromatographic capability (right).

bination of a gas chromatograph, a magnetic field mass spectrometer and a powerful data system. Finnigan Instruments, Sunnyvale, Calif., introduced their new low-cost GC–MS system, Model 1020, equipped with a Perkin-Elmer SIGMA-3 gas chromatograph.

Varian showed the new Model 212 GC–MS combination from their German factory in Bremen. The difference with their previous Model 112 is the large improvement in resolution, the so-called open connection interface for capillary-column GC work and the standard use of the Model 3700 gas chromatograph. A negative-ion chemical-ionization accessory should be available shortly.

Hewlett-Packard introduced the negative-ion chemical-ionization option for the 5985 GC–MS system.

In the LC field, Varian Associates showed the new LC 5000 system with a reciprocating pump, microprocessor control and video screen display.

Varian also introduced a hardware interface between the new 5000 HPLC and a 3700 gas chromatograph. The interface allows the user to stop the flow in the HPLC system, automatically inject a sample from the detected LC peak in the capillary 3700 system, wait until the GC analysis is completed, and start the HPLC flow again. This system is of restricted general importance, but nevertheless, offers an interesting new tool to the analyst in the chromatography laboratory.

The Micromeritics Instrument Corporation of Norcross, Ga., introduced the new Model 7500 HPLC system with microprocessor control, a newly designed dual-piston pump and the possibility of ternary gradient elution. Chromatix, Sunnyvale, Calif., showed their Model 600/200 HPLC pump and a GPC data system.

Hewlett-Packard's B version of the low-cost 1081 HPLC system was also new for the U.S. market. The Schoeffel division of Kratos Instruments, Westwood, N.J., exposed the Series 250 liquid chromatograph, the fluorometric detector FS 950 and the variable wavelength UV–VIS detector SF 740. Furthermore, Schoeffel showed the new SD-3000 spectrodensitometer for TLC applications.

Spectra-Physics' Autolab division, Santa Clara, Calif., showed their scanning UV–VIS detector for the SP-8000 HPLC system and the new single-channel computing integrator, SP 4100, with an alpha-numerical keyboard, a block of preprogrammed keys and a very fast printer/plotter.

The Dionex Corporation (formerly Durrum), Sunnyvale, Calif., showed an improved version of the ion chromatograph, Model 16, and a new automated ion chromatograph AutoIon System 12.

Tracor Instruments, Austin, Texas, exposed the improved versions of the Model 965 photoconductivity detector and of the Model 970A variable-wavelength detector.

Laboratory Data Control, Riviera Beach, Fla., introduced a microprocessor-controlled chromatographic control module equipped with a video tube. ChemResearch (formerly ISCO), Lincoln, Nebr., showed the series 2000 HPLC and the Model 1560 automatic sampler for HPLC.

Waters Associates, Milford, Mass., exposed their WIIP, the Waters Intelligent Information Processor, equipped with a video tube, the new Waters Data Module: a computing integrator with a printer/plotter. Also was new the low-cost M-45 pumping system, the high-temperature (150°C) HPLC/GPC system 150C and the radial compression system for the preparation of HPLC columns with the Radial-Pak cartridges and the RCM-100 Radial Compression Module.

This year's Pittsburgh Conference revealed a continued trend towards the use of microprocessors in all kinds of laboratory equipment. Instrument developmental groups in the instrumentation industry are progressively trying to provide the user of the microprocessor-controlled instrument with more of the useful capabilities of the magic chip.

The new programmable instruments showed are mostly programmable in BASIC by means of an alpha-numerical keyboard. Besides these facilities, several newly introduced instruments are equipped with a series of preprogrammed keys. Sometimes these preprogrammed functions can be changed within certain limits to other useful purposes by the user. The use of built-in video tubes and of floppy disk drives is rapidly growing.

In gas chromatography there is still much interest in capillary work and in splitter systems.

Finally, in HPLC, there is increased interest in gradient elution work and in autosampling systems.

KLAAS H.P. BROER

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (e.g., Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the authors' expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3] and proceedings [4] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford. New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography/Journal of Chromatography, Biomedical Applications, Elsevier Scientific Publishing Company, P.O. Box 330, 1000 AH Amsterdam, The Netherlands.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.