JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 17 NUMBER 5 1994

JOURNAL OF LIQUID CHROMATOGRAPHY

March 1994

Aims and Scope. The journal publishes papers involving the applications of liquid chromatography to the solution of problems in all areas of science and technology, both analytical and preparative, as well as papers that deal specifically with liquid chromatography as a science within itself. Included will be thin-layer chromatography and all models of liquid chromatography.

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

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

Your order must be prepaid by personal check or may be charged to MasterCard, VISA, or American Express. Please mail payment with your order to: Marcel Dekker Journals, P.O. Box 5017, Monticello, New York 12701-5176.

CODEN: JLCHD8 17(5) i-iv, 957-1202 (1994)
ISSN: 0148-3919

Printed in the U.S.A.

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography* are selectively indexed or abstracted in:

■ Analytical Abstracts ■ ASCA ■ Berichte Pathologie ■ BioSciences Information Service of Biological Abstracts (BIOSIS) ■ Cambridge Scientific Abstracts ■ Chemical Abstracts ■ Chemical Reactions Documentation Service ■ Current Awareness in Biological Sciences ■ Current Contents/Life Sciences ■ Current Contents/Physical and Chemical Sciences ■ Engineering Index ■ Excerpta Medica ■ Journal of Abstracts of the All-Union Institute of Scientific and Technical Information of the USSR ■ Physikalische Berichte ■ Reference Update ■ Saltykov-Shchedrin State Public Library ■ Science Citation Index

Manuscript Preparation and Submission. See end of issue.

This journal is also available on CD-ROM through ADONIS™ beginning with the 1991 volume year. For information contact: ADONIS, Marketing Services, P.O. Box 839, Molenwerf 1, 1000 AV Amsterdam, The Netherlands, Tel: +31-20-6842206, Fax: +31-20-6880241.

The Journals of Marcel Dekker, Inc. are available in microform form: RESEARCH PUBLICATIONS, 12 Lunar Drive, Drawer AB, Woodbridge, Connecticut, 06525, (203) 397-2600 or Toll Free 1-800-REACH-RP(732-2477). Outside North and South America: P.O. Box 45, Reading, RG1 8HF, England, 0734-583247.

Contributions to this journal are published free of charge.

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

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 957-980 (1994)

MICELLAR LIQUID CHROMATOGRAPHY WITH HYBRID ELUENTS

M. L. MARINA* AND M. A. GARCÍA
Departamento de Química Analítica
Facultad de Ciencias
Universidad de Alcalá de Henares
28871 Alcalá de Henares, Madrid, Spain

ABSTRACT

The characteristics of Micellar Liquid Chromatography with hybrid eluents are described. The influence of the addition of an organic modifier to the mobile phase on the retention, eluent strength, efficiency and selectivity is discussed. The application of MLC with hybrid eluents for predicting solutes hydrophobicity is studied.

I. INTRODUCTION

The combination of hydrophobic and hydrophilic properties in the molecules of surfactants confers to micellar systems some special characteristics in aqueous solution and this has made these systems applicable in different areas (**1-8**). The ability of micellar systems to solubilize hydrophobic compounds in aqueous solution (**9**) or improve

different analytical methodologies (**7,10**) should be emphasized. The use of surfactant solutions above their critical micellar concentration or c.m.c. as mobile phases in Reversed Phase High Performance Liquid Chromatography (RPLC) originates Micellar Liquid Chromatography (MLC) which is an interesting alternative to the use of hydro-organic mobile phases in chromatography (**11-13**).

The great number of interactions that are possible in the separations by MLC techniques, as electrostatic, hydrophobic and esteric (**14-16**) and the modification of the stationary phase by adsorption of monomeric surfactants (**17,18**) make these systems more complicated than conventional RPLC with hydro-organic mobile phases. In fact in MLC, three different equilibria can be considered (**19**): the distribution equilibrium of the solute (1) between the micellar mobile phase and the aqueous mobile phase, (2) between the micellar mobile phase and the stationary phase and (3) between the stationary phase and the aqueous mobile phase. From these equilibria, different equations have been developed to describe the chromatographic behavior of eluted solutes (**19-21**). These equations have allowed the determination of the solute-micelle association constants of several solutes with different micellar systems (**15,19,20,22-27**). This method can be applied to a great variety of solutes without the limitations or pre-requisites needed when other methods are employed (**28**).

MLC techniques also present other advantages such as: a) micellar mobile phases have low cost and low toxicity as compared with hydro-organic mobile phases since they are mainly composed of water (**11,19**). b) It is possible to separate ionic and nonionic solutes due to some special characteristics of micelles (**11**). c) Luminiscence detection can be improved in MLC because many solutes show enhanced fluorescence (**10,29-33**) and in some cases, room temperature liquid phosphorescence (**29,33,34**)

when associated with micelles. Furthermore, many metal-dye complexes show increased absorbance in the presence of micelles (**10,35**). d) It is possible to inject biological fluids directly into the chromatographic system because of the solubilization of the proteins by anionic surfactants as sodium dodecylsulphate (SDS) or nonionic surfactants such as polioxietilen[23]dodecanol (Brij-35) (**36-44**). e) Rapid elution gradients can be achieved in MLC because micellar gradients do not require reequilibration time (**45**). This is due to the amount of surfactant adsorbed on the stationary phase which remains practically constant after the equilibrium is reached and the surfactant concentration in mobile phase is above the c.m.c. (**46-48**). f) The control of separation selectivity is exerted through a great number of parameters such as nature (type and charge) and concentration of the surfactant in the mobile phase, the presence of additives as organic modifiers and salts and the pH (**16,23,49-52**). g) The correlation between chromatographic retention of several organic compounds in MLC and their logarithm of octanol-water partition coefficient (**53,54**) or their bioactivity (**55**) has been shown so this technique can be considered interesting in the evaluation of solute hydrophobicity.

An important drawback of MLC techniques is the decrease in chromatographic efficiency (**56**) as compared to that obtained in conventional RPLC with hydro-organic mobile phases. This efficiency loss can be precluded by adding an organic modifier to the mobile phase and increasing the working temperature.

Because the addition of alcohols to micellar mobile phases can increase the efficiency and selectivity (**57**) and reduce the analysis time, the use of micellar mobile phases modified by organic modifiers has acquired importance in last years. The term hybrid is used for the ternary eluents of water-organic solvent-micelles throughout the text.

Some of the most significant features of MLC techniques with hybrid eluents and their applications are described in this work.

II. EFFICIENCY

One of the main drawbacks of MLC techniques is the loss observed in the chromatographic efficiency as compared with that obtained in RPLC with hydro-organic mobile phases. This efficiency loss is attributed to the increase in the resistence of mass transfer of the solute from the mobile phase to the stationary phase (**58**).

However, the addition of small quantities of organic modifier to the mobile phase (3% propanol) and the increase in working temperature (40ºC) have shown to allow the obtainment of efficiencies similar to those obtained in RPLC with hydro-organic mobile phases (**59,60**). Other authors suggest working with low flow rates, high work temperatures, and low surfactant concentration in mobile phase (**58**). In fact, it has been shown that the use of an elevated surfactant concentration in mobile phase can cause a chromatographic efficiency loss (**61**).

Surfactant adsorption on the stationary phase seems to have a great influence on the efficiency (**62-65**). The addition of a short or medium chain alcohol causes surfactant desorption out of the stationary phase and improves efficiency (**66**). This effect increases with increasing concentration and hydrophobicity of the modifier (**27,57,64**).

Alcohols may also improve the efficiency obtained in MLC with micelles of ionic surfactants because their presence can reduce the net electrical charge density of the ionic micellar surface decreasing the repulsive barrier (**61**). In fact, the addition of alkanes does not affect the

surface charge density and does not improve the efficiency obtained for very hydrophobic solutes. This explains why an efficiency enhancement is not observed with alcohols for nonionic surfactants as Brij-35 that are not charged. In fact, the efficiency observed for very hydrophobic solutes with a Brij-35 micellar mobile phase was better than that obtained with ionic micelles.

III. INFLUENCE OF MICELLE CONCENTRATION ON RETENTION

As previously stated, three different equilibria can be considered in MLC: the distribution of the solute between the micelle and bulk water, with the corresponding P_{MW} partition coefficient; the partitioning of the solute between the stationary phase and the micelle, with P_{SM} as partition coefficient, and the distribution of the solute between the stationary phase and water, with P_{SW} as partition coefficient.

According to these equilibria, several equations have been developed relating chromatographic retention in MLC and micelle concentration in mobile phase. Armstrong and Nome (**19**) reported the following equation:

$$V_s/(V_e - V_m) = \{v(P_{MW} - 1)/P_{SW}\}\, C_M + 1/P_{SW} \qquad [1]$$

where V_s, V_e, and V_m are the stationary phase volume, elution volume of the solute and the void volume of the column, respectively; v is the molar volume of the surfactant and C_M is the micellized surfactant concentration in the mobile phase (C_M = C - c.m.c., C being the total surfactant concentration in solution). A plot of $V_s/(V_e - V_m)$ vs C_M is linear and the term

$v(P_{MW} -1)$ can be obtained from the slope: intercept ratio. Since from the Berezín treatment (**67**), $v(P_{MW} -1)$ is equal to the solute-micelle association constant, this parameter can be obtained from this treatment and also the partition coefficient of solute between bulk water and micelle, P_{MW}, if the surfactant molar volume,v, is known.

Arunyanart and Cline-Love (**20**) have derived a similar equation that correlates the capacity factor, k', to micellized surfactant concentration, C_M, in the form:

$$1/k' = \{K_2/\Phi\ [L_s]K_1\}\ C_M + 1/\Phi\ [L_s]K_1 \qquad [2]$$

where K_2 is the solute-micelle association constant, Φ is the phase ratio (the ratio of the stationary phase volume, V_s, to the volume of the mobile phase, V_m, in the column), $[L_s]$ is the stationary phase concentration, and K_1 is the binding constant for the solute between the bulk solvent and the stationary phase. Again, a plot of $1/k'$ vs C_M should result in a straight line and the value of the solute-micelle binding constant K_2, can be obtained from the slope: intercept ratio.

The solute micelle association constant obtained in this way is called the association constant per monomer. If this constant is multiplied by the aggregation number of the micelle, the association constant per micelle is obtained. Likewise, the P_{MW} and K_2 values only depend on the solute and the micellar system employed but not on the stationary phase (**15**).

Equations [1] and [2] show how the retention of a solute in MLC decreases when micelle concentration in mobile phase increases. This is in contrast to reversed-phase ion-interaction chromatography where the surfactant concentration is below the c.m.c., that is, no micelles exist, and the addition of an ionic surfactant will increase retention for compounds which interact electrostatically with it (**23**).

Equations [1] and [2] have frequently been employed with the aim of determining solute-micelle association constants in purely micellar systems (**15,19,20,22-27**). However, its validity for hybrid eluents has been shown (**15,27,68**). This has allowed the determination of the solute-micelle association constants in micellar media modified by alcohols. The addition of an organic modifier to a micellar solution can modify the characteristics of the micellar system (c.m.c. and the aggregation number) and this can originate a variation of the solute-micelle interactions (**69-71**) which, in turn, can change the chromatographic retention.

On the other hand, the error obtained during the determination of K_2 increases with solute hydrophobicity since P_{sw} values for these compounds are elevated (intercept very small, see equation [1]). With hybrid eluents, the value of P_{sw} decreases and the error in the determination of the solute-micelle association constants for very hydrophobic compounds also decreases (the intercept in equation [1] increases).

Although the validity of equations [1] and [2] has been shown for octylsilica and octadecylsilica stationary phases, cyano bonded columns have also been employed. In these columns, the retention for hydrophobic compounds considerably decreases especially when anionic surfactants as sodium dodecylsulphate (SDS) are used. This has allowed the determination of the solute-micelle association constants with similar or lower errors than those obtained for octadecylsilica columns but in considerably less time (**72**).

Solute-micelle interactions generally decrease in media modified by alcohols. In fact, solute-micelle association constants for a group of benzene and naphthalene derivatives with SDS and hexadecyltrimethylammonium bromide (CTAB) are greater in purely

micellar media than in solutions modified by a 5% or 10% n-butanol (**68**). This result has been attributed to the existence of a competing effect between the solute and the alcohol to interact with the micelle. However, the addition of a salt as NaCl can increase the interactions between the above-mentioned solute and SDS micelles. This is shown by obtaining of similar or higher association constants in the NaCl modified solution than in a purely micellar medium (**68**).

IV. INFLUENCE OF THE ORGANIC MODIFIER PERCENTAGE ON RETENTION

Khaledy et al. (**57**) proposed the following equation to relate solute retention (lnk') in MLC and volume fraction of organic modifier (Φ_{org}):

$$\ln k' = -S_{hyb}\,\Phi_{org} + \ln k'_{o} \qquad [3]$$

where S_{hyb} is the solvent strength parameter and $\ln k'_{o}$ is the retention of the solute in a purely micellar mobile phase.

This equation is simillar to that used to describe the retention variation with fraction volume of modifier in RPLC where lnk' linearly varies with Φ_{org} over a limited range. The slope of this straight line is called solvent strength parameter, S, and is generally proportional to the retention and molecular weight of the solute (**73,74**).

Equation [3] shows how solute retention in MLC decreases when Φ_{org} increases. However, in the same article where equation [3] is proposed, it was observed that the variation of lnk' with Φ_{org} for some amino acids and alkylbenzenes in SDS and CTAB mobile phases was not

linear. In other articles, a deviation from linearity was also observed as is the case of a group of benzene and naphthalene derivatives in a MLC system with SDS - n-butanol mobile phases (**75**). For other groups of solutes, the linear variation of lnk' with Φ_{org} was only found when methanol was used as organic modifier (**76**).

Recently, Torres-Lapasió et al. (**76**) have proposed a new model to describe the variation of solute retention in MLC with Φ_{org}. In this model, retention can be expressed by the following equation:

$$1/k' = A\mu + B\Phi + C\mu\Phi + D \qquad [4]$$

where μ and Φ are the surfactant and alcohol concentrations in mobile phase, respectively. The validity of this model has been shown for several solutes as catecholamines, amino acids, peptides, and other aromatic compounds with organic modifiers different from methanol (**76**).

Equation [4] shows that for a constant surfactant concentration in mobile phase, the term 1/k' should linearly vary with Φ_{org}:

$$1/k' = (A\mu + D) + (B + C\mu)\,\Phi \qquad [5]$$

On other hand, in purely micellar mobile phases ($\Phi = 0$):

$$1/k' = A\mu + D \qquad [6]$$

and an equation similar to that obtained by Arunyanart and Cline-Love (equation [2]) is obtained.

More work is required for different solutes, different surfactants, and different organic modifiers to show the validity range of equations [3] and [4].

V. SOLVENT STRENGTH OF HYBRID MICELLAR ELUENTS

In MLC, purely micellar eluents can have a quite small eluent strength (**57**). Eluent strength of purely micellar eluents increases when micelle concentration in mobile phase also increases (**57**). However, an increase in micelle concentration in mobile phase generally causes an efficiency loss.

For these reasons, the addition of organic modifiers to micellar mobile phases is of great interest: it is possible to increase both eluent strength and efficiency.

Solvent strength (S_{hyb}) in MLC with hybrid eluents has been defined as the slope of straigth line resulting from the variation of lnk' as a function of Φ_{org}. The value for S_{hyb} has been calculated for fourteen alkylbenzenes in micellar phases of CTAB modified by methanol (MeOH), 2-propanol (PrOH) and butanol (BuOH) (**57**). S_{hyb} values can be ranked as $S_{BuOH} > S_{PrOH} > S_{MeOH}$ which is similar to conventional hydro-organic systems as BuOH is the strongest solvent and MeOH is the weakest. The larger S_{hyb} for BuOH and PrOH indicate that these solvents interact more with micelles and, consequently, can solvate more effectively and/or can better compete with micelles for solute interactions. However, all values obtained for S_{hyb} for the group of compounds studied are still smaller than for those in absence of micelles, as S_{hyb} for BuOH is even smaller than S values for MeOH in conventional hydro-organic eluents.

Another consideration which also demonstrates the impact of micelles is the fact that the ranking of S_{hyb} for different solutes is different for MeOH, PrOH and BuOH. On the contrary, in conventional hydro-organic systems the same ranking of S values can be anticipated for different solutes. This is because in MLC, solvents interact differently with micelles and, therefore, their own microenvironment in micelles is different.

Since S values reflect the extent of solvation of solutes by organic solvents, the location of solutes and/or organic solvents in micelles can greatly influence the sensitivity of retention to changes in the concentration of organic solvent. In a conventional hydro-organic system, S significantly varies with solute molecular weight and functional groups. As an example, anthracene has a large S value and its retention in conventional RPLC with hydro-organic mobile phases (methanol-water) is more sensitive to variations in the concentration of organic solvent than other compounds with a minor S value. However, in the presence of CTAB micelles, the S_{hyb} value for anthracene in methanol is small and, therefore, its retention is less affected by the addition of organic solvents. This is because this compound strongly interacts with micelles and is less accessible to a polar solvent such as methanol. However, the relationship between S_{hyb} and solutes' structural properties cannot be easily recognized and it cannot be concluded that S_{hyb} is inversely related to hydrophobicity of solute (**57**).

VI. SELECTIVITY

Solute retention in MLC generally decreases when micelle concentration increases, as indicated in section III. The rate of change in retention of different solutes varies with charge and hydrophobicity of solutes as well as the length of alkyl chain, charge, and concentration of micelles (**77**). This fact causes inversions of elution order that are the result of two competing equilibria: solute-micelle association characterized by K_2 and solute-stationary phase interaction characterized by P_{sw}. The parameters K_2 and P_{sw} have a different effect on retention. When P_{sw} increases, retention also increases but when K_2 increases, retention decreases. When the surfactant concentration in mobile phase increases,

the effect that K_2 has on retention also increases and reversals in elution order can be obtained if the difference in K_2 values for two solutes is quite different (**23**). Therefore, separation selectivity in MLC can be controlled by modifying surfactant nature and concentration. Furthermore, when organic modifiers are added to the mobile phase, the solvent strength parameter S_{hyb} for a group of compounds does not have the same ranking for different alcohols due to the different interaction of these modifiers with micelles. For these reasons, MLC techniques are very interesting for chromatographic separation.

Although the conditions to optimize separation selectivity in MLC can vary with solutes' nature, several works show an increase in separation selectivity for aromatic compounds in MLC with hybrid eluents when the micelle concentration in the mobile phase decreases (**57,75,77**). However, for a group of amino acids and peptides, an increase in micelle concentration can cause an increase or decrease in selectivity (**57**).

The effect of the organic modifier content in mobile phase seems to be clearer. Generally, separation selectivity in MLC is improved in the presence of an organic modifier and increases with the volume fraction of the modifier in mobile phase (**57,75,77**). This result is opposed to that observed in conventional RPLC with hydro-organic mobile phases in which an increase in organic modifier content causes a decrease in solute retention and selectivity. Recently, a comparative study on the influence of organic modifier content and surfactant concentration on solvent strength and selectivity in Ion Pair Chromatography and in MLC has been completed (**78**). The selectivity enhancement observed in MLC when the solvent strength increases has been attributed to the competing partitioning equilibria in micellar systems and/or to the unique characteristics of micelles to compartmentalize solutes and organic solvents (**57**).

Although separation selectivity is generally improved when the volume fraction of organic modifier is increased, for some amino acids and peptides selectivity can decrease with the content of 2-propanol of a SDS micellar mobile phase (**77**). In this case, it has been shown that for pairs of peaks whose selectivities were reduced with increasing 2-propanol concentration, a selectivity enhancement was observed as a result of increasing micelle concentration and vice versa. These observations suggest that solvent strength increases with concentrations of both micelle and organic solvent, the effect of these two parameters on selectivity could be quite different, even opposite. Micelles and 2-propanol compete to interact with solutes and, as a result, they influence the role of one another in controlling retention and selectivity.

As a consequence of these results, a model has been developed which explains the dependence of the solvation ability of organic solvents in MLC (represented by solvent strength parameter, S_{hyb}, of solutes) and the degree of solute interactions with micelles. Whenever the difference in solvent strength parameter values of two solutes in micellar eluents, dS_{hyb}, was positive, maximum selectivity was observed at the weakest eluent strength. When dS_{hyb} was negative, an inverse relationship between retention and solvent strength parameter exists so that selectivity increases with volume fraction of organic solvent in micellar eluents (**77**).

The mutual effects of micelles and organic modifiers on one another would also require a simultaneous optimization of these two parameters. Like in the study of the separation selectivity of 15 benzene and naphthalene derivatives in MLC with SDS and CTAB mobile phases modified by methanol, n-propanol, and n-butanol, it was found that selectivity was better in SDS than in CTAB and that it increases when surfactant concentration in mobile phase decreased. Regarding organic

modifier content, selectivity was better in the presence of n-propanol or n-butanol at medium percentages, but the latter had the advantage of decreasing analysis time with respect to n-propanol (**75**). Obtaining maximum selectivities at medium alcohol percentages can be justified by the existence of pairs of compounds whose selectivity increases when eluent strength decreases.

VII. QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS (QSAR)

Another interesting possibility of MLC techniques is their application to the quantitation of physicochemical properties of biologically active compounds in QSAR studies, specifically for the prediction of hydrophobicity.

Hydrophobicity is commonly understood as a measure of the relative tendency of a solute to prefer a nonaqueous rather than an aqueous environment. Biological activity of many compounds, bioaccumulation of organic polutants, and soil sorption of environmental contaminants have all been correlated to the lipophilic character of molecules (**13**). The quantitation of hydrophobicity has both diagnostic and predictive value in various disciplines such as drug design, toxicology, and environmental monitoring (**79-82**). When comparing behavior of various solutes in the same environment, a quantitative scale can be used to demonstrate the abilities of individual solutes to participate in hydrophobic interactions. Octanol-water partitioning is a common reference system that provides the most recognized hydrophobicity measure: the logarithm of the partition coefficient, log P_{ow} (**83**). The standard "shake-flask" method for determining partition coefficients in liquid-liquid systems has several serious

disadvantages (**84**). Despite numerous efforts using a variety of methods, the measurement of P_{ow} is still difficult. In 1977 publications began to appear on what is now termed quantitative structure-retention relationships (QSRRs) (**84**). QSRRs result from applying the methodology used for quantitative structure-biological activity relationships (QSARs) (**83**) to the analysis of chromatographic data.

Following the first reports on reversed-phase TLC and HPLC methods of hydrophobicity parameterization, hundreds of reports on the application of chromatographically derived hydrophobicity descriptors in medicinal, agricultural, and environmental chemistry have appeared (**84**). In reversed-phase HPLC with hydro-organic mobile phases, a representative relationship has been obtained between the chromatographic measure of hydrophobicity (logk') determined on a deactivated phase for a noncongeneric series of nonionized basic, acidic, and neutral solutes, as well as their log P_{ow} values. Thus the advantages of the log P_{ow} hydrophobicity scale -its universality and continuity- are challenged by a more convenient, reproducible, fast, and inexpensive chromatographic approach. A systematic study could produce a large chromatographic hydrophobicity database similar to the one collected laboriously for log P_{ow} (**85**).

Another chromatographic approach used to evaluate octanol-water partition coefficients is countercurrent chromatography (CCC) with an octanol-water biphasic solvent system (**86,87**). The mobile phase is water saturated with octanol, and the stationary phase is octanol saturated with water. The measurable P_{ow} range was 0.003 to 300. A liquid stationary octanol phase permits the development of a dual-mode elution method using CCC which extends the measurable P_{ow} range to 5000. The co-current CCC method was developed to extend the range to P_{ow} = 20.000.

In co-current CCC, both the water and the octanol phase move in the same direction at different rates.

However, the use of a bulk solvent such as octanol as a model for complex systems such as biomembranes has been occasionally criticized. On the other hand, micelles have long been known as simple chemical models for biomembranes (**54**). Several workers have demostrated that the solubilization (or partitioning of solutes in micelles) closely resembles that of lipid bilayers and that both of these are different from the two-phase octanol-water system (**88-91**). Both micelles and biomembranes have amphiphilic properties and are anisotropic media. Molecular size and shape are significant factors in the partitioning of solutes in anisotropic environments while they are not determinant for the partition process in an isotropic solvent such as 1-octanol (**92**). These reports provide interesting examples confirming the suitability of micelles for representing biomembranes as far as hydrophobic interactions are concerned (**54**). A shake flask method has been presented for the determination of the partition coefficients involved in the distribution of polar solutes between octanol and aqueous micellar solutions (**93**).

Several studies have appeared in literature in which the correlation between retention in MLC and octanol-water partition coefficient or carbon number is studied. These works can be divided in two groups. In a first group are the studies in which a linear relationship is found between the logarithm of the capacity factor (logk') of compounds and log P_{ow} or number of carbom atoms (n_c) in the molecule. Like, a linear correlation logk' = f(log P_{ow}) has been found for a group of monosubstituted benzenes with mobile phases of sodium dodecylsulphate (SDS), hexadecyltrimethylammonium bromide (CTAB), and polyoxyethylene(23)dodecanol (Brij-35) (**53**). The same correlation has

been obtained for a group of phenols and other group of monosubstituted benzenes with mobile phases of SDS and CTAB that can be modified by alcohols (**94**), and for a series of aromatic polycyclic hydrocarbons with mobile phases of SDS, CTAB and polyoxyethylene(23)lauryl ether (Brij-35) (**95**). Also, this first group can include works in which a linear relationship is found for the variation of log P_{ow} with the logarithm of the solute-micelle association constants ($logK_z$) (**94,96**) or in which a linear variation is found for the transfer free energies from water to micelle as a function of the transfer free energy from octanol to water (**97,22,68**).

The second group includes studies in which a linear relationship between capacity factors (k') (and not logk') and log P_{ow} or n_c. For example, a linear relation has been found for k'-n_c in the case of groups of n-alkylbenzenes and n-alkylphenones with purely or hybrid SDS and CTAB mobile phases (**27**) and for a series of alkylbenzenes with mobile phases of SDS and Brij-35 (**98**). In the same way, a linear relationship has been found between k' and log P_{ow} for sixteen aromatic compounds in purely and hybrid SDS and CTAB mobile phases (**54**) and between k' and the bioactivity of 26 para-substituted phenols with tetradecyltrimethylammonium bromide (**55**). In this case, the addition of 10% 2-propanol to the micellar system (hybrid system) proved the best chromatographic system for the best estimation of the phenols bioactivity.

Regarding the nonlinearity observed for the variation of logk' as a function of carbon atoms (n_c), an equation has been derived recently which explains this apparently anomalous result (**99**). The equation is simply based on partitioning between moving and stationary phases. Experimental results on a variety of systems have displayed the nonlinearity seen previously. These data are adequately fit by the equation.

In summary, MLC appears as an interesting alternative to evaluate log P_{ow} and bioactivity of organic compounds, especially for hybrid

systems, utilized to extract the systematic information from diversified yet often highly intercorrelated sets of data, modern multivariate chemometric methods of data analysis must be used (**84**).

ACKNOWLEDGEMENTS

The authors thank the DGICYT (Spain) for the project PS90-0026 and the Comunidad Autónoma de Madrid for the project C102/91.

REFERENCES

1. J.H. Fendler, E.J. Fendler, Catalysis in Micellar and Macromolecular Systems, Academic Press, New York, 1975.

2. E.J. Fendler, Membrane Mimetic Chemistry, Willey, New York, 1982.

3. A. Berthod, J.Chim.Phys., 80, 407 (1983).

4. L.J. Cline-Love, J.G. Habarta, J.G. Dorsey, Anal.Chem., 56, 1132A (1984).

5. E. Pelizzetti, E. Pramauro, Anal.Chim.Acta, 169, 1 (1985).

6. D.W. Armstrong, Sep.Purif.Methods, 14, 213 (1985).

7. W.L. Hinze, D.W. Armstrong, Eds., Ordered Media in Chemical Separations, ACS Symposium Series, Vol. 342, Whasington, 1987.

8. J. Georges, Analusis, 17, 231 (1989).

9. P. Lianos, J. Lang, C. Strazielle, R. Zana, J.Phys.Chem., 86, 1019 (1982).

10. F. Fernández-Lucena, M.L. Marina, A.R. Rodríguez in Vibrational Spectra and Structure, R. Durig, Ed., Elsevier, Amsterdam, 1991, Cap.3.

11. J.G. Dorsey, Adv.Chromatogr., 27, 167 (1987).

12. M.G. Khaledi, Biochromatography, 3, 20 (1988).

13. M.G. Khaledi, Trends Anal.Chem., 7, 293 (1988).

14. W.L. Hinze, Sep.Purif.Methods, 10, 159 (1981).

15. A. Berthod, I. Girard, C. Gonnet, Anal.Chem., 58, 1359 (1986).

16. M.J. Medina Hernández, M.C. García Alvarez-Coque, Analyst, 117, 831 (1992).

17. A. Berthod, I. Girard, C. Gonnet, Anal.Chem., 58, 1362 (1986).

18. A. Berthod, I. Girard, C. Gonnet in Ordered Media in Chemical Separations, W.L. Hinze y D.W. Armstrong, Eds., ACS Symposium Series, Vol 342, Whasington 1987, pp.130.

19. D.W. Armstrong, F. Nome, Anal.Chem., 53, 1662 (1981).

20. M. Arunyanart, L.J. Cline-Love, Anal.Chem., 56, 1557 (1984).

21. J.P. Foley, Anal.Chim.Acta, 231, 237 (1990).

22. M.L. Marina, S. Vera y A.R. Rodríguez, Chromatographia, 28, 379 (1989).

23. P. Yarmchuk, R. Weinberger, R.F. Hirsch, L.J. Cline-Love, Anal.Chem., 54, 2233 (1982).

24. E. Pramauro, E. Pelizzetti, Anal.Chim.Acta, 154, 153 (1983).

25. E. Pramauro, G. Saini, E. Pelizzetti, Anal.Chim.Acta, 166, 233 (1984).

26. M. Arunyanart, L.J. Cline-Love, Anal.Chem., 57, 2837 (1985).

27. M.G. Khaledi, E. Peuler, J. Ngeh-Ngwainbi, Anal.Chem., 59, 2738 (1987).

28. C. Hirose, L. Sepúlveda, J.Phys.Chem., 85, 3689 (1981).

29. D.W. Armstrong, W.L. Hinze, K.H. Bui, H.N. Singh, Anal.Lett., 14, 1659 (1981).

30. W.L. Hinze, Solution Chemistry of Surfactants, K.L. Mittal, Ed., Plenum Press, New York, 1979, Vol.1.

31. H. Singh, W.L. Hinze, Anal.Lett., 15, 221 (1982).

32. H. Singh, W.L. Hinze, Analyst(London), 107, 1073 (1982).

33. R. Weinberger, P. Yarmchuk, L.J. Cline-Love, Anal.Chem., 54, 1522 (1982).

34. L.J. Cline-Love, M. Skrilec, J.G. Habarta, Anal.Chem., 52, 754 (1980).

35. J.H. Callahan, K.D. Kook, Anal.Chem., 54, 59 (1982).

36. F.J. DeLuccia, M. Arunyanart, L.J. Cline-Love, Anal.Chem., 57, 1564 (1985).

37. M. Arunyanart, L.J. Cline-Love, J.Chromatogr., 342, 293 (1985).

38. L.J. Cline-Love, S. Zibas, J. Noroski, M. Arunyanart, J.Pharm.Biomed.Anal., 3, 511 (1985).

39. Y.N. Kim, P.R. Brown, J.Chromatogr., 384, 209 (1987).

40. P. Menéndez Fraga, E. Blanco González, A. Sanz-Medel, Anal.Chim.Acta, 212, 181 (1988).

41. F. Palmisano, A. Guerrieri, P.G. Zambonin, T.R.I. Cataldi, Anal.Chem., 61, 946 (1989).

42. K.B. Sentell, J.P. Clos, J.G. Dorsey, BioChromatography, 4, 35 (1989).

43. L.J. Cline-Love, J. Fett, J.Pharm.Biomed.Anal., 9, 323 (1991).

44. I. Carretero, M. Maldonado, J.J. Laserna, E. Bonet, G. Ramis Ramos, Anal.Chim.Acta., 259, 203 (1992).

45. J.S. Landy, J.G. Dorsey, J.Chromatogr.Sci., 22, 68 (1984).

46. C.T. Hung, R.B. Taylor, J.Chromatogr., 209, 175 (1981).

47. J.G. Dorsey, M.G. Khaledi, J.S. Landy, J.L. Lin, J.Chromatogr., 316, 183 (1984).

48. A. Berthod, I. Girard, C. Gonnet, Anal.Chem., 58, 1356 (1986).

49. D.W. Armstrong, G.Y. Stine, Anal.Chem., 55, 2317 (1983).

50. F.G.P. Mullins, G.F. Kirkbright, Analyst, 111, 1273 (1986).

51. J. Podcasy Berry, S.G. Weber, J.Chromatogr.Sci., 25, 307 (1987).

52. M.G. Khaledi, Anal.Chem., 60, 876 (1988).

53. F. Gago, J. Alvarez-Builla, J. Elguero, J.C. Díez-Masa, Anal.Chem., 59, 921 (1987).

54. M.G. Khaledi, E.D. Breyer, Anal.Chem., 61, 1040 (1989).

55. E.D. Breyer, J.K. Strasters, M.G. Khaledi, Anal.Chem., 63, 828 (1991).

56. A. Berthod, M.F. Borgerding, W.L. Hinze, J.Chromatogr., 556, 263 (1991).

57. M.G. Khaledi, J.K. Strasters, A.H. Rogers, E.D. Breyer, Anal.Chem., 62, 130 (1990).

58. P. Yarmchuck, R. Weinberger, R.F. Hirsch, L.J. Cline-Love, J.Chromatogr., 283, 47 (1984).

59. J.G. Dorsey, M.T. De Echegaray, J.S. Landy, Anal.Chem., 55, 924 (1983).

60. J.S. Landy, J.G. Dorsey, Anal.Chim.Acta, 178, 179 (1985).

61. M.F. Borgerding, R.L. Williams, Jr., W.L. Hinze, F.H. Quina, J.Liq.Chromatogr., 12, 1367 (1989).

62. M.F. Borgerding, W.L. Hinze, Anal.Chem., 57, 2183 (1985).

63. D.W. Armstrong, T.J. Ward, A. Berthod, Anal.Chem., 58, 579 (1986).

64. M.F. Borgerding, W.L. Hinze, L.D. Stafford, G.W. Fulp, W.C. Hamlin, Anal.Chem., 61, 1353 (1989).

65. R. Bailey, R.M. Cassidy, Anal.Chem., 64, 2277 (1992).

66. A. Berthod, A. Roussel, J.Chromatogr., 449, 349 (1988).

67. I.V. Berezín, K. Martinek, A.K. Yatsimirskii, Russ.Chem.Rev., Eng. Transl., 42, 787 (1973).

68. M.A. García, S. Vera, M.L. Marina, Chromatographia, 32, 148 (1991).

69. M. Almgren, S. Swarup, Surfactants in solution, K.L. Mittal y B. Lindman, Eds., Plenum, New York, 1984, pp.613.

70. L.G. Ionescu, L.S. Romanesco, F. Nome, Surfactants in solution, K.L. Mittal y B. Lindman, Eds., Plenum, New York, 1984, pp.789.

71. A. Berthod, J. Georges, Nouv.J.Chim., 9, 101 (1985).

72. M.A. García, S. Vera, M.L. Marina, unpublished results.

73. L.R. Snyder, M.A. Quarry, J.L. Glajch, Chromatographia, 24, 33 (1987).

74. P.M.J. Coenegracht, H.J. Metting, A.K. Smilde, P.J.M. Coenegracht-Lamers, Chromatographia, 27, 135 (1989).

75. M.A. García, S. Vera, M. Bombín, M.L. Marina, J.Chromatogr., (in press).

76. J.R. Torres-Lapasió, R.M. Villanueva-Camañas, J.M. Sanchís-Mallols, M.J. Medina-Hernández, M.C. García-Alvarez-Coque, J.Chromatogr., 639, 87 (1993).

77. A.S. Kord, M.G. Khaledi, Anal.Chem., 64, 1894 (1992).

78. A.S. Kord, M.G. Khaledi, Anal.Chem., 64, 1901 (1992).

79. C. Hansch, in Drug Design, Ariens, E.J., ed., Academic Press, New York, 1971, Vol.1, pp.271.

80. Structure-Activity Correlation as a Predictive Toal in Toxicology, Goldberg, L., ed., Hemisphere Publishing, Whashington, DC, 1983.

81. C. Hansch, A. Leo, Substituent Constants for Correlation Analysis in Chemistry and Biology, Willey-Interscience, New York, 1979.

82. C.T. Chiou, Environ.Sci.Technol., 19, 57 (1985).

83. C. Hansch, T. Fujita, J.Am.Chem.Soc., 86, 1616 (1964).

84. R. Kaliszan, Quantitative Structure-Chromatographic Retention Relationships, Wiley, New York, 1987, pp.1.

85. R. Kaliszan, Anal.Chem., 64, 619A (1992).

86. A. Berthod, V. Dalaine, Analusis, 20, 325 (1992).

87. A. Berthod, R.A. Menges, D.W. Armstrong, J.Liq.Chromatogr., 15, 2769 (1992).

88. K.W. Miller, L. Hammond, E.G. Porter, Chem.Phys.Lipids, 20, 229 (1977).

89. C. Treiner, J.Colloid Interface Sci., 93, 33 (1983).

90. C. Treiner, A.K. Chattopadhyay, J.Colloid Interface Sci., 109, 101 (1986).

91. J.M. Diamond, Y. Katz, J.Membr.Biol., 17, 121 (1974).

92. T. Braumann, J.Chromatogr., 373, 191 (1986).

93. G.M. Janini, S.A. Attari, Anal.Chem., 55, 659 (1983).

94. B.K. Lavine, A.J. White, J.H. Han, J.Chromatogr., 542, 29 (1991).

95. V. González, M.A. Rodríguez-Delgado, M.J. Sánchez, F. García-Montelongo, Chromatographia, 34, 627 (1992).

96. K.T. Valsaraj, L. Thibodeaux, Sep.Sci.Technol., 25, 369 (1990).

97. E. Pramauro, C. Minero, G. Saini, R. Graglia, E. Pelizzetti, Anal.Chim.Acta, 212, 171 (1988).

98. M.F. Borgerding, F.H. Quina, W.L. Hinze, J. Bowermaster, H.M. McNair, Anal.Chem., 60, 2520 (1988).

99. W.L. Hinze, S.G. Weber, Anal.Chem., 63, 1808 (1991).

Received: June 24, 1993
Accepted: September 28, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 981-998 (1994)

SIMULTANEOUS DETERMINATION OF PHOSPHOLIPID CLASSES AND THE MAJOR MOLECULAR SPECIES OF LECITHIN IN HUMAN AMNIOTIC FLUID BY HPLC

BURKHARD H. KLEIN AND JOACHIM W. DUDENHAUSEN

Freie Universität Berlin
Universitätsklinikum Rudolf Virchow
Frauenklinik und Poliklinik Charlottenburg
Arbeitsgruppe Perinatale Medizin
Pulsstraße 4
D 14059 Berlin, Germany

Abstract

Phospholipid classes in human amniotic fluid, whose quantitative determination plays an important role in obstetrics for diagnosing lung maturity in the fetus, are separated through normal phase chromatography by HPLC. In this study an already known chromatographic system is described with which not only the phospholipids, but also phosphatidylcholine partly after being combined with both its fatty acid residues, is separated. This separation implies the fatty acid residues which are of greatest interest to the obstetrician. A mass or evaporative light-scattering detector was used for the detection.

Introduction

The determination of surface active phospholipids in amniotic fluid is of great importance in obstetrics for diagnosing lung maturity in the fetus. A number of quantitative or semiquantitative methods of determination exist today [1]. Tests which measure the physical (surface) properties of lung surfactant, tests which measure the chemical components of amniotic fluid (AF) or tests which provide an index of fetal maturity and thus an approximation of fetal lung maturity are used in clinical routine. In the "chemical" methods three phospholipids in particular are measured - phosphatidylcholine or lecithin, sphingomyelin and phosphatidylglycerol.

The most versatile method of determination is chromatography [2,3], in particular HPLC which can quantitatively detect up to 11 classes of phospholipids in one analysis. This is achieved exclusively with normal phase chromatography either in silica gel columns [4] or in chemically modified silica gel, particularly the diol phase [5]. The individual phospholipids, like for example lecithin, are not chemically homogenous substances but are in fact a mixture of an homogenous basic frame with one or two fatty acid residues which can vary according to the method of production [6]. A large number of saturated and unsaturated fatty acids are attached to the lipids; however their surface active effect can be very different. Using reversed phase chromatography in ODS phases these individual lipid classes can be separated according to the single fatty acid residue and/or combinations [2,3]. In the case of lecithin the chromatogram can sometimes show more than 20 peaks [7].

In this study a HPLC separation system is described that separates not only the phospholipid classes but also the major molecular species of lecithin from each other in one chromatography run. It concerns normal phase chromatography with chemically non-modified silica gel. Detection is achieved using a mass or evaporative light-scattering detector.

Materials

Chemicals

The phospholipid standards, phosphatidylglycerol (PG, ammonium salt, from egg yolk lecithin), diphosphatidylglycerol (DPG, cardiolipin, sodium salt, from bovine

heart), phosphatidylinositol (PI, ammonium salt, Sigma: from soybean and Fluka: from bovine liver), phosphatidylserine (PS, from bovine brain), phosphatidylethanolamine (PE, from egg yolk), phosphatidylmonomethylethanolamine (PMME), phosphatidyldimethylethanolamine (PDME), lysophosphatidylethanolamin (LPE, from egg yolk), phosphatidylcholin (PC, lecithin, from frozen egg), didecanoyl- (DD-PC), dilauroyl- (DLa-PC, dihydrate), dimyristoyl- (DM-PC, monohydrate), dipalmitoyl- (DP-PC), distearoyl- (DS-PC), dioleoyl- (DO-PC), 2-oleoyl-1-palmitoyl- (OP-PC), 2-linoleoyl-1-palmitoyl-lecithin (LiP-PC), lysolecithin (LL, from soybeans), sphingomyelin (SP, from bovine brain), N-palmitoyl- (P-SP, from bovine brain) and N-stearoylsphingomyelin (S-SP, from bovine brain) stem from the firm Sigma, Deisenhofen/FRG, and also from Fluka, Neu-Ulm/FRG. Most of the substances are delivered in the form of solutions in the solvents chloroform, methanol or a mixture of chloroform-methanol.

The solvents used - chloroform, methanol, n-hexane and 2-propanol (all of grade pro analysi) - are obtained from the Merck Company, Darmstadt/ FRG, and the water "Chromasolv" from Riedel de Haen, Seelze/FRG. The charcoal activated granular about 2.5 mm is also procured from Merck, Darmstadt/FRG.

Amniotic Fluids

The amniotic fluid samples are obtained on the one hand during amniocentesis and on the other hand on delivery. They are frozen in portions of about 2 cm^3 and are stored at a temperature of between -18°C to -24°C.

HPLC Equipment

The modular equipment consists of apparatus produced by the following firms: Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin/FRG (Programmer 50 B and two pumps type 64.00, a dynamic mixing chamber, a column oven with control unit and a recorder); Gynkotek, Germering/FRG (an injection valve with built-in reed relay and a C-R3A integrator with keyboard, monitor and floppy disk drive); Hewlett Packard, Frankfurt/Main/FRG (digital thermometer 2802A with PT 100 sensor) and Zinnser, Frankfurt/Main/FRG (mass or evaporative light-scattering detector, model ACS 750/14).

As separation columns we used Vertex-ECO-columns with dimensions of 120 mm in length and 4 mm inner diameter filled with Nucleosil 100, 3 μm, Nucleosil 120, 3 μm, and Nucleosil 50, 5 μm, supplied by Säulentechnik Dr. Ing. H. Knauer GmbH, Berlin/FRG.

Methods

When the individual standard substances are not delivered in the form of a solution with the concentration of 10 mg/cm^3, solutions are correspondingly composed with a chloroform-methanol mixture (2:1, v:v), or the concentrated solutions are diluted. Then the standard injection solvents which are listed in Table 1 are produced with these solutions.

The extraction of amniotic fluid to obtain the injection solutions has already been described [8]. 5 μl lysolecithin solution (10 mg/cm^3) was added as internal standard to the 1,5 cm^3 amniotic fluid, which was used for extraction. This lysolecithin serves to measure the reaction degree of the extraction and also to measure the peak identification of the chromatogram. The completely dried extractions are diluted in 0.2 cm^3 chloroform/methanol (2:1, v:v) and can then be injected into the HPLC equipment.

HPLC

The selection of the columns, the eluents and the composition of the gradients was made according to the studies made by Breton et al. [9], Letter [10], Dugan [11] and Geilen [12].

For the gradients a mixture of n-hexane and 2-propanal (60:40, v:v) is used as eluent A and as eluent B, a mixture of 500 cm^3 solution A with 29 cm^3 water. At first it is started in one liter of eluent A, half of which is then further used for B. Both eluents are degassed in an ultrasonic bath for at least 15 minutes. The composition of the gradient is given in Table 2. The flow amounts to 1.0 cm^3/min. A chromatogram takes either 45 or 50 minutes depending on which column is being used. The column has a temperature of 30°C in order to exclude fluctuations in the temperature in our laboratory. The detector works with compressed air purified with

TABLE 1

Standard solutions used with partial concentration for the substances (for abbreviations see Materials section Chemicals)

Solution No.	Substances	Concentration in g/cm^3
1	PG, PI, PS, PE, PC, SP, LL	0.25
1A	No. 1 + DPG, PMME, PDME, LPE	0.25
2	DD-PC, DLa-PC, DM-PC, DP-PC, DS-PC	0.25
3	DO-PC, OP-PC, LiP-PC	0.25
3A	No. 3 + DD-PC, DS-PC	0.25
4	DD-PC, DS-PC	4.00

TABLE 2

Composition of the mobile phase, eluent A: n-hexane/2-propanol (60:40) and eluent B: 500 cm^3 eluent A with 29 cm^3 water

Time in min	Portion A in %	Portion B in %
0	50	50
6	50	50
11	22	78
20	22	78
30	0	100
50	0	100
55	50	50
60	50	50

charcoal activated granular as nebulizing gas, pressure approximately 1.4 bar (20 psi) at 5 bar first pressure. The temperature in the evaporator is set at 50°C.

The measuring data are recorded over the integrator on the diskette for later evaluation. The integrator and also the programmer are started by injection by hand through the contact point of the reed relay.

The calibrations were done according to the one point method and were stored in various files in the integrator. One calibration contains various phospholipid classes (standard solution 1), a second one the lecithins with the various fatty acids residues (standard solution 2 and 3, respectively 3a). No calibration was made with sphingomyelin on its own.

Results and Discussion

By using the mass/light-scattering detector and due to its function [3] no solution peaks and no changes in the base line through the gradients are to be found in the chromatogram. The peak occurring at the point of the solution does at least represent the proteins present in the solution, if not any other substances. The presence of the proteins could be proved by an injection of a purified protein solution with a portion of phospholipid.

The temperature of the columns has no influence on the separation as regards the retention time, the resolution and the form of the peaks. The chromatograms undergo no changes whether they are being produced at room temperature or whether the column oven has a temperature of 45°C.

Separation of the Phospholipids Classes

In figure 1 chromatograms of the standard solutions 1 and 1A can be seen. Chromatogram (A) serves as concentration calibration. The individual substances, with the exception of PI and PS are separated from each other on the base line. The PS peak is set upon the PI peak with its characteristic form, the strong fronting through the sixring sugar in the molecule. Sphingomyelin is represented by its typical double peak, here also separated on the base line.

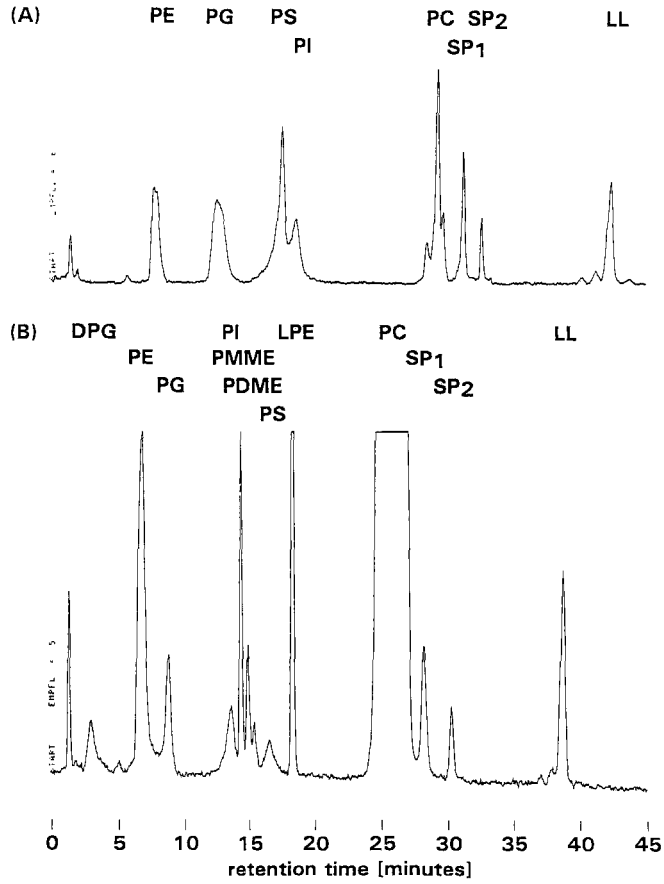


FIGURE 1: Chromatogram of the standard solution (A) No. 1 on a Nucleosil 100/3 and (B) No. 1A on a Nucleosil 120/3

Chromatogram (B) shows a separation of the extended standard solution with substances that possibly may also be present in amniotic fluid. All 11 substances are clearly separated from each other. In this column - a different one than in (A) - PI and PS are widely separated from each other. The separation of these two substances is very strongly dependent on the column and can only result in one peak or in the two forms described.

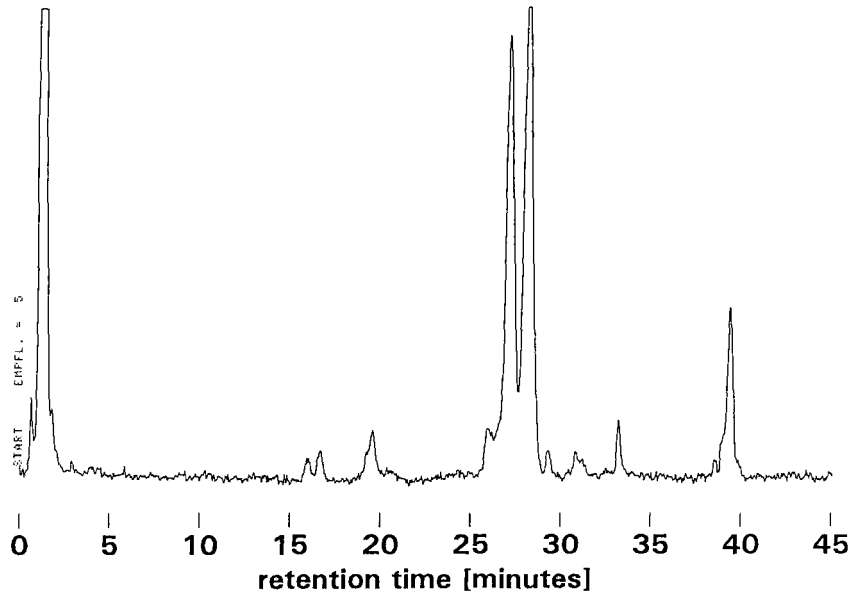


FIGURE 2: Phospholipid separation of the extract of amniotic fluid (AF 244, week of gestation 39/1) on Nucleosil 100/3

An exemplary chromatogram of an extract of mature amniotic fluid is given in figure 2. The last peak at about 39 minutes is lysolecithin, the "internal standard". Lecithin with approximately 70 to 80% proportion of the complete phospholipid content of amniotic fluid [13] forms the "main" peak between 26 and 29 minutes. Furthermore in this amniotic fluid, SP (31 and 33 minutes) and PI (19.5 minutes) and PG (16 minutes) are clearly detected. PE was not found in this sample. These other phospholipids together form only 20 to 30% of the total and are therefore partially very difficult to determine since the mass detector has a very high detection limit at 0.5 to 1 μg of the injected quantity.

Separation of the Molecular Species of the Lecithin

If we consider the lecithin peak in the previous chromatogram and in figure 3 (frozen egg lecithin), then, in contrast to the study made by van der Meeren et al.

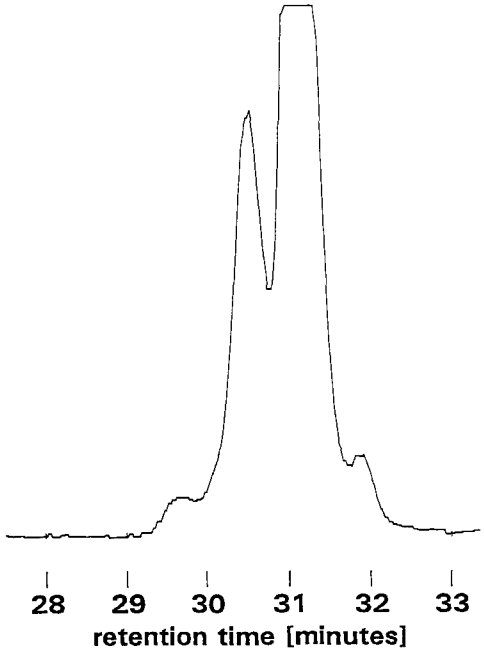


FIGURE 3: Part of a chromatogram of lecithin (from frozen egg) from Sigma, 5 μg injected

[14], who found a deformed lecithin peak, a clear separation into up to 4 peaks is striking. It is assumed that here a separation is to be seen according to the individual combinations of both the fatty acid residues of the lecithin. To prove this the PC standard solutions 2 and 3 were composed and injected. In order to detect retention time fluctuations due to the charge-like insertion of the eluents and - if necessary - to correct these, solution 3 was modified in 3A after making certain that the substances were separated enough from each other, to accurately measure the retention times of the maxima. Figure 4 shows the result of the dependence of the retention time and of the capacity factor as natural logarithm from the total of the number of the carbon atoms in the fatty acid residues. It can be seen that both dependencies are to be described as straight lines in mathematical terms. As a result of this for later calculation of the amount of carbon, only the relationship retention time to the

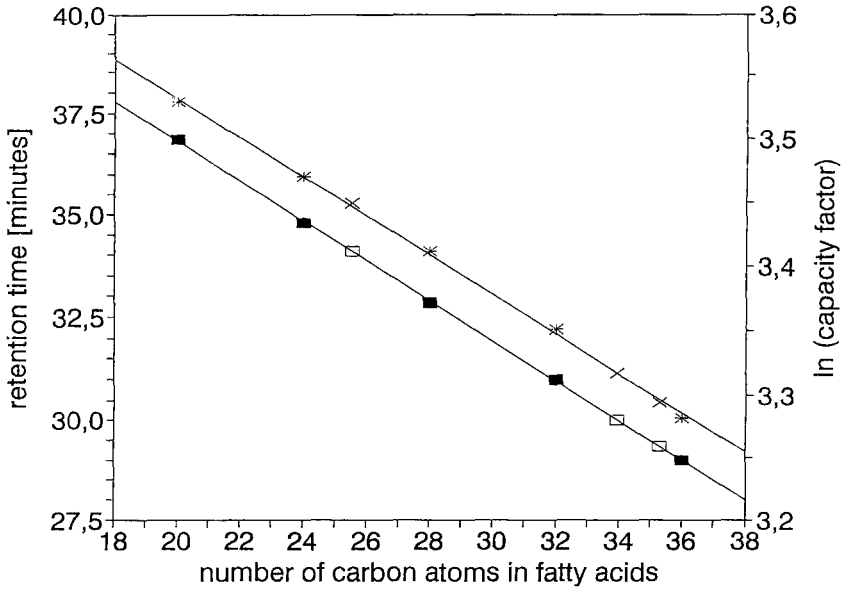


FIGURE 4: Dependence of the retention (time - continuous line and capacity factor - broken line) from the total of the carbon atoms of the saturated (filled in square, star) and unsaturated (empty square, plus sign) fatty acid residue of the lecithin

amount of carbon was always used. Also in the case of lecithin with unsaturated residues, the retention time was calculated back to the amount of carbon. Table 3 gives the numerical values for all the Nucleosils used. Within the framework of the measuring accuracy of the equipment used for example, the amounts of carbon given for the oleoyl residue (18:1 = number of carbon atoms: double bond amounts in the chain), for DO-PC and PO-PC correlate very well. As can be seen in figure 5, this is also true of all the PCs used with unsaturated residue.

Lecithin in Amniotic Fluid

As very large variations in the concentration of lecithin occur in amniotic fluid a calibration was performed with 6 concentrations. As can be seen in figure 6, the

TABLE 3

Calculated number of carbon atoms in unsaturated fatty acids of lecithin from retention times for the three Nucleosil used

Fatty acid residues		Number of carbon atoms of the residues					
		Nucleosil 120/3		Nucleosil 100/3		Nucleosil 50/5	
1	2	1	2	1	2	1	2
18:1	18:1	17.3	17.3	17.6	17.6	17.4	17.4
16:0	18:1	16.0	17.7	16.0	17.9	16.0	17.5
16:0	18:2	16.0	9.7	16.0	9.5	16.0	8.9

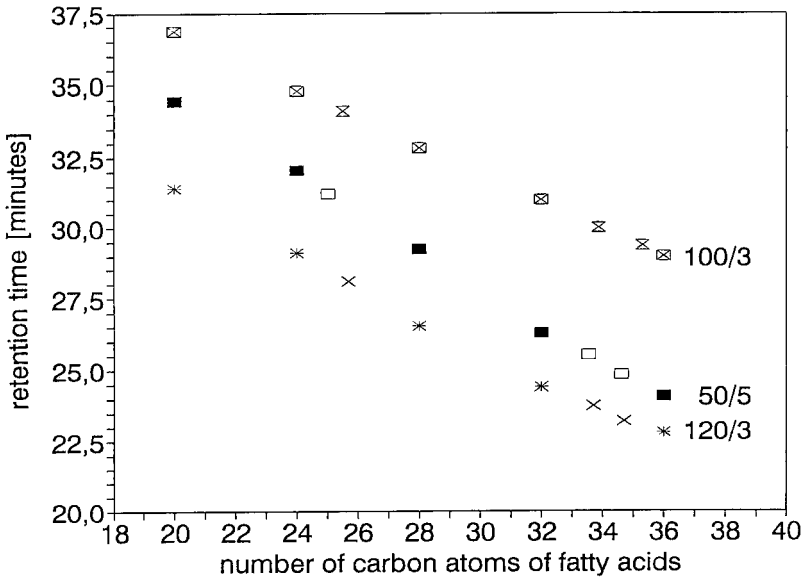


FIGURE 5: Separation of the lecithin with saturated (filled in square, square with X inside and star), and unsaturated (empty square, sand glass) on the three Nucleosils used

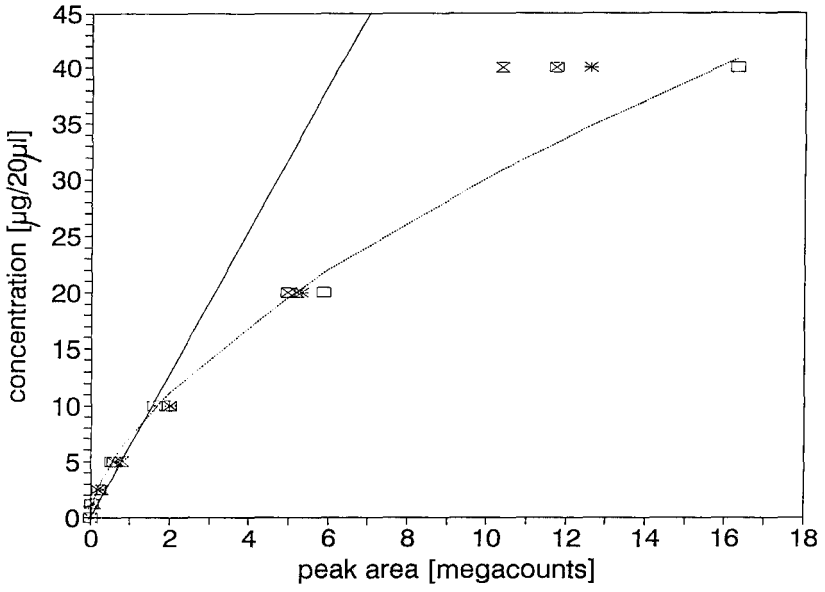


FIGURE 6: Calibration curve for the mass detector with PC (first - square with X inside and second measurement - empty square) and DD-PC, (first - sand glass and second measurement - star) one point method of the integrator (continuous line) and exposed function (broken line)

one point calibration with the help of the integrator (continuous line) can be used for the lower concentrations in the first approximation. Above an amount of 5 μg injected substance however increasing fluctuations occur. The broken line is used to calculate the portions of the total of the individual PC peaks. With the exception of the concentration 40 μg/20 μl the peak surface is independent of the substance (PC or DD-PC) and is easily reproducable. To calculate the curve the median value of the peak surfaces on 40 μg/20 μl is used.

Figure 7 shows standardized chromatograms of two samples of amniotic fluid. The addition of the standard solution 4 - peak at 24 respectively 32 minutes in chromatogram (A) - serves to calculate the carbon atom count n(C) from the retention times of the lecithin peaks. The results of the calculations for this amniotic fluid are given in Table 4. The margin of error of the carbon count amounts to a maximum of plus minus 0.5 atoms.

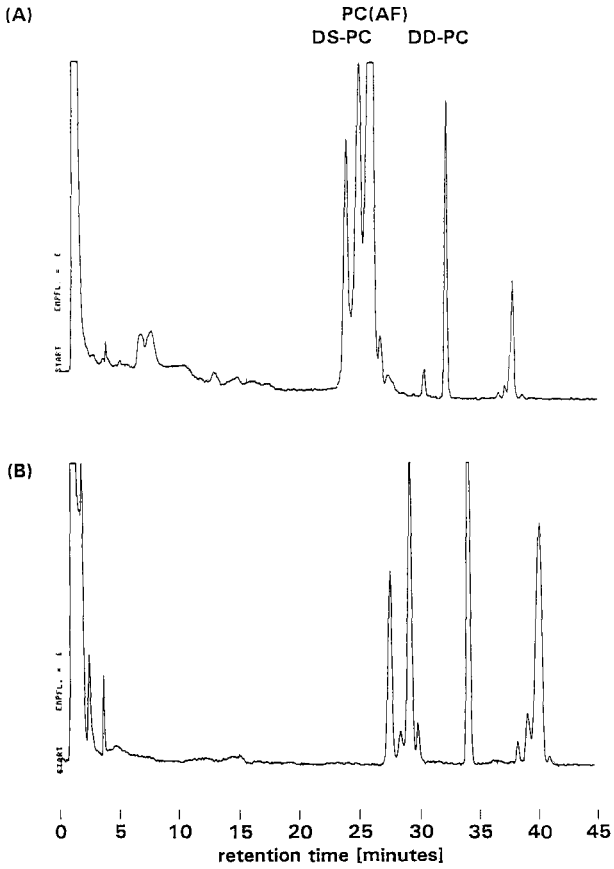


FIGURE 7: Chromatogram of (A) AF 432 (40/0 weeks) and (B) AF 491 (40/5 weeks), each with addition of standard solution 4

The average results of the measurements of 40 amniotic fluid samples are illustrated in figure 8 in an example with a Nucleosil 100 column. In all the amniotic fluid peaks were found, to which a total of 5 carbon atom counts could be attributed. Three of these five values (36.0, 33.9 and 32.1) are congruent with the established standards (DS-PC, PO-PC and DP-PC). These three fatty acids occur in amniotic fluid with at least 8 others [15,16]. The determination of further lecithins with

TABLE 4

Results of composition of lecithin in two amniotic fluids, AF 432 [40/0 weeks, figure 7 (A)] and AF 491 [40/5 weeks, figure 7 (B)]

Peak	AF 432		AF491	
No.	n(C)	Portion in %	n(C)	Portion in %
1	33.9	36.2	34.0	16.8
2	32.1	51.9	32.0	67.6
3	30.5	9.2	30.5	15.5
4	29.3	2.7		

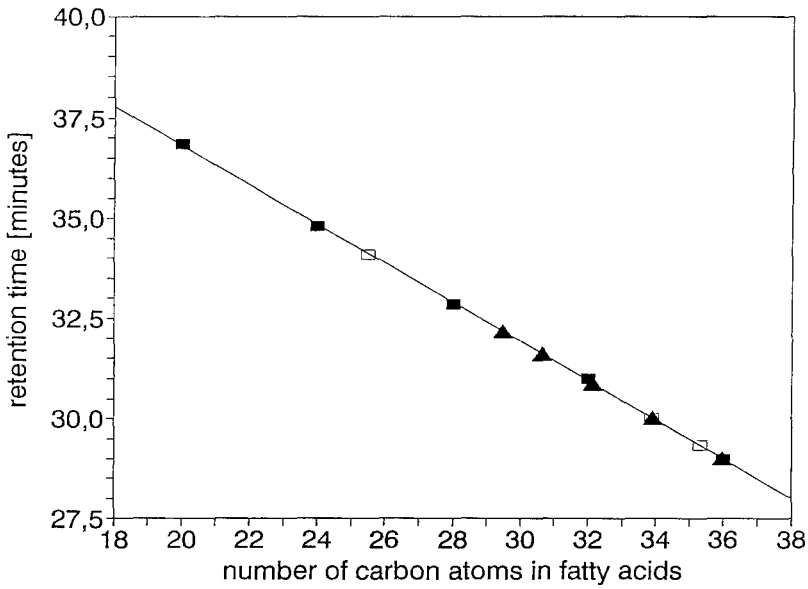


FIGURE 8: Retention of the lecithin with saturated (filled in square) and unsaturated (empty square) fatty acid residues as the peaks of amniotic fluid-lecithin (filled in triangle) in an example of a Nucleosil 100 column

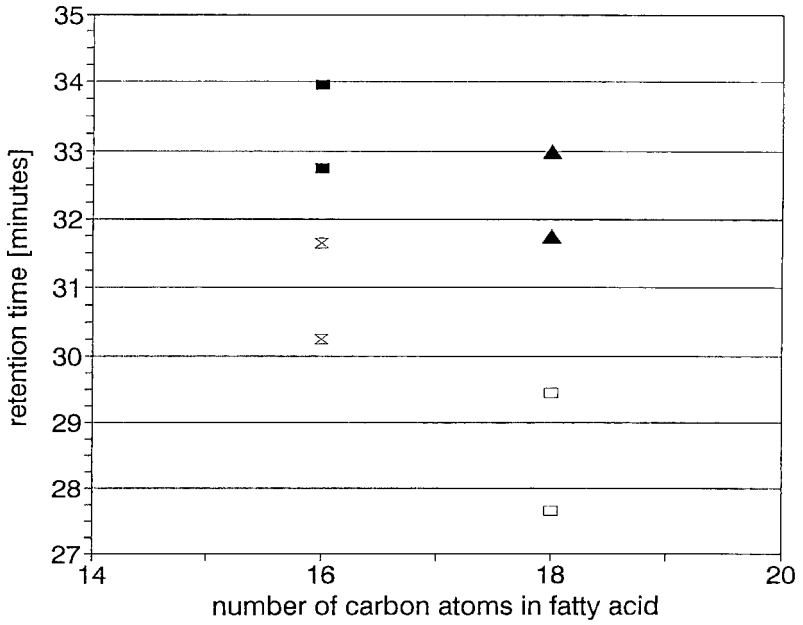


FIGURE 9: Retention times of the double peak of the sphingomyelin with a defined fatty acid residue on Nucleosil 100/3 (filled in square, or triangle) and Nucleosil 120/3 (sand glass, empty square)

unsaturated fatty acid residues (18:2, 18:3, 20:4 and 22:6) is hardly possible as they also elute in the range of sphingomyelin. Furthermore they are to be found in very small quantities in amniotic fluid so that they lie below the detection limit. The other two carbon counts determined to the peaks (30.7 and 29.2) only allow speculation which is not carried out here, on the possible combinations of the fatty acids in PC.

This chromatographic system can and ought not to replace the separation through reversed phase chromatography [15] to determine the combinations of the fatty acid residues particularly in lecithin. However the obstetrician is also especially interested in DP-PC, since its concentration gives information on lung maturity [15]. In addition to the total concentration of lecithin just the portion of this particular species is clearly measurable. After closer study of the chromatogramms given in the

literature, for example in Breton et al. [9], describing the analogous systems, the separation of lecithin can be observed in the same way. However this, with the exception of van der Meeren et al. [14] who presumably observed a peak widening with scatter formation due to a high water content, has apparently not been researched further.

The described separation into several peaks does not only apply to lecithin. Only in phospholipids that elute earlier can a visible peak widening be effected. On the other hand more than one peak is observed in SP and LL that elute later on.

Separation of Sphingomyelin

The double peak that is almost always observed in SP allows one to presume the separation according to various fatty acid residues is the cause. This should be examined by injecting P-SP and S-SP. In figure 9 the results are given on a Nucleosil 100 and a Nucleosil 120 column. The sphingomyelins also with a defined fatty acid residue each form a double peak. So - in contrast to the descrip-tion by Christie [3] - it cannot be a separation according to the chain length of the fatty acids. A much more probable solution seems to be that the separation is due to nonhydroxy and hydroxy fatty acids of equal chain length [17].

Conclusion

Also using normal phase chromatography by means of which the individual phospholipid classes in human amniotic fluid can be separated from each other, phosphatidylcholine can at least be separated in several particular fatty acid combinations which are of interest to the obstetrician. The obvious conclusion that the double peak of sphingomyelin is based on the same effect could not be confirmed.

Acknowledgements

We wish to express our hearty thanks to the Maria-Sonnenfeld-Gedächtnis-Stifung, Berlin for financing the mass or evaporative light-scattering detector.

Further thanks go to the company Säulentechnik Dr. Ing. H. Knauer GmbH in Berlin for the umcomplicated help given when problems arose with the columns. We wish to thank Dr. J. Reusch, the managing director of the company, for the stimulating and helpful discussions.

References

1) C. E. Parkinson and D. Harvey, "Amniotic fluid and fetal pulmonary maturity", in Amniotic fluid and its clinical significance, M. Sandler eds., Marcel Dekker New York 1981, pp. 229-252

2) W. W. Christie, Z. Lebensm. Unters. Forsch. 181: 171-182 (1985)

3) W. W. Christie, HPLC and Lipids - A practical guide, Pergamon Press Oxford 1987

4) P. R. Redden and Y.-S. Huang, J. Chromatogr. 567: 21-27 (1991)

5) T. Heinze, G. Kynast, J. W. Dudenhausen, C. Schmitz and E. Saling, Chromatographia 25: 497-503 (1988)

6) R. L. Sanders, "Introduction to lipid biochemistry" in Lung development: biological and clinical perspectives - volume 1 - biochemistry and physiology, P. M. Farrell Eds., Academic Press New York 1982, pp. 167-178

7) N. Sotirhos, C. Thörngren and B. Herslöf, J. Chromatogr. 331: 313-320 (1985)

8) G. Kynast and C. Schmitz, Z. Ernährungswiss. 27: 203-212 (1988)

9) L. Breton, B. Serkiz, J.-P. Volland and J. Lepagnol, J. Chromatogr. 497: 243-249 (1989)

10) W. S. Letter, J. Liq. Chromatogr. 15: 253-266 (1992)

11) L. L. Dugan, P. Demediuk, C. E. Pendley and L. A. Horrocks, J. Chromatogr. 378: 317-327 (1986)

12) C. C. Geilen, Freie Universität Berlin, Institut für Biochemie, personal communication

13) F. Possmayer, "Biochemistry of pulmonary surfactant during fetal development and in the perinatal period", in Pulmonary surfactant, B. Robertson, L. M. G. Van Golde and J. J. Batenburg, eds., Elsevier Science Publishers Amsterdam 1984, pp. 295-355

14) P. Van der Meeren, J. Vanderdeelen, G. Huyghebaert and L. Baert, Chromatographia 34: 557-562 (1992)

15) L. Gluck, M. V. Kulovich, R. C. Borer, P. H. Brenner, G. G. Anderson and W. N. Spellacy, Am. J. Obstet. Gynecol. 3: 440-445 (1971)

16) S. J. Robins and G. M. Patton, J. Lipid Res. 27: 131-139 (1986)

17) R. S. Lutzke and J. M. Braughler, J. Lipid Res. 31: 2127-2130 (1990)

Received: June 24, 1993
Accepted: September 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 999-1009 (1994)

DETERMINATION OF THE CONSTANTS OF THE SNYDER-SOCZEWINSKI EQUATION BY MEANS OF GRADIENT MULTIPLE DEVELOPMENT

W. MARKOWSKI[1], K. L. CZAPINSKA[1],
M. BLASZCZAK[2]
[1]*Department of Inorganic and Analytical Chemistry*
Medical Academy
20-081 Lublin
Staszica 6, Poland
[2]*Department of Mathematics*
Medical Academy
20-059 Lublin
Al. Raclawickie 1, Poland

ABSTRACT

Two - parameter equation which follows from the Snyder - Soczewinski competitive adsorption model describes the retention - eluent composition relationships for numerous solutes both in normal phase as well as reversed-phase systems. Its parameters for a given system can be determined from series of isocratic experiments or by reversed gradient multiple development.
In the present paper a two-step gradient development was applied. A method of solution of sets of equations leading to calculation of parameters k_o and m of the equation:
$\log k = \log k_o - m \log c$
is reported.

INTRODUCTION

In recent years an increasing popularity of high performance thin-layer chromatography has been observed; this is due to low cost, simplicity, short time of analysis, possibility of parallel

analysis of numerous samples. Modern densitometers transformed HPTLC in an accurate method of microanalysis; the same chromatogram can be scanned at several wavelengths which facilitates at different wavelengths. A new dimension is the combination of HPTLC with other chromatographic methods to increase the effectiveness of separation.

The optimization of the separation process is important especially in the case of complex samples and can be carried out by many methods [1-3]. One of the elements of the integral process of optimization is the choice of the eluent and its composition. In most methods multicomponent eluents are used. To choose the optimal composition of a multicomponent system the knowledge of the behaviour of the solutes in simpler - binary solvent systems is required [4]. From the characteristics of solute retention in binary systems the optimal composition of eluent in ternary system can be found, e.g., by the PRISMA model [5]. The behaviour of solutes in a binary solvent system may frequently be described by a two-parameter equation [6,7]. The constants of this equation are usually determined from log k′ vs. c plots for a series of isocratic experiments (c - concentration of modifier in mole or volume fraction). For multicomponent samples of wide range of polarities of the components a more promissing technique is gradient elution which also requires the knowledge of retention vs. composition relationships. In the present paper we describe an attempt to apply the principle of reverse gradient in version of two-step development to characterize the components of multicomponent samples and to determine the retention-eluent composition relationships. These relationships can then be used for further optimization of the separation depending on the purpose of analysis and criteria assumed.

THEORETICAL CONSIDERATIONS

The process of multiple development with increasing development distances and reverse gradient (decreasing elution strength for consecutive developments, evaporations of solvent after each step) has been discussed in detail in earlier papers [8,9]. The final R_F value for the simplest case of reverse - gradient double development and increasing development distance can be calculated from the following equation [8]:

$$R_{FGh(j)} = z_{(1)} R_{F(1,j)} + [1 - R_{F(1,j)} z_{(1)}] R_{F(2,j)} \quad (1)$$

Let us assume that two experiments are carried out. In the first the development distance was $z_{(1)}$ with an eluent of concentration of the modifier $c_{(1)}$; then the plate is dried. In the second stage the distance was $z_{(2)}=1.0$, with an eluent of $c_{(2)} < c_{(1)}$. In the second experiment the first development distance is $z_{(3)}$ with an eluent of concentration $c_{(3)}$, the plate is dried as in the first experiment and then developed with eluent of concentration $c_{(4)}$ to distance of $z_{(4)}=1.0$. In both experiments the same plates are used and eluents $c_{(1)}$ - $c_{(4)}$ have the same qualitative composition and differ only in the concentrations of the modifier. We have thus two equations for the final R_F of the solute j:

$$R_{FG1(j)} = z_{(1)} R_{F(1,j)} + [1 - R_{F(1,j)} z_{(1)}] R_{F(2,j)} \quad (2)$$

$$R_{FG2(j)} = z_{(3)} R_{F(3,j)} + [1 - R_{F(3,j)} z_{(3)}] R_{F(4,j)} \quad (3)$$

The R_F values of individual solutes j can be expressed by their characteristic parameters. If the relationships are given by two - parameters equation which follows the Snyder-Soczewinski model [6,7]:

$$\log k_{(i,j)} = \log_{o(j)} - m_{(j)} \log c_{(i)} \quad (4)$$

then the R_F value is equal to:

$$R_{F(i,j)} = \frac{1}{1 + \frac{k_{o(j)}}{c_{(i)}^{m_{(j)}}}} \quad (5)$$

If eq.(5) is substituded to eqs., (2,3) then after transformation we have the following set of equations:

$$R_{FG1(j)} k_{o(j)}^2 + k_{o(j)} [R_{FG1(j)} c_{(2)}^{m(j)} + R_{FG1(j)} c_{(1)}^{m(j)} - z_{(1)} c_{(1)}^{m(j)}] - [R_{FG1(j)} - 1] c_{(1)}^{m(j)} c_{(2)}^{m(j)} \quad (6)$$

$$R_{FG2(j)} k_{o(j)}^2 + k_{o(j)} [R_{FG2(j)} c_{(4)}^{m(j)} + R_{FG2(j)} c_{(3)}^{m(j)} - z_{(3)} c_{(3)}^{m(j)}] - [R_{FG2(j)} - 1] c_{(3)}^{m(j)} c_{(4)}^{m(j)} \quad (7)$$

in which k_o and m are unknown. The remainning values are known and the values of $R_{FG1(j)}$ and $R_{FG2(j)}$ are determined experimentally in two experiments as described above. The set of eq., (6,7) can be solved

by approximation methods; no analytical solution exists. The following procedure is applied. Eqs., (6,7) are quadratic with respect to k_o. Therefore, we determine for eq., (6) for which values of m the equation is sensible, i.e $\Delta \geq 0$. Then its solution are given by equations:

$$k_o^{(1)} = \frac{(-b+\sqrt{\Delta})}{2a} \quad (8a)$$

$$k_o^{(2)} = \frac{(-b-\sqrt{\Delta})}{2a} \quad (8b)$$

Since the root given by eq.(8b) is always negative, it is discarded. The root calculated from eq. (8a) for a given m values is calculated assuming that $\Delta \geq 0$. The root is introduced to eq.(7) and the polynom obtained is checked:

$$F(m) = R_{FG2(j)} k_{o(j)}^2 + k_{o(j)} [R_{FG2(j)} c_{(4)}^{m(j)} + R_{FG2(j)} c_{(3)}^{m(j)} - z_{(3)} c_{(3)}^{m(j)}] - [R_{FG2(j)} - 1] c_{(3)}^{m(j)} c_{(4)}^{m(j)} \quad (9)$$

The polynom F is checked relative to the change of sign: if for a certain value of m1, $F_1 >= 0$, and for another m2 >m1, $F_2 <= 0$ or if the opposite is true, that is, $F_1 <= 0$ and $F_2 >= 0$, then the polynom can be solved, e.g., by the bisection method [10]. Defining the accuracy of solution of the polynom (ϵ),we find the value of m, and then from eq. (6) or (8) the corresponding value k_o,thus obtaining the two necessary parameters of eqs. (5) and (9). The procedure was applied to elaborate a computer program. The starting data for a given solute are the values of R_{FG1} and R_{FG2} obtained in two different gradient experiments and development distances $z_{(1)}$ and z(3), modifier concentration $c_{(1)}$ and $c_{(4)}$ in the individual stages of development and the epsilon value, i.e., the accuracy with which the zero value of polynom is determined. If there is no solution, an appropiate answer is given. The program has been written in Pascal (version 6.0).

EXPERIMENTAL

The chromatografic experiments were carried out using a horizontal sandwich chamber DS (Chromdes, Lublin, Poland) [11,12]. Precoated 10 x 5 cm plates covered with 0.25 mm layer of silica Si 60 (E. Merck, Darmstadt, FRG) were used. As model solutes, lipophilic mixture of test dyes (E. Merck), azobenzene and 2-nitroaniline were used, chromatographed in the system heptane - diisopropyl ether. The total development distance was 80 mm.

RESULTS AND DISCUSSION

As stated in the Introduction, the relationship between retention and eluent composition is frequently represented by a straight line in the system of cordinates log k' vs. log c. The equation (4) is characterized by two parameters: the slope m and the value k_o (k' for pure modifier, c=1). It follows from numerous experimental results [4] that the equation is approximate and that deviations from linear relationships are observed especially for low and high concentration of modifier. The log k' vs. log c plots for a whole group of substances investigated permits the estimation of selectivity and range of k' values which allows for the choice of suitable modifier concentration for a given set of solutes; this constitutes the basis of the optimization process.

The solutes were chromatographed in isocratic runs for several concentrations of diisopropyl ether in heptane. From the R_M (log k') vs. log c plots the k_o and m vaues were determined by the least squares method (Table 1).

Since reverse - gradient two - stage development was used, the final R_{FG} values were calculated (Table 3) using an equation and computer program derived earlier [8] for the gradient programs applied (Table 2 - development distances and modifier concentrations).

Table 1

The comparison of coefficients k_o and m calculated from isocratic measurement of R_F (in the range of modifier from 0.2 to 0.4 volume fraction) and calculated from gradient data.

CODE	Isocratic mode			Gradient mode	
	k_o	m	r	k_o	m
Azobenzene 1	0.139	0.99	0.9981	0.116	1.25
Azobenzene 2	0.241	1.44	0.9409	0.208	1.55
4-Dimethylamino-azobenzene	0.336	1.15	0.9555	0.192	1.58
Indophenole Blue	0.447	1.82	0.9424	0.214	2.33
Sudan Red G	0.751	1.51	0.9672	0.286	2.22
Orto-nitroaniline	0.698	1.49	0.9407	0.203	2.55
Meta-nitroaniline	1.309	1.52	0.7824	0.639	2.25
Para-nitroaniline	2.53	1.66	0.9603	3.52	1.75

Two gradient experiments permitted to obtained pairs of R_{FG1} and R_{FG2} values for each solute (Table 3).

Applying the method of solution of the pair of equations described above and the computer program (Fig.1) the constants k_o and m for each solute were calculated (Table 1). The values thus obtained can be used to determine log k' vs. log c lines and to charactrize the system (sample, eluent, adsorbent).

Table 2.

The gradient program used in two - stage development.

RUN	STEP	DISTANCE	CONCENTRATION
#1	1	0.25	0.40
	2	1.00	0.20
#2	1	0.50	0.40
	2	1.00	0.30

Table 3

The comparison of the final values of R_{FG} obtained by simulation of two stage development and in experiments.

CODE	$R_{FG(calc.)}$		$R_{FG(exp.)}$	
	Run #1	Run #2	Run #1	Run #2
Azobenzene 1	0.63	0.77	0.62	0.78
Azobenzene 2	0.34	0.54	0.38	0.58
4-Dimethylamino-azobenzene	0.36	0.54	0.39	0.56
Indophenole Blue	0.14	0.29	0.18	0.36
Sudan Red G	0.13	0.26	0.16	0.32
Orto-nitroaniline	0.14	0.27	0.15	0.32
Meta-nitroaniline	0.08	0.16	0.08	0.17
Para-nitroaniline	0.04	0.08	0.03	0.06

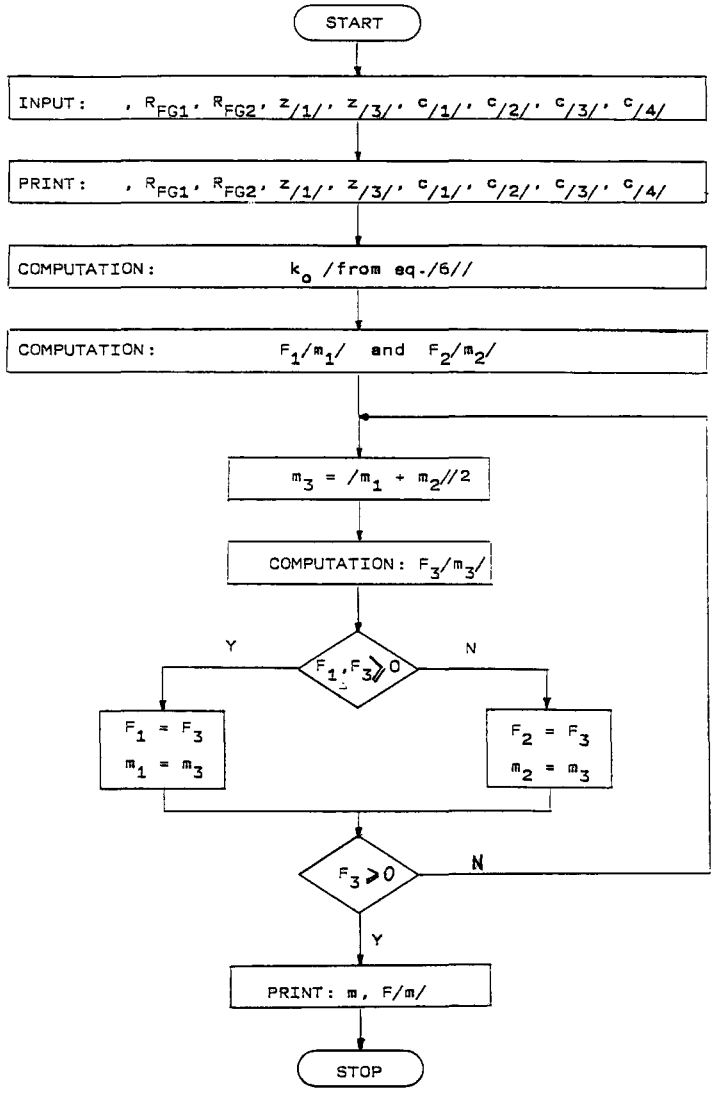


Fig.1 The flow diagram of the computer program for calculation ko and m values

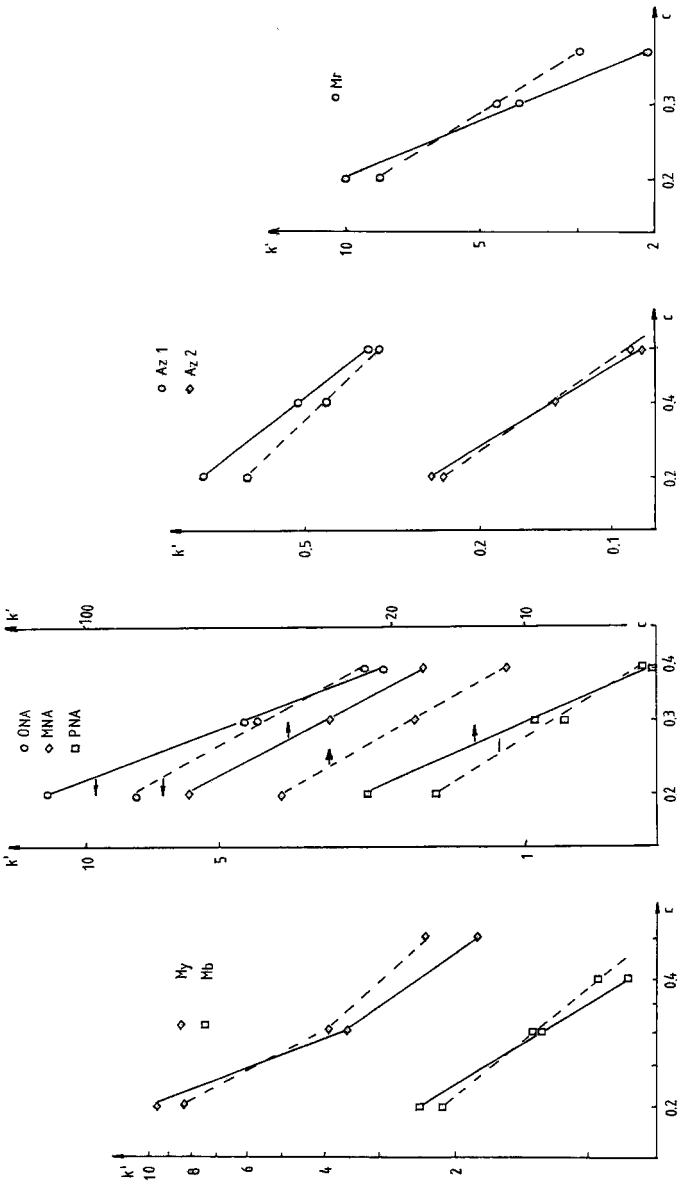


Fig.2 The comparison of log-log plots determined by the isocratic method (the dashed line) and by the two-step gradient (the continuous line) for investigated solutes: Mr - Sudan Red G, Mb - Indophenol blue, My - 4-Dimethylaminoazobenzen, Az1 - Azobenzene 1, Az2 - Azobenzene 2, ONA Orto-nitroanilina, PNA Para-nitroanilina, MNA Meta-nitroanilina

The comparison of the retention - eluent composition relationships obtained by isocratic and stepwise gradient methods is presented in Fig.2. It can be seen from Fig.2 and comparison of the Table 3 and Table 1, that the data from gradient experiment indicated somewhat higher values of slopes m that those obtained from isocratic experiments. Except for 4 - nitroaniline the remaining straight lines are close to each other; sometimes they cross. Taking into account the differences in experimental techniques (isocratic and gradient multiple development) the similarity of results obtained can be considered satisfactory.

The retention - eluent composition relationships can be used for futher optimization procedure.

References

1. S. Habibi-Goudarzi, K.J. Ruterbories, J.E. Steinbrunner and D. Nurok, Computer-Aided Survey of Systems for Separating Steorides: J. of Planar Chromatogr., 1 , (1988), 162

2. G. Matysik, W. Markowski, E. Soczewinski and B. Polak: Computer-Aided Optimization of Stepwise Dradient Profiles in Thin-Layer Chromatography, Chromatographia, 34, No. 5-8, (1992), 302

3. Q.-S. Wang and H.-Y. Wang: Computer-Assisted Comprehensive Optimization of Mobile Phase Selectivity in HPTLC, J. of Planar Chromatogr., 3, (1990), 15

4. W. Markowski: Computer-Assisted Selection of the Optimum Gradient in Thin-Layer Chromatography, J. of Chromatogr., 485, (1989), 517

5. S. Nyiredy, K. Dallenbach-Talke and A. Sticher: The "Prizma" Optimization System in Planar Chromatography, J. of Planar Chromatogr., 1, (1988), 336

6. E. Soczewinski: Solvent Composition Effects in Thin-layer Chromatography System of the Silica Gel-electron donor solvent, Anal. Chem., 41, (1969), 179

7. E. Soczewinski: Coadsorption Effects in Liquid-Solid Systems of the Type Silica-Heptane + Dioxane. I Theoretical Considerations, J. of Chromatogr., 388, (1987), 97

8. W. Markowski and E. Soczewinski: Computer-aided Optimization of Gradient Multiple-development Thin-layer Chromatography, I Two-stage Development, J. of Chromatogr., 623, (1992), 139,

9. W. Markowski: Computer-aided Optimization of Gradient Multiple-development Thin-layer Chromatography, II Multi-stage Development, J. of Chromatogr., accepted for publication

10. E. Slavicek: Computing Techniques for Chemists, (in polish), WNT, Warsaw 1991

Received: April 29, 1993
Accepted: August 10, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1011-1028 (1994)

FLOW FLUCTUATIONS IN GPC-VISCOMETRY*

JAMES LESEC
CNRS URA#278 - ESPCI
10 rue vauquelin
75231 - Paris cedex 05 - France

INTRODUCTION

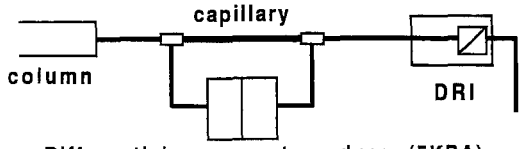
GPC-Viscometry requires a molecular weight calibration curve, usually a "Universal calibration curve" (1) Log ([η]*M) = f (Ve). With classical GPC, using only one concentration detector, perfect control of solvent flow rate is required since molecular weights of broad polymers are calculated by comparison of their sliced distributions with elution volumes of narrow standards. Data acquisition being performed as a function of time, this comparison can be achieved accurately only when both experiments are run at exactly the same flow rate. A small error in flow rate introduces a significant error in molecular weight because of the logarithmic scale of the calibration curve.

The purpose of this paper is not to discuss this kind of problem, but to study the consequence of very small flow fluctuations. These fluctuations are unable to introduce a significant error in molecular weights when referring to a calibration curve, but they lead to very small peak distortions with flow-sensitive detectors like the Single Capillary Viscometer (SCV) (2-6) and, consequently, to errors in data interpretation.

THE SINGLE CAPILLARY VISCOMETER.

The Single Capillary Viscometer (SCV) used in the WATERS GPC 150CV instrument (7) is described in Figure 1. It is composed of a capillary tube

* paper presented at the WATERS Int'l GPC symposium, SAN FRANCISCO, october 1991.



Capillary characteristics
Diameter = 15/1000"
Length = 6" (15 cm)
Internal volume = 18 µl

Performance in THF at 1 ml/mn and 35°C
Shear rate = 2800 s^{-1}
Reynolds = 120
Differential pressure = 2 KPA (20 mbars)
Working pressure = 200 KPA (2 bars)

Figure 1. The Single Capillary Viscometer (SCV).

with the following characteristics (length=6", internal diameter=0.014") and of a differential pressure transducer connected to both capillary ends to measure the pressure drop across the capillary.

SCV obeys Poiseuille's law and the pressure drop P across the capillary depends on the capillary geometry (radius r and length l), on the flow rate Q and on the viscosity of fluid η according to:

$$P = 8/\pi * l/r^4 * \eta * Q$$

At constant flow rate Q, the pressure drop is proportional to viscosity η and at constant viscosity η, the pressure drop is proportional to flow rate Q. Consequently, in order to use the SCV as an accurate viscometer, the flow rate must be maintained absolutely constant during the GPC experiment. Conversely, SCV allows perfect control of flow rate and can also be used as a very powerful troubleshooting tool.

The purpose of this paper is to demonstrate that, when using a SCV for viscometry measurements and assuming a very constant flow that is not strictly observed, the viscometer function may be corrupted by a very small flow fluctuation, leading to erroneous interpretation of viscometry data.

EVIDENCE OF A FLOW FLUCTUATION.

Origin of the flow fluctuation.

Figure 2 represents the block diagram of the GPC/Viscometry experiment. For our purpose, the important part of the design is the detector area where restrictions occur because of the presence of capillary tubes (0.009"). These low diameter tubings are usually used as connecting tubes but also as detector inlet tubings (Differential Refractive Index detector - DRI) in order to minimize dead volumes.

In GPC, the problem is we are using high molecular weight samples that increase the viscosity of solvent. As the viscosity of pure solvent is η_0, when a polymer is dissolved it becomes η using the following relationship in a first approximation:

$$\eta = \eta_0 * (1 + [\eta] * C)$$

C is the sample concentration and $[\eta]$ the sample intrinsic viscosity that varies with molecular weight M according to:

$$[\eta] = K * M^{\mathbf{a}}$$

K and **a** being the Mark-Houwink coefficients ($0.5 > \mathbf{a} > 0.8$ for coil polymers).

The parameter $[\eta]*C$ represents the increase of solvent viscosity due to the presence of polymer. Consequently, when the polymer solution enters the detector area, the viscosity of fluid increases, and, according to the Poiseuille's law above-described, the pressure drop in detectors increases proportionally, leading to an increase of the total back pressure in the system. This increase is very weak, since polymers are very diluted, but depends on the parameter $[\eta]*C$; nevertheless this is the origin of the flow fluctuation.

The flow fluctuation.

When the polymer comes across the columns, the system is in pressure equilibrium. When the polymer comes across the detectors, the previous equilibrium is disturbed according to two phases:

- phase #1 - When the polymer enters into the detectors, there is an increase of pressure leading to a momentary decrease of flow rate to reach another equilibrium. The pumping system being at constant flow rate, the system

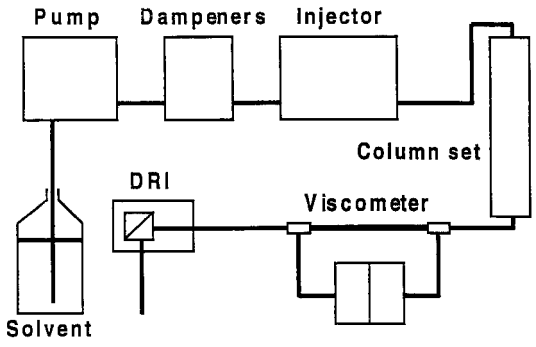


Figure 2. Block diagram of the GPC/Viscometry experiment.

(dampeners and columns) accumulates some solvent and gives a negative wave in flow rate at the outlet.

- phase #2 - When the polymer elutes from the detectors, there is a decrease of pressure leading to a momentary increase of flow rate. The system (dampeners and columns) which has previously accumulated some solvent, releases the solvent and now gives a positive wave in flow rate at the outlet to reach another equilibrium.

In fact, the detector volume being very small with regard to the peak volume, the two phenomena (phase #1 and phase #2) occur quite simultaneously. Consequently, it is impossible to obtain the two waves separately but only their resultant overlay, since they occur at different times that correspond only to the detector volumes. This is represented in Figure 3.

The consequence of this flow fluctuation on the viscometer profile is represented in figure 4. The real peak and the flow fluctuation are overlaid in 4a. The composition of the two signals is represented in 4b where the dashed line corresponds to the real peak and the solid line to the experimental peak. Obviously, a peak distortion occurs which leads to an experimental peak that looks to be moved downstream. It is a peak distortion but it looks like a peak shift, this is why this effect conflicts with interdetector volume correction, as we shall see later. It should be noted here that a very small fluctuation is enough to produce a significant apparent peak shift.

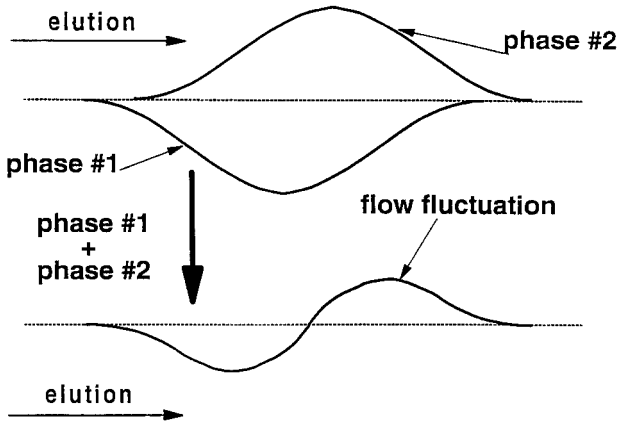


Figure 3. The flow fluctuation, summation of two fluctuations.

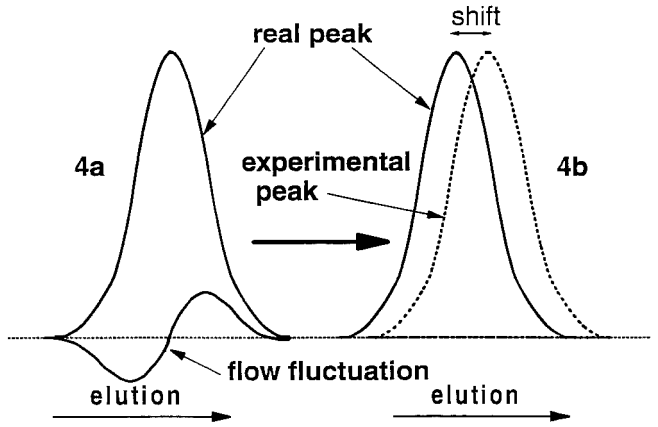


Figure 4. Viscometer profile distortion by a flow fluctuation.

Computer simulation.

A computer simulation was performed in order to verify the hypothesis of the flow fluctuation. The chromatographic system is represented as slices of solvent, each slice having a volume of 20 µl. The following conditions were used:

- the column set is represented by a long tubing with an internal diameter of 0.017" based on the equivalence of a volume of 11 ml and a pressure drop of 6 bars which is the normal behavior of a column set at 1 ml/mn of THF. (high number of 20 µl slices).

- the viscometer is represented by a tubing with 0.014" I.D. and one 20 µl slice (which is very close to the reality 18 µl).

- the refractometer inlet capillary is represented by a tubing with 0.009" I.D. and five 20 µl slices (which represents approximately its common internal volume, 80-100 µl).

- the refractometer outlet tube is represented by a tubing with 0.040" I.D. with a large number of 20 µl slices (the pressure drop in this tubing is negligible anyway).

In the simulation, these four volumes are connected in series and in this sequence. A gaussian viscosity profile is entered into the simulation to simulate the detection of a viscometer peak. The total pressure drop is then computerized for pure THF using the Poiseuille's relationship by the summation of individual pressure drops of each slice. From the viscosity profile, three different parameters can be calculated: the pressure profile, the flow profile and the distorted viscosity profile. They are represented in Figure 5.

- the pressure profile - The excess of pressure starts at a non-zero value because of the previous presence of the polymer in the columns. Then it increases when the polymer enters into the DRI inlet tubing then decreases when it enters the outlet tubing. We can observe that the final pressure value is smaller than the starting pressure value, since the system was first at equilibrium with the polymer in a 0.017" tubing and at the end it is in a 0.040" tube (which is roughly zero excess of pressure).

- the flow profile - It is represented in arbitrary units and starts at a value of 4, which corresponds to the nominal value of flow rate (the system being at equilibrium). It decreases to a value of 2 when the polymer enters into the DRI inlet tubing, then it increases to approximately a value of 6.6 and returns to the

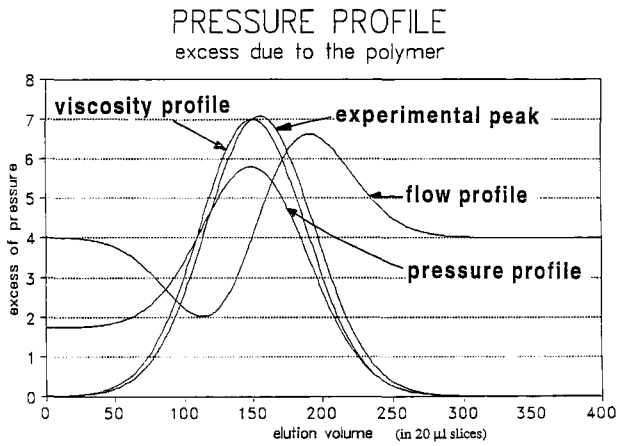


Figure 5. Computer simulation of the flow fluctuation and the viscometric signal distortion on a standard instrument.

previous equilibrium value of 4 (nominal flow rate). THIS IS THE TYPICAL FLOW FLUCTUATION. We can observe that this fluctuation is not symmetrical, due to the fact that the pressure fluctuation profile is not symmetrical either. This reason has been previously explained.

- the distorted viscosity profile (experimental peak) - It is calculated by adding the viscosity profile to the flow fluctuation profile using an attenuation coefficient to take into account the difference in scale (the flow profile being expanded to become visible since it is a weak effect). We can see in Figure 5 that the distortion of the viscosity profile looks like a small shift towards high elution volumes as previously predicted.

Experimental evidence.

In order to evidence this phenomenon experimentally and, as it was impossible to eliminate this effect in a first step, two sets of experiments were performed with two different settings represented in Figure 6.

- instrument #1 is the standard Waters model 150CV.

- instrument #2 is the same instrument with a restrictor (long 0.009" I.D. capillary tube) inserted between the column set and the detectors. This device does not

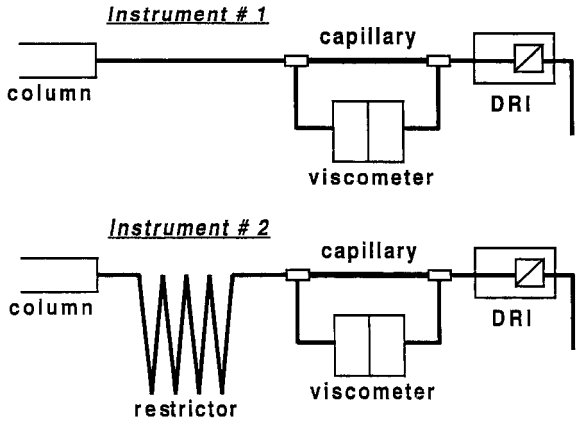


Figure 6. Schematics of instrument #1 and instrument #2.

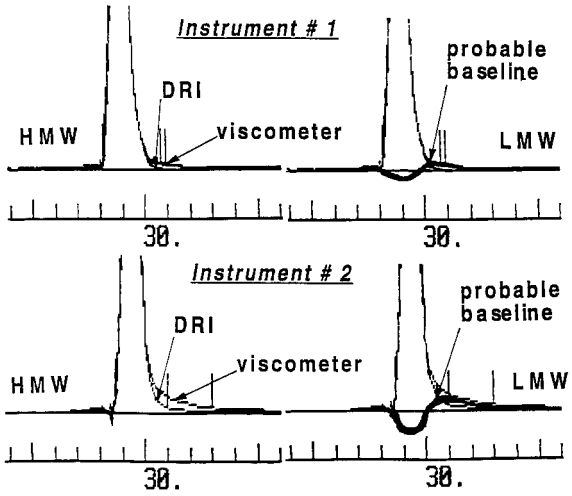


Figure 7. Influence of the flow fluctuation on the elution profile of the narrow polystyrene standard 355K.

change the interdetector volume at all and, with regard to the DRI peak, the viscometer peak has no reason to move. The advantage of this setting is not to correct the effect but to amplify it, since the presence of the restrictor should normally increase the pressure drop in the detector area and, consequently, the intensity of the pressure fluctuation.

The results are represented in Figure 7 where a narrow polystyrene standard 355K has been injected into both instruments under exactly the same conditions. On instrument #1, we can observe on peak feet (top, left) an abnormal behavior of the viscometer peak since, in the low molecular weight region, the viscometer response is a little bit stronger than the DRI one, which is impossible. The DRI response being as C and the viscometer response being as $C^*[\eta]$, the viscometer response should be smaller than the DRI response in the low molecular weight region. Also, we can suspect a flow fluctuation represented (top, right).

On instrument #2, the phenomenon is much more obvious. We can observe that the viscometer signal (bottom, left) is significantly above the DRI signal and, even at the beginning of the peak, there is a small sharp decrease of the signal that is very probably the beginning of the flow fluctuation. It is easy to imagine the probable baseline under the peak that is drawn in Figure 7 (bottom, right). So, increasing the pressure drop in the detector area increases the effect. That demonstrates that the pressure drop in detectors is really responsible for the flow fluctuation.

The interdetector volume correction.

The interdetector volume correction is indirectly involved here since the consequence of the flow fluctuation is an apparent peak shift. The two detectors are connected in series and the slice data acquisition is usually performed simultaneously on both detectors. In order to match the slice information coming from the two detectors, it is then necessary to correct the viscometer slice retention volumes to take into account the time necessary for one molecule to move from the viscometer to the DRI. This is what is called the "interdetector volume correction".

As the flow fluctuation abnormally moves the viscometer peak downstream, it is possible to use the interdetector volume correction to study the consequence of the flow fluctuation. As we shall see, using the correct value of interdetector volume leads to erroneous results, but using an underestimated value may lead to correct results, the flow fluctuation shift being corrected by this too-small interdetector volume correction.

Consequence on data interpretation.

The consequence of the flow fluctuation on data interpretation is exactly the same as a wrong interdetector volume correction. There is a mismatch between the slice concentration information C_i from the DRI and the slice pressure information $[\eta]_i*C_i$ from the viscometer. $[\eta]_i$ being calculated by dividing $[\eta]_i*C_i$ by C_i; the mismatch leads to a wrong calculation of $[\eta]_i$. As this effect is very weak, the errors on $[\eta]_i$ are almost negligible and the average $[\eta]$ is generally calculated well. BUT, AND THIS IS THE MAIN ISSUE, THE ERRORS ARE NOT SYMMETRICAL. When the viscometer peak is moved downstream for example, $[\eta]_i$ is calculated a little bit too small in the HMW region and a little bit too high in the LMW region, leading to a slight rotation of the Mark-Houwink plot and a slight decrease of the Mark-Houwink **a** exponent. THIS IS THE MAIN CONSEQUENCE OF THE FLOW FLUCTUATION.

Unfortunately, when running a GPC-Viscometry experiment, we are very concerned with the exponent **a** value that has a physical meaning (**a**=0.5 for poor solvents, **a**=0.8 for good solvents, higher value for rigid polymers). Also, for branched polymers, the branching distribution g'_i being calculated by dividing the experimental intrinsic viscosity $[\eta]_i$ by the $[\eta]_i$ value of the corresponding linear polymer at the same MW, the calculation of the branching distribution would be also affected by this effect. For these reasons, the flow fluctuation must be corrected if possible or eliminated by a modification of the hardware.

EXPERIMENTAL RESULTS.

The evidence of the flow fluctuation was firstly described in 1991 (8-9) using the analysis of several broad distribution polystyrene samples. Table 1 shows typical results obtained with the polystyrene DOW 1683 using a standard Model 150 CV having an interdetector volume (offset) of around 80 µl (geometrical value). The Mark-Houwink K and **a** coefficients were measured with 11 polystyrene narrow standards (**a**=0.71, logK=-1.8775).

This result shows that, when using the correct value of interdetector volume, a too small value is obtained for **a**, the correct value being obtained using

Table 1 - Mark-Houwink **a** determination on DOW 1683.

Offset used (in µl)	**a** exponent
80 (geometrical)	0.6
-20 (corrected)	0.71

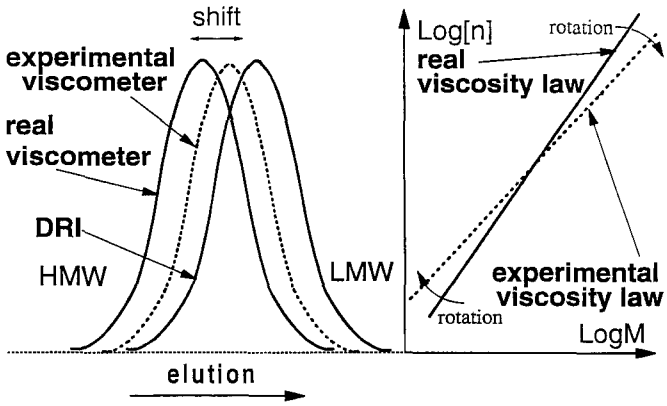


Figure 8. Viscometer peak shift and rotation of the Mark-Houwink law.

an interdetector volume correction of -20 µl. This means that the flow fluctuation has moved the viscometer peak downstream by 100 µl, which is a small effect but which is enough to make a decrease of the **a** Mark-Houwink exponent from 0.71 to 0.6.

It is important to notice that this 100 µl apparent shift, and consequently, the decrease of the **a** exponent from 0.71 to 0.6, is induced by a very weak flow fluctuation. Its maximum intensity has been estimated to be around 4% of the maximum deviation of the viscometer peak. In the DOW 1683 conditions, at a flow rate of 1 ml/mn, the maximum deviation of the viscometer signal corresponds to an equivalent flow variation of 0.01 ml/mn. 4% of this value leads to a maximum flow fluctuation value of 0.0004 ml/mn.

Nevertheless, this extremely weak variation in flow (0.4 µl/mn, that is 0.04%) significantly disturbs the viscometry calculation and leads to a decrease of the **a** exponent from 0.71 to 0.6.

Obviously, this kind of extremely weak fluctuation 0.04% cannot lead to any problems in the calculation of molecular weights when referring to the calibration curve; only the Mark-Houwink plot rotates a little bit. This effect demonstrates the very high general quality of the flow control in the Waters GPC 150CV instrument, based on the use of the Single Capillary Viscometer as a flow controller.

Other evidence is given in Table 2 where results on instrument #1 and instrument #2 (previously described) are compared.

Table 2 - Mark-Houwink **a** determination function of pressure drop.

Offset used (in µl)	**a** (instrument #1)	**a** (instrument #2)
80 (geometrical)	0.6	0.52
- 20 (corrected)	0.71	0.63

Table 3 - Measurements of peak elution volumes in ml.

Difference (DRI-Visco)	NBS 706	DOW 1683	Offset
Theoretical	0.847	1.224	0
Instrument #1	0.747	1.125	0.100
Instrument #2	0.691	1.079	0.150

Table 4 - Measurements of broad polystyrene samples.

Samples	Mw_{ps}	Mw_{univ}	[η]	**a**	LogK
DOW1683-labo	242,600	245,800	81.8	0.709	-1.873
DOW1683-elf	241,300	241,900	82.5	0.708	-1.866
NBS706-labo	261,300	257,000	90.7	0.715	-1.888
NBS706-elf	258,800	256,900	88.6	0.715	-1.895
BASF168N-labo	303,700	302,500	99.6	0.682	-1.712
BASF168N-elf	316,900	319,200	101	0.689	-1.760
PS-IUPAC	217,600	213,600	77.9	0.705	-1.831
PS1240-elf	305,600	300,000	101.5	0.707	-1.840

For instrument #1, we get the same result than in Table 1. For instrument #2, we observe a stronger effect leading to smaller a values. It has not been possible to correct the used offset to obtain the right value 0.71 for instrument #2, the software not allowing too negative offset values.

A further evidence is reported in Table 3 by the measurement of the differences in retention volumes at the peak apex between the viscometer and the DRI for DOW 1683 and NBS 706 on both instruments.

The measurements of peak retention volumes confirm a viscometer peak shift downstream depending upon pressure drop in the detector area: 100 µl for instrument #1 and 150 µl for instrument #2.

Nevertheless, it was possible to analyze several broad distribution polymers using the corrected value of interdetector volume of -20 µl. Instrument

used is a Waters 150 CV at a temperature of 40°C with THF as eluent at 1 ml/mn. The column set was composed of four Waters Ultrastyragel 10^3, 10^4, 10^5 and 10^6 Å. For each sample, 400 µl were injected at a concentration of 0.1%. Broad polystyrene samples were analyzed using a polystyrene calibration to check the behavior of universal calibration. Mw_{ps} are molecular weights in PS units (classical GPC) and Mw_{univ} are molecular weights in real units by GPC-Viscometry. They are listed in Table 4 with viscometry results. The viscosity law obtained using 11 narrow distribution standards was: **a**=0.710, LogK=-1.8775.

We observe a good correlation of molecular weights and viscosity parameters. Nevertheless, Table 4 contains original results of the very first experiments in March 1990. With regard to more recent results, the molecular weight values listed in Table 4 are too low by approximately 4% due to an imperfect calibration curve. The same holds true for intrinsic viscosity [η] values that are also too low by approximately 4%, but because the injector volume was not properly calibrated. The 150CV injector is a very reproducible injection system but needs to be carefully calibrated to know the exact amount of injected polymer, this one being used in viscosity calculations.

DISCUSSION.

Parameters influencing the flow fluctuation.

As we have seen, the flow fluctuation effect is induced by viscosity problems. It depends, accordingly, upon the specific viscosity of the polymer solution, that is [η]*C. This means that the effect depends upon both sample concentration and molecular weight (proportional to intrinsic viscosity [η]); the higher the parameter [η]*C, the stronger the fluctuation and the stronger the peak shift, as shown in Figure 9.

Conversely, the length of the perturbation will depend upon the length of the viscosity profile, that is roughly the sample polydispersity. The higher the polydispersity, the longer the flow fluctuation profile and the stronger the peak shift, as shown in Figure 10.

This can be observed by carefully looking at the a values in Table 4 where the offset value -20 µl was adjusted for the DOW 1683. Obviously, for this sample, the right **a** value is perfectly determined (0.709, 0.708). For NBS 706, which has a smaller polydispersity (for the main body), the effect is smaller and the value -20 µl overcorrects the effect (0.715, 0.715). For BASF 168N, which has a broader distribution and a higher molecular weight, the effect is much stronger and the value -20 µl undercorrects the effect (0.682, 0.689). Finally, PS-IUPAC, which has a smaller molecular weight but a broader distribution, is

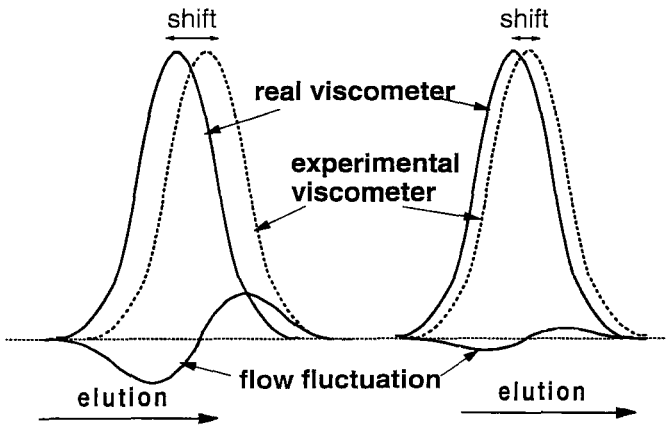


Figure 9. Influence of specific viscosity on apparent peak shift.

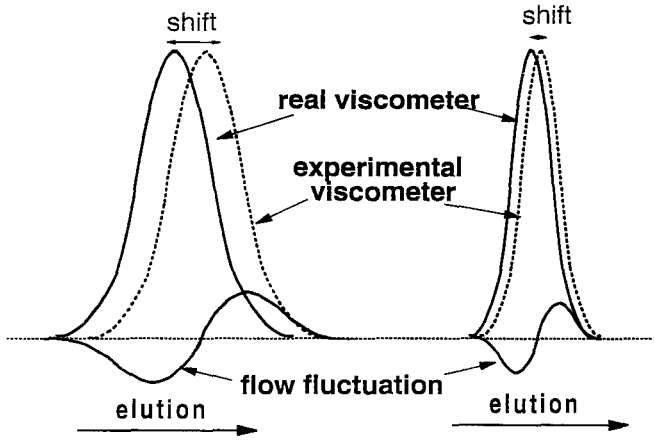


Figure 10. Influence of polydispersity on apparent peak shift.

calculated quite well (0.705), there is compensation between molecular weight and polydispersity for this sample.

The results of Table 4 ([η], a), in addition to the polydispersity "dis" and the concentration C, were entered into a regression software (Eureka from Borland) in order to determine if there was a relationship between those parameters. The following general relationship was obtained:

$$[\eta]^*C * dis^{0.5} * a^4 = 31.25$$

This relationship is valid only for polystyrene and under our experimental conditions. It means that the fluctuation depends directly upon the [η]*C value and upon the square root of the sample polydispersity.

Is it possible to correct this effect ?

This effect, depending simultaneously on three important parameters, the sample concentration C, the molecular weight (intrinsic viscosity [η]) and the polydispersity "dis", should have a different intensity for every sample. It appears quite impossible to set a perfect correction procedure with the software. Furthermore, we have just analyzed the situation in terms of peak shift when it is really a very small peak distortion.

What is the solution ?

The solution is obviously in the hardware. Every 0.009" capillary tube must be removed from the detector area and be replaced by tubes with larger internal diameter. This is true not only for the DRI inlet tubing, but also for every connecting tube between the columns and the several series-connected detectors. This is particularly valid when an external detector is used, such as a UV detector or a light scattering detector. The usual design uses long connecting capillaries to go into and out of the model 150CV, 0.009" tubes being used to minimize dead volumes. This can produce strong flow fluctuations. To avoid this, tubes with higher internal diameters must be used, but with a much shorter length in order to maintain minimal dead volumes. This requires more compact designs.

The internal geometry of the DRI detector must also be changed according to the same rules, i.e., higher internal diameter and shorter length. This has already been done and several DRI prototypes were tested successfully (9,10).

To check this proposed solution, the same simulation program was used, with different parameters, to study the behavior of DRI prototypes:

- the column set is still represented by a large number of 20 μl slices.

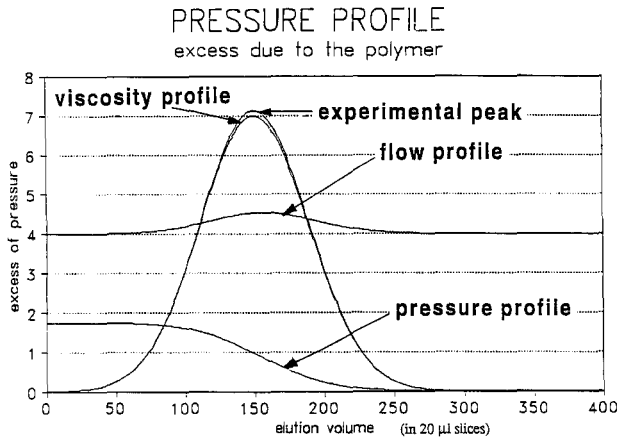


Figure 11. Computer simulation of the flow fluctuation and the viscometric signal distortion on an instrument equipped with a DRI prototype.

- the viscometer is still one slice of 0.014" tubing.
- the DRI inlet tubing is six slices (120 µl) of 0.020" tubing.
- the DRI outlet tube is still a great number of 0.040" slices.

The result of the simulation is represented in Figure 11; it should be compared to the one represented in Figure 5.

- the pressure profile is drastically changed. There is now a smooth decrease of pressure, corresponding to the polymer coming out the system. The pressure increase has completely disappeared.

- the flow profile is also completely changed. The flow fluctuation completely disappeared and there is now only a very small, quite negligible variation, corresponding to the polymer coming out the system. It is important to notice that the scale of the flow profile was amplified to show the fluctuation. The flow profile in Figure 11 must be compared to the one in Figure 5 where the fluctuation intensity is 0.04% of flow. They are plotted at the same scale factor.

- the experimental peak is now quite identical to the viscosity profile and there is no evidence of a peak shift; there is only a very small increase in intensity corresponding to the polymer coming out the system. This kind of profile is obviously within the standard error in detection and must certainly lead to a perfect determination of the viscosity law and the correct values of the Mark-Houwink K and **a**.

CONCLUSION.

The flow fluctuation, when the polymer peak comes across the detectors, is produced by the variation of pressure drop in the detectors, due to the specific viscosity of the polymer solution. It may occur in every system using capillary tubes (0.009") in the detector area. It is particularly visible on flow-sensitive detectors such as the SCV.

This effect is not reproducible from one instrument to another, since it depends on the internal diameter of the capillary tubes to the 4th power (Poiseuille's law), and capillaries are not reproducible. The example given in this paper is probably the worst case never encountered and the largest intensity never recorded. Many other 150CVs have been tested with a much smaller effect.

The intensity of the effect depends upon three main parameters (concentration, molecular weight and polydispersity of the sample); it is quite impossible to control it and to correct it with a software procedure. The only way is to change the hardware to reduce pressure drop in the detector area. Some DRI prototypes have been successfully built and tested with a geometry based on the rule: higher internal diameter and smaller length of connecting tubes. The results will be published in further papers (9-11).

The effect does not involve only the DRI detector but also any detector having a strong pressure drop or any arrangement between several detectors that involves long capillary tubes. Of course, the problem occurring with 0.009" tubings, any arrangement using smaller tubings like the 0.005" capillaries will produce much stronger effects.

A good practice for GPC using several detectors is to use more compact arrangements and connecting tubes with the highest possible internal diameters and the shortest possible length.

REFERENCES.

1. H. Benoit, P. Rempp and Z. Grubisic, J. Polym. Sci., B5, 753 (1967).
2. A.C. Ouano, J. Polym. Sci., Part A1, 10, 2169 (1972).
3. J. Lesec and C. Quivoron, Analusis, 4, 399 (1976).
4. D. Lecacheux, J. Lesec and R. Prechner, French Pt. 82402324.6 (1982), US Pt. 4478071 (1984), J. Appl. Polym. Sci., 27, 4867 (1982).
5. J. Lesec, D. Lecacheux and G. Marot, J. Liq. Chromatogr., 11, 2571 (1988).
6. C. Kuo, T. Provder, M.E. Koehler and A.F. Kah, ACS symposium series No 352, T. Provder Ed., 130 (1987).

7. J.L. Ekmanis, Waters Int'l GPC Symposium 89, Newton (1989).
8. J. Lesec, 1st Int'l Symposium on GPC/Viscometry, Houston (1991).
9. T. Huard, W.A. Dark, J.L. Ekmanis, T.J. Havard, R. Nielson and J. Lesec, 1st Int'l Symposium on GPC/Viscometry, Houston (1991)
Waters Int'l GPC Symposium 91, San Francisco (1991).
J. Lesec, M. Millequant and T.J. Havard, Am. Chem. Soc., Polym. Mater. Sci. Eng., NEW-YORK, 65, 138 (1991); Gel Permeation Chromatography: Characterization by SEC and FFF, ACS series 521, T. Provder ed., 220 (1993).
10. J. Lesec, M. Millequant and T. Havard, Waters Int'l GPC Symposium 91, San Francisco (1991).
11. J. Lesec and T. Havard (to be published).

Received: October 12, 1993
Accepted: October 19, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1029-1055 (1994)

HIGH TEMPERATURE GPC WITH A SINGLE CAPILLARY VISCOMETER*

JAMES LESEC[1], MICHÈLE MILLEQUANT[1], AND TREVOR HAVARD[2]**
[1]CNRS URA#278 - ESPCI
10 rue vauquelin
75231 - Paris cedex 05 - France
[2]Millipore - Waters Chromatography Division
34 Maple Street
Milford, Massachusetts 01757

INTRODUCTION

The purpose of this paper is to discuss the results obtained with high temperature GPC using a WATERS GPC 150CV equipped with a single capillary viscometer (SCV) and a differential refractive index detector (DRI) prototype. The reason for using a DRI prototype is that it has been demonstrated that a standard DRI detector may lead, under certain conditions, to erroneous results in viscosity calculations because of the occurrence of a very small flow fluctuation so-called "Lesec effect" when the polymer flows across the detectors (1-3). This very small fluctuation is enough to produce a significant apparent shift of the viscometer peak and leads to a small rotation of the viscosity law. The consequence is an abnormal small decrease of the Mark-Houwink **a** exponent. This phenomenon is caused by the specific viscosity of the polymer solution increasing the pressure drop in the detector area (1-3).

Furthermore, GPC-Viscometry requires a calibration curve in molecular weight, usually a "universal calibration curve" $Log([\eta]*M)=f(Ve)$ (4). As for classical GPC, perfect control of solvent flow rate is required, since molecular weights of broad polymers are calculated by comparison of their sliced distribution with elution volumes of narrow standards. The use of the SCV allows

* presented at the WATERS Int'l GPC Symposium 91, SAN FRANCISCO, October 1991.
** to whom correspondence should be addressed.

perfect control of the flow rate and this small flow fluctuation is not sufficient to introduce a significant error in molecular weight calculations when referring to a calibration curve. It only leads to a very small viscometer peak distortion and a decrease of the viscosity law **a** exponent.

The purpose of this study is to check the behavior of a DRI prototype at high temperature. This prototype has been designed to avoid the occurrence of the flow fluctuation that may occur with a standard DRI.

INSTRUMENTATION AND GENERAL CONSIDERATIONS.

The single capillary viscometer.

The Single Capillary Viscometer (SCV) is used inside the WATERS GPC 150CV instrument (5). It is composed of a capillary tube with the following characteristics (length=6", internal diameter=0.014", volume=18 µl) and of a differential pressure transducer (5 KPA full scale) connected to both capillary ends to measure the pressure drop across the capillary.

SCV obeys Poiseuille's law and the pressure drop P across the capillary depends upon the capillary geometry (radius r and length l), on the flow rate Q and on the viscosity η according to:

$$P = 8 / \pi * l / r^4 * \eta * Q$$

At constant flow rate Q, the pressure drop is proportional to viscosity η and, at constant viscosity η, the pressure drop is proportional to flow rate Q. Consequently, in order to use the SCV as an accurate viscometer, the flow rate must be maintained absolutely constant during the GPC experiment. Conversely, SCV allows perfect control of flow rate and is also used as a very powerful troubleshooting tool.

The flow fluctuation ("Lesec effect").

It has been shown that the standard DRI detector, but also every instrument using long capillary connecting tubes in the detector area, may lead to an abnormal behavior of the viscometric detection (1-3) but also possibly with other detectors. A flow fluctuation occurs when the polymer solution, with a higher specific viscosity than solvent, flows through the detectors, increasing the pressure drop. This weak flow fluctuation slightly distorts the viscometer peak and produces an apparent peak shift downstream. This apparent shift introduces a mismatch between the slice concentration information from the DRI and the slice pressure information from the viscometer. This mismatch leads to a slightly

incorrect calculation of the slice intrinsic viscosity $[\eta]_i$. As this effect is very weak, the errors on intrinsic viscosity are almost negligible but they lead to a slight rotation of the Mark-Houwink plot and a slight decrease of the Mark-Houwink **a** exponent.

Unfortunately, with GPC-Viscometry, the exponent **a** value has a physical meaning (**a**=0.5 for poor solvents, **a**=0.8 for good solvents, higher value for rigid polymers) and it must be determined very accurately. Also, for branched polymers, the branching distribution g'_i being calculated by dividing the experimental intrinsic viscosity $[\eta]_{br_i}$ by the $[\eta]_{lin_i}$ value of the corresponding linear polymer at the same molecular weight, the calculation of the branching distribution would be also affected by this effect. For these reasons, as the flow fluctuation cannot be readily corrected, it must be eliminated by an appropriate modification of the hardware.

The differential refractive index detector prototype.

For this study, a DRI prototype has been built with a geometry designed to avoid the flow fluctuation. The 0.009" inlet tubing has been replaced by a 0.020" tubing. The ratio in diameter being 2.22, we can expect, according to Poiseuille, a decrease of the pressure drop by a factor of 24 for the same length, but at the same time, we get an increase of internal volume by a factor of 5 that is unacceptable. For this reason, the internal design has been modified to reduce this volume. This detector, called prototype #1, having an internal volume around 150 µl, was first successfully tested at room temperature with THF (2). Nevertheless, the results obtained with this prototype show a small discrepancy of 25 µl between the interdetector volume value and the value necessary to run broad sample perfectly, probably due to excessive internal volume (interdetector volume being 150 µl plus the half of detector cells 13 µl, i.e. around 165 µl).

In order to improve the detector performance, the internal geometry of prototype #1 has been modified to give prototype #2. The inlet tubing having an internal volume of around 87 µl, interdetector volume is 87µl plus the half of detector cells 13 µl, i.e. around 100 µl. This is the DRI prototype that has been used in this high temperature study.

EXPERIMENTAL RESULTS.

Experimental conditions.

A WATERS GPC 150CV, equipped with the DRI prototype #2 described above, was used for this study. The solvent was 1,2,4-trichlorobenzene (TCB) at a temperature of 145°C and a flow rate of 1 ml/mn. TCB was filtered through

basic alumina to remove acidity and through Millipore membrane type FH 0.45µ. Then, a stabilizer (Irganox 1010) was added at a concentration of 0.1 %. The sample solutions were prepared at a concentration of approximately 0.001 g/ml at 170°C for 1 hour without stirring to avoid mechanical degradation, then at 150°C during 2 hours with stirring. They were overprotected at a concentration of 0.2 % of Irganox 1010 to avoid chemical degradation.

The columns used were a set of WATERS Ultrastyragel (10^3, 10^4, 10^5 and 10^6 Å). The narrow standards used were a set of polymethylmethacrylate (PMMA) from POLYMER LABORATORIES and a set of polystyrene (PS-TSK) from TOYO SODA.

The GPC software.

The software is the "Multidetector GPC software", a PC-DOS package written by J. Lesec (6) for triple detection GPC. For data acquisition, the PC computer is connected to the 150 CV through a CEC IEEE board (CAPITAL EQUIPMENT CORPORATION) and a 199 scanner/multimeter (KEITHLEY). Molecular weights are calculated either using a universal calibration curve or a combination of the classical molecular weight calibration curve and the viscosity law of the standards.

The data area is determined by the extreme bounds of both peaks to run calculations on the totality of peak information, a special procedure of extrapolation being used to recover missing data at both ends of chromatograms (6). The same holds true for intrinsic viscosity $[\eta]_i$ versus elution volume and versus Log(M), the central part of data being used to extrapolate and to re-build noisy data out of bounds at both ends of $[\eta]_i$ data. In addition to the average molecular weights, intrinsic viscosity $[\eta]$, Mark-Houwink K and **a** coefficients, DRI area constant and refractive index increment dn/dc are calculated. For branched polymers, the branching distribution g'_i is calculated by dividing the experimental intrinsic viscosity $[\eta]_{br_i}$ by the $[\eta]_{lin_i}$ value of the corresponding linear polymer at the same molecular weight. Average g' $<g'>$, g'_i at Mn, g'_i at Mw, g'_i at Mz and molecular weight where branching begins to occur are also calculated.

Another available advantage is the ability to calculate the number average molecular weight Mn from the viscometer without using the concentration detector data. A calculation method was originally described by J.M. Goldwasser (7). The GPC software uses a different method similar to the one described later by W. Yau (8). As Mn is defined as:

$$Mn = \Sigma C_i / \Sigma (C_i / M_i)$$

when multiplying (C_i / M_i) by $[\eta]_i$ at numerator and denominator, it comes:

$$(C_i / M_i) = (C_i*[\eta]_i / M_i*[\eta]_i)$$

and $$Mn = \Sigma C_i / \Sigma (C_i*[\eta]_i / M_i*[\eta]_i)$$

ΣC_i is the sample concentration Conc (using the injection concentration C_i, the injection volume and the slice volume). $M_i*[\eta]_i$ is the hydrodynamic volume HV_i coming from universal calibration. $C_i*[\eta]_i$ is the viscometer response when using the following formula to calculate $[\eta]_i$ at zero concentration:

$$[\eta]_i = [\ 2/C_i * (\eta_{redi} - \eta_{inhi})\ ]^{0.5}$$

using: $\eta_{redi} = 1/C_i * ((P_i - P_0) / P_0)$ and $\eta_{inhi} = 1/C_i * Log\ (P_i / P_0)$

it comes that the viscometer value $Visco_i = C_i*[\eta]_i$ with:

$$C_i*[\eta]_i = [\ 2 * (\ (P_i-P_0)/P_0 - Log\ (P_i/P_0)\ ]^{0.5}$$

Finally:

$$Mn = Conc / \Sigma\ (\ Visco_i / HV_i)$$

Using this formula, it is possible to calculate Mn by an absolute procedure using only the sample concentration Conc, the viscometer signal $Visco_i$ and a universal calibration curve HV_i without the need of any constant or any assumption and without need of the concentration detector. This feature is particularly useful to check the validity of results and when studying copolymers with a variable composition for which the DRI response is not a concentration signal, the refractive index increment dn/dc not being constant.

The interdetector volume correction.

The interdetector volume correction is indirectly involved here since the consequence of the flow fluctuation is an apparent peak shift. The two detectors being connected in series, the slice data acquisition is performed simultaneously on both detectors. In order to match the slice information coming from the two detectors, a correction of the viscometer slice elution volumes is performed to take into account the time necessary for each polymer molecule to move from the viscometer to the DRI.

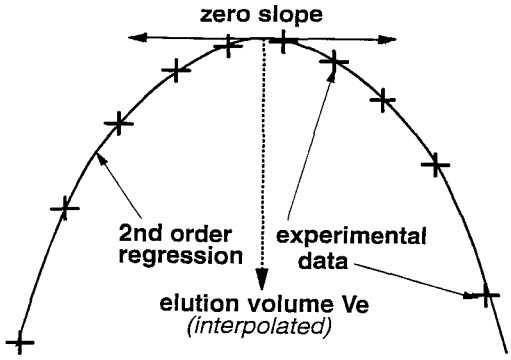


Figure 1 - Peak apex determination.

As the flow fluctuation slightly moves the viscometer downstream, this effect can be measured using the interdetector volume correction. When using an adjusted value for interdetector volume correction to obtain the appropriate value of the Mark-Houwink **a** exponent with a broad polymer, the difference between the theoretical value of the correction and the adjusted value corresponds to the viscometer peak shift and, consequently, to the intensity of the flow fluctuation. This is how the 100 µl apparent peak shift was measured in a standard 150 CV (1-3).

Interdetector volume determination.

In order to very carefully determine the interdetector volume, the software uses a special procedure for peak apex determination. A second order regression is performed around the highest data value, then the derivative is calculated. The exact value (interpolated) of the peak apex corresponds to the elution volume for which this derivative is equal to zero. This is represented in Figure 1.

Interdetector volume was measured using the Irganox 1010 peak that elutes around 42.5 ml with both detectors (Figure 2). This peak occurs because of the higher level of Irganox 1010 in the sample solution than in the solvent. Measuring the difference in elution volumes between the viscometer and the DRI must normally provide the interdetector volume, this molecule being assumed to be strictly monodisperse. The results obtained using a more efficient method will be published soon (9).

Interdetector volume was also measured using the PMMA standard peaks that are not strictly monodisperse; this is represented in Figure 3. A value of 95 µl

CNRS URA 278 * UNIVERSITE P.& M. CURIE - PARIS VI * E.S.P.C.I.

Version 3.00a - MULTIDETECTOR GPC SOFTWARE - revised 03/17/93 J.Lesec

CALIBRATION SYSTEM : Polyolefines # 1 Tue 23 MAR 1993 15:29:09
C:\GPC\E (Thu 11 APR 1991) - 150CV - new RI#1 modif - 4 ustyr. TCB 145°C

RUN # 8 DATE : Thu 20 JUN 1991 TIME : 14:59:48 SAMPLES : 12
INJECTION # 11 CODE : INJ 91 TIME : 01:11:52

SAMPLE PARAMETERS		CALCULATION PARAMETERS	
Name : NBS 1475 - linear polyethylene		Flow rate adjustment	: 1
Concentration (g/ml)	: .001389	Concentrat.adjust (0-1)	: 1
Injected volume (ml)	: .414	Index incr.adjust (0-1)	: 0
Alpha (Mark-Houwink)	: 0	CALCULATION MODES (Yes=1,No=0)	
LOG(K) (Mark-Houwink)	: 0	Refractometer active	: 1
Index increment (dn/dc)	: .1085	Spectrometer active	: 0
A2 - 2nd virial coeff.	: 0	Viscometer active	: 1
DETECTOR PARAMETERS		LALLS detector active	: 0
Refractom. sensitivity	: 256	Calibration active	: 1
Refractom. constant (K)	: 11.98	Universal Mark-Houwink	: 0
Spectrom. sensitivity	: 0	Please, do not use	: 0
Spectrometer constant	: 0	Branching viscometer	: 0
LALLS Gain value	: 0	Branching LALLS-Viscom.	: 0

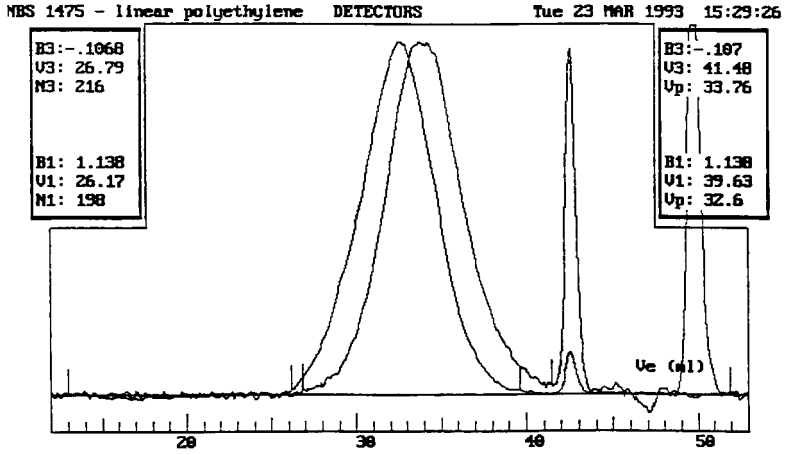


Figure 2 - Irganox 1010 peaks on a chromatogram of the NBS 1475.

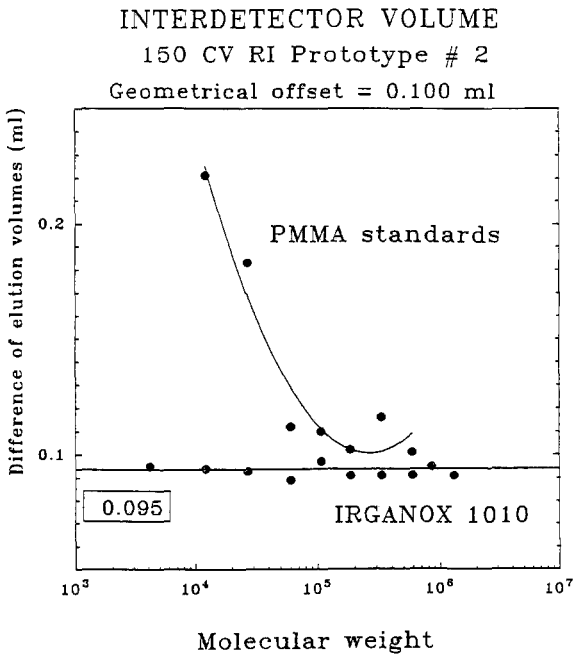


Figure 3 - Interdetector volume measured using PMMA standards and Irganox 1010 for DRI prototype #2.

was found with Irganox 1010 when the geometrical value was 100 µl, which is in very good agreement. For PMMA standards, the value is a little bit higher except for low molecular weight samples. This demonstrates that the standards are not strictly monodisperse, the low molecular weight ones exhibiting a higher polydispersity.

By comparison, the same study was run with prototype #1 and is represented in Figure 4. A value of 170 µl was found instead of the geometrical value 165 µl, which is in very good agreement, and the same behavior is observed for PMMA standards.

Polystyrene standards have also been used for the same measurements. The results are represented in Figure 5. Again, a value of 95 µl is found with Irganox 1010 but PS standards, except for the lowest and the highest molecular weight ones whose polydispersities are significantly greater than unity, exhibit an

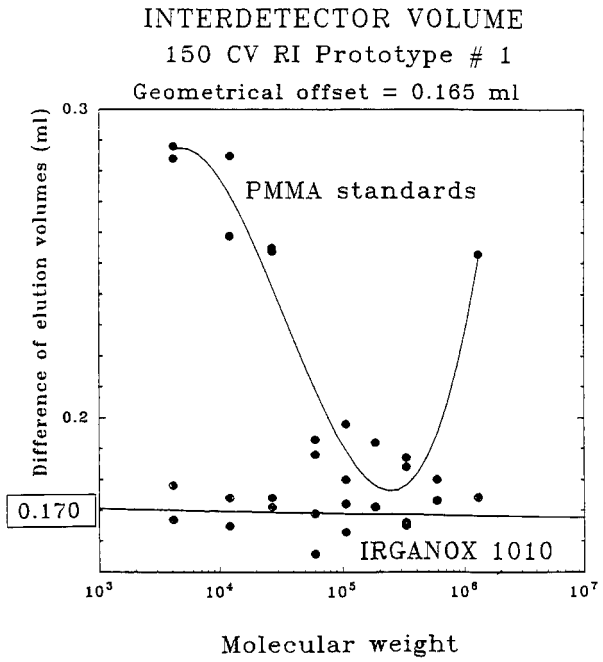


Figure 4 - Interdetector volume measured using PMMA standards and Irganox 1010 for DRI prototype #1.

abnormal behavior since they give a value a little bit smaller than Irganox 1010, which is theoretically impossible. It was not possible to find an explanation for this phenomenon. This discrepancy is extremely small and may come from a non perfect monodispersity of Irganox 1010 or a small residue of the flow fluctuation effect that was significantly weakened, but may not be completely eliminated.

In any case, these results are to be compared with the ones obtained with a standard DRI and represented in Figure 6. They were obtained at room temperature with THF using the water impurity peak to determine the interdetector volume. Again, a very good agreement was found between the geometrical volume (85 µl) and the water peak (90 µl). Conversely, the values obtained with PS standards are much smaller than expected (around 60 µl). This difference corresponds to the apparent shift of the viscometer peak downstream resulting from the flow fluctuation occurring in this unit.

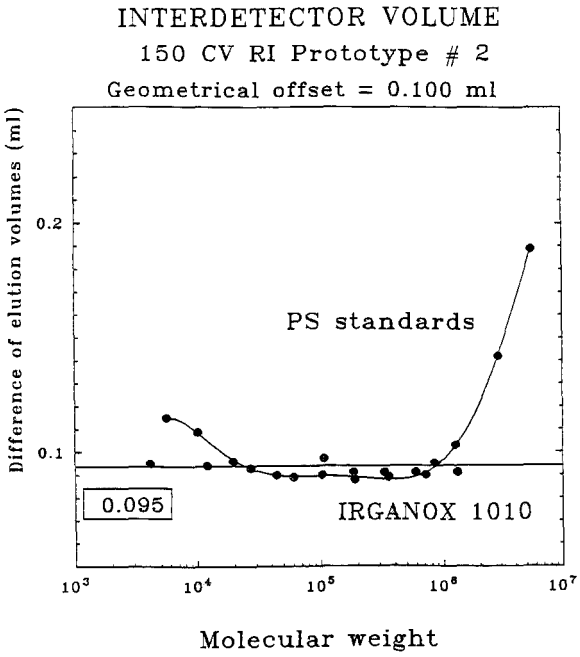


Figure 5 - Interdetector volume measured using PS standards and Irganox 1010 for DRI prototype #2.

The comparison between Figure 6 (standard DRI) and Figures 3, 4 and 5 (DRI prototypes) gives a good demonstration of the difference of behavior between a standard DRI and the DRI prototypes. It shows that the problem has been solved with the DRI prototypes and that the consequences of the flow fluctuation are eliminated.

Numerical results of polymer analysis.

Column calibration was performed with PMMA standards, using universal calibration. For this study, the combination of a LogM calibration curve and the viscosity law of standards has been used; they are represented in Figure 7. In the parameter table obtained after running the calibration and containing the analytical representation of both LogM calibration and viscosity law, it is important to note that the viscometer offset value, used to correct the

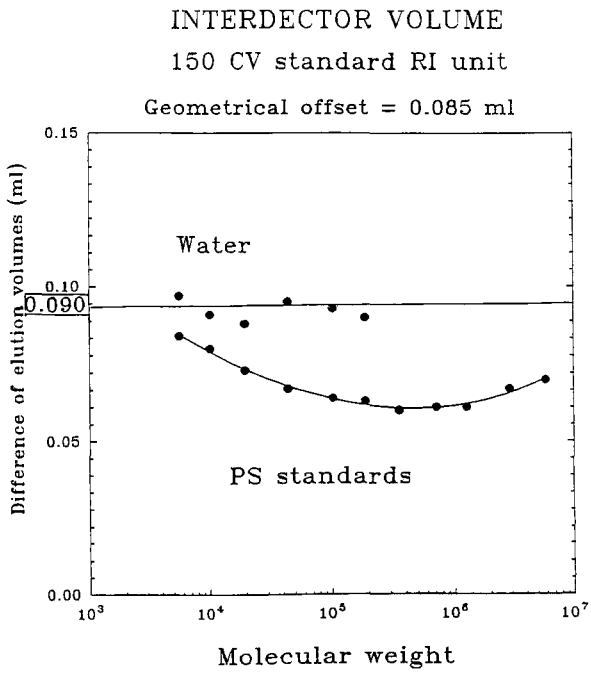


Figure 6 - Interdetector volume measured using PS standards and water peak for a standard DRI.

interdetector volume, was 85 µl. This will be discussed later. In order to check the performance of the system, several polymers were analyzed using this calibration.

Two polyethylene samples (NBS 1475 & 1476) were also studied. The chromatograms of NBS 1475, linear polymer, are represented in Figure 8 and the viscosity variations are represented in Figure 9. A very straight Mark-Houwink plot is obtained with an exponent **a** value of 0.715, which is correct. Numerical results and molecular weight distribution are represented in Figure 10. The first column of molecular weight results is in standard units (PMMA) and the second column in real units (UNIVERSAL). A value of 52,800 is found for Mw and 21,000 for Mn, which is extremely close to the expected values. The viscometer gives an intrinsic viscosity of 105 ml/g and an Mn value of 22,500, very close to the Mn value by the universal calculation.

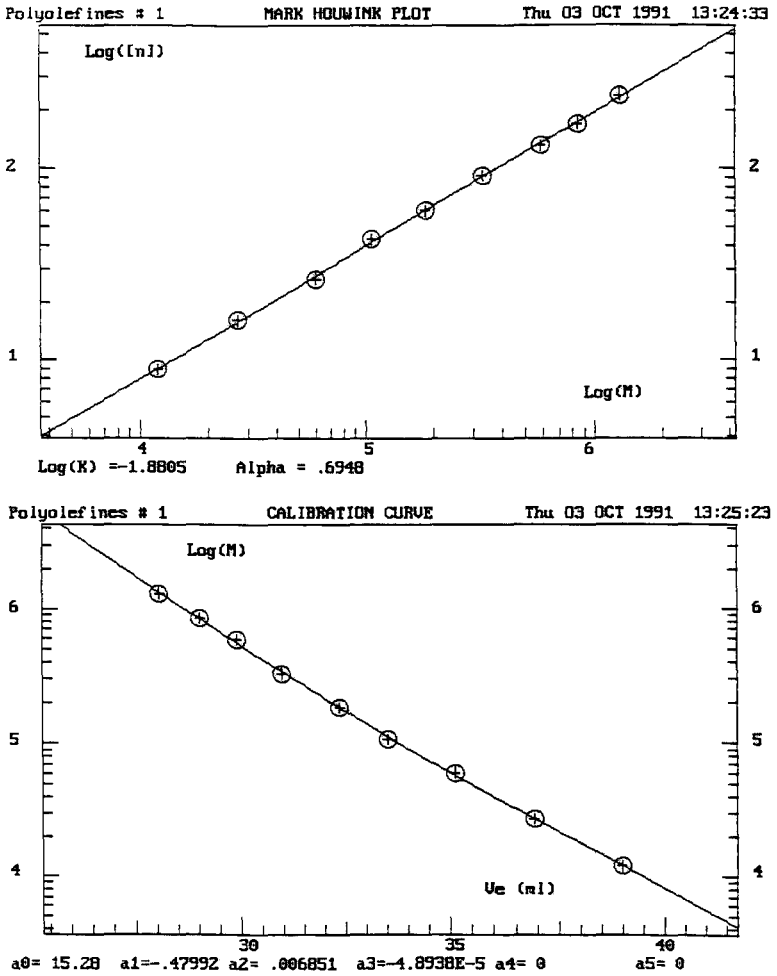


Figure 7 - Viscosity law and LogM calibration with PMMA standards.

CNRS URA 278 * UNIVERSITE P.& M. CURIE - PARIS VI * E.S.P.C.I.
Version 2.20c - MULTIDETECTOR GPC SOFTWARE - revised 09/10/91 J.Lesec

CALIBRATION SYSTEM : Polyolefines # 1 Thu 03 OCT 1991 13:38:53
C:\GPC\E (Thu 11 APR 1991) - 150CV - new RI#1 modif - 4 ustyr. TCB 145°C

RUN # 15 DATE : Wed 02 OCT 1991 TIME : 17:16:51 SAMPLES : 16
INJECTION # 15 CODE : INJ 161 TIME : 07:32:59

SAMPLE PARAMETERS		CALCULATION PARAMETERS	
Name : NBS 1475		Flow rate adjustment	: 1
Concentration (g/ml)	: .002993	Concentrat.adjust (0-1)	: 1
Injected volume (ml)	: .414	Index incr.adjust (0-1)	: 0
Alpha (Mark-Houwink)	: 0	CALCULATION MODES (Yes=1,No=0)	
LOG(K) (Mark-Houwink)	: 0	Refractometer active	: 1
Index increment (dn/dc)	: .1085	Spectrometer active	: 0
A2 - 2nd virial coeff.	: 0	Viscometer active	: 1
DETECTOR PARAMETERS		LALLS detector active	: 0
Refractom. sensitivity	: 256	Calibration active	: 1
Refractom. constant (K)	: 9.22	Universal Mark-Houwink	: 0
Spectrom. sensitivity	: 0	Please, do not use	: 0
Spectrometer constant	: 0	Branching viscometer	: 0
LALLS Gain value	: 0	Branching LALLS-Viscom.	: 0

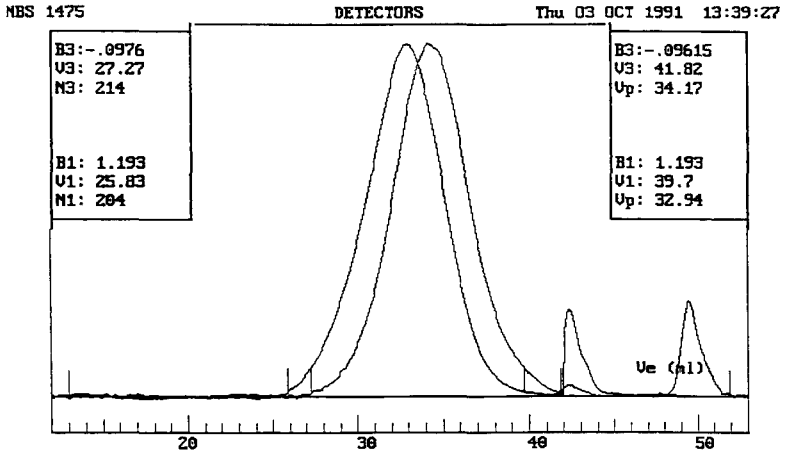


Figure 8 - Chromatograms of polyethylene NBS 1475.

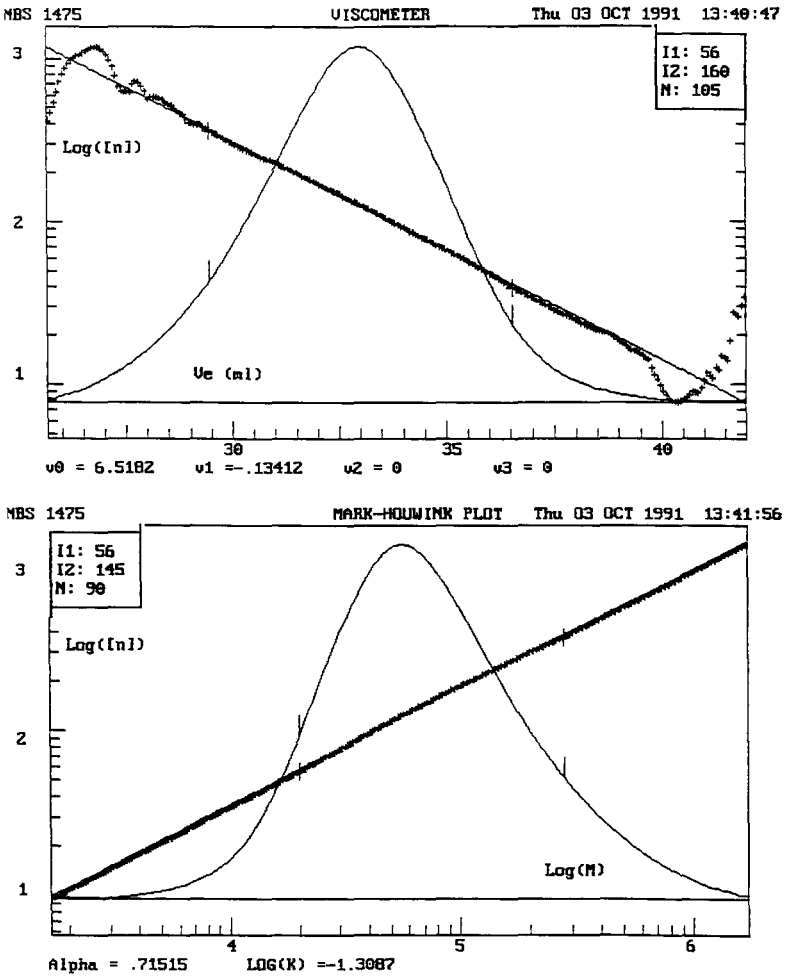


Figure 9 - Viscosity variations of polyethylene NBS 1475.

CNRS URA 278 * UNIVERSITE P.& M. CURIE - PARIS VI * E.S.P.C.I.

Version 2.20c - MULTIDETECTOR GPC SOFTWARE - revised 09/10/91 J.Lesec

NBS 1475	RESULTS		Thu 03 OCT 1991 13:42:41
Polyolefines # 1	RUN # 15 Inj # 15		CODE : INJ 161
DATE : Wed 02 OCT 1991	TIME : 07:32:59		Manual integration
Calibration # 1.33	Number of points: 239		Axial dispersion: YES

MOLECULAR WEIGHTS	STANDARD	UNIVERSAL
Peak mol. wt Mp :	80280	32580
Number aver. Mn :	50520	21020
Viscos. aver. Mv :	112800	45680
Weight averag.Mw :	131300	52810
Z average Mz :	400600	159400
Polydispersity :	2.6	2.51
[n] (ml/g) :	105.4	105.6
Log(K) (M-H) :	-1.548	-1.309
Alpha (M-H) :	.707	.715

REFRACTOMETER C/c : 1.068	Peak elution : 34.299	Baseline :-.096876
Area constant : 1.068	Conc. (g/ml) : .0031956	dn/dc : .1085
VISCOMETER Mn : 22500	Peak elution : 32.966	Baseline : 1.1928
[n]area (ml/g): 105.2	[n]peak(ml/g): 125	[n]exp (ml/g): 105.2

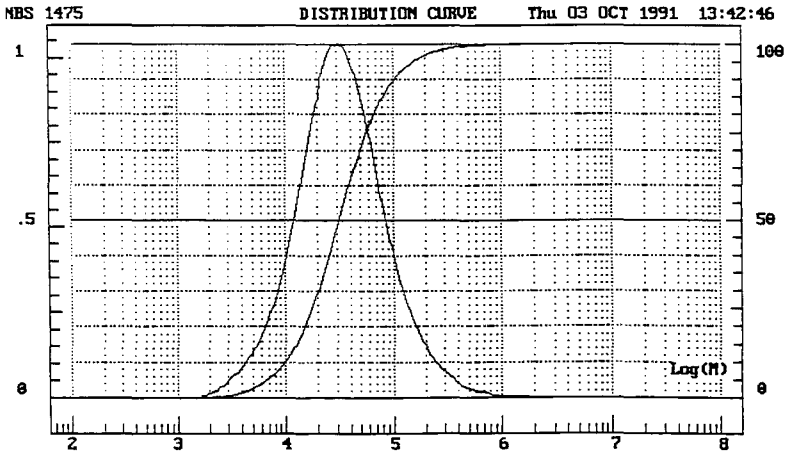


Figure 10 - Numerical results and molecular weight distribution of polyethylene NBS 1475.

The chromatograms of the branched polyethylene NBS 1476 are represented in Figure 11 and the viscosity variations in Figure 12. Contrary to the NBS 1475, the Mark-Houwink plot is curved because of branching and it is compared to the linear viscosity law obtained with the NBS 1475 (Figure 9) to provide the long-chain branching distribution $g'_i=[\eta]_{br_i}/[\eta]_{lin_i}$, plotted as small crosses. Figure 13 represents the numerical results and molecular weight distribution. A value of 81,300 is found for Mw and 25,800 for Mn, which is extremely close to the expected values. The viscometer gives an intrinsic viscosity of 93 ml/g and an Mn value of 29,100, close to the Mn value by the normal calculation. The average $<g'_i>$ value is found to be 0.65 with branching beginning to occur at very low molecular weight (1,600).

To check the validity of results, some broad polystyrene samples with known molecular weights (DOW 1683 and NBS 706) were also analyzed. The chromatogram of DOW 1683, linear polystyrene, is represented in Figure 14. A linear Mark-Houwink plot is obtained with an exponent value of 0.65. The "Standard" column is in PMMA units and the "Universal" column in real units. Mn and Mw were found to be 104,400 and 240,200 respectively, which is correct. The viscometer gives an intrinsic viscosity of 76.2 ml/g and an Mn value of 100,600, extremely close to the expected Mn value. Also NBS 706, represented in Figure 15 gives good values, slightly low in molecular weights: Mn=102,000, Mw=252,400. The Mark-Houwink exponent is found 0.64 (very close to the DOW value 0.65) and the viscometer gives an intrinsic viscosity of 80.7 ml/g and an Mn value of 103,700.

DISCUSSION.

All of these results where obtained with an interdetector volume correction of 85 µl instead of the 95 µl value determined using the Irganox 1010 peak (Figure 3). The reason is that this value has been adjusted with a broad linear PMMA sample to determine the exact same **a** exponent value 0.695 as with the kit of PMMA standards (Figure 7). Figure 16 represents the viscosity variations of the broad linear PMMA sample, using a correction of 85 µl, which gives the right **a** exponent 0.696. Under these conditions, the molecular weights are well calculated. The "Standard" column in PMMA units and the "Universal" column in real units are very similar, which is expected, since the sample has the same nature as the calibration standards. Again, the Mn by viscometry is found to be very close (60,550) to the "universal" value (61,930).

As a comparison, Figure 17 represents the same viscosity variations but using the Irganox 1010 correction of 95 µl which gives the underestimated value of 0.682. The difference between 0.696 and 0.682 is very small and introduces only very small variations in polymer analysis. A comparison of results obtained

CNRS URA 278 * UNIVERSITE P.& M. CURIE - PARIS VI * E.S.P.C.I.
Version 2.20c - MULTIDETECTOR GPC SOFTWARE - revised 09/10/91 J.Lesec

CALIBRATION SYSTEM : Polyolefines # 1 Thu 03 OCT 1991 13:44:55
C:\GPC\E (Thu 11 APR 1991) - 150CV - new RI#1 modif - 4 ustyr. TCB 145°C

RUN # 15 DATE : Wed 02 OCT 1991 TIME : 17:16:51 SAMPLES : 16
INJECTION # 16 CODE : INJ 162 TIME : 08:34:02

SAMPLE PARAMETERS		CALCULATION PARAMETERS	
Name : NBS 1476		Flow rate adjustment	: 1
Concentration (g/ml)	: .002369	Concentrat.adjust (0-1)	: 1
Injected volume (ml)	: .414	Index incr.adjust (0-1)	: 0
Alpha (Mark-Houwink)	: .71515	CALCULATION MODES (Yes=1,No=0)	
LOG(K) (Mark-Houwink)	:-1.3087	Refractometer active	: 1
Index increment (dn/dc)	: .1085	Spectrometer active	: 0
A2 - 2nd virial coeff.	: 0	Viscometer active	: 1
DETECTOR PARAMETERS		LALLS detector active	: 0
Refractom. sensitivity	: 256	Calibration active	: 1
Refractom. constant (K)	: 9.22	Universal Mark-Houwink	: 0
Spectrom. sensitivity	: 0	Please, do not use	: 0
Spectrometer constant	: 0	Branching viscometer	: 1
LALLS Gain value	: 0	Branching LALLS-Viscom.	: 0

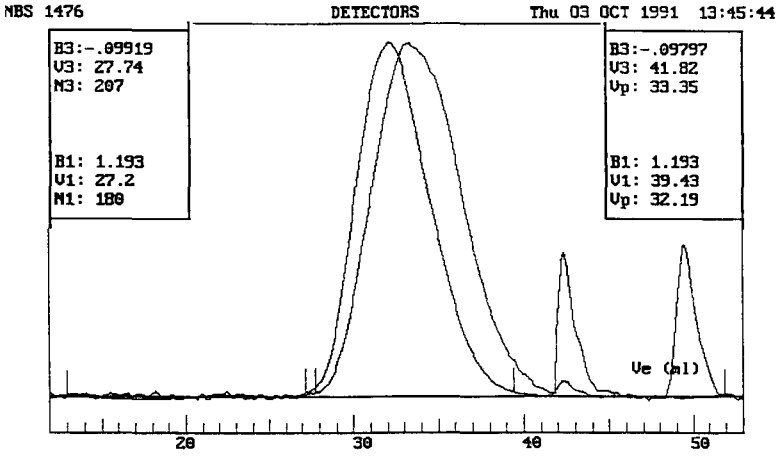


Figure 11 - Chromatograms of polyethylene NBS 1476.

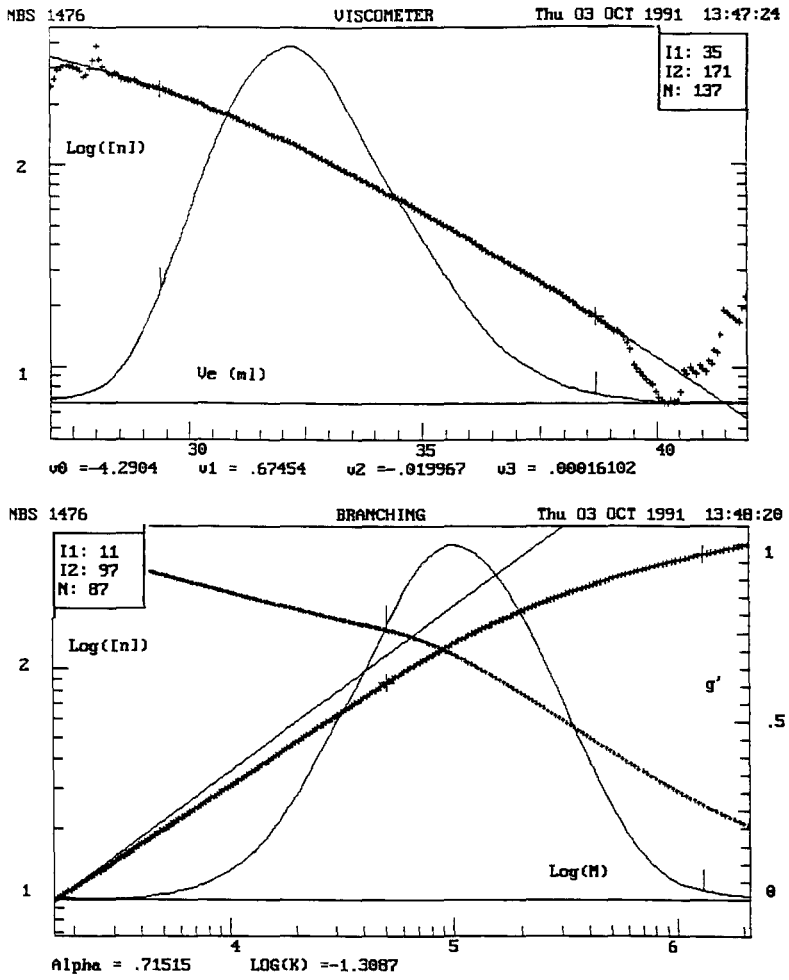


Figure 12 - Viscosity variations of polyethylene NBS 1476.

CNRS URA 278 * UNIVERSITE P.& M. CURIE - PARIS VI * E.S.P.C.I.

Version 2.20c - MULTIDETECTOR GPC SOFTWARE - revised 09/10/91 J.Lesec

NBS 1476		RESULTS		Thu 03 OCT 1991 13:49:10
Polyolefines # 1		RUN # 15	Inj # 16	CODE : INJ 162
DATE : Wed 02 OCT 1991		TIME : 08:34:02		Manual integration
Calibration # 1.33		Number of points: 219		Axial dispersion: YES
MOLECULAR WEIGHTS	STANDARD	UNIVERSAL	g'BRANCH.	
Peak mol. wt Mp :	124000	50050	.751	
Number aver. Mn :	56470	25770	.8	
Viscos. aver. Mv :	119700	69290	.732	
Weight averag.Mw :	151600	81340	.714	
Z average Mz :	332700	234300	.547	
Polydispersity :	2.68	3.16	< g' > :	
[n] (ml/g) :	98.83	142.3	.654	
Log(K) (M-H) :	-.4474	-1.309	g' mass:	
Alpha (M-H) :	.481	.715	1643	
REFRACTOMETER C/c : .9772		Peak elution : 33.276		Baseline :-.098578
Area constant : .9775		Conc. (g/ml) : .0023149		dn/dc : .1085
VISCOMETER Mn : 29090		Peak elution : 32.148		Baseline : 1.1928
[n]area (ml/g): 91.84		[n]peak(ml/g): 128.3		[n]exp (ml/g): 93.03

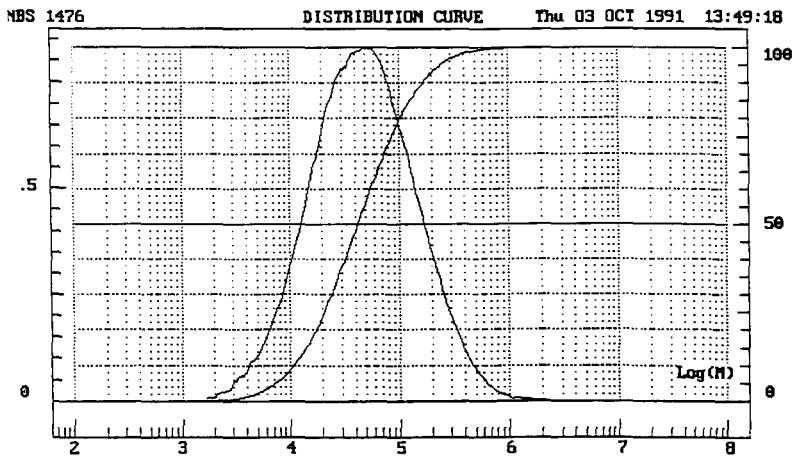


Figure 13 - Numerical results and molecular weight distribution of polyethylene NBS 1476.

CNRS URA 278 * UNIVERSITE P.& M. CURIE - PARIS VI * E.S.P.C.I.

Version 2.20c - MULTIDETECTOR GPC SOFTWARE - revised 09/10/91 J.Lesec

DOW 1683 - 3	RESULTS		Thu 03 OCT 1991 16:28:49
Polyolefines # 1	RUN # 15	Inj # 12	CODE : INJ 158
DATE : Wed 02 OCT 1991	TIME : 04:29:51		Manual integration
Calibration # 1.33	Number of points: 182		Axial dispersion: YES

MOLECULAR WEIGHTS	STANDARD	UNIVERSAL
Peak mol. wt Mp :	190800	173200
Number aver. Mn :	117300	104400
Viscos. aver. Mv :	229500	210400
Weight averag.Mw :	258300	240200
Z average Mz :	484400	467700
Polydispersity :	2.2	2.3
[n] (ml/g) :	76.59	76.85
Log(K) (M-H) :	-1.701	-1.583
Alpha (M-H) :	.669	.652

REFRACTOMETER C/c : .9987	Peak elution : 32.283	Baseline :-.12113
Area constant : .5414	Conc. (g/ml) : .0016379	dn/dc : .0588
VISCOMETER Mn : 100600	Peak elution : 30.961	Baseline : 1.1927
[n]area (ml/g): 74.63	[n]peak(ml/g): 99.44	[n]exp (ml/g): 76.17

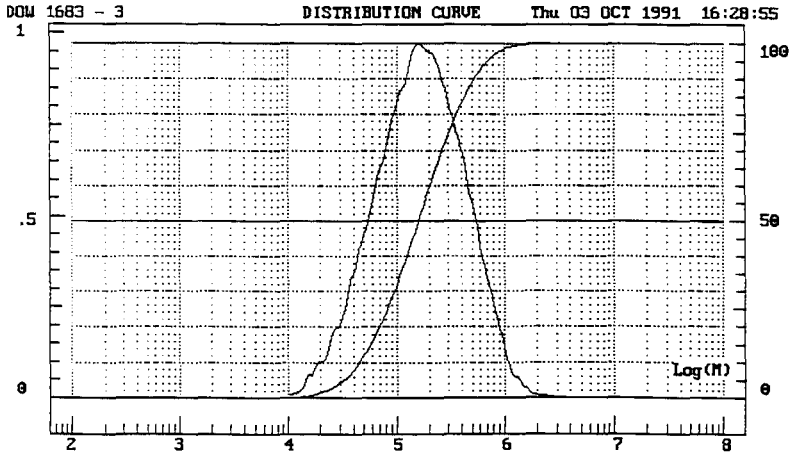


Figure 14 - GPC analysis of broad polystyrene DOW 1683.

CNRS URA 278 * UNIVERSITE P.& M. CURIE - PARIS VI * E.S.P.C.I.

Version 2.20c - MULTIDETECTOR GPC SOFTWARE - revised 09/10/91 J.Lesec

NBS 706 - 3	RESULTS		Thu 03 OCT 1991 16:33:39
Polyolefines # 1	RUN # 15	Inj # 13	CODE : INJ 159
DATE : Wed 02 OCT 1991	TIME : 05:30:53		Manual integration
Calibration # 1.33	Number of points: 203		Axial dispersion: YES

MOLECULAR WEIGHTS	STANDARD	UNIVERSAL
Peak mol. wt Mp :	243300	220100
Number aver. Mn :	117400	102000
Viscos. aver. Mv :	246400	223800
Weight averag.Mw :	273600	252400
Z average Mz :	462100	442400
Polydispersity :	2.33	2.48
[n] (ml/g) :	81.08	81.28
Log(K) (M-H) :	-1.655	-1.51
Alpha (M-H) :	.661	.639

REFRACTOMETER C/c : 1.012	Peak elution : 31.733	Baseline :-.11949
Area constant : .5484	Conc. (g/ml) : .0017864	dn/dc : .0588
VISCOMETER Mn : 103700	Peak elution : 30.906	Baseline : 1.1926
[n]area (ml/g): 79.94	[n]peak(ml/g): 102.2	[n]exp (ml/g): 80.75

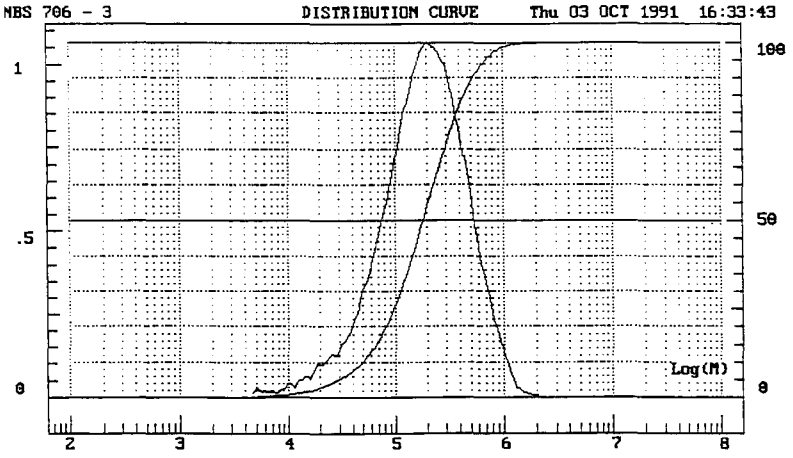


Figure 15 - GPC analysis of broad polystyrene NBS 706.

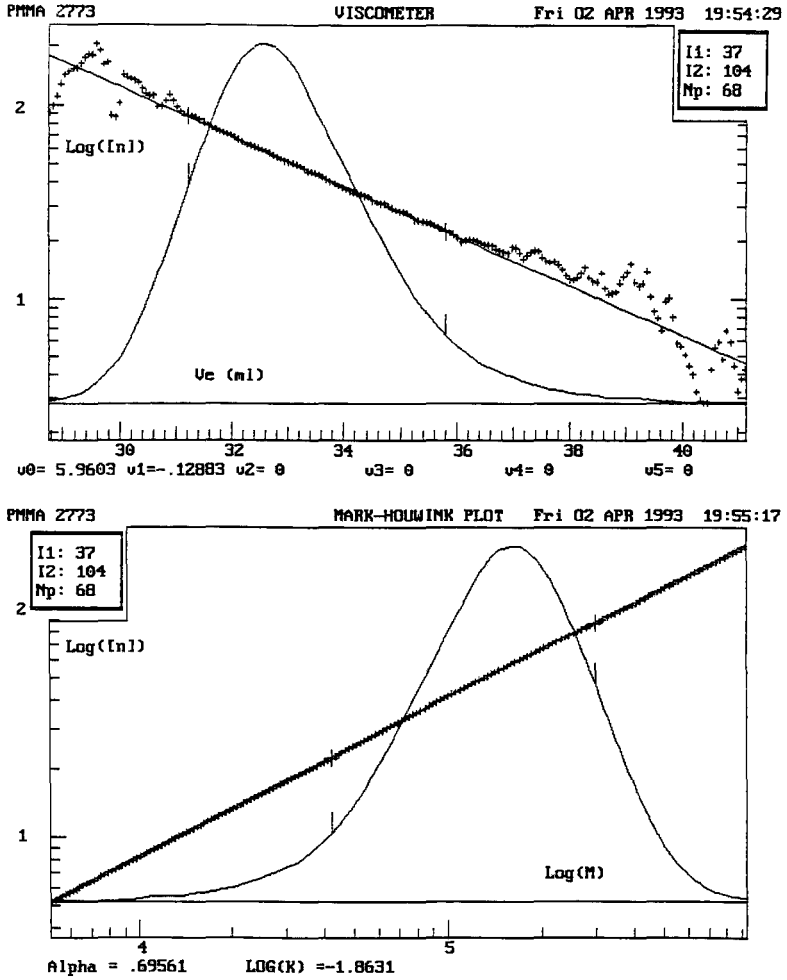


Figure 16 - Viscosity variations of a broad PMMA using 85 µl.

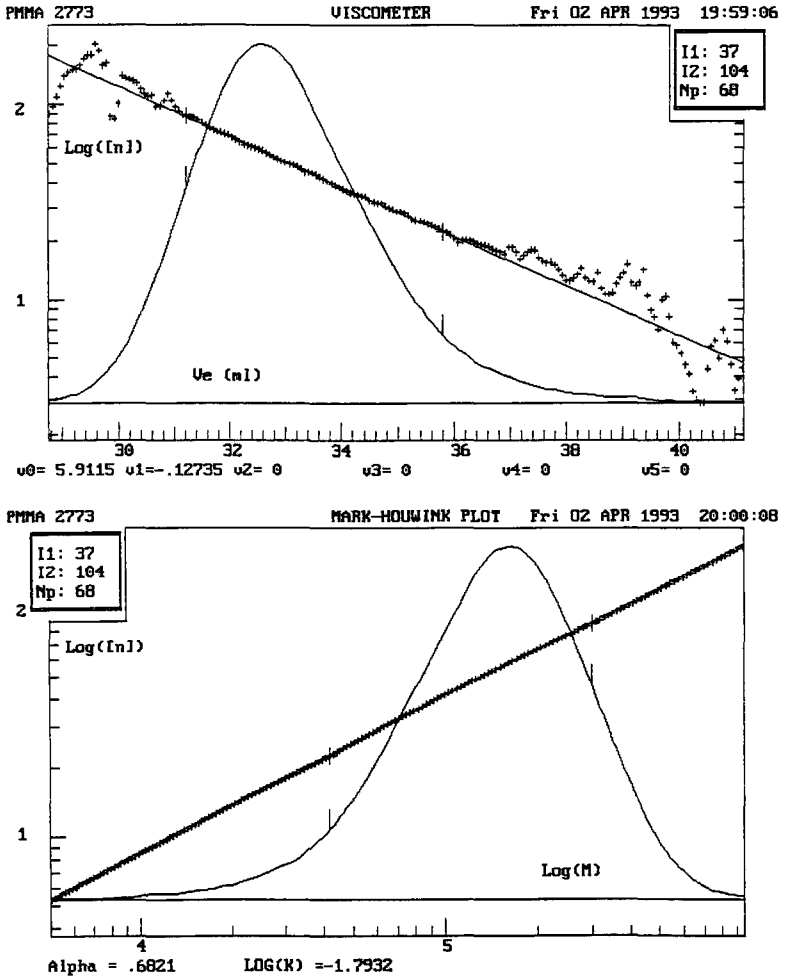


Figure 17 - Viscosity variations of a broad PMMA using 95 µl.

CNRS URA 278 * UNIVERSITE P.& M. CURIE - PARIS VI * E.S.P.C.I.

Version 3.00a - MULTIDETECTOR GPC SOFTWARE - revised 03/17/93 J.Lesec

PMMA 27000 * 2	RESULTS		Fri 02 APR 1993 20:06:18
Polyolefines # 1	RUN # 14 Inj # 3		CODE : INJ 133
DATE : Tue 01 OCT 1991	TIME : 19:54:56		Manual integration
Calibration # 1.33	Number of points: 85		Axial dispersion: YES
MOLECULAR WEIGHTS	STANDARD	UNIVERSAL	
Peak mol. wt Mp :	27120	26090	
Number aver. Mn :	23870	22540	
Viscos. aver. Mv :	25870	24770	
Weight averag.Mw :	26290	25320	
Z average Mz :	28280	27590	
Polydispersity :	1.1	1.12	
[n] (ml/g) :	16	16	
Log(K) (M-H) :	-1.443	-1.203	
Alpha (M-H) :	.6	.548	
REFRACTOMETER C/c : 1	Peak elution : 36.944		Baseline :-.10396
Area constant : .3006	Conc. (g/ml) : .001814		dn/dc : .0307
VISCOMETER Mn : 21480	Peak elution : 36.839		Baseline : 1.1905
[n]area (ml/g): 16.19	[n]peak(ml/g): 16.83		[n]exp (ml/g): 16

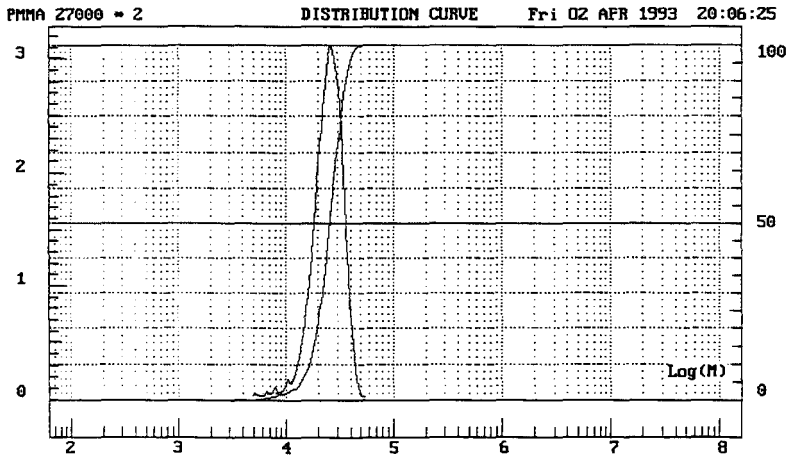


Figure 18 - GPC analysis of PMMA standard 27,000 using 85 µl.

Table 1-Comparison of NBS 1475 and 1476 values using either 85 µl or 95 µl.

	1475(85µl)	1475(95µl)	1476(85µl)	1476(95µl)
Mn	21,020	20,970	25,770	25,290
Mw	52,810	52,340	81,340	81,300
[η]	105.2	105.3	93.03	92.9
a	0.715	0.710	-	-
LogK	-1.309	-1.2876		
<g'>	-	-	0.654	0.652

using either 85 µl or 95 µl for interdetector volume correction is given in Table 1 for NBS 1475 and 1476.

These differences are very small and quite negligible on broad polymers; only the viscosity law coefficients change slightly. For narrow polymers, this change is more significant. Figure 18 represents the analysis of the PMMA standard 27,000 using 85 µl. Both "standard" and "universal" columns are quite identical and the determination of molecular weights is good. The Mark-Houwink **a** exponent was found 0.55, different from the calibration value 0.695 (Figure 7), but, still an acceptable value for a standard since band broadening occurs. By comparison, when using 95 µl, the exponent value decreases from 0.55 to 0.46 which shows that narrow standards are much more sensitive to the correction. They may be used to carefully adjust the interdetector offset, but a very accurate band broadening correction has to be used.

The system has also been checked with polystyrene standards. Polystyrene samples are very sensitive to chemical degradation in TCB; the solutions need to be very well protected by Irganox 1010 and also against degradation by light. When the proper precautions are not taken, degradation may occur and this is probably the reason for underestimated values obtained with DOW 1683 and NBS 706 (Figures 14 and 15). A single universal calibration curve was obtained using the two sets of standards (PS + PMMA) and is plotted in Figure 19. There is excellent agreement between both sets of standards, which shows that the system was running well.

CONCLUSION.

The performance of the DRI prototype #2 is excellent. The changes in internal geometry correct for the occurrence of the flow fluctuation and its consequences when the polymer solution flows across the detectors. Contrary to the previous experiments using a standard DRI, where a peak shift of 100 µl was observed, the difference between the measured interdetector volume using

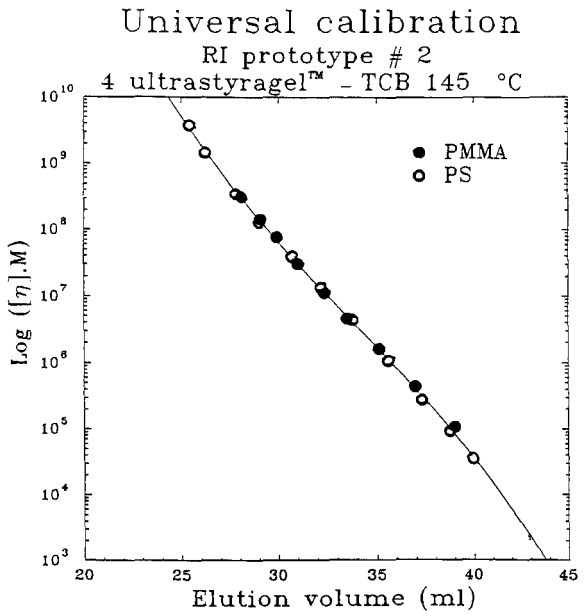


Figure 19 - Single Universal calibration with PS and PMMA standards.

Irganox 1010 (95 µl) and the adjusted value (85 µl), to obtain exactly the same Mark-Houwink exponent with a set of standards and with a broad sample with the same chemical nature, is very small (10 µl).

The interpretation of this 10 µl value is very difficult since this is, at least, the experimental error on interdetector volume measurement using Irganox 1010 and also the experimental error when adjusting the Mark- Houwink exponent with a broad sample on the set of standards. This small discrepancy may come from a very weak residual flow fluctuation but the experimental errors do not allow conclusive explanation.

Anyway, this very weak difference does not introduce any significant error on results. Table 1 shows only very minor changes in molecular weights and other parameters when using either 95 µl or 85 µl for interdetector volume correction. Even the Mark-Houwink exponent variation, which is at the origin of this study and of the DRI prototype design, does not vary significantly (from 0.710 to 0.715). The discrepancy value 10 µl is probably the extreme limit of precision we can expect experimentally, even under the best conditions, and it certainly has no physical meaning.

REFERENCES.

1. J. Lesec, 1st Int'l Symposium on GPC/Viscometry, Houston (1991).
2. T. Huard, W. A. Dark, J. L. Ekmanis, T. J. Havard, R. Nielson and J. Lesec, 1st Int'l Symposium on GPC/Viscometry, Houston (1991); Waters Int'l GPC Symposium 91, San Francisco (1991). J. Lesec, M. Millequant and T. Havard, Am. Chem. Soc., Polym. Mater. Sci. Eng., NEW-YORK, 65, 138 (1991); Gel Permeation Chromatography: Characterization by SEC and FFF, ACS series 521, T. PROVDER ed., 220 (1993).
3. J. Lesec, Waters Int'l GPC Symposium 91, San Francisco (1991).
4. H. Benoit, P. Rempp and Z. Grubisic, J. Polym. Sci., B5, 753 (1967).
5. J. L. Ekmanis, Waters Int'l GPC Symposium 89, Newton (1989).
6. J. Lesec and G. Volet, J. Liq. Chromatogr., 13, 831 (1990); J. Appl. Polym. Sci. appl. polym. Symp., 45, 177 (1990).
7. J. M. Goldwasser, Waters Int'l GPC Symposium 89, Newton (1989).
8. W. W.Yau and S. W. Remeter, J. Liq. Chromatogr., 13, 627 (1990).
9. J. Lesec and T. Havard (to be published).

Received: October 22, 1993
Accepted: October 29, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1057-1064 (1994)

SIMULTANEOUS DETERMINATION OF MONO-, DI-, AND TRINUCLEOTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING *N*-(DANSYL)ETHYLENEDIAMINE AS A FLUORESCENT DERIVATIZING REAGENT

S. SONOKI, A. SANDA, AND S. HISAMATSU
College of Environmental Health
Azabu University
Sagamihara, Kanagawa 229, Japan

ABSTRACT

A method for simultaneous determination of mono-, di- and trinucleotides with high sensitivity has been developed. This based on the fluorescent derivatization of nucleotides by the reaction of 5-dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide (dansylEDA) with the phosphoric acid moiety of nucleotide, and then the resolution of fluorescent derivatives by high-performance liquid chromatography with a spectrofluoro monitor. The fluorescent dansylEDA derivatives of nucleotides were separated sharply each other. The detection limits for dansylEDA derivatives of nucleotides were between 4.7 and 20.3 pmol per 10-μl injection. This method was applied to analyze the yeast RNA after the generation of ribonucleoside 5'-monophosphate by nuclease P1 digestion. As a result, no more than 40 ng of RNA was needed for one analysis, and so such sensitivity of this method gives an advantage to analyze the RNA in a small scale.

INTRODUCTION

The analysis of purine and pyrimidine bases, the corresponding nucleosides and nucleotides is an essential step in many areas of biochemical study. The development of instrumental analyses such as gas chromatography - mass spectrometry and high - performance liquid chromatography (HPLC) has made it possible to analyze precisely the bases, nucleosides and nucleotides (1-8). Especially, various HPLC methods have been reported for the determination of these compounds, however, these methods have some disadvantages with respect to the sensitivity and specificity.

Recently we have reported the procedure with which 2'-deoxynucleoside 5'-monophosphate (dXmp) was detected with high sensitivity and selectivity (9). This procedure is based on the fluorescent derivatization by the reaction of 5-dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide (dansylEDA) at the phosphoric acid moiety of dXmp, then the resolution of these fluorescent compounds by HPLC. This procedure was successfully applied to the determination of guanine+cytosine content of DNA (10).

In this report, we describe the application of the procedure to analyze ribonucleoside 5'-mono-, di- and triphosphates which are essential in biological metabolism as constituents of RNA and high-energy phosphates.

MATERIALS AND METHODS

Apparatus

A Japan Spectroscopic (JASCO) Model 800-MP-15 high-performance liquid chromatograph with a JASCO FP-210 spectrofluoro monitor was used. Chromatograms were recorded on a JASCO Model 805-GI graphic integrator, while fluorescence spectra were obtained on a JASCO FP-770 spectrofluorometer. Reversed phase octadecyl-bonded polyvinyl alcohol gel column, Finepak ODP-50 (250 mm x 4.6 mm; Asahikasei, Tokyo, Japan) was used for the separation of dansylEDA derivatives.

Chemicals

Adenosine 5'-monophosphate (AMP), Guanosine 5'-monophosphate (GMP), Cytidine 5'-monophoaphate (CMP), Uridine 5'-monophosphate (UMP), Adenosine 5'-diphosphate (ADP), Guanosine 5'-diphosphate (GDP), Adenosine 5'-triphosphate (ATP) and Guanosine 5'-triphosphate (GTP) were obtained from Sigma (St. Louis, MO, U.S.A.). 1-Methylimidazole was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, U.S.A.). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) was obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan). 5-Dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide (dansylEDA) was synthesized by the method described in the previous paper (9). All other chemicals used were of analytical grade from commercial sources. Standard solutions were prepared separately by dissolving each of the ribonucleoside 5'-monophosphate (Xmp), ribonucleoside 5'-di- or triphosphates in distilled water to a concentration of 30 mM. Standard mixture solution was prepared by mixing standard solutions each other to a final concentration of 3 mM.

Preparation of DansylEDA Derivatives of Mono-, Di- and Trinucleotides

Unless specified otherwise, the following procedure was used. A 10 μl of the standard mixture solution or biological sample in 200 μl of 1-methylimidazole buffer (0.1 M, pH 7.5) was reacted with 10 μl of 0.1 M EDAC in 1-methylimidazole buffer and 40 μl of 50 mM dansylEDA in dimethylsulfoxide. The reaction was carried out in dark for 18 hr at 27℃.

Chromatographic Conditions

Chromatographic separations of dansylEDA derivatives were performed at a flow rate of 0.6 ml / min at 40℃. The eluting solvents were: A, 10 mM phosphate buffer (pH 10.3) - acetonitrile (88 : 12, v / v); B, 10 mM phosphate buffer (pH 10.3) - acetonitrile (78 : 22, v / v); C, 10 mM phosphate buffer (pH 10.3) - acetonitrile (60 : 40, v / v). Elution was carried out for 10 min with solvent A, followed by the linear gradient elution system from solvent A to solvent B in 18 min, then solvent C was used for another 10 min. The column effluent was

monitored fluorometrically at an excitation wavelength of 270 nm and at an emission wavelength of 546 nm. The separation of non-derivatized Xmp, ATP, ADP, GTP and GDP was performed at a flow rate of 0.6 ml / min at 25℃ with 10 mM phosphate buffer (pH 3.5) as the eluting solvent.

RESULTS AND DISCUSSION

Fluorescence Spectra of DansylEDA Derivatives

After the derivatization of 10 µl of the standard mixture solution containing 30 nmol each of the Xmp, ATP, ADP, GTP and GDP, each fluorescent derivative, which was separated on the silica TLC plate, was extracted with 70% methanol, then analyzed. Each derivative of Xmp, ATP, ADP, GTP, and GDP exhibited similar fluorescence excitation and emission spectra patterns as shown in FIGURE 1. As a result, the fluorescence intensity was measured using excitation at 270 nm and emission at 546 nm.

Separation of DansylEDA Derivatives by HPLC

The separation of dansylEDA derivatives of Xmp, ATP, ADP, GTP and GDP is shown in FIGURE 2. The dansylEDA derivatives were sharply separated from each other.

Assay Linearity and Detection Limit

The fluorescence intensity of each derivatives was linear over a range of detection limit up to 580 pmol per 10-µl injection. The detection limits for dansylEDA derivatives were between 4.7 pmol of dansylEDA-ATP and 20.3 pmol of dansylEDA-CMP per 10-µl injection at a signal-to-noise ratio of about five..

Assay Precision

Relative standard deviations obtained in 5 measurements for 58 pmol / 10 µl of dansylEDA derivatives were between 0.89 % of dansylEDA-GTP and 8.56 % of dansylEDA-CMP, and between 0.97 % of dansylEDA-GTP and 4.78 % of dansylEDA-CMP for 580 pmol / 10 µl of dansylEDA derivatives.

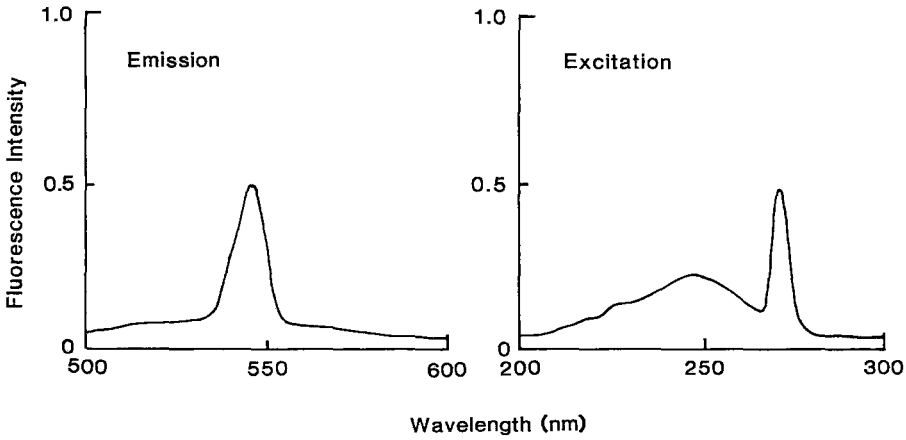


FIGURE 1 Fluorescence spectra of the dansylEDA derivative of AMP. All other derivatives of Xmp, ATP, ADP, GTP and GDP exhibited similar fluorescence spectra patterns. Emission was measured at an excitation wavelength of 270 nm, while excitation was measured at an emission wavelength of 546 nm.

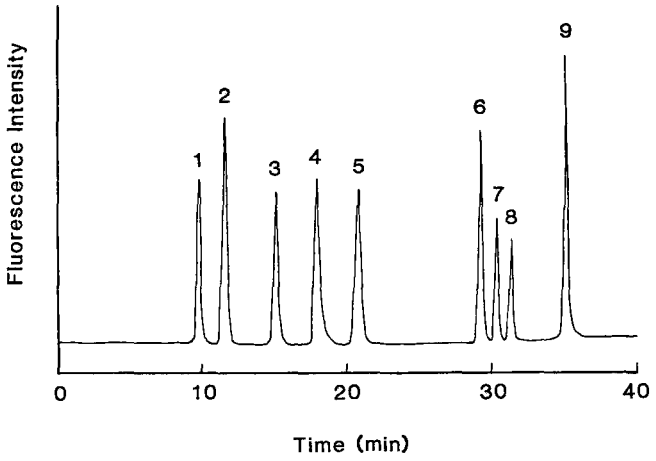


FIGURE 2 Chromatogram of dansylEDA derivatives of mono-, di- and trinucleotides. Peaks: 1 = dansylEDA-GTP; 2 = dansylEDA-ATP; 3 = dansylEDA-GDP; 4 = reaction by-product; 5 = dansylEDA-ADP; 6 = dansylEDA-GMP; 7 = dansylEDA-UMP; 8 = dansylEDA-CMP; 9 = dansylEDA-AMP.

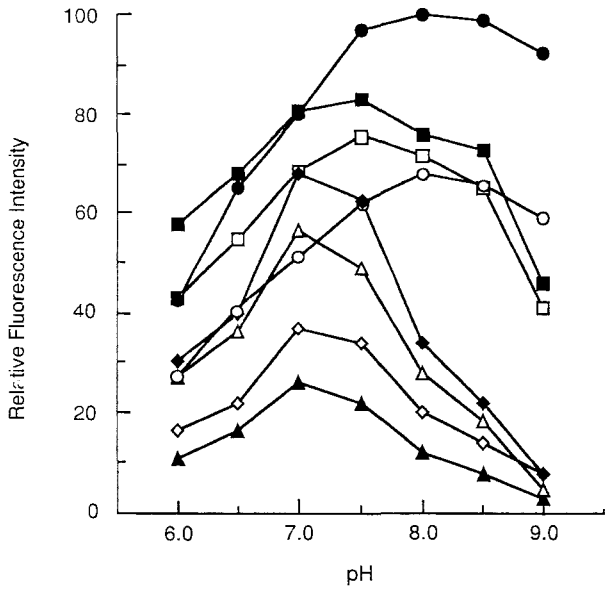


FIGURE 3 Effect of the pH in the reaction mixture on dansylEDA derivatization of mono-, di- and trinucleotides. O, dansylEDA-GTP; ●, dansylEDA-ATP; □, dansylEDA-GDP; ■ dansylEDA-ADP; △, dansylEDA-GMP; ▲, dansylEDA-UMP; ◇, dansylEDA-CMP; ◆, dansylEDA-AMP. Each point represents the mean of triplicate determinations of the fluorescence intensity of each peak of the dansylEDA derivative separated on the Asahipak ODP-50 column.

Effect of pH on the Derivatization

The derivatization with dansylEDA was carried out using the procedure described in the MATERIALS AND METHODS, except that the pH was varied from 6 to 9. As shown in FIGURE 3, pH 7 in the reaction mixture was found to be most effective on the derivatization of mononucleotides, on the other hand, pH 7.5 and pH 8 were most effective for dinucleotides and trinucleotides, respectively. Following an increase of the number of phosphoric acid moiety in the nucleotide, an increasing of the most effective pH values on the derivatization was noted, therefore a value of 7.5 was selected as the pH on the derivatization for the simultaneous analysis of all nucleotides.

Effect of Reaction Time on the Derivatization Yield

The effect of the reaction time on the derivatization at 27℃ was examined over a period of 8 days. The result shows that the fluorescence intensities of dansylEDA derivatives increased with the reaction time, then reached the plateau in one day. Every dansylEDA derivatives were stable throughout the investigation period without any decomposition.

Application to the analysis of RNA base composition

Yeast RNA in 10 μl of distilled water was heated in a boiling bath for 5 min, then rapidly cooled on ice. To this was added 10 μl of nuclease P1 (Yamasa, EC 3.1.30.1.) solution prepared by dissolving 2 units of nuclease P1 in 1 ml of sodium acetate buffer (40 mM, pH 5.3) containing 0.2 mM $ZnCl_2$. After incubation at 50 ℃ for 1h, Xmp, which was generated by nuclease P1 digestion, was derivatized directly and analyzed by HPLC. As a result, no more than 40 ng of RNA was enough for one analysis. This means that this method gives an advantage to the analysis of RNA which is hard to be prepared from some biological materials.

REFERENCES

1. R. Meyer, K.G. Wagner, Anal. Biochem., 148: 269-276 (1985)

2. R. Boulier, C. Bory, J. Chromatogr., 339: 380-387 (1985)

3. K. Nakano, K. Shindo, T. Yasaka, H. Yamamoto, J. Chromatogr., 343: 21-33 (1985)

4. P. Rotllan, A. Liras, P. Llorente, Anal. Biochem., 159: 377-385 (1986)

5. S. Sonoki, Y. Tanaka, S. Hisamatsu, T. Kobayashi, J. Chromatogr., 475: 311-319 (1989)

6. C. Randt, M. Linscheid, Fresenius Z Anal. Chem., 335: 865-868 (1989)

7. A. N. Al-Deen, D. C. Cecchini, S. Abdel-Baky, N. M. A. Moneam, R. W. Giese, J. Chromatogr., 512: 409-414 (1990)

8. W. Furst, S. Hallstrom, J. Chromatogr., 578: 39-44 (1992)

9. S. Sonoki, Y. Kadoike, M. Kiyokawa, S. Hisamatsu, J. Liq. Chromatogr., in press

10. S. Sonoki, S. Hisamatsu, A. Kiuchi, Nucleic Acids Res., 21(11): 2776 (1993)

Received: August 24, 1993
Accepted: August 31, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1065-1088 (1994)

AN IMPROVED HPLC PROCEDURE FOR THE QUANTITATION OF DICLOFENAC IN PLASMA

F. A. MOHAMED[1], H. W. JUN[1]*, T. H. ELFAHAM[2],
H. A. SAYED[2], AND E. HAFEZ[2]
[1]*Department of Pharmaceutics*
College of Pharmacy
University of Georgia
Athens, Georgia 30602
[2]*Department of Pharmaceutics*
Faculty of Pharmacy
Assiut University
Assiut, Egypt

ABSTRACT

A rapid, simple and sensitive high performance liquid chromatographic (HPLC) assay for the quantitation of diclofenac (DF) in dog plasma has been developed. Mefenamic acid (MA) was used as the internal standard. After acidification, DF and MA were extracted from plasma into chloroform. Separation was achieved using a C18 reversed phase column. The retention times of DF and MA were 3.8 and 6.3 min., respectively at the flow rate of 1.5 ml/min. The DF interday standard plots (n=4) were highly linear (r>0.99) over the concentration range of 0.01 to 10 μg/ml. DF mean recovery was 98% ± 5.5, and the % CV of intra- and inter-day sample analyses ranged from 2.6 to 10.8% for the entire calibration range. The limit of quantification of DF in plasma was 0.01 μg/ml with the CV of 9.4%. The method was applied for the determination of the pharmacokinetic parameters of DF given by oral and iv bolus administration to dogs.

*Corresponding author

INTRODUCTION

Diclofenac sodium (voltaren, [0-(2,6-dichloroanilino)-phenyl]acetate) is a nonsteroidal anti-inflammatory drug (NSAID), available in many countries since 1974. It is currently marketed in the USA for treatment of various inflammatory conditions such as rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and other related symptoms (1-2). The drug has also analgesic and antipyretic activities, and is used for the relief of dental or minor surgical pain and headache. The anti-inflammatory activity of the drug and its other pharmacological effects are generally thought to be related to its inhibition of prostaglandin synthesis (3). Diclofenac competes with arachidonic acid for binding to cyclo-oxygenase, thereby decreasing the synthesis of prostaglandin (3,4).

Diclofenac is rapidly and efficiently absorbed after oral administration when given as a solution or as an enteric coated tablet (5,6). The drug undergoes a significant first-pass metabolism and only about 60% of the drug reaches the systemic circulation unchanged (5). The peak plasma concentrations of the drug usually occur 1.5 to 2.5 hrs after oral ingestion in fasting subjects, while peak times vary widely (2.5-12 hrs) under fed conditions (7). Diclofenac is eliminated principally by hepatic metabolism and subsequent urinary and biliary

excretion (8). In healthy volunteers, the mean plasma clearance of diclofenac is 16 L/hr and the elimination half-life is approximately 1.5 hr (5,6).

In view of its wide clinical use, different analytical methods have been developed for the quantitation of diclofenac in pharmaceutical preparations, blood, plasma, urine and other biological matrices. These methods include spectrophotometry (9,10), high performance liquid chromatography (HPLC) (11-21), gas-liquid chromatography with electron-capture detector (GC) (22-28), thin layer chromatography (TLC) (29), radioactive isotope (30) and GC/mass spectrophotometry (GC/MS) (31,32). The HPLC methods reported in the literature (11-21) were often developed with a simplified sample preparation steps and direct UV detection without derivatization. Some of these methods employ a single extraction step (11-15,18), multiple extraction (16,20) or an automated robotic extraction (19,21). The HPLC methods that depend on either repeated extraction or reextraction, although yielding higher sensitivity, are generally more complex, time consuming and involve tedious extraction steps (16,20). The GC with electron-capture detection methods are based on the formation of an indolone of a methyl or ethyl ester derivative (22-28). The reported TLC method (29), beside being less accurate and time-consuming, lacks the

sensitivity required for pharmacokinetic and bioavailability studies. The use of radioactive isotopes (30) is limited because of the potential hazards of radioactivity in man and lack of specificity for the intact compound.

The aim of this work was to develop an improved assay of unchanged diclofenac in plasma using HPLC. The new method is simple and highly sensitive with a limit of quantitation of 10 ng/ml of DF in plasma, rapidity with the assay time of approximately 2 hrs for 10 replicate samples and essentially complete recoveries (>98%) for the entire calibration range of 10 ng to 10 μg of DF in ml of plasma. The method also offers specificity without any interfering peaks near the drug or the internal standard from common anti-inflammatory drugs or endogenous plasma components. The inter- and intra-day reproducibilities as shown by % CV were excellent ranging from 2.6 to 10.8 for the entire calibration range. The method was successfully applied for the determination of the pharmacokinetic parameters of DF in dogs for up to 24 hrs after receiving a single dose of the drug (5 mg/kg) given by oral and i.v. bolus doses.

EXPERIMENTAL

Chemicals and Reagents

Diclofenac sodium, mefenamic acid, ketoprofen, ibuprofen, naproxen sodium and indomethacin were

purchased from Sigma Chemical, St. Louis, MO. Aspirin was obtained from Amend Drugs and Chemical, Irvington, NJ. Acetonitrile, methanol, chloroform and phosphoric acid were purchased from J.T. Baker Chemical, Inc., Phillipsburg, NJ. The solvents were all of HPLC grade. Water was deionized and filtered.

Chromatographic Conditions and Equipment

The chromatographic system used consists of a Beckman Pump (Model 110A), operated at a flow-rate of 1.5 ml/min, a variable-wavelength detector (SpectroMonitor III, Model 1204 A) set at 278 nm, and a fixed volume (50 μl) injector (Rheodyne, Model 7125) and the separation was achieved on a stainless steel reversed phase Novapak C_{18} (150 x 3.9 mm) column (Phenomenex, Model PP/9400 A) with a C_{18} pre-column (30-40 μm pellicular packing). A mobile phase consisting of acetonitrile and water (50:50% V/V) adjusted to pH 3.5 with glacial acetic acid was degassed using an ultrasonicator (Fisher Scientific, Model 14). The samples were centrifuged using IEC-Centrifuge (Damon ISC, Model CU-5000). Vortexing was achieved by Fisher Mini-Shaker (Fisher Scientific, Model 58). Chromatograms were recorded on a strip chart recorder (Esterline Anug, Rainin Instrument Model) at a speed of 2.5 mm/min.

Standard Calibration Curves

In screw-capped centrifuge tubes, 0.5 ml of dog plasma was mixed with 10 μl of the DF reference solutions

to produce concentrations of 10 ng to 10 μg of DF in ml of plasma. The tubes were vortexed for 10 sec. After adding 10 μl of the MA stock solution to each tube to yield the final MA concentration of 1 μg/ml, acidification was achieved by adding 50 μl of phosphoric acid (85.2%) to each tube, then the tubes were vortexed for 10 sec. The extraction of DF and MA was carried out by the addition of 3 ml of chloroform to each tube followed by vortexing for 1 min, then the tubes were centrifuged for 5 min at 6000 rpm. The organic layer was transferred into a 4 ml glass tube and evaporated to dryness under a stream of nitrogen at 45°C. The residue was reconstituted in 200 μl of the mobile phase and vortexed for 10 sec. A 50 μl of this solution was loaded into the HPLC sample loop. The calibration curves were obtained by plotting the peak height ratios of DF/MA versus their respective concentrations of DF.

Assay of Dosed Samples

The same extraction and chromatographic procedures described for the preparation of the calibration plots were used for the quantitation of DF in the dosed dog plasma samples excluding the addition of diclofenac sodium.

Animal Studies

A single dose of 5 mg/kg of diclofenac sodium was administered to two beagle dogs via bolus intravenous

injection after dissolving in 3 ml of normal saline, and orally in a gelatin capsule to two other beagle dogs. After two weeks of washout period, the animals were crossed-over to receive the other formulation. The dogs were fasted overnight with water freely available prior to dose in the early morning and continued fasting for 4 hrs after drug administration. The jugular vein was used to collect the blood samples (4-5 ml) into commercial blood collecting tubes containing a standard amount of anticoagulant (sodium heparin). The blood samples were obtained at the intervals of 0, 0.33, 0.67, 1, 2, 3, 5, 7, 9, 12 and 24 hrs post dose. Plasma was separated by centrifugation for 10 min at 2000 rpm and stored in refrigerator at -20°C until analysis which was usually done within one week.

RESULTS AND DISCUSSION

Chromatographic Specificity and Sensitivity

Typical chromatograms of (a) blank dog plasma spiked with mefenamic acid (1 μg/ml) and plasma spiked with the internal standard and diclofenac sodium (b) 5 ng/ml and (c) 10 ng/ml are shown in Figure 1. The chromatograms of plasma sample taken (a) before administration of DF and plasma taken (b) 12 hrs and (c) 24 hrs after oral administration of DF powder (5 mg/kg) to a beagle dog are shown in Figure 2. The specificity of the method was

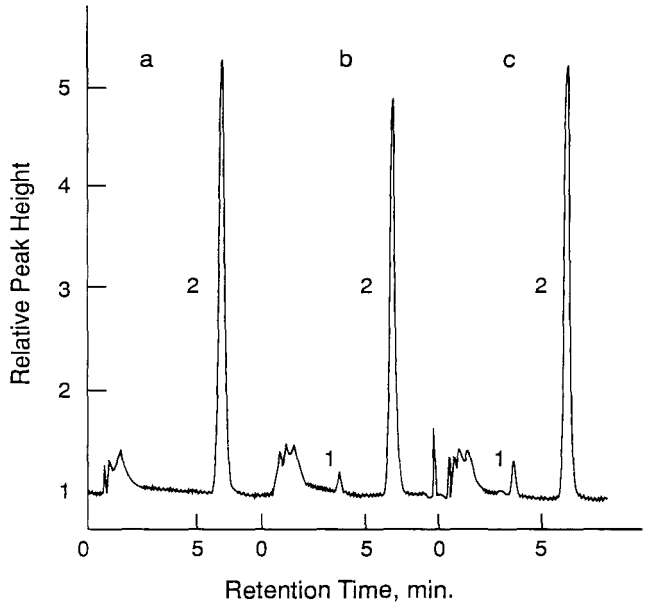


Figure 1. Typical chromatogram of blank dog plasma spiked with (a-2) mefenamic acid (1 μg/ml) and plasma spiked with the internal standard and diclofenac sodium (b-1) 5 ng/ml and (c-1) 10 ng/ml.

demonstrated by the lack of interferences at the retention times of DF (3.8 min) and MA (6.3 min). Both peaks were sharp and symmetrical with good baseline resolution, thus facilitating accurate measurement of the peak height ratio. The sensitivity of assay defined as the minimum concentration that can be quantitated with a statistically acceptable coefficient of variation (10%) in the peak height ratio was 10 ng/ml with the CV equal to 9.4% (see Table 1-A). The minimum detectable amount

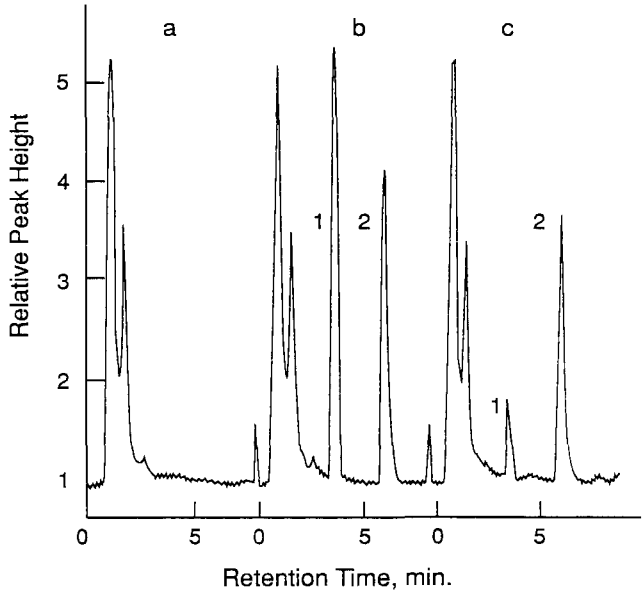


Figure 2. Chromatogram of plasma sample taken at time zero (a) and plasma taken (b-1) 12 hrs and (c-1) 24 hrs after oral dose of DF powder (5 mg/kg) to a beagle dog.

defined as the amount in nanograms that gives peak height of DF equal to twice the background noise at the most sensitive instrument setting used in the study was 5 ng of DF.

Selection of the Mobile Phase

A mobile phase consisting of acetonitrile-water (50:50% V/V) adjusted to pH 3.5 with glacial acetic acid gave the optimum resolution of DF and MA with a flow rate of 1.5 ml/min. According to Sayed et al. (18), the composition and pH of the mobile phase drastically

affected the retention times of the compounds. In this study, similar results were observed. Increasing the percentage of acetonitrile decreased the retention times of the analytes and visa versa. However, an opposite effect was observed with the pH change, where the retention times of DF and MA were increased with increasing the pH.

Selection of the Extraction Solvent

Several organic solvents were initially tested for the extraction of DF and MA from plasma samples. The solvents tested were: benzene, chlorobenzene, ethyl ether, petroleum ether, chloroform, and n-hexane. Of these solvents, chloroform showed the highest recovery without interfering peaks at the retention times of both DF and MA. When plasma samples (1 μg/ml) of both DF and MA were extracted using the above solvents, chloroform consistently gave the highest peaks for DF and MA at given concentrations.

Retention Times and Selectivity of the Assay

By using the assay procedure, the retention times of DF and MA were 3.8 and 6.3 min, respectively. As shown in Figure 1, there were no interferences due to the endogeneous plasma components at the retention times of the drug and the internal standard. The intercepts of the calibration plots showed no significant deviation

from zero, indicating that blank plasma has negligible interference for the analyte. Potential interferences to the peaks were also evaluated by injecting some common anti-inflammatory drugs to the HPLC system. Solutions of the following drugs (aspirin, ketoprofen, ibuprofen, naproxen, diclofenac, mefenamic acid and indomethacin were separately prepared in the mobile phase and directly injected onto the HPLC column, and their respective retention times were 1, 2, 4.3, 2, 3.8, 6.3, and 3.8 min. Except indomethacin, no compounds tested exhibited the same retention times of DF and MA.

Linearity of the Calibration Plots

Least-squares analyses of the inter-day calibration curves gave excellent linear responses to the tested concentration range (10 ng to 10 μg/ml) of DF in plasma (Table 1). A typical standard plot of DF in plasma can be described by the equation: $y= 1.87x + 0.049$ with a correlation coefficient (r) of greater than 0.99, indicating a good fit to the least-squares linear regression model.

Reproducibility of the Assay

The inter-day reproducibility of the assay was evaluated by comparing the linear regression analyses of the four standard plots obtained from spiked dog plasma samples at four different days over a period of two weeks (Table 2). Least-squares regression analyses of the

Table 1-A: Inter-day Reproducibility[1] of Calibration Curves of Diclofenac in Dog Plasma.

Plasma Conc (μg/ml)	Mean Peak Height Ratio (SD)[2]	% Coefficient of Variation, n=4
0.01	0.064 (0.006)	9.37
0.025	0.105 (0.011)	10.47
0.05	0.150 (0.008)	6.00
0.1	0.245 (0.019)	7.75
0.2	0.388 (0.002)	5.67
0.5	0.983 (0.077)	7.83
1	1.921 (0.063)	3.27
2	3.800 (0.198)	5.21
5	9.433 (0.491)	3.94
10	18.756 (0.670)	3.57

[1] Determined from four sets of standard curves on four different days over a period of two weeks.
[2] Standard deviation

Table 1-B: Inter-day Calibration Data.

Calibration Curve[1]	Slope[2]	Intercept	Correlation Coefficient, r
1	1.856	0.027	>0.99
2	1.914	0.086	>0.99
3	1.914	0.008	>0.99
4	1.778	0.096	>0.99

[1] Prepared over a period of two weeks.
[2] Mean slope (% CV) = 1.872 ml/μg (3.85)

calibration curves gave linear responses over the tested concentration range of DF (10 ng to 10 μg/ml). The average slope of the four standard plots was 1.87 ml/μg with a standard deviation (SD) equal to 0.064, and a day-to-day coefficient of variation (CV%) of 3.43, indicating excellent inter-day reproducibility. The correlation coefficient (r) was typically higher than 0.99.

Table 2-A: Intra-Day Reproducibility[1] of Calibration Curves of Diclofenac in Dog Plasma.

Plasma Conc (μg/ml)	Mean Peak Height Ratio (SD)[2]	% Coefficient of Variation, n=3
0.01	0.063 (0.005)	7.46
0.025	0.101 (0.010)	10.89
0.050	0.154 (0.012)	7.79
0.1	0.243 (0.013)	3.26
0.2	0.398 (0.022)	5.52
0.5	1.014 (0.061)	6.01
1	1.924 (0.050)	2.59
2	3.766 (0.098)	2.60
5	9.258 (0.561)	6.05
10	18.238 (0.770)	4.22

[1] Determined from three sets of standard curves on the same day.
[2] Standard deviation.

Table 2-B. Intra-Day Calibration Data.

Calibration Curve[1]	Slope[2]	Intercept	Correlation Coefficient, r
1	1.778	0.092	>0.99
2	1.746	0.073	>0.99
3	1.821	0.077	>0.99

[1] Calibration curves prepared on the same day.
[2] Mean slope (% CV) = 1.782 ml/μg (2.11)

The intra-day reproducibility was determined by comparing the linear regression analyses of three standard plots obtained from spiked dog plasma samples in the same day (Table 2). Least-squares regression analyses of the three calibration curves gave linear responses in the tested concentration range of DF (10 ng to 10 μg/ml). The average slope of the three plots were

1.78 ml/μg with a standard deviation (SD) of 0.038 and a coefficient of variation of 2.12, indicating good within-day reproducibility. The correlation coefficient (r) was also higher than 0.99 for each standard plot.

Recovery of Diclofenac from Plasma

Absolute recoveries of DF and MA from plasma were determined by comparing peak height ratios of DF/MA from the extracted plasma samples containing DF (0.01 to 10 μg/ml) to those ratios (DF/MA) obtained from direct injection of standard solutions prepared in the mobile phase at equivalent concentrations of DF (0.01 to 10 μg/ml) and the internal standard (1 μg/ml). In Table 3, the overall mean recovery of the assay was shown to be 98% with a standard deviation of 5.47 and overall coefficient of variation (CV%) of 5.58, indicating excellent extraction efficiency and reproducibility.

Accuracy and Precision of the Assay

Relative recovery data (Table 4) were used to assess the overall accuracy of the assay. Relative recovery of DF and MA was calculated by comparing peak height ratios of DF/MA from the spiked plasma samples containing DF (0.01 to 10 μg/ml) to those (DF/MA) obtained from the spiked water at the equivalent concentrations of DF and MA which were treated with the same procedure. Inter-day reproducibility of the recovery data were used to determine the precision of the method. The overall mean

Table 3: Recoveries of Diclofenac in Spiked Dog Plasma.

Plasma Conc (μg/ml)	% Recovery[1,2]	Coefficient of Variation, %
0.01	98.2 (9.89)	10.07
0.025	102.8 (7.09)	6.90
0.05	104.1 (3.65)	3.51
0.1	95.3 (7.67)	8.04
0.2	95.4 (5.37)	5.63
0.5	92.7 (7.30)	3.39
1	108.9 (3.69)	7.87
2	92.2 (4.94)	5.36
5	95.1 (5.65)	5.94
10	95.4 (3.87)	4.06

[1] Overall mean recovery (% CV) = 98.0% (5.58)
[2] Each value represents the mean % recovery (± SD) of 4-6 samples

Table 4: Inter-day Accuracy and Precision Data for Diclofenac Assay in Dog Plasma.

Plasma Conc. (μg/ml)	% Recovery[1,2]	Coefficient of Variation, %
0.01	95.81 (4.73)	4.94
0.025	102.93 (6.01)	5.84
0.05	101.87 (3.40)	3.33
0.1	94.42 (7.06)	7.48
0.2	98.45 (5.68)	5.77
0.5	102.93 (5.21)	5.06
1	98.92 (4.18)	4.23
2	97.36 (5.05)	5.18
5	93.41 (6.82)	7.30
10	96.18 (4.99)	5.19

[1] Overall mean relative recovery (% CV) = 98.2% (5.43)
[2] Each value represents the mean % recovery (± SD) of 3 samples

recovery for diclofenac for the calibration samples was 98.2% ± 5.31 (SD). In Table 4, the day-to-day coefficients of variation were 5.41% at the lower limit of quantitation (10 ng/ml) and 9.4-3.3% over the entire concentration range (10 ng-10 μg/ml).

The validation of the method was evaluated by analyzing DF in spiked plasma samples over the entire calibration concentration range (10 ng to 10 μg/ml) in a blind fashion where the analyst did not know the concentrations of the sample (Table 5). The peak height ratios of DF/MA were determined and the corresponding concentrations were calculated using the standard plot of DF in plasma (Table 1-A). In the blind study, accuracy and precision were remarkably good as measured by the overall mean recovery of 99.9% with a mean coefficient of variation (CV%) of 6.61% in the concentration range of 10 ng-10 μg/ml in plasma as shown in Table 5.

Table 6 summarizes the sensitivity, retention times, type of extraction solvents, recoveries and the coefficients of variation (CV%) of selected recent publications describing HPLC analysis of diclofenac in biological matrices. The proposed method clearly offers advantages over the existing HPLC procedures with respect to the extraction times, overall recoveries and inter- and intra-day variations. For example, Chan et al. (13) described a HPLC method which was claimed to be superior

Table 5: Validation of Assay for Diclofenac in Spiked Dog Plasma.

Actual Conc (μg/ml)	Measured Conc (μg/ml)	% Recovery	% Error[1]	% CV
0.01	0.009	90.0	10	9.35
0.025	0.028	112.0	12	4.12
0.05	0.053	106.0	6	4.12
0.1	0.103	103.0	3	5.80
0.2	0.180	90.0	10	1.75
0.5	0.493	98.0	1.5	6.40
1	0.999	99.9	0.1	3.36
2	1.999	99.9	0.05	1.82
5	5.014	100.3	0.28	6.10
10	9.995	99.9	0.05	4.20

[1] % Error = (Actual Conc - Measured Conc)/Actual Conc x 100
[2] % CV = Standard Deviation/Mean x 100

Table 6: Comparison of the Proposed Method with Published HPLC Methods of Analysis of Diclofenac in Biological Matrices.

Method	Sensitivity (ng/ml)	Retention Time (min)	Extraction Time (min)	Extraction Solvent	Mean Recovery (%)	CV (%)
Proposed Assay	10	3.8	6(1[a]+5[b])	Chloroform	98	5.5
Nielsen-Kudsk (1980)	-	10.1	5	ppt[c] with acetonitrile	-	-
Yaginuma (1981)	-	5.8	12(10[a]+2[b])	Benzene	-	-
Chan (1982)	5	3.9	25(15[a]+10[b])	Hexane-IPA[d]	95	8.2
Godbillon (1985)	10	5.8	20(10[a]+10[b])	Hexane-IPA[d]	98	3.1
Battista (1985)	-	~28	repeated	Diethyl ether	-	-
El-sayed (1988)	25	6.5	11(1[a]+10[b])	ppt[c] with acetonitrile	90-98	2.5-4.6
Mascher (1989)	10	~1.5	reextraction	Heptane IPA[d]	78	2-12
Grandjean (1989)	20	2	8(3[a]+5[b])	Hexane-IPA[d]	-	-
Brunner (1991)	5	2	30(15[a]+15[b])	Hexane-IPA[d]	99.8	0.5-11.1

[a] Shaking Time
[b] Centrifugation Time
[c] Precipitation
[d] Isopropyl Alcohol

to other methods for analysis of DF in plasma. However, the reproducibilities as indicated by the coefficients of variation which were 20% at 5 ng/ml (three replicate samples analyzed) and 22% at 10 ng/ml (four replicates) were relatively low. The recoveries reported for the calibration range varied widely between 67% and 144%. In addition, the analytical procedure was relatively tedious involving 15-min shaking for extraction prior to freezing the aqueous layer by dipping the tubes into a dry ice bath. The method described by Godbillon et al. (15), Battista et al. (16), Sayed et al. (18) and Mascher (20), despite their high sensitivity, are rather complex and time consuming as compared to the proposed assay (Table 6). While having very high sensitivity (20 ng/ml and 5 ng/ml, respectively), the automated methods described by Grandjean et al. (19) and Brunner et al. (21) required longer extraction times than the current method.

In Vivo Application of the Assay

Figure 3 shows the individual plasma concentration-time profile of a single dose (5 mg/kg) of DF given to four adult beagle dogs via oral route. The drug was rapidly absorbed after oral administration as indicated by the short peak time (~20 min). After the peak concentration, the plasma levels of DF exponentially declined following both iv bolus and oral administration. The pharmacokinetic parameters of DF in the dogs after iv

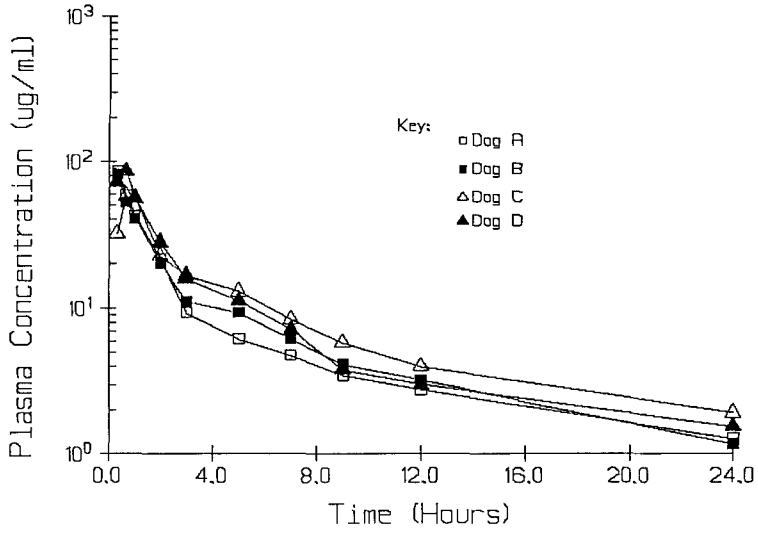


Figure 3. Plasma concentration versus time profile of diclofenac after a single oral dose (5 mg/kg) to four dogs.

bolus administration were calculated using the computer program (R-Strip) for the equation:

$$C = A_1 e^{-\alpha t} + A_2 e^{-\beta t}$$

The two half lives of the biexponential equation were 0.54 and 6.98 hr for α and β phases, respectively.

The pharmacokinetic parameters after the oral administration of DF to four dogs were calculated after fitting to the two-compartment model using the same computer program. These data are shown in Table 7. The mean peak plasma concentration (C_{max}) was found to be 78.2 μg/ml ($\pm$ 12.5 SD). The time required to reach this

Table 7: Pharmacokinetic Parameters of Diclofenac after Oral Dose (5 mg/kg) to Dogs Based on the Two-Compartment Model.

Parameter	Dog A	Dog B	Dog C	Dog D	Mean	SD[1]
AUC[2] (μg.hr/ml)	183.84	199.79	223.69	199.48	199.48	17.41
MRT (hr)	4.35	4.65	5.89	4.43	4.83	0.71
CL_t (L/hr/kg)	0.14	0.15	0.16	0.16	0.15	0.01
Vd_{ss} (L/kg)	1.12	1.03	1.36	0.94	1.11	0.18
F[3] (%)	57.38	71.29	69.64	68.42	66.68	6.31
C_{max} (μg/ml)	86.17	82.41	58.10	86.13	78.21	12.52
t_{max} (hr)	0.33	0.33	0.66	0.01	0.44	0.19

[1] Standard deviation
[2] AUC over 0-24 hr
[3] $F = AUC_{oral}/AUC_{iv} \times 100$

concentration (t_{max}) was 0.4 hr (± 0.19 SD). The absolute bioavailability (%F) was calculated from the equation:

$$F = AUC_{oral}/AUC_{iv} \times Dose_{iv}/Dose_{oral} \times 100$$

The mean % F was 66.7 (± 6.31 SD). The relatively low value of F is attributed to a significant first pass metabolism of the drug (2,3).

CONCLUSIONS

The HPLC method described herein has sufficient sensitivity to determine the pharmacokinetic parameters of diclofenac in plasma following a single oral or iv bolus dose in a usual therapeutic range. The method is simple, rapid, accurate, and reproducible, representing a significant improvement over many of the recently published HPLC methods for the quantitation of this drug

in plasma. The pharmacokinetic parameters of diclofenac after a single oral or iv bolus administration (5 mg/kg) in dogs were determined.

ACKNOWLEDGEMENTS

Financial support to Fergany A. Mohamed from the Egyptian Cultural and Educational Bureau in Washington, DC is greatly appreciated.

REFERENCES

1. Mark Abramowicz: Diclofenac. Medical Letter, 30 (Dec):109-111 (1988).

2. Peter, A.T. and Eugene, M.S.: Diclofenac Sodium; A reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy. Drugs, 35, 244-285 (1988).

3. Menasse, R., Hedwall, P.R., Kraetz, J., Pericin, C., Riesterer, L., Sallmann, A., Ziel, R., and Jaques, R.: Pharmacological properties of diclofenac sodium and its metabolites. Scand. J. Rheumatology, 22(Suppl):5-16 (1978).

4. Small, R.E.: Drug Reviews, Diclofenac Sodium. Clinical Pharmacy, 8:545-548 (1989).

5. Willis, J.V., Kendall, M.J., Flinn, R.M., Thornhill, D.P. and Welling, P.G.: The pharmacokinetics of diclofenac sodium following intravenous and oral administration. Eur. J. Clin. Pharmacol., 16:405-410 (1979).

6. Brogden, R.N., Heel, R.C., Pakes, G.E., Speight, T.M. and Avery, G.S.: Diclofenac sodium: A review of its pharmacological properties and therapeutic use in rheumatic diseases and pain of varying origin. Drugs, 20:24-48 (1980).

7. Willis, J.V., Kendall, M.J., and Jack, D.B.: The influence of food on the absorption of diclofenac after single and multiple oral doses. Eur. J. Clin. Pharmacol., 19:33-37 (1981).

8. Stierlin, H., Faigle, J.W., Sallmann, A. and Kung, W.: Biotransformation of diclofenac sodium (voltaren) in animals and in man. I-Isolation and identification of principle metabolites. Xenobiotica, 9(10):601-610 (1979).

9. El-Sadek, M., Baraka, M. and Aboul Kheir A.: Determination of diclofenac sodium through the formation of charge transfer complex with chloranil. Egypt J. Pharm. Sci. 29(1-4):367-379 (1988).

10. El-Sadek, M.: Spectrophotometric determination of diclofenac sodium in the presence of its impurities via chalcone formation. Ibid, 32(3-4):457-463 (1991).

11. Nielsen-Kudsk, F.: HPLC-determination of some anti-inflammatory, weak analgesic and uricosuric drugs in human blood and its application to pharmacokinetics. Acta Pharmacol. et. Toxicol., 47:267-273 (1980).

12. Yaginuma, H., Nakata, T., Toya, H., Murakami, T., Yamazaki, M. and Kamada, A.: Rectal delivery of antiinflammatory drugs on rectal absorption of β-lactam antibiotics. Chem. Pharm. Bull., 29(10):2974-2982 (1981).

13. Chan, K.K.H., Vyas, K.H. and Wnuck, K.: A rapid and sensitive method for the determination of diclofenac sodium in plasma by high-performance liquid chromatography. Anal. Lett., 15(B21 and 22):1649-1663 (1982).

14. Chan, K.K.H. and Vyas, K.H.: Determination of diclofenac sodium in synovial fluid by high performance liquid chromatography. Ibid., 18(B20):2507-2519 (1985).

15. Godbillon, J., Gauron, S. and Metayer, J.P.: High-performance liquid chromatographic determination of diclofenac sodium and its monohydroxylated metabolites in biological fluids. J. Chromatogr. Biomedical Appl., 338:151-159 (1985).

16. Battista, H.J., Wehinger, G. and Henn, R.: Separation and identification of non-steroidal antiinflammatory drugs containing a free carboxyl function using high-performance liquid chromatography. Ibid., 345:77-89 (1985).

17. Sane, R.T., Samant, R.S. and Nayak, V.G.: High performance liquid chromatographic determination of diclofenac sodium from pharmaceutical preparation. Drug Develop. Ind. Pharm., 13(7):1307-1314 (1987).

18. Sayed, Y.M., Abdel-Hameed, M.E., Suleiman, M.S. and Najib, N.M.: A rapid and sensitive high performance liquid chromatographic method for the determination of diclofenac sodium in serum and its use in pharmacokinetic studies. J. Pharm. Pharmacol., 40:727-729 (1988).

19. Grandjean, D., Beolor, J.C., Quincon, M.T. and Savel, E.: Automated robotic extraction and subsequent analysis of diclofenac sodium in plasma samples. J. Pharm. Sci., 78(3):247-249 (1989).

20. Mascher, H.: The pharmacokinetics of a new sustained-release form of diclofenac sodium in humans. Drug Design and Delivery, 4:303-311 (1989).

21. Brunner, L.A. and Luders, R.C.: An automated method for the determination of diclofenac sodium in human plasma. J. Chromatogr. Sci., 29(7):287-291 (1991).

22. Geiger, U.P., Degen, P.H. and Sioufi, A.: Quantitative assay of diclofenac in biological material by gas liquid chromatography. J. Chromatogr., III:293-298 (1975).

23. Ikeda, M., Kawase, M., Hiramatsu, M., Hirota, K., and Ohmori, S: Improved gas chromatographic method of determining diclofenac in plasma. J. Chromatogr. Biomed. Appl., 183:41-47 (1980).

24. Schweizer, A., Willis, J.V., Jack, D.B. and Kendall, M.J.: Determination of total monohydroxylated metabolites of diclofenac in urine by electron-capture gas liquid chromatography. J. Chromatogr., 195:421-424 (1980).

25. Schneider, W. and Degen, P.H.: Simultaneous determination of diclofenac sodium and its hydroxy metabolites by capillary column gas chromatography with electron-capture detection. Ibid., 217:263-271 (1981).

26. Jack, D.B. and Willis, J.V.: Letter to the Editor. Comments to the article, "Improved gas chromatographic method of determining diclofenac in plasma". J. Chromatogr. Biomed. Appl., 223:484-485 (1981).

27. Ikeda, M., Kawase, M., Kishie, T. and Ohmori, S.: Letter to the Editor. "Supplementary data for improved gas chromatographic method for determining diclofenac in plasma". Ibid., 223:486-491 (1981).

28. Schneider, W. and Degen, P.H.: Note. Simultaneous determination of diclofenac sodium and its metabolites in plasma by capillary column gas chromatography with electron-capture detection. Ibid., 383:412-418 (1986).

29. Schumacher, A., Geissler, H.E. and Mustschler, E.: Quantitative bestimmung von diclofenac-natrium aus plasma durch absorptionsmessung mit hilfe der direkten auswertung von dunnschichtchromatogrammen. J. Chromatogr., 181:512-515 (1980).

30. Riess, W., Stierlin, H., Degen, P.H., Faigle, J.W., Gerardin, A., Moppert, J., Sallmann, A., Schmid, K., Schweizer A., Sule, M., Theobald, W., and Wagner, J.: Pharmacokinetics and metabolism of the antiinflammatory agent voltaren. Scand. J. Rheumatol. Suppl., 22:17-29 (1978).

31. Kadowaki, H., Shiino, M., and Uemura, I.: Sensitive method for the determination of diclofenac in human plasma by gas chromatography-mass spectrometry. J. Chromatogr. Biomed. Appl., 308:329-333 (1984).

32. Del Puppo, M., Cighetti, G., Kienle, M.G., Paroni, R., and Borghi, C.: Determination of diclofenac in human plasma by selected ion monitoring. Biolog. Mass Spectrom., 20:426-430 (1991).

33. Gibaldi, M. and Perrier, D.: Pharmacokinetics. Marcel Dekker, New York and Basel, 84-109 (1982).

Received: March 20, 1993
Accepted: August 10, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1089-1104 (1994)

RAPID REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY METHOD FOR RANITIDINE HYDROCHLORIDE IN DOSAGE FORMS

C. A. LAU-CAM, M. RAHMAN, AND R. W. ROOS*
College of Pharmacy and Allied Health Professions
St. John's University
Jamaica, New York 11439

ABSTRACT

A HPLC method with photometric detection has been developed for the rapid assay of ranitidine hydrochloride in dosage forms and samples from tablet dissolution testing. This method also separates ranitidine from its related compound ranitidine S-oxide. Analyses were carried out on a Microsorb-MV C18 column, with a (1:1) mixture of methanol-0.01 M Na_2HPO_4 (pH 7.0) as the mobile phase, and detection at 320 nm. At a flow rate of 1.0 mL/min, typical retention times for ranitidine and its S-oxide compound were 3.50 min and 1.95 min, respectively. Detector responses were linearly related to on column concentrations of ranitidine and ranitidine S-oxide in the ranges 0.035-9.000 μg and 0.005-0.320 μg, respectively. Recoveries of ranitidine from spiked synthetic formulations simulating tablets, injections and syrups ranged from 99.7 ± 0.5% to 100.5 ± 0.5% of the added amount (n = 2). For assay purposes, tablets were extracted into or liquid samples (injections, syrups) were quantitatively diluted with methanol-water (1:1), and the solutions were injected onto the column. Samples from tablet dissolution tests required no preliminary preparation. Assay values by the proposed method were found to agree closely with those obtained using methods in the USP XXII.

*Present address: Food and Drug Administration, New York Regional Laboratory, Brooklyn, NY 11232.

INTRODUCTION

Among analytical techniques suggested for the determination of the quality and purity of the H_2-receptor antagonist ranitidine hydrochloride as a raw material, in commercial dosage forms and in extemporaneously-made parenteral solutions, HPLC is probably the one that has received the most extensive use (1-8). For example, the stability of ranitidine hydrochloride in total parenteral nutrition (TPN) solutions has been verified with a reversed phase (RP) HPLC method entailing a C_2 column, various ratios of acetonitrile-phosphate buffer (pH 6.8) as the eluent, and detection in the ultraviolet range (1). By switching to a C_{18} column and adding an ion-pairing reagent to the mobile phase, this method was subsequently modified to monitor the stability of ranitidine in TPN solutions (2) and in frozen and refrigerated solutions for injection (3). For both methods, however, neither full experimental details nor their applicability to the analysis of commercial dosage forms or of ranitidine related compounds have been described. A later, simpler approach has been based on the use of a C_{18} RP-HPLC column, a methanol-water mobile phase and detection at 221 nm. Although this method permitted a complete analysis of ranitidine in tablets and injections to be completed in less than 3 minutes, it required the column to be maintained at 50°C throughout the experiment to ensure resolution of the analyte from the recommended internal standard; in addition, the ability of this method to separate ranitidine

from any of its related compounds has not been documented (4). An alternative method, possessing both quantitative and stability-indicating capabilities, has relied on the use of an nonpolar column and photometric detection (5), but the utility of this approach in the analysis of dosage forms other than tablets and injections or of related compounds has not been investigated.

More recently, two HPLC methods have been described for the quantification of ranitidine and ranitidine related compounds in raw material samples (6,7) and tablets (7). One of these methods (7), requiring the use of a methanol-0.1 M aqueous ammonium acetate mobile phase, an ODS column, and detection at 322 nm, has been adopted by the USP XXII (8) for the assay of ranitidine and its tablets and injections. In addition, this method permits the separation of ranitidine from related compound C (ranitidine S-oxide), reported to occur both as a degradation product (6) and as a metabolite (9). For optimal resolution between the ranitidine peak and the ranitidine S-oxide peak, it is specified that the ratio of the mobile phase components as well as the flow rate be kept within certain limits. In our experience, this method is specific and accurate; but changing the brand of C_{18} column used may, in some cases, necessitate the composition ratio and flow rate of the original mobile phase to be significantly altered in order to maintain baseline resolution, reasonable elution times and minimal peak tailing. The purpose of this report is to describe a simple and specific isocratic RP-HPLC method for the determination of ranitidine

hydrochloride in pharmaceutical samples. This method separates ranitidine from its S-oxide compound in less than 5 minutes, shows good reproducibility from column to column, and is well suited for the assay of ranitidine in commercial liquid and solid dosage forms.

EXPERIMENTAL

Samples and Materials

Ranitidine hydrochloride and ranitidine S-oxide were a generous gift from the manufacturer (Glaxo Pharmaceuticals, Research Triangle Park, NC). They were dried to constant weight prior to use. Several lots of ranitidine hydrochloride tablets (150 and 300 mg), injections (25 mg/mL), and syrups (15 mg/mL) were obtained from local commercial sources. Solvents for chromatographic analysis were of HPLC grade (J.T. Baker); the methanol for dilutions, H_3PO_4, and Na_2HPO_4 were of analytical reagent grade (Mallinckrodt).

Sample Preparations

a. Ranitidine hydrochloride standard preparation - An accurately weighed quantity of ranitidine hydrochloride (about 75 mg) was dissolved in and quantitatively diluted with methanol-water (1:1), to obtain a preparation containing 75 μg/mL of ranitidine hydrochloride. The standard preparation for tablet dissolution testing assays was prepared in identical manner, except that the final concentration of ranitidine hydrochloride was 150 μg/mL.

b. Ranitidine hydrochloride and ranitidine S-oxide preparation - Accurately weighed quantities of ranitidine hydrochloride and ranitidine S-oxide were dissolved in methanol-water (1:1), to yield a preparation containing these compounds in concentrations of about 75 μg/mL and 2 μg/mL, respectively.

c. Tablet preparation - A group of 10 ranitidine hydrochloride tablets was weighed and ground to a fine powder. An accurately weighed quantity of powder, equivalent to about 150 mg of ranitidine hydrochloride, was transferred to a 100 mL volumetric flask, extracted with about 50 mL of methanol-water (1:1) with the aid of sonication for 10 min, and diluted to volume with the same solvent. The suspension was filtered, and a 5 mL portion of the filtrate was quantitatively diluted with methanol-water (1:1) to 100 mL.

d. Liquid dosage form (injection, syrup) preparation - An accurately measured volume of injection or syrup, equivalent to about 75 mg of ranitidine hydrochloride, was transferred to a 50 mL volumetric flask, diluted to volume with methanol-water (1:1), and mixed. A 5 mL portion of this solution was transferred to a 100 mL volumetric flask, diluted with methanol-water (1:1) to volume, and mixed.

e. Tablet dissolution testing preparation - Six tablets from each lot were tested according to the dissolution method for ranitidine hydrochloride tablets of USP XXII, i.e., apparatus 2, 900 mL of water as the dissolution medium, 37°C, and stirring at 50 rpm for 45 min. A

portion of the dissolution medium was filtered through a 0.45 μm membrane filter prior to HPLC analysis.

Synthetic Preparations

a. Synthetic tablet preparation - Prepared by sonicating ranitidine hydrochloride (75 or 150 mg), a (1:1:1) mixture of lactose-starch-magnesium stearate (250 mg) and 50 mL of methanol-water (1:1) in a 100 mL volumetric flask for 10 min. After dilution to volume with the same solvent, and mixing, the suspension was filtered. A portion of the filtrate was quantitatively diluted with methanol-water (1:1), to give a preparation containing a concentration of ranitidine hydrochloride similar to that of the standard preparation.

b. Synthetic injection preparation - Prepared in a volumetric flask with water, to contain 25 mg of ranitidine hydrochloride, 5 mg of phenol, 2.4 mg of Na_2HPO_4, and 0.96 mg of KH_2PO_4 in each mL. A portion of this solution was quantitatively diluted with methanol-water (1:1) to a final concentration of ranitidine hydrochloride similar to that of the standard preparation.

c. Synthetic syrup preparation - Prepared in a volumetric flask, by dissolving ranitidine hydrochloride in simple syrup to a concentration of 15 mg/mL. A portion of this solution was quantitatively diluted with methanol-water (1:1), to give a preparation containing a concentration of ranitidine hydrochloride similar to that of the standard preparation.

HPLC Method

a. Apparatus - Consisting of a Series 10 liquid chromatograph and a LC 90 UV spectrophotometric detector (Perkin-Elmer Corporation), connected either to a strip chart recorder (Knauer) or a ChromJet electronic integrator (Spectra-Physics). Samples were introduced through a sample injection valve fitted with a 20 μL sample loop (Rheodyne).

b. Chromatographic conditions - Separations were performed on a 15 cm x 4.6 mm i.d., Microsorb-MV C18, 5 μm, column (Rainin). Elutions were carried out with a (1:1) mixture of methanol and 0.01 M Na_2HPO_4 (previously adjusted to pH 7.0 with H_3PO_4), filtered and degassed prior to use. The flow rate was 1.0 mL/min, and the detection wavelength was 320 nm.

c. Calculations - The quantity of ranitidine hydrochloride in the sample preparation analyzed was calculated using one of the following equations:

$$\text{mg/tablet} = (R_{sp}/R_{st}) \times C \times 2 \times (A/W)$$

$$\text{mg/mL liquid dosage form} = (R_{sp}/R_{st}) \times (C/V)$$

$$\% \text{ dissolved} = (R_{sp}/R_{st}) \times S \times M \times (0.1/D)$$

where R_{sp} and R_{st} = the peak responses for the sample preparation and the standard preparation, respectively; C = the amount of ranitidine hydrochloride in the standard preparation, i.e., 75 μg/mL; A = the average tablet weight, mg; W = the weight of sample taken for the assay, mg; V = the volume of injection or syrup taken for the assay, mL; S = the amount of ranitidine in the standard preparation for the tablet dissolution test assay, i.e.,

150 μg/mL; M = the total volume of dissolution medium used, mL; and D = the amount of ranitidine hydrochloride declared per tablet, mg.

RESULTS AND DISCUSSION

The spectrophotometric detection of ranitidine and ranitidine S-oxide in HPLC analysis has been performed usually at 320-322 nm (1-3,5-8), but also at 220-228 nm (4,9). Although the present study verified peak responses at 320 nm not to be significantly different from those obtained at 228 nm, the former wavelength was preferred over the latter to ensure greater selectivity in the presence of potentially interfering peaks from excipients or additives in the dosage form. Moreover, elutions could also be monitored at 254 nm but with a 2-fold (ranitidine S-oxide) to 2.7-fold (ranitidine) decrease in sensitivity.

The effects of certain mobile phase-related factors such as pH of the buffer component, the ratio of the organic component to the buffer component, and the flow rate on resolution, R, and peak tailing, T, were systematically evaluated to achieve optimum resolution between ranitidine and its S-oxide compound. Ranitidine S-oxide is a degradation product of ranitidine which has also been used as part of the system suitability test for the HPLC analysis of ranitidine in pharmaceutical samples (7,8). As shown in Figure 1, by maintaining the ratio of methanol to phosphate buffer in the mobile phase constant at (1:1) and increasing the pH of the phosphate buffer in a step-

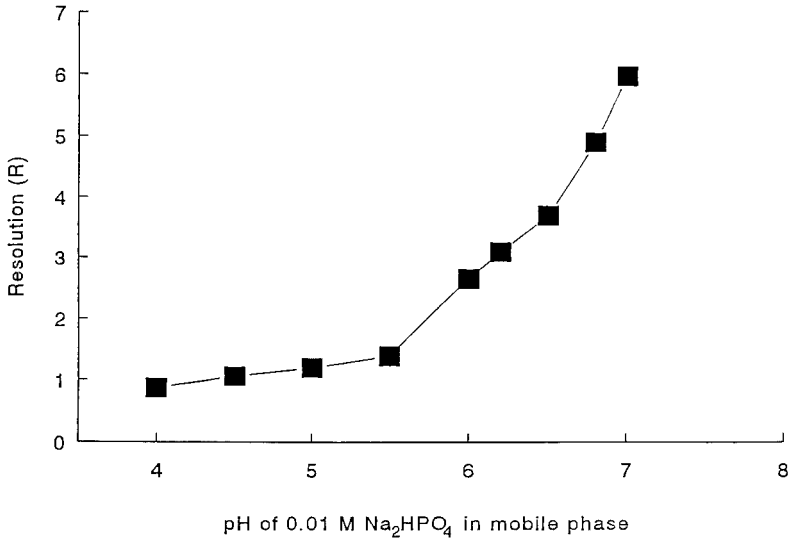


Figure 1. Effect of the pH of the 0.01 M Na_2HPO_4 solution on resolution, R, between ranitidine and ranitidine S-oxide peaks. All experiments were conducted using a (1:1) methanol-0.01 M Na_2HPO_4 mixture flowing at 1.0 mL/min.

wise manner, R increased with increasing pH of the phosphate component, to become ≥ 3.0 at pH ≥ 6.0 and maximal at pH 7.0. The effect of the concentration of methanol on R was studied using ratios of methanol to phosphate buffer (pH 7.0) in the mobile phase between (40:60) and (65:35). In this manner, separations with R > 2.5 and requiring less than 5 min were obtainable with a mobile phase composed of methanol-0.01 M Na_2HPO_4 (pH 7.0) in a (1:1 to 3:2) ratio (Figure 2) and flowing at the rate of 1.0-2.0 mL/min. The use of lower ratios resulted in elutions that were much slower and in some peak tailing. Assays were

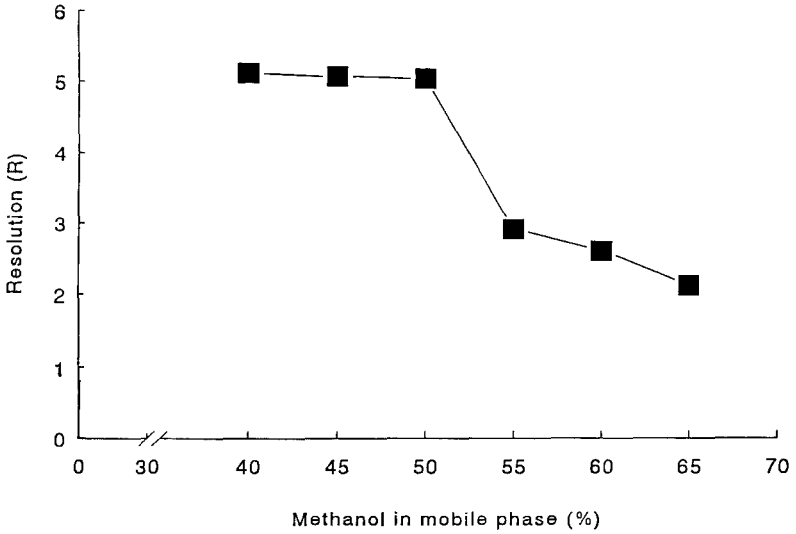


Figure 2. Effect of the percentage of methanol in the mobile phase on resolution, R, between ranitidine and ranitidine S-oxide peaks. All experiments were conducted at a flow rate of 1.0 mL/min.

routinely performed using a methanol to 0.01 M phosphate buffer (pH 7.0) ratio of (1:1) and a flow rate of 1.0 mL/min. These conditions were found to produce equivalent results when the analysis was repeated on either a μBondapak C_{18} (Waters) or an Econosphere C_{18} (Alltech) column. Typical chromatograms, obtained using the recommended conditions, are shown in Figure 3.

To verify the linearity of the proposed method, peak responses (heights or areas) at 320 nm were assessed using serial dilutions of stock solutions of ranitidine hydrochloride (450 μg/mL) and ranitidine S-oxide (16 μg/ mL) in methanol-water (1:1). Peak responses were found to be

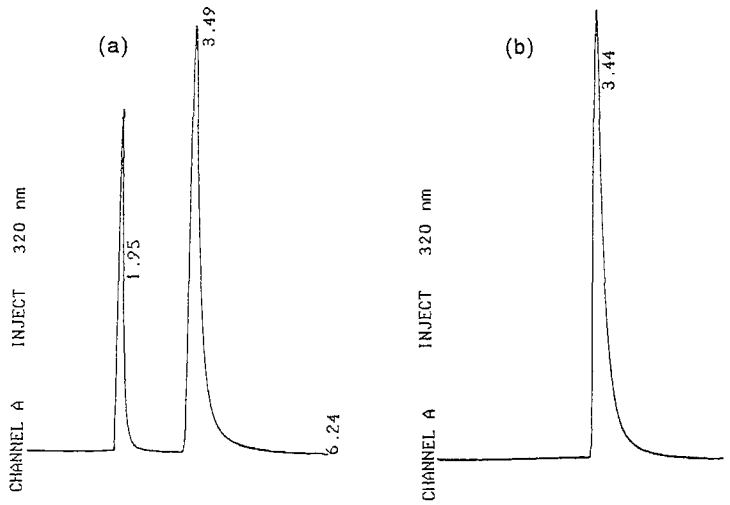


Figure 3. Typical high-performance liquid chromatograms of (a) a standard preparation of ranitidine plus ranitidine S-oxide, and (b) a tablet sample. Flow rate was 1.0 mL/min; for comparative purposes, a chromatogram recorded at 1.5 mL/min is shown in the inset. Ranitidine is the slower eluting peak.

linearly related to on column concentrations of ranitidine and ranitidine S-oxide between 0.035-9.000 μg (n = 2, r = 0.999) and 0.005-0.32 μg (n = 2, r = 0.999), respectively, with the curves passing through the origin. The precision of the proposed method was evaluated by measuring peak heights and peak areas following multiple injections of a standard preparation containing about 56 μg/mL of ranitidine and 1 μg/mL of ranitidine S-oxide. Based on peak heights from 6 consecutive injections, the RSD values for ranitidine and its S-oxide were 0.92% and 0.02%, respectively; the RSD values for the corresponding areas

were 1.40% and 1.26%, respectively. Accuracy was evaluated by spiking synthetic formulations, prepared to simulate tablets, injections and syrups, with known amounts of ranitidine and subjecting the samples to the assay procedure described for commercial dosage forms. As shown in Table 1, mean recoveries (n = 2) of ranitidine hydrochloride from the various synthetic formulations were, in all cases, not less than 99.0% of the added amount.

The application of the proposed method to the assay of commercial dosage forms yielded the results summarized in Table 2. These results, representing the mean values of duplicate determinations, were in turn compared with those obtained by the HPLC method of USP XXII (8). Since the syrup sample is not listed in the official compendium, this dosage form was analyzed in the same manner as the injections. Intermethod differences for 150 and 300 mg tablets ranged from 0.7-2.6% of declared; for injections and syrups, the differences were about 1.45% and 1.35% of declared, respectively. All samples complied with the official requirements for drug potency, and were found to be free of contamination with the S-oxide or any other reported related compound (6,8). Furthermore, the proposed method was also useful to measure the amount of ranitidine released during the course of the USP XXII tablet dissolution test. The assay results for two sets of 150 mg tablets and one set of 300 mg tablets are shown in Table 3. A comparison of these results with those derived using the official spectrophotometric method indicated the existence of a close agreement, with the intermethod differences

Table 1

Results of recovery studies of ranitidine hydrochloride from synthetic dosage forms by the proposed HPLC method

Synthetic formulation	Ranitidine hydrochloride found, % of added			
	Run 1	Run 2	Mean	SD
Tablet, 150 mg	99.0	100.0	99.5	± 0.5
Tablet, 300 mg	100.5	99.5	100.0	± 0.5
Injection, 25 mg/mL	99.0	98.9	98.9	± 0.2
Syrup, 15 mg/mL	101.0	100.0	100.5	± 0.5

ranging from 0.2-1.7% of dissolved in 45 min. All tablets conformed to the USP XXII dissolution requirements. Tablet excipients or additives did not interfere with the proposed assay method.

In conclusion, the HPLC method presented here will permit the analysis of ranitidine hydrochloride in commercial solid and liquid dosage forms and in tablet dissolution samples in a rapid, specific and quantitative manner, and by using uniform experimental conditions. In comparison with compendial HPLC methods for tablets, injections and tablet dissolution samples of the title drug, in the proposed method all samples for analysis were prepared in methanol-water rather than in the mobile phase, the new chromatographic conditions met all of the official

Table 2

Determination of ranitidine hydrochloride in commercial dosage forms by proposed HPLC method and USP XXII HPLC method[a,b]

Lot No.	Ranitidine hydrochloride. % of declared					
	Proposed method			USP XXII method		
	Run 1	Run 2	Mean	Run 1	Run 2	Mean
	Tablets, 150 mg/tablet					
1	99.5	101.3	101.3	99.3	98.7	99.0
2	103.0	103.7	103.7	101.0	100.4	100.7
	Tablets, 300 mg/tab					
1	102.9	101.8	102.3	102.3	100.5	101.4
2	98.9	99.9	99.4	100.1	100.1	100.1
	Injections, 25 mg/mL					
1	108.6	108.6	108.6	107.6	106.8	107.2
2	106.0	106.0	106.0	105.0	104.0	104.5
	Syrups, 15 mg/mL					
1	100.0	102.4	101.2	101.7	102.5	102.6
2	103.2	102.6	102.9	102.5	100.7	101.6

[a]USP XXII ranges for tablets and injections = 90.0 to 110.0%
[b]The syrup is not listed in USP XXII.

Table 3

Assay results (% found) for tablet dissolution test samples by proposed HPLC method and USP XXII spectrophotometric method[a]

Tablet No.	150 mg (Lot 1)		150 mg (Lot 2)		300 mg	
	HPLC	USP	HPLC	USP	HPLC	USP
1	99.5	99.8	94.4	97.6	93.4	93.1
2	90.5	91.9	90.0	93.0	101.7	99.0
3	92.8	92.6	98.9	99.7	104.2	102.6
4	100.4	101.8	92.8	94.9	96.1	95.5
5	98.0	94.8	94.7	94.7	99.5	99.7
6	97.8	96.5	95.7	94.0	99.5	98.6
Mean	96.4	96.2	93.9	95.6	99.1	98.1
SD	3.91	3.96	3.41	2.50	3.86	3.33

[a]USP XXII tolerances: not less than 80% of the labeled amount of ranitidine is dissolved in 45 min.

system suitability requirements for the HPLC assay of ranitidine, and peak elution times at similar flow rates were shorter. Moreover, the proposed method yielded assay results that agreed well with those obtained using methods in the USP XXII.

REFERENCES

1. Walker, S. and Bayliff, C.D., Stability of ranitidine hydrochloride in total parenteral nutrient solution, Am. J. Hosp. Pharm., 42, 590-592 (1985).

2. Bullock, L., Parks, R.B., Lampasona, V. and Mullins, R.E., Stability of ranitidine hydrochloride and amino acids in parenteral nutrient solutions, Am. J. Hosp. Pharm., 42, 2683-2687 (1985).

3. Lampasona, V., Mullins, R.E. and Parks, R.B., Stability of ranitidine admixtures frozen and refrigerated in minibags, Am. J. Hosp. Pharm., 43, 921-925 (1986).

4. Ficarra, P., Ficarra, R. and Tommasini, A., High-performance liquid chromatography of ranitidine in pharmaceuticals, J. Pharm. Biomed. Anal., 2, 119-123 (1984).

5. Das Gupta, V., Quantitation of ranitidine hydrochloride in tablets and injections using high-performance liquid chromatography, Drug Dev. Ind. Pharm., 14, 1647-1655 (1988).

6. Beaulieu, N., Lacroix, P.M., Sears, R.W. and Lovering, E.G., High-performance liquid chromatographic methods for the determination of ranitidine and related substances in raw materials and tablets, J. Pharm. Sci., 77, 889-892 (1988).

7. Evans, M.B., Haywood, P.A., Johnson, D., Martin-Smith, M., Munro, G. and Wahlich, J.C., Chromatographic methods for determining the identity, strength and purity of ranitidine hydrochloride both in the drug substance and its dosage forms - an exercise in method selection, development, definition and validation, J. Pharm. Biomed. Anal., 7, 1-22 (1989).

8. The United States Pharmacopeia, 22nd Rev., U.S. Pharmacopeial Convention, Inc., Rockville, MD, 1990, pp. 1209-1211.

9. Mihaly, G.W., Drummer, O.H., Marshall, A., Smallwood, R.A. and Louis, W.J., High-pressure liquid chromatographic determination of ranitidine, a new H_2-receptor antagonist, in plasma and urine, J. Pharm. Sci. 69, 1155-1157 (1980).

Received: May 9, 1993
Accepted: August 25, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1105-1110 (1994)

DIRECT ENANTIOMERIC SEPARATION OF PHENGLUTARIMIDE BY CHIRAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY*

HASSAN Y. ABOUL-ENEIN[1]**, SOLIMAN A. BAKR[1], AND PAUL J. NICHOLLS[2]

[1]*Bioanalytical and Drug Development Laboratory*
Biological & Medical Research Department
MBC-03
King Faisal Specialist Hospital and Research Centre
P.O. Box 3354
Riyadh 11211, Kingdom of Saudi Arabia
[2]*Welsh School of Pharmacy*
University of Wales
P.O. Box 13
Cardiff CF1 3XF, United Kingdom

ABSTRACT

Enantiomeric separation of racemic phenglutarimide (PG) by chiral high performance liquid chromatography is reported using cellulose tris (3,5-dimethylphenyl carbamate) known as Chiralcel OD chiral stationary phase. Maximum resolution (R_S) of 1.26 is obtained for the enantiomers of PG. The method could be applied for preparative scale chromatography and in the assay of PG in biological fluids.

I. INTRODUCTION

Phenglutarimide (PG), chemically known as (±)-3-(2-diethylaminoethyl)-3-phenyl-piperidine-2,6-dione, is an anticholinergic agent with actions and uses similar to those of

* Presented at the 44th Pittsburgh Conference and Exposition, Atlanta, Georgia, USA, 8-12 March 1993.

** Author to whom correspondence should be addressed

benzhexol. It was formerly used also in the treatment of Parkinsonism [1]. Phenglutarimide possesses a chiral carbon at C_3 and is administered as a racemic mixture of (+)-S and (-)-R enantiomers as shown in Figure 1. Most of the pharmacological clinical efficacy and metabolism in rat and man has been studied on the racemic mixture and not on the individual enantiomers [2-10]. Recently, Lambrecht et al [11] reported the extremely high stereoselectivity and high affinities for the (+)-S-enantiomer PG as compared to the (-)-R-enantiomer to three muscarinic receptor subtypes namely, M_1, M_2 and M_3. It was found that the (+)-S-PG to be a potent M_1-selective antagonist as compared to (-)-R-PG.

Described here is a simple, isocratic method for the enantioseparation of the racemic mixture of PG. Effective resolution is achieved on cellulose tris (3,5-dimethylphenyl carbamate) chiral stationary phase (CSP), known as Chiralcel OD. Furthermore, preliminary evaluation of the chiral recognition-structure relationships, involved in the enantioseparation of structurally related drugs having piperidine-2,6-dione ring system on this cellulose CSP is discussed.

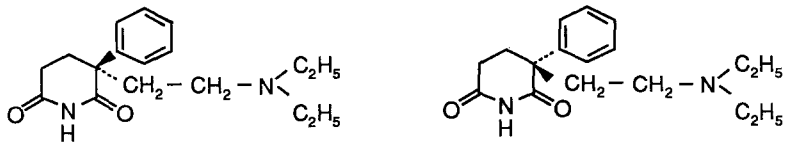
2. EXPERIMENTAL

2.1 Apparatus

The Waters Liquid Chromatography System (Waters Associates, Milford, MA, USA) consisted of a Model M-45 pump, a U6K injector, and a Lambda-Max Model 481 LC spectrophotometer UV detector operated at 257 nm. The stationary phase Chiralcel OD analytical column of cellulose tris-3,5-dimethylphenyl carbamate, (25cm x 0.46cm, i.d.; Daicel Chemical Industries, Tokyo, Japan) coated on silica gel with particle size 10 μm was used.

2.2 Chemicals

Racemic PG was obtained from Professor P. J. Nicholls, Welsh School of Pharmacy, Cardiff, U.K. and the corresponding (+)-S and (-)-R PG as hydrochloride salts were supplied by Professor Dr. J. Knabe, Institute of Pharmaceutical Chemistry, University of Saarland, Saarbrücken, Germany. HPLC grade hexane, 2-propanol were obtained from Fisher Scientific, Fairlawn, NJ, USA.



(-)- (R) (+)- (S)

Fig. 1. The structures of the enantiomers of phenglutarimide.

2.3 Chromatographic Conditions

The maximum and symmetrical stereochemical resolution of PG was obtained using hexane and 2-propanol (70:30) on Chiralcel OD column. Flow rate was 1.0 ml/min. and chart speed was 0.5 cm/min. A temperature of 23^{O}C was maintained throughout the experiment. Detection was obtained at UV 257 nm with a sensitivity range 0.01 aufs. Sample amount injected was 2.0 nmole for racemic PG and 1.0 nmol for its corresponding individual enantiomers.

2.4 Determination of Enantiomeric Elution Order

The enantiomeric elution was determined by chromatographing the individual enantiomers (-)-R-and (+)-S-PG separately under the same conditions. Thus, the peak that eluted with the lower capacity factor was identified as (+)-S-PG and the one that eluted with the higher capacity factor was identified as (-)-R-PG.

3. RESULTS AND DISCUSSION

The method described here is the first reported in literature where PG enantiomers can be directly resolved using cellulose (tris 3,5-dimethylphenyl carbamate) CSP. A typical chromatogram of the enantioseparation of racemic PG is shown in Fig. 2. By comparison the chromatogram and the capacity factor of individual enantiomers, the peak which eluted at a lower capacity factor (k_1=1.11) was identified as the (+)-S-enantiomer, (Fig. 3a) and the peak with the higher capacity factor (k_2=1.66) as the (-)-R-PG (Fig. 3b). The stereochemical separation factor (α) was 1.50. The maximum stereochemical resolution factor (R_S) was 1.26.

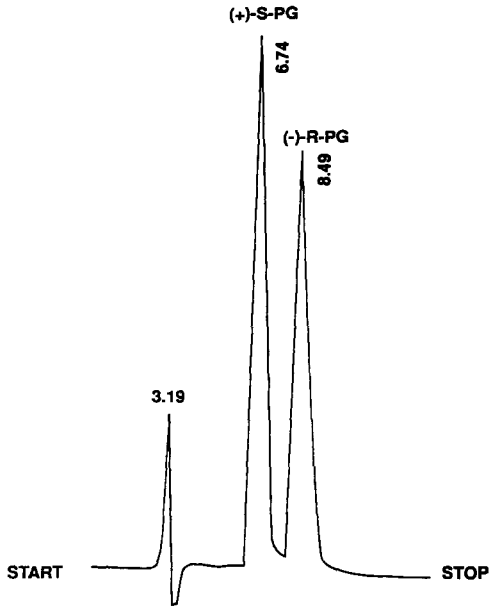


Fig. 2 Enantiomeric HPLC separation of racemic phenglutarimide. Column: Chiralcel OD (250 x4.6mm i.d.); mobile phase: hexane: 2-propanol (70:30); flow rate: 1 ml/min; chart speed: 0.5cm/min.; temperature: 23°C; detector: UV 257nm; sensitivity 0.01 AUFS; sample amount 2 nmol.

This cellulose-derived chiral stationary phase has been successfully used for direct separation of several piperidine-2,6-dione analogs namely aminoglutethimide [12], cyclohexylaminoglutethimide [13], 1991), pyridoglutethimide [14] and glutethimide [15]. It is of interest to mention that regardless of the absolute configuration of the substituents at C_3 of the piperidine 2,6-dione drug chromatographed the levorotatory enantiomers did elute first followed by the dextrorotatory enantiomer with the exception of PG where the (+)-S enantiomer did elute first followed by (-)-R enantiomer. This could be rationalized by the effect of non-aromatic basic substituent 2-diethylaminoethyl group at C_3 of the piperidine-2,6-dione ring system of PG which might have caused the reversal enantiomeric elution order of PG enantiomers contrary to the other analogs separated on the same CSP where the non-aromatic substituent at C_3 are more hydrophobic in nature being of hydrocarbon residue. It is obvious that the non-aromatic substituent and C_3 in this

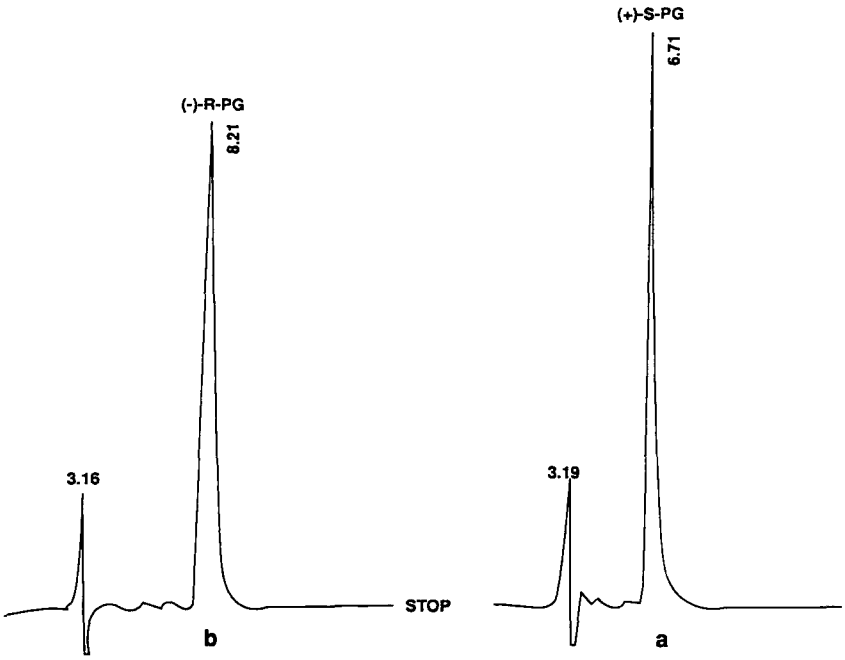


Fig. 3 (a) Chromatogram of (+)-(S)-phenglutarimide.
(b) Chromatogram of (-)-(R)-phenglutarimide
Conditions were the same as in Fig. 1 except sample amount was1 nmol.

piperidine-2,6-dione series does play an important role as to the determination of the elution order of the individual enantiomers.

4. CONCLUSION

The direct stereochemical separation of racemic PG was achieved on commercially available cellulose 3,5-dimethylphenyl carbamate under isocratic conditions. This method could be applied on a preparative scale for the preparation of a large quantity of PG enantiomers by using the preparative Chiralcel OD column which is commercially available. Furthermore, since the method is simple and fast it can be adopted to quantitate the enantiomers of PG in biological fluids for further pharmacokinetic and pharmacodynamic studies which is in progress.

ACKNOWLEDGEMENT

The authors (H.Y.A.E and S.A.B.) thank the Administration of King Faisal Specialist Hospital and Research Centre for their continuous support to the Bioanalytical and Drug Development research program. This investigation was supported financially under Project No. 88-0015 by King Faisal Specialist Hospital and Research Centre.

REFERENCES

[1] E. Tagmann, E. Sury and K. Hoffmann, *Helv. Chim. Acta.* **35** (1952) 1235.

[2] H. J. Bein and J. Tripod, *Schweitz. Med. Wschr.* **88** (1958) 1160.

[3] E. Frommel, *Presse Méd.* **66** (1958) 1745.

[4] R. O. Gillhespy, *Br. Med J.* **1** (1958) 1542.

[5] R. O. Gillhespy, *Br. J. Clin. Pract.* **14** (1960) 287.

[6] W. Hughes, J. H. Keevil and I. E. Gibbs, *Br. Med. J.* **1** (1958) 928.

[7] J. Peremans, *Gastroenterologia* **90** (1958) 29.

[8] D. G. Wenzel and G. H. Emick, *J. Am. Pharm. Ass.* **45** (1956) 414.

[9] G. M. Wyant and F.C. Haley, *Anesthesiology* **20** (1959) 581.

[10] J. S. Douglas and P. J. Nicholls, *Xenobiotica* **3** (1973) 605.

[11] G. Lambrecht, R. Feifel and E. Mutschler, *Chirality* **1** (1989) 170.

[12] H. Y. Aboul-Enein and M. R. Islam, *Chromatographia* **30** (1990) 223.

[13] H. Y. Aboul-Enein and S. A. Bakr, *Chirality* **3** (1991) 204.

[14] H. Y. Aboul-Enein, S. A. Bakr, P. J. Nicholls, *J. Liq. Chromatogr.* **15** (1992) 123.

[15] H. Y. Aboul-Enein and V. Serignese, Unpublished results.

Received: October 25, 1993
Accepted: November 4, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1111-1124 (1994)

SEPARATION OF PORPHYRINS USING A γ-CYCLODEXTRIN STATIONARY PHASE

WENHONG WU AND APRYLL STALCUP*
Department of Chemistry
2545 The Mall
University of Hawaii at Manoa
Honolulu, Hawaii 96822

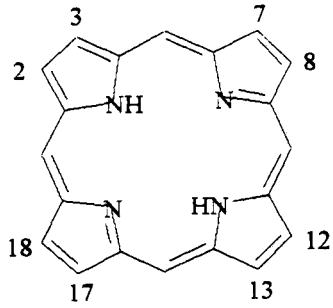
ABSTRACT

An isocratic separation of five porphyrin carboxylic acids using conventional mobile phase conditions was achieved on a commercially available γ-cyclodextrin bonded phase within 30 minutes. The effects of pH, ionic strength, organic modifier as well as the retention and separation mechanisms are discussed. The direct determination of porphyrin levels in blood serum is also discussed.

INTRODUCTION

Polycarboxylated porphyrins are intermediate metabolites of heme biosynthesis (1). Excessive production and excretion of porphyrins in biological fluids is a sign of disturbances of the heme biosynthetic pathway (2). These disorders can be inherited or caused by exposure to some toxic chemicals. Determination of relative levels of porphyrins in biological fluids, e.g. urine, stool, or blood, is essential for diagnosis of various porphyrias, lead poisoning, and iron deficiency anemia (3). In normal urine, five porphyrins may be found, containing four to eight carboxylic acid groups, namely copro-, pentacarboxyl-, hexacarboxyl-, heptacarboxyl-, and uro-porphyrin (Table 1).

Table 1. Structures of porphyrins used in this study.



Porphyrins	Side-chain substitution							
	2	3	7	8	12	13	17	18
Uroporphyrin I	A	P	A	P	A	P	A	P
Heptacarboxylporphyrin I	M	P	A	P	A	P	A	P
Hexacarboxylporphyrin I (cis)	M	P	M	P	A	P	A	P
Hexacarboxylporphyrin I (trans)	M	P	A	P	M	P	A	P
Pentacarboxylporphyrin I	M	P	M	P	M	P	A	P
Coproporphyrin I	M	P	M	P	M	P	M	P

A = $-CH_2COOH$; P = $-CH_3CH_2COOH$; M = $-CH_3$

Various chromatographic techniques have been developed to determine free porphyrin carboxylic acids. Among these methods, high-performance liquid chromatography (HPLC) is a preferred technique due to its efficiency, sensitivity, simplicity and easy quantitation (2,4).

Both normal phase and reversed-phase modes have been tried. In the normal phase mode, an aminopropyl-bonded silica phase was used for the separation of

porphyrins (5). Routinely, normal phase procedures require esterification of the porphyrin carboxylic acids before analysis. Reversed-phase systems have been tried using octadecylsilane stationary phases (2, 6-9). These reversed-phase separations almost exclusively used linear gradient elutions.

Native and derivatized cyclodextrin (CD) bonded stationary phases are best known for their ability of separating enantiomers. In addition, CD bonded stationary phases are also used for separating structural isomers (10). Many of these separations are difficult or impossible to achieve on conventional HPLC columns.

Under reversed-phase conditions, formation of an inclusion complex of the solute with the relatively nonpolar interior of the CD cavity is suggested to be an essential step to achieve separations (11). Furthermore, the hydroxyl groups lining the mouth of the CD cavity can form hydrogen bonds with polar segments of the solute molecules. The stability of an inclusion complex is a function of the size and shape of a molecule relative to a given CD (10). Separation due to inclusion complexation may not be possible if the diameter of a molecule is significantly larger than the mouth of the CD cavity. However, as long as a portion of the molecule is tightly complexed, the analytes may be effectively separated (12). Recent studies suggest that some chiral analytes may be separated by sitting atop of the CD cavity like a lid, without forming a conventional inclusion complex (13).

In the normal phase mode, the nonpolar component of the mobile phase is thought to compete with the solute to occupy the CD cavity, thereby preventing the analyte from forming an inclusion complex. In this case, the separation is more likely due to hydrogen bonding between the solute and the hydroxyl groups at the top and bottom of the CD toroid (14).

The structural similarities of free porphyrin carboxylic acids make the isocratic separation of these compounds challenging. Recently, an isocratic separation method for free porphyrin carboxylic acids on a β-CD bonded stationary phase was reported (1, 15). The isocratic elution on the β-CD bonded stationary phase was completed in less than eight minutes (15). However, this method employed a mobile phase containing 18-crown-

6 ether, phosphate buffer, acetonitrile and pyridine. The suggested mechanism for the separation of carboxylated porphyrins was retention via adsorption rather than inclusion complexation, because the cavities of β-CDs were too small to accommodate the bulky porphyrins.

The γ-CD column is not as widely used as the β-CD column, because the γ-CD cavity is thought to be too big to tightly complex with most analytes. The current work shows that a γ-CD bonded stationary phase in conjunction with a conventional mobile phase can achieve the isocratic separations of carboxylated porphyrins within 30 minutes.

EXPERIMENTAL

Chemicals

Uroporphyrin I, heptacarboxylporphyrin I, hexacarboxylporphyrin I, pentacarboxylporphyrin I, coproporphyrin I were all purchased from Porphyrin Products, Inc.(Logan, UT). Sodium phosphate monobasic was bought from J. T. Baker Chemical Co. (Phillipsburg, NJ). Sodium phosphate dibasic was purchased from Matheson Coleman & Bell Manufacturing Chemists (Norwood, OH). HPLC grade acetonitrile and water were obtained from Fisher Scientific (Tustin, CA).

Apparatus

The HPLC system consisted of a Shimadzu LC-600 Liquid Chromatograph, a SPD-10A variable wavelength UV/vis detector, and a CR601 Chromatopac data acquisition system. The Cyclobond II Gamma column (250 x 4.6 mm i.d. stainless steel, 5μm particle diameter) was obtained from Advanced Separation Technologies, Inc. (Whippany, NJ).

Chromatographic conditions

The separations were performed with isocratic elution. The mobile phases used were acetonitrile/phosphate buffer (Na_2HPO_4, NaH_2PO_4; *ca.* 100 mM). The isocratic elution was carried out at 18 °C at a flow rate of 0.8 mL/min. The eluates were monitored at 400 nm.

Appropriate amounts of the porphyrin samples were dissolved in methanol and stored in the dark at 4°C.

RESULTS AND DISCUSSION

Isocratic separation of five porphyrin carboxylic acids was achieved within 30 minutes using a γ-CD bonded stationary phase. The chromatographic data is summarized in Table 2.

Effect of organic modifier

Methanol was not investigated as extensively as an organic modifier as acetonitrile because preliminary results showed that selectivities and peak shapes obtained by using methanol were not as good as those obtained using acetonitrile. Therefore optimization focused on acetonitrile as an organic modifier. The effect of acetonitrile concentration on capacity factors is shown in Figure 1.

At low acetonitrile concentrations (<30%), as the volume fraction of organic modifier increases, the capacity factors tend to decrease. However, at higher acetonitrile concentrations (>40%), as the organic modifier increases, the capacity factors start to increase. The same trend exists for all five porphyrins but with apparently different "turning points". For instance, the turning point for uroporphyrin is approximately 30% acetonitrile; for coproporphyrin it is *ca.* 40% acetonitrile.

The appearance of a capacity factor minimum as a function of the composition of the organic modifier in the mobile phase suggest that there might be two retention mechanisms involved in the separations. When the mobile phase contains a low volume fraction of organic modifier, the retention mechanism is likely due to hydrophobic interactions with stationary phase. The porphyrin macrocycle is planar, highly conjugated with significant aromatic and hydrophobic character (16). The macrocycle part of the porphyrin may enter or sit on the top of the relatively nonpolar γ-CD cavity, allowing the carboxylated side chains of the porphyrins to associate with the hydroxyl groups lining the

Table 2. Chromatographic results of isocratic separation of polycarboxylated porphyrins on γ-CD bonded phase. Mobile phase: 25/75 ACN/buffer (140 mM phosphate; pH 6.9).

Porphyrins	k'	α
Uroporphyrin I	0.521	1.960
Heptacarboxylporphyrin I	1.021	1.427
Hexacarboxylporphyrin I (1)	1.457	1.339
Hexacarboxylporphyrin I (2)	1.951	1.240
Pentacarboxylporphyrin I	2.615	1.336
Coproporphyrin I	3.493	

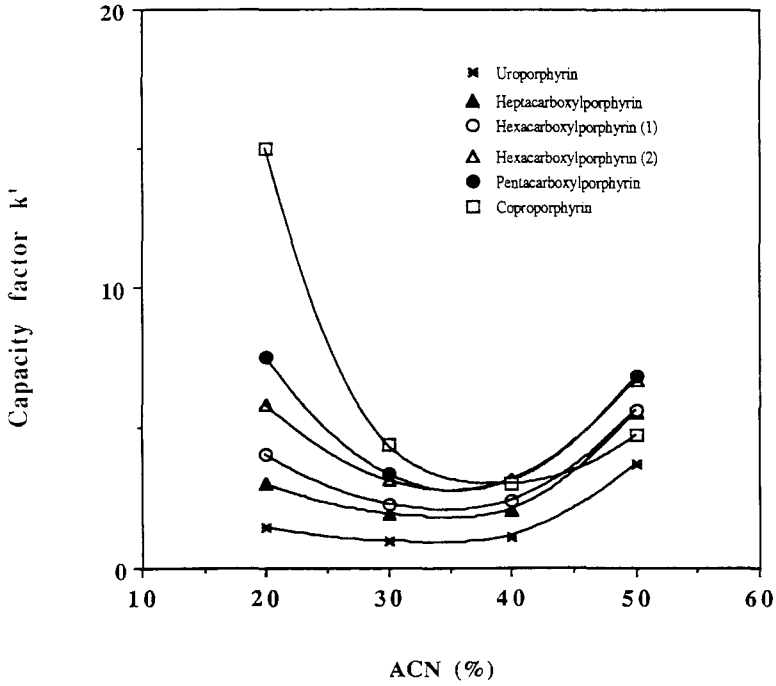


Figure 1. Effect of acetonitrile concentration on capacity factors of polycarboxylated porphyrins. Mobile phase: ACN/buffer (140 mM phosphate; pH 6.7).

mouth of the CD cavity. As the organic modifier concentration increases, retention decreases which is typical of a reversed phase mode of retention. In this region of mobile phase composition, the porphyrins elute according to polarity, with the most polar porphyrin, uroporphyrin, eluting first, and followed by heptacarboxylporphyrin, hexacarboxylporphyrin, pentacarboxylporphyrin, with the least polar porphyrin, coproporphyrin, eluting last. This elution order at low organic modifier concentration is also consistent with a reversed phase mode of retention.

The increase in retention when the concentration of acetonitrile increases beyond the "turning point" may be due to interaction between the porphyrin carboxylated side chains and CD hydroxyl groups. The least polar porphyrin, coproporphyrin, was eluted before hepta-, hexa- and penta-carboxylporphyrin.

Effect of ionic strength

The effect of ionic strength on retention was studied and the results are shown in Figure 2. As can be seen from the figure, when the concentration of phosphate buffer increases, the capacity factors of all five polycarboxylated porphyrins decrease. In addition, it was found that better peak shapes and resolution were obtained at high buffer concentration. However, the concentration of phosphate buffer was limited to *ca.* 140 mM, because acetonitrile miscibility decreases as phosphate buffer increases.

Effect of pH

Given the presence of carboxylic acid (-COOH) side chains, pyrrole (=NH) groups, and pyrrolenine (≡N) groups, it is not too surprising that the elution of porphyrin carboxylic acids is pH sensitive (Figure 3). The retention of all five porphyrins increase when pH decreases. As pH decreases, porphyrins tend to be protonated, therefore the solubility in the mobile phase decreases. Complete elution of the five porphyrins within 60 minutes requires a pH of 6.5 or higher.

Other considerations

Optimum conditions for the separation was found to be 25/75 (140 mM) of acetonitrile/phosphate buffer (Na_2HPO_4, NaH_2PO_4). The pH of the aqueous portion was 6.9. A typical chromatogram is shown in Figure 4.

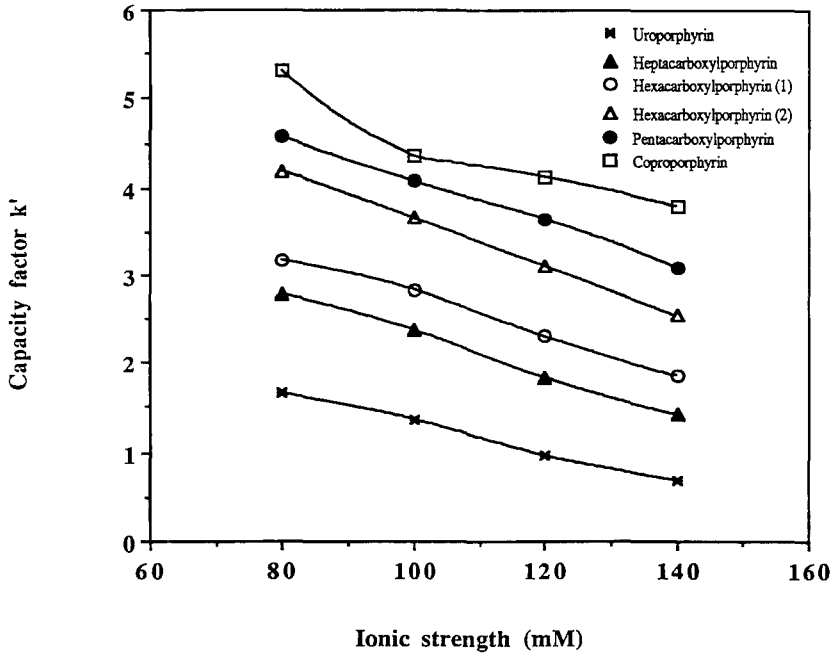


Figure 2. Effect of ionic strength on the retention of polycarboxylated porphyrins. Mobile phase: 30/70 ACN/buffer (phosphate; pH 6.7).

It is interesting to note the presence of an additional peak upon injection of standard hexacarboxylporphyrin I (Figure 5). Further testing by the manufacturer precluded the presence of an impurity. Various measures were taken to ensure that the additional peak was not an artifact of the experiment protocol. The retention times and the peak areas of these two peaks were found to be very reproducible even from various lots of hexacarboxylporphyrin from the same manufacturer.

Hexacarboxylporphyrin I is manufactured by decarboxylation of uroporphyrin I. There are two potential isomers, cis-and trans-hexacarboxylporphyrin (Figure 6) resulting from the decarboxylation process. One is formed by decarboxylation of two acetic acid

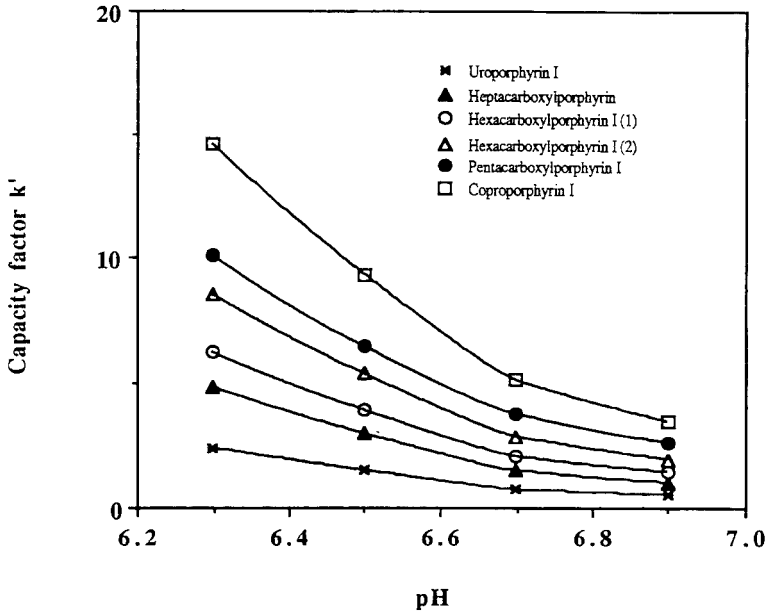


Figure 3. Effect of mobile phase pH on the retention of polycarboxylated porphyrins. Mobile phase: 25/75 ACN/buffer (140mM phosphate).

groups on two adjacent pyrroles, and the other by the decarboxylation of two acetic acid groups on two opposite pyrroles (17). Chu & Chu also observed two isomers as their ester derivatives using paper chromatography(18). For each of the remaining four porphyrins studied, only a single isomer results from decarboxylation. From statistical estimation, the ratio of cis to trans isomers produced in the decarboxylation of uroporphyrin is approximately 2:1. Assuming that the extinction coefficients of the two isomers are similar, the larger area of the second peak suggests that this peak may correspond to the cis isomer. Further confirmation of this interpretation is still under investigation. To the best of our knowledge, this may well be the first report of a chromatographic separation of the two native hexacarboxylporphyrin I isomers.

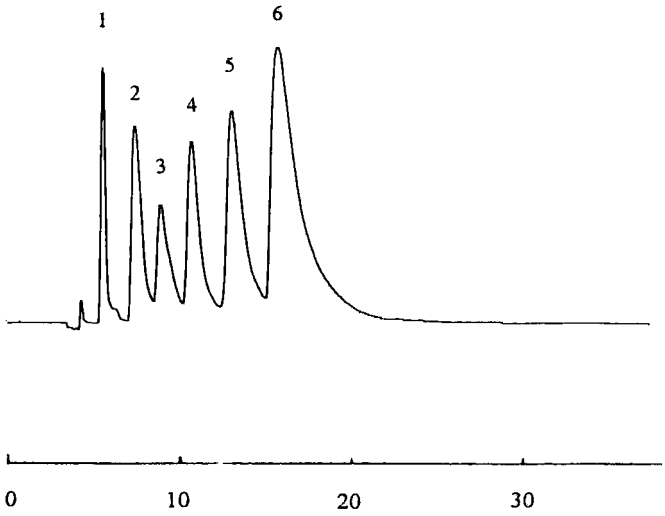


Figure 4. Chromatogram of optimized isocratic separation of polycarboxylated porphyrins on a 25 cm x 4.6 mm I.D. column. Mobile phase: 25/75 ACN/buffer (140 mM phosphate; pH 6.9). Flow rate 0.8 mL/min. 1. Uroporphyrin; 2. Heptacarboxylporphyrin; 3. Hexacarboxylporphyrin (1?); 4. Hexacarboxylporphyrin (2?); 5. Pentacarboxylporphyrin; 6. Coproporphyrin.

Biological samples

For clinical purposes, the determination of porphyrin is carried out in biological samples, such as urine and blood. There have been several reports of the direct injection of urine samples (6-9). However, direct injection of serum sample for the analysis of porphyrins without prior extraction procedures has not been reported. Recently, Stalcup & Williams (19) successfully applied direct enantiomeric resolution of chiral drugs in human serum using a commercially available β-CD column. They attributed the feasibility

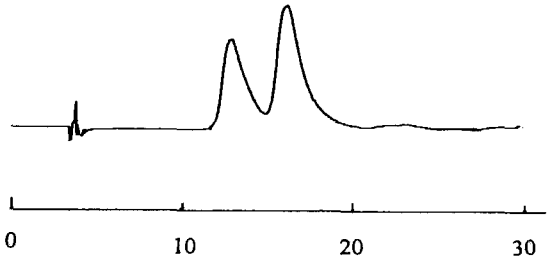


Figure 5 Chromatogram of hexacarboxylporphyrin I standard. Mobile phase: 30/70 ACN/buffer (100 mM phosphate; pH 6.7). Flow rate 0.8 mL/min.

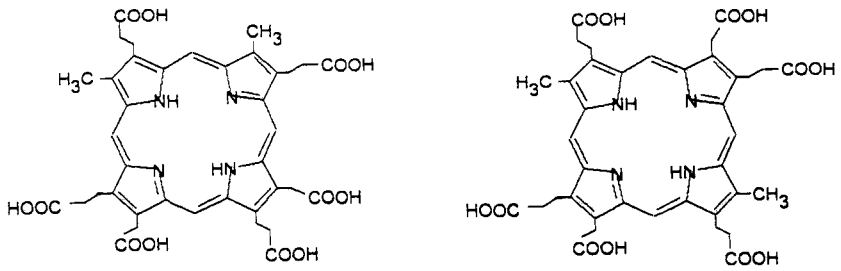


Figure 6. Structures of hexacarboxylporphyrin I isomers. a) cis-isomer b) trans-isomer.

of direct injection of serum onto the CD column to the high aqueous content of mobile phase and the diol character of the stationary phase. The high aqueous mobile phase composition for the separation of the porphyrins suggested that it might also be possible for direct injection of human serum on the γ-CD column which is synthesized analogously to the β-CD column.

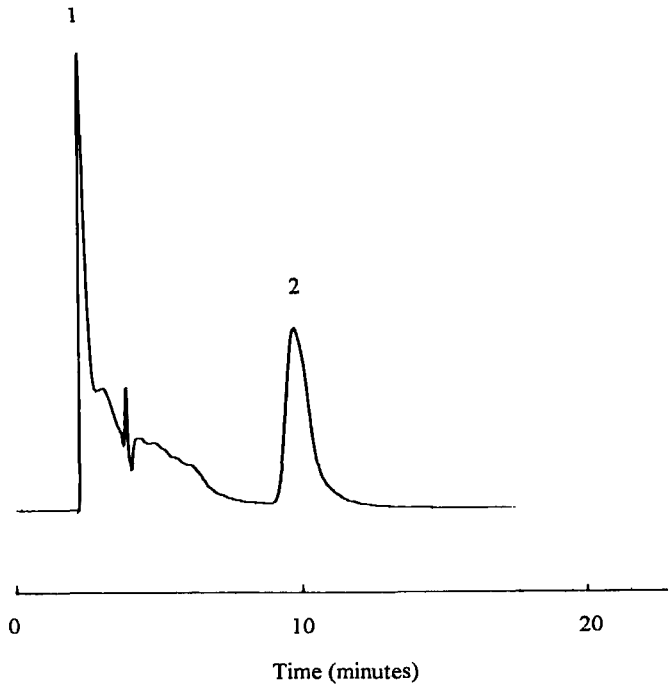


Figure 7a. Chromatogram of direct injection of serum. Mobile phase: 25/75 ACN/buffer (140 mM phosphate; pH 6.7). 1. Protein; 2. Unknown.

Human serum samples were obtained in the same way as previously reported (19). Uroporphyrin and coproporphyrin were spiked into the serum. The chromatographic results (Figure 7 a, b) showed that the detection of uroporphyrin and coproporphyrin were not affected by serum interferences. Thus, direct injection of serum onto the γ-CD column has the potential to facilitate quantitation of porphyrin levels in human blood.

CONCLUSIONS

Separations of porphyrin carboxylic acids on the γ-CD column takes advantage of the large cavity of the γ-CD. The isocratic separation of five porphyrin carboxylic acids

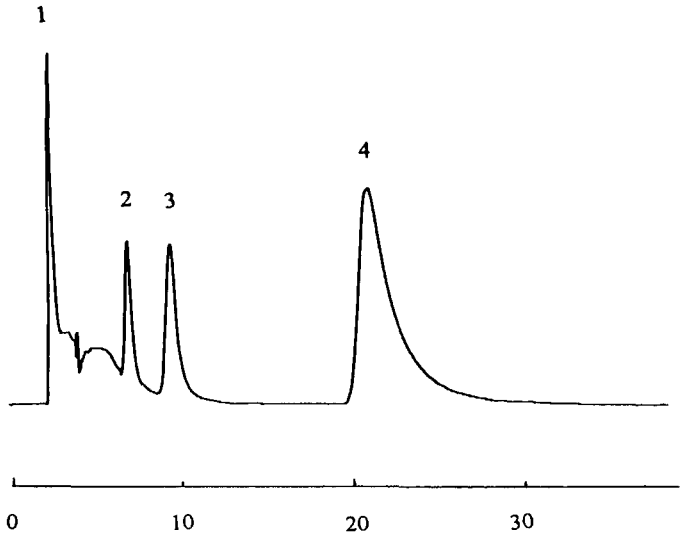


Figure 7b. Chromatogram of direct injection of serum sample spiked with uro- and copro-porphyrin (400 nm). Mobile phase: 25/75 ACN/buffer (140 mM phosphate; pH 6.7). 1. Protein; 2. Uroporphyrin; 3. Unknown; 4. Coproporphyrin.

has been achieved. The experimental conditions used may facilitate the clinical investigation of porphyrin related diseases.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Randy W. Larsen (University of Hawaii) and Jeffrey Yearyean (Porphyrin Products, Inc.) for their helpful discussions and suggestions. The authors would also like to acknowledge the support of National Institutes of Health.

REFERENCES

1. J. W. Ho, J. Liq. Chromatogr., **13/11**, 1990, 2193-2205.
2. H. D. Meyer, W. Vogt and K. Jacob, J. Chromatogr., **290**, 1984, 207-213.
3. J. W. Ho, Anal. Biochem., **183**, 1989, 134-138.
4. S. W. Kennedy and A. L. Maslen, J. Chromatogr., **493**, 1989, 53-62.
5. P. Kotal, B. Porsch, M. Jirsa and V. Kordac, J. Chromatogr., **333**, 1985, 141-151.
6. E. Englert Jr., A. W. Wayne, E. E. Wales Jr., and R. C. Straight, J. HRC & CC, **2**, 1979, 570-574.
7. R. E. Ford, C. Ou, and R. D. Ellefson, Clin. Chem., 27/3, **1981**, 397-401.
8. P. M. Johnson, S. L. Perkins, and S. W. Kennedy, Clin. Chem., **34/1**, 103-105.
9. H. D. Meyer, K. Jacob, W. Vogt, and M. Knedel, J. Chromatogr., **199**, 1980, 339-343.
10. S. M. Han, and D. M. Armstrong, in **Chiral Separation by HPLC**, A. M. Krstulovic, Ed., John Wiley & Sons: New York, 1989, pp 208-287.
11. D. W. Armstrong, A. M. Stalcup, M. L. Hilton, J. D. Duncan, J. R. Faulkner, Jr., and S. -C. Chang, Anal. Chem., **62**, 1990, 1610.
12. D. W. Armstrong, and W. Li, Chromatography, **2**, 1987, 43-48.
13. D. W. Armstrong, S. Chen, C. Chang, and S. Chang, J. Liq. Chromatogr., **15/3**, 1992, 545-556.
14. C. A. Chang, Q. Wu, and D. W. Armstrong, J. Chromatogr. **354**, 1986, 454.
15. J. W. Ho, J. Chromatogr., **508**, 1990, 275-381.
16. K. M. Smith, in **Porphyrins and Metalloporphyrins**, K. M. Smith, Ed, Elsevier/North-Holland Biological Press, Amsterdam, 1975, pp 3-27.
17. T. K. With, Biochem. J., **147**, 1975, 249-251.
18. T. C. Chu, and J-H Chu, J. Biol. Chem., **234/10**, 1959, 2747-2750.
19. A. M. Stalcup and K. L. Williams, J. Liq. Chromatogr., **15/1**, 1992, 29-37.

Received: July 10, 1993
Accepted: September 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1125-1139 (1994)

SEPARATION OF THE DIASTEREOISOMERS OF ETHYL ESTERS OF CAFFEIC, FERULIC, AND ISOFERULIC ACIDS BY THIN-LAYER AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

M. FERNANDA M. BORGES AND MADALENA M. M. PINTO
Laboratório de Química Orgânica
Faculdade de Farmácia do Porto
Rua Aníbal Cunha, 4000 - Porto, Portugal

ABSTRACT

The separation of the diastereoisomers of cinnamoyl derivatives have been studied by thin-layer (TLC) and high performance liquid chromatography (HPLC). TLC separations on two different layers and with two different solvent systems are described. HPLC separation was carried out on a reversed-phase column (Lichrosorb RP-8, 5 μm) using an isocratic elution [acetonitrile : water-acetic acid (95 : 5)]. The series of compounds includes the *cis/trans* isomers of ethyl esters of caffeic, ferulic and isoferulic acids.

INTRODUCTION

During our work on the biomimetic synthesis of simple coumarins the *cis/trans* isomers of ethyl caffeate, ethyl ferulate and

ethyl isoferulate (**Fig. 1**) were identified as side products of the reactions [1].

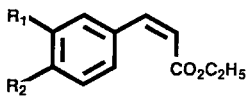
Although a large variety of cinnamic acids derivatives could be found in the literature, only a few papers concerned especially with the separation of their diastereoisomers by HPLC and/or TLC have been published. The present paper reports the chromatographic analysis of some Z/E ethylcinnamates which was carried out by TLC and HPLC. The HPLC analysis developed allows a quick identification and estimation of the relative percentage of the components in solution. Consequently it is of great value for monitoring the development of reactions, performed in our lab, for the light-induced biomimetic synthesis of coumarins.

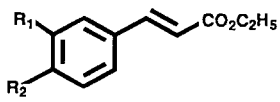
MATERIAL AND METHODS

Thin-Layer Chromatography

Commercially available pre-coated TLC plates were used : silica gel 60 F254 (S_1) and cellulose (S_2), without fluorescent indicator, from Merck, Darmstadt. The layer thickness was 0.2 and 0.1 mm, respectively.

The following solvent systems were used: F_1 - petroleum benzine 40-60 ^{0}C/diethyl ether/ formic acid (5 : 5 : 0.1); F_2 - water/acetic acid (9 : 1). Before development the chambers were allowed to saturate 1h and 3 h, respectively. The time of elution was 1h and 30 min for system S1/F1 and 3 h and 30 min for system S2/F2.





	R_1	R_2		R_1	R_2
1	OH	OH	2	OH	OH
3	OCH_3	OH	4	OCH_3	OH
5	OH	OCH_3	6	OH	OCH_3

FIGURE 1

Structures of the diastereoisomers of the ethylcinnamates
1. Ethyl *cis*-caffeate **2.** Ethyl *trans*-caffeate
3. Ethyl *cis*-ferulate **4.** Ethyl *trans*-ferulate
5. Ethyl *cis*-isoferulate **6.** Ethyl *trans*-isoferulate

Before detection the plates were air-dried (30 min for system S1/F1 and 2 h for system S2/F2). The compounds were visualized under UV light (254 and 366 nm) before and after spraying with 10 ml of an ethanolic solution of KOH (5%), without and with heating (in an oven at 100 °C, during 10 min).

Samples (2.5 µl) were spotted onto the TLC plates by means of a graduated microsyringe. TLC separations were performed at room temperature. Details of other chromatographic conditions can be found in reference [2]. All the analysis were performed in triplicate.

The TLC data are shown in Results and Discussion.

High Performance Liquid Chromatography

A Jasco model Liquid Chromatographic System equipped with a loop injector, a Jasco 875 variable wavelength UV photometric detector

and a Varian 4270 integrator was used. The reversed-phase analytical column was RP-8 with 5 μm particle size (250 x 4.0 mm I.D) from E. Merck, Darmstadt, Germany. The UV detector was set at 290 nm. Samples of each standard (5 μl) were injected in the column, equilibrated with the mobile phase at a flow rate of 1.0 ml /min..

The system was operated at room temperature. A chart speed of 5 mm /min. was used. The mobile phase was acetonitrile/aqueous acetic acid (95:5): system L1 - 30/70 %,v/v; system L2 - 25/75 %,v/v. All the analysis were performed in triplicate.

The HPLC data are shown in Results and Discussion.

Other conditions

The ^1H NMR spectra were recorded on a Bruker pulse (300 MHz) instrument. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The infrared spectra (IR) were measured with a Perkin Elmer-257 spectrophotometer. Mass spectra (MS) were recorded on a Hitachi Perkin Elmer RMU 6M spectrometer, using a direct inlet probe and an electron energy of 70 eV.

Reagents

All the chemicals used were of analytical-reagent grade. *Trans*-caffeic acid and *trans*-ferulic acid were obtained from Fluka AG, Chemische Fabrik CH-9470 Buchs, Switzerland. *Trans*-isoferulic acid was obtained from Aldrich Chemical Company, Inc., Wisconsin USA . Acetonitrile was Lichrosolv for chromatography (E. Merck). Water

used in the chromatographic mobile phase was distilled, desionized and filtered through a 0.2 μm membrane filter. The aqueous solutions were fleshly prepared and degassed by vacuum and sonication before use.

Synthesis

The ethyl esters used in this study were synthesized using a modification of the Pearl and Beyer method [3].

General method for the preparation of compounds **2, 4** and **6** :

The corresponding *trans*-cinnamic acid (2.5 mM) was dissolved in EtOH (50 ml) and H_2SO_4 (0.5 ml) was added. The mixture was refluxed for 3 h. After cooling, the solvent was partially evaporated under reduced pressure. The cooled solution was poured into cold water and neutralized with a sodium bicarbonate solution.

The TLC control of the reaction was carried out with the system S1/F1 and S2/F2 (see Experimental).

Ethyl *trans* 3-(3,4-dihydroxyphenyl)-2-propenoic acid (2). The yellow precipitate obtained was filtered, washed and dried. The residue was recrystallized from aqueous MeOH to give 400 mg of the ester (77 %), as light yellow needles. M.p. 144-147 °C . IRυ_{max} (cm^{-1}) (KBr): 3450, 1660, 1610, 1600, 1520, 1515. ^{1}H NMR (CD_3COCD_3): 7.52 (1H, *d*, J= 15.9, Hb), 7.15 (1H, *d*, J= 2.1, H-2Ar), 7.02 (1H, *dd*, J= 2.1, 8.2, H-6Ar), 6.85 (1H, *d*, J= 8.2, H-5Ar), 6.26 (1H, *d*, J= 15.9, Ha), 4.17 (2H, *q*, J=7.1, OCH_2), 1.25 (3H, *t*, J=7.1, CH_3). MS: m/z 208 (M^+).

Ethyl *trans* 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid (4) and **ethyl *trans* 3-(3-hydroxy-4-methoxyphenyl)-2-propenoic acid(6)**. The white precipitates obtained immediately turn to colorless oils. The aqueous emulsions were then extracted with diethyl ether (3x50 ml).

The extracts were washed with H_2O (3x50 ml), dried over Na_2SO_4 and concentrated under reduced pressure. The oil residues were crystallized from diethyl ether/n-hexane to give 440 mg of the ester **4** (79 %) and 490 mg of the ester **6** (88 %), as white needles.

Compound **4** m.p. 49-52 °C. IRυ_{max} (cm^{-1}) (KBr): 3180, 1670, 1630, 1580, 1510. ^{1}H NMR ($CDCl_3$): δ: 7.58 (1H, *d*, J= 15.9, Hb), 7.04 (1H, *dd*, J= 1.9, 8.2, H-6Ar), 6.99 (1H, *d*, J= 1.9, H-2Ar), 6.88 (1H, *d*, J= 8.2, H-5Ar), 6.26 (1H, *d*, J= 15.9, Ha), 5.99 (1H, *s*, OH), 4.23 (2H, *q*, J= 7.1, OCH_2), 3.88 (3H, *s*, OCH_3), 1.30 (3H, *t*, J=7.1, CH_3). MS: m/z 222 (M^+).

Compound **6** m.p. 53-55 °C . IRυ_{max} (cm^{-1}) (KBr): 3360, 1690, 1640, 1610, 1580, 1510. ^{1}H NMR ($CDCl_3$): 7.56 (1H, *d*, J= 15.9, Hb), 7.11 (1H, *d*, J= 2.1, H-2Ar), 6.99 (1H, *dd*, J= 2.2, 8.3, H-6Ar), 6.81 (1H, *d*, J= 8.3, H-5Ar), 6.26 (1H, *d*, J= 16.0, Ha), 5.71 (1H, *s*, OH), 4.22 (2H, *q*, J=7.1, OCH_2), 3.89 (3H, *s*, OCH_3), 1.30 (3H, *t*, J=7.1, CH_3). MS: m/z 222 (M^+).

Sample Solutions

The sample solutions of the ethyl *trans*-cinnamates were prepared by dissolution of the synthesized compounds in ethanol (1 mg/ml and 0,1 mg /ml for TLC and HPLC, respectively).

The ethyl *cis*-cinnamates were obtained by exposure of an aliquot of the sample solutions of ethyl *trans*-cinnamates to diffused daylight for 2 hours. As expected, a mixture of *cis* and *trans* isomers was

formed [4]. The solutions were stored in sealed containers, at 4 °C in darkness.

RESULTS AND DISCUSSION

During the study on the biomimetic synthesis of simple coumarins by a photochemical process, besides the Z/E isomers of the cinnamic acids, another pair of fluorescent compounds has been found to appear on the TLC control (system L2/F2) of some solutions. Separation by column chromatography and preparative TLC together with spectroscopic determination have led to the identification of the two compounds as the Z/E isomers of the ethyl esters of the cinnamic acids, used as building blocks for coumarins synthesis. However, these traditional methods of purification are quite tedious and consequently ineffective for our purpose. Eventhough the chromatographic processes were carried out in subdued light, a mixture of the referred Z/E isomers were always found. The long time of analysis for an estimation of the Z/E isomers formed in the reaction made them cumbersome for a systematic study. For this reason an improvement in the chromatographic method, for a quick identification and/or estimation of the diastereoisomers of the cinnamoyl derivatives, was developed.

Thin-Layer Chromatography

Table 1 shows the Rf values of the diastereoisomers of the ethyl cinnamates obtained on silica gel (S1) and cellulose (S2), developed on

TABLE 1. TLC and HPLC Data of Ethyl Cinnamates

		TLC [a]		HPLC [b]	
Nº	Compound	S_1/F_1	S_2/F_2	L_1	L_2
1	Ethyl *cis*-caffeate	39	67	8.29	11.40
2	Ethyl *trans*-caffeate	34	38	9.21	13.32
3	Ethyl *cis*-ferulate	62	67	15.89	24.23
4	Ethyl *trans*-ferulate	47	43	17.88	28.68
5	Ethyl *cis*-isoferulate	46	68	14.36	22.32
6	Ethyl *trans*-isoferulate	44	36	16.95	28.36

a) For TLC systems see Experimental. The Rf values are in Rf .100.

b) For HPLC systems see Experimental. The Rt are in min.

the system F1 and F2, respectively. The system S2/F2 gave a better separation of isomers than the system S1/F1, although it was quite time consuming (see Experimental).

Concerning the pair of diastereoisomers **3-4** it was found that their chromatographic behaviour, i.e. in Rf and fluorescence color, seems to be similar in both chromatographic systems (**Tables 1, 2** and **3**). The pairs of diastereoisomers **1-2** and **5-6** seem to have similar chromatographic separation (**Table 1**).

Table 2 and **Table 3** show the fluorescence colours of the compounds (UV light 366 nm), before and after KOH treatment. It was observed that the original fluorescence produced by the compounds changed after spraying with the chromogenic reagent. The effect of the spraying reagent on the fluorescence colours of the cinnamates, both before or after heating, is undoubtedly helpful for a rapid detection and characterization of the ethylcinnamates.

High Performance Liquid Chromatography

In order to observe the chromatographic behaviour of the Z/E isomers of ethyl esters a study was carried out with sample solutions (see Experimental).

Using the binary solvent system, acetonitrile/aqueous acetic acid and the reversed-phase octylsilane packing used in the analysis of the corresponding cinnamic acids [5-7] it was possible to obtain the separation and estimation of the diastereoisomers of the cinnamates derivatives.

The results of this chromatographic study were shown in **Table 1**. These findings allow us to conclude that with the mobile phase L1 the

TABLE 2. Colour of Fluorescence of the Ethyl Cinnamates Spots on TLC, System $S_1/F1$[a], With/Without the Chromogenic Reagent.

Nº	Compound	Spot appearance [b]		
		UV	KOH/UV	
			before heating	after heating[c]
1	Ethyl *cis*-caffeate	lt-b	bn	bn-y
2	Ethyl *trans*-caffeate	lt-b	bn-y	bn-y
3	Ethyl *cis*-ferulate	b	lt-gn	lt-b
4	Ethyl *trans*-ferulate	b	lt-gn	lt-b
5	Ethyl *cis*-isoferulate	lt-b	bn	lt-bn
6	Ethyl *trans*-isoferulate	lt-b	lt-bn	lt-bn

a) For TLC system see Experimental.

b) Colour of the fluorescence at 366 nm : b, blue; lt-b, light blue; bn-y, brownish yellow; lt-gn, light green; lt-bn, light brown; bn, brown

c) The plates were sprayed with the chromogenic reagent and observed immediately, after which the colours tend to darken.

TABLE 3. Colour of Fluorescence of the Ethyl Cinnamates Spots on TLC, System S2/F2[a], With/Without the Chromogenic Reagent.

Nº	Compound	Spot appearance [b]		
		UV	KOH/UV	
			before heating	after heating[c]
1	Ethyl *cis*-caffeate	b	y-gn	y
2	Ethyl *trans*-caffeate	b	y-gn	y
3	Ethyl *cis*-ferulate	lt-b	lt-gn	b
4	Ethyl *trans*-ferulate	lt-b	lt-gn	b
5	Ethyl *cis*-isoferulate	p	y	y
6	Ethyl *trans*-isoferulate	p	y	y

a) For TLC system see Experimental.

b) Colour of the fluorescence at 366 nm : b, blue; lt-b, light blue; p, purple, y-gn, yellowish green; lt-gn, light green; y, yellow.

c) The plates were sprayed with the chromogenic reagent and observed immediately, after which the colours tend to darken.

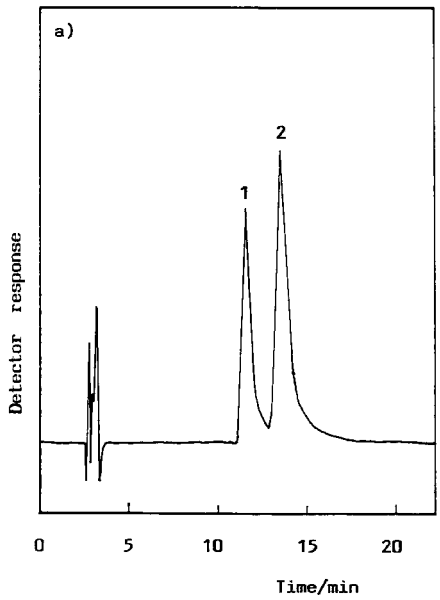


FIGURE 2

Chromatogram of the sample solutions containing:

a) **1.** Ethyl *cis*-caffeate ; **2.** Ethyl *trans*-caffeate (Mobile phase : L2)

b) **3.** Ethyl *cis*-ferulate ; **4.** Ethyl *trans*-ferulate (Mobile phase : L1)

c) **5.** Ethyl *cis*-isoferulate ; **6.** Ethyl *trans*-isoferulate(Mobile phase : L1)

Other conditions described in Experimental

separation between the pairs of the diastereoisomers **3-4** and **5-6** was achieved. To improve resolution the polarity of the mobile phase was increased (system L2), leading to an excellent separation between the geometrical isomers **1-2**. In this system the separation between the Z/E isomers of the methoxy cinnamate derivatives was also improved, although it involved a longer time of analysis.

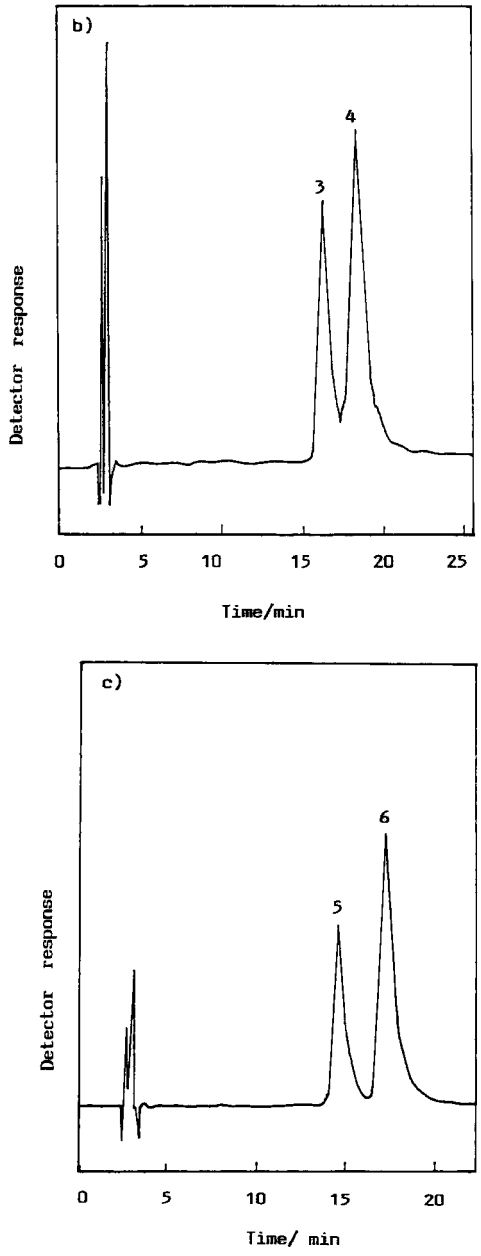


FIGURE 2 (continued)

Although the differences in the observed selectivities are not striking (1.11; 1.12; 1.18 with L1 and 1.16; 1.18; 1.27 with L2) the variation found on the retention times is relevant when a simultaneous isocratic separation and identification of a mixture of natural or synthetic compounds was carried out.

The expected elution pattern [5-7] in this reversed-phase system is observed : the *cis* isomers are first eluted , while the *trans* isomers have longer retention times. **Fig. 2** shows the isocratic elution profile of the Z/E isomers of the cinnamic acid derivatives. The separation took less than 20 min.

The HPLC method presented appears to be a good alternative to TLC. It provides a mean for a fast, simultaneous, sèmi-quantitative screening of the composition of the solutions performed in our work.

Although designed specifically for the simultaneous determination of the diastereoisomers of cinnamic acid ethyl esters of synthetic mixtures, the chromatographic systems developed herein could be a valuable contribution for food chemistry [8].

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support from Instituto Nacional de Investigação Científica (INIC).

REFERENCES

1. Borges, M. F. M. and Pinto, M. M. M., Helv. Chim. Acta, 75: 1061 (1992).

2. Borges, M. F. M., in "Síntese de Cumarinas Simples *ortho* Diidroxiladas ", Provas de Aptidão Pedagógica e Capacidade Científica, Faculdade de Farmácia da Universidade do Porto, 1987.

3. Pearl, I. A. and Beyer, D. L., J. Org. Chem. , 16: 12, 217 (1951).

4. Hartley, R. D. and Jones, E. C., J. Chromatogr., 107: 213 (1975).

5. Borges, M. F. M. and Pinto, M. M. M., J. Liq. Chromatogr., 12: 12, 2345 (1989).

6. Borges, M. F. M., Roleira, F. M. F., Pinto, M. M. M., J. Liq. Chromatogr., 14: 12, 2307 (1991).

7. Borges, M. F. M., Roleira, F. M. F., Pinto, M. M. M, J. Liq. Chromatogr., 16: 1, 149 (1993).

8. Versini, G. and Tomasi, T., *L'Enotecnico*, 19, 9, 595 (1983).

Received: November 18, 1993
Accepted: November 24, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1141-1146 (1994)

CORTICOSTEROID ANALYSIS BY HPLC WITH INCREASED SENSITIVITY BY USE OF PRECOLUMN CONCENTRATION

C. VALENTA AND H. JANOUT
Institute of Pharmaceutical Technology
University of Vienna
Währinger Straße 25
1090 Vienna, Austria

ABSTRACT

In this paper ttwo HPLC methods are described which offer the possibility of increasing the sensitivity for a couple of corticosteroids The increase is achieved by precolumn concentration with C-18 cartridges from 5 to 20 fould. The concentration step is in principle applicable to all other steroids.

INTRODUCTION

Many HPLC methods for determination of corticosteroids have been reported (1-7). For drug release testing of topical forms extremely sensitive methods are required. In this paper the possibility of increasing the sensitivity by precolumn concentration is described.

MATERIALS

Hydrocortisone, Hydrocortisone-21-acetat, Prednisolon, Dexamethason, Betamethasone-17-valerate and Flumethason-21-acetat were from Sigma (St.Louis, USA). C-18 Cartridges, Bond elut, Vac-elut by Analytichem International I.C.T.; all solvents are of HPLC grade.

EQUIPMENT

HPLC: Perkin Elmer Series 10; detector: Perkin Elmer LC 235, automatic sample injector: ISS-100 Perkin Elmer, Software: Omega-2 Vers.2.50, Column: Nucleosil C-18 (150x4 mm ID), 5 µm particle size.

METHODS

Assay procedure:
A Bond elut C-18 cartridge, which has been prepared for use by rinsing with 4 ml of water followed by 3 ml of methanol, is inserted onto a vac-elut system. An aliquot of the aqueous corticosteroid solution is added and sucked through the cartridge. The corticosteroid is absorbed by the apolar sorbent and is eluted from the cartridge with less methanol than the aqueous corticosteroid solution. The concentration was increased from five to twenty fould.
The precolumn concentration offers the possibility of increasing the sensitivity more than 20 fould.
Method 1:
The mobile phase consisted of a CH_3OH/H_2O (7+3). The flow rate was set 1,0 ml/min. After precolumn concentration as described before the injection volume for the sample preparations was maintained at 20 µl. This method was used for Hydrocortison (240 nm), Hydrocortison-21-acetat (240 nm) and Prednisolon (242 nm). The corticosteroids were quantitated by comparing the peak area of the

TABLE 1
Recovery of Hydrocortison (HC), Hydrocortisonacetat (HCac) and Prednisolon (Pred) after precolumn concentration (method 1)

HC µg/ml spiked	5 fould conc. µg/ml	Recovery %	µg/ml
1.46	7.3	110	8.0
1.44	7.2	102	7.3
1.34	6.7	93.5	6.32

HCac µg/ml spiked	10 fould conc. µg/ml	Recovery %	µg/ml
0.55	5.5	105,4	5.8
0.65	6.5	103.1	6.7
0.85	8.5	94.1	8.2

Pred µg/ml spiked	10 fould conc. µg/ml	Recovery %	µg/ml
0.835	8.35	104.1	8.7
0.64	6.4	101.2	6.4
0.504	5.04	98.5	5.1

unknown with standard curves (for each corticosteroid 5 points) prepared by adding known amounts of each corticosteroid. Standard solutions containing 4-14 mg/ml. Linear regression analysis of the peak areas gave correlation coefficients (r) between 0,98 and 0,99. The detection limit was 3,5µg/ml.

Method 2:
The mobile phase consisted of acetonitril/H_2O (7+3). The flow rate and injection volume were the same as in method 1.

TABLE 2
Recovery of Dexamethason (Dex) , Flumethason-21-acetat (Flu) and Betamethason-17-valerat (Bet) after precolumn concentration (method 2)

Dex	10 fould conc.	Recovery	
µg/ml spiked	µg/ml	%	µg/ml
0.6	6.0	105.5	6.33
0.33	3.3	117.3	3.87
0.3	3.0	88.4	2.65

Flu	20 fould conc.	Recovery	
µg/ml spiked	µg/ml	%	µg/ml
0.14	2.8	91.4	2.56
0.115	2.3	100.0	2.3
0.067	1.35	108.1	1.46

Bet	20 fould conc	Recovery	
µg/ml spiked	µg/ml	%	µg/ml
0.285	5.7	105	5.98
0.180	3.6	98.1	3.52
0.045	0.9	88.9	0.8

This method was used for Dexamethason (239 nm), Betamethason-17-valerate (239 nm) and Flumethason-21-pivalat (237 nm). The corticosteroids were quantitated by comparing the peak area of the unknown with standard curves (for each corticosteroid 5 points) prepared by adding known amounts of each corticosteroid. Standard solutions containing 3-18 mg/ml. Linear regression analysis of the peak areas gave correlation coefficients (r) between 0,98 and 0,99. The detection limit was 0,12 µg/ml.

RESULTS

Recovery:
Recovery data for the solid phase extraction sample preparation were generated by spiking acceptorphase (phosphate buffer 10 mM, pH 7,4, 150 mM sodium chloride) with known amounts of the corticosteroids. The recovery rates are listed in table.1 and 2.

DISCUSSION

For release experiments of corticosteroids from topical forms (ointments, gels, lotions) extremely sensitive analytical methods are required. The corticosteroid in the aqueous acceptor phase may be less than 0,5 µg/ml for Hydrocortison, Hydrocortison-21-acetat and Prednisolon and about 0,02 µg/ml for Betamethasone-17-valerate and Flumethason- 21-acetat. Solid phase C-18 cartridges were used to concentrate the corticosteroids till 20 fould. The concentration step is in principle applicable to all other steroids.

REFERENCES

1. J.A.Mollica, R.F.Strusz, J.Pharm.Sci., 61: 444-447 (1972)

2. M.C.Olson, J.Pharm.Sci., 62: 2001-2006 (1973)

3.A.R.Lea, J.M.Kennedy, G.K.-C. Low, J.Chromatogr., 198: 41-47 (1980)

4. F.Huber, M.Wiedemann, G.Heinrich, Z.Salama, H.Jaeger, Drug Res., 40: 926-931 (1990)

5. J.Girault, B.Istin, J.M. Malgouyot, A.M.Brisson, J.B.Fourtillan, J.Chromatogr. 564: 43-53 (1991)

6.G.R.Cannell, R.H. Mortimer, D.J. Maguire, R.S.Addison, 563: 341-347 (1991)

7. J.Noma, N.Hayashi, K.Sekiba, J.Chromatogr. 568: 35-44 (1991)

Received: August 21, 1993
Accepted: August 31, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1147-1169 (1994)

OPTIMIZATION OF HPLC CONDITIONS TO ANALYZE WIDELY DISTRIBUTED ETHOXYLATED ALKYLPHENOL SURFACTANTS

N. MÁRQUEZ[1]*, R. E. ANTÓN[1], A. USUBILLAGA[2], AND J. L. SALAGER[1]
[1]*Laboratorio FIRP, Ingeniería Química*
[2]*Instituto de Investigaciones, Facultad de Farmacia*
Universidad de Los Andes, Mérida, Venezuela

ABSTRACT

Commercial ethoxylated alkylphenol surfactants are always a mixture of oligomers with different ethylene oxide number (EON). The different oligomers can be separated by various HPLC techniques. Isocratic mode with mixed solvent on silica column allows to separate oligomers up to EON = 10; gradient programming moves the limit up to EON = 15. For higher EON values (up to 25) a NH_2 column has to be used, either with isocratic or gradient mode. Applications to the analysis of microemulsion systems and to the separation of tributyl phenol ethoxylates are discussed. Extreme separation of wide range EON distribution is attained with two columns (Si and NH_2) in series, and a solvent programming.

*also Facultad Experimental de Ciencias, Universidad del Zulia, Maracaibo, Venezuela

INTRODUCTION

The polyethoxylated alkylphenols have held a prominent position in the field of nonionic surfactants in the past forty years. They are used in household products such as liquid dish washing formulations and hard surface cleaners, cleansers for textiles, leather, and plastics, particularly in systems with high electrolyte content.
They are used extensively in the oil industry, e. g., as emulsifiers in drilling fluids and heavy crude emulsions (1), and have been proposed as tension-lowering surfactants for enhanced oil recovery (2-3).

Nonylphenol is by far the most widely used base material, followed by the octyl, dodecyl, and n,n-dinonyl counterparts; recently tri-butyl phenol ethoxylates have been also available.

Since the addition of ethylene oxide to an alkylphenol is a polycondensation process, the final product is not a pure substance, but rather a mixture of homologues with different ethylene oxide number (EON), so-called oligomers (4).

As a consequence of its ethylene oxide number (EON) distribution, which often follows a Poisson law (4), a commercial surfactant may contain substances with widely different properties; for instance a commercial nonylphenol with an average of 5 EO groups per nonylphenol molecule contains about 50% of substances which are not water soluble. In presence of both an oil and a water phase this can result in an independent solution behavior of each sustance. When all the mixture surfactant species do not behave collectively, the low EON ones tend to migrate into the oil phase, i.e., a severe fractionation can

occur. As a consequence the effective surfactant mixture at interface departs considerably from the surfactant mixture which was introduced in the first place in the system (5). This situation is often worsened by a current practice in surfactant handling, i. e., mixing different surfactants in order to attain some average value or some synergistic effect. Such mixing may result in EON distributions with very wide range and two or more modes.

The surfactant users need to know the oligomer composition of alkylphenol nonionics in different phases, generally oil and water, and sometimes a third one, so-called a microemulsion; this knowledge can be processed to calculate the real interfacial surfactant mixture (5-6), an important information since it has been recently found that the physico-chemical conditions at interface are linked with the properties of the associated emulsions (7-10) and foams (11).

ANALYSIS OF ETHOXYLATED ALKYLPHENOLS

Various chromatographic procedures have been tried to separate the commercial ethoxylated alkylphenols into their different oligomers.

Thin layer and paper chromatography (12-15) are limited to qualitative analysis. Gas chromatography (16-21) has been used only for the low average EON mixtures, since it fails to separate higher oligomers (EON>8), because of their low volatility and the thermal degradation that can take place. For these reasons, high performance liquid chromatography (HPLC) appears to be the choice method for the separation and characterization of ethoxylated alkylphenols (22-25).

Ethoxylated monoalkylphenol surfactants have been separated by both reverse and normal phase HPLC. In reverse phase chromatography the separation depends upon the alkyl group in the surfactant molecule and the retention mechanism is governed by hydrophobic interactions between the alkyl tail of the surfactant and the bonded stationary phase; octadecyl or octyl-silane columns have been used for such separations (26-30). However, the alkylate base material is often monoisomeric, and the main analysis problem is to separate the oligomers according to their ethylene oxide number; for such a separation, normal phase chromatography is the logical choice; silica gel (26, 31-32) and silica with chemically bonded nitrile (31, 34), diol (32), and amino phases (23, 24, 30, 35-39) have been tested as column packing in normal phase HPLC separations.

In a recent report, several column packings for normal phase separation were tested with aliphatic alcohols and hydrocarbon solvents (40), in order to compare the chromatographic behavior of underivatized ethoxylated nonylphenols on different stationary phases. A normal phase HPLC separation of ethoxylated nonylphenol oligomers has been described by Zhou et al. (41), who used a silica-diol column and a nonpolar solvent gradient elution. HPLC analysis of oligomer distribution of low EON ethoxylated alkylphenols was selected for interlaboratory testing, using diol bonded phase column under isocratic and gradient conditions (42, 43). In a recent paper (44), we reported that the selection of a proper three-component solvent mixture allows to separate the ethoxylated nonylphenol oligomers up to EON = 12 on a silica column.

In most of the cited reports, detection is carried out by measuring the UV absorbance at a wavelength corresponding to the substituted benzene ring, i. e., at 270-280 nm. It has been shown that the UV absorbance leads to the molar concentration in alkylphenol base and is independent on the EON (24, 37, 45).

This review indicates that HPLC is the choice method to analyze commercial polyethoxylated alkylphenol surfactants, and that there exists a variety of stationary phases and solvent mixtures to do so. However, the previous publications deal with the analysis of a sample of a (single) commercial surfactant, i. e., a mixture with a narrow range single mode EON distribution.

In this paper, we will address the problem of analyzing wide EON distribution mixtures found either in microemulsion systems, or by purposely blending two commercial surfactants, so that a highly bimodal EON distribution is attained. In such a case, the difficulty is to separate oligomers with very different poly-EO chain lengths, ranging from oil soluble to very water soluble, which can be present in very different amounts. The path leading to the optimum column/solvent conditions is discussed.

Additionally, the optimized HPLC separation is applied to the separation of a new commercial surfactant of the same family but with three alkyl groups on the benzene ring : the tri-butyl phenol ethoxylates.

HPLC EQUIPMENT AND EXPERIMENTAL PROCEDURE

HPLC separations are performed on a liquid chromatography equipment consisting of two M6000A pumps and a U6K injector from

Waters Associates, an UV variable wavelength detector model Dupont model 837 operated at 270 nm, and an integrator Varian model 4270.

Whenever it is used, the solvent gradient elution is carried out with a Waters model 660 solvent programmer. All flow rates are set to 1 ml/min.

Two column are used : (1) a silica column, stainless steel, 250 mm x 4.6 mm, Lichrosorb Si 60 - 10 μm, manufactured by Hibar-Merck. (2) an intermediate polarity column, stainless steel, 250 mm x 4.6 mm, Adsorbosphere NH_2 - 10 μm, made by Alltech. The precolumns were filled with the same material than the analytical columns.

Surfactants are analyzed either as received from the manufacturer, or as extracted from a water, oil or microemulsion phase, according to a procedure discussed elsewhere (44). Whatever the origin of the surfactant sample, it is evaporated to dryness and then diluted with methanol down to a concentration in the 0.05 mol per liter range. Aliquots (5 or 10 μl) of these methanol solutions are injected for chromatographic analysis.

N-heptane, chloroform and methanol are HPLC grade solvents from Baker Chemicals. Whenever a gradient elution is carried out solvent A refers to the initial solvent, while solvent B is the secondary one. The solvent mixture composition is indicated on a volume percentage basis.

NPX refers to an ethoxylated nonylphenol oligomer with X ethylene oxide groups per nonylphenol molecule; by extension and for a commercial product, it represents a mixture of ethoxylated nonylphenols with an average of X ethylene oxide group per molecule;

commercial nonylphenols received from Stepan Chemicals (Makon brand) and Hoechst gmbh (Arkopal trade name) were found to exhibits a Poisson distribution with average EON very close to the one claimed by the manufacturer. TBPX stands for tri-butyl phenol ethoxylate, where X is the average number of EO group per molecule; they were received from Hoechst gmbh which manufactures them under the trade name Sapogenat T. The surfactant mixture composition is indicated on a mol percentage basis.

ISOCRATIC HPLC ON A SILICA COLUMN

Isocratic HPLC is the first alternative to be considered because of its attractive features for routine analysis: simplicity, low cost, and rapidity. Such a technique is applied to the separation of oligomers of commercial ethoxylated nonylphenols with different average EON. The silica column tends to retain the different species according to their EON. Thus the solvent must exhibits a low polarity.

The optimization variable is the solvent polarity, which can be changed by mixing different amounts of an apolar solvent (heptane) with a polar one (methanol), together with a third solvent (chloroform) which is added to insure miscibility of the others. A heptane-chloroform-methanol 70-10-20 mixture is found to produce the best compromise between separation and retention, as discussed elsewhere (44). The method is applied to an equimolar mixture of commercial products NP4 + NP10 so that the covered EON spectrum ranges from 1 to 15. Figure 1 (left) shows that the resolution is good up to EON = 10,

but that the retention becomes too severe beyond EON = 12. Actually this method should be the prefered one up to an average EON = 8, i.e., when there is no oligomer with more than 12 EO groups. Above this value, something should be done to reduce the retention time of high EON oligomers.

SOLVENT GRADIENT HPLC ON A SILICA COLUMN

In order to reduce the retention time of high EON oligomers, without affecting the retention time of low EON oligomers, a solvent gradient technique must be used. The column is still the same, but this time the optimization variables are the solvents A and B nature and the mixing programming.

Since the heptane-chloroform-methanol 70-10-20 mixture exhibited the best separation in the isocratic mode, the gradient program is set to scan from a slightly less polar solvent to a slightly more polar solvent.

Base solvent A is a heptane-chloroform-methanol 75-10-15 mixture, which would allow an excellent separation of low EON oligmers. The second solvent B must be more polar, but its mixture with solvent A must not become too polar, because it would elute all high EON oligomers together. Trial and error experiments were carried out on a NP4 + NP10 surfactant mixture, and the best performance was attained with a linear gradient from 0% to 10% of solvent B (chloroform-methanol 50-50), over a 15 minutes period. Figure 1 (right)

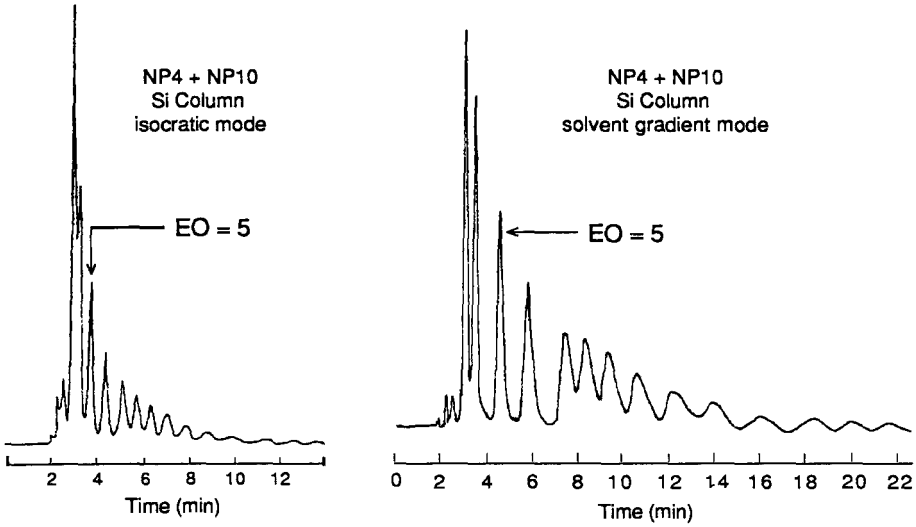


FIGURE 1 : Chromatograms from the analysis of equimolar mixtures of NP4 and NP10 on a silica column. Left : Isocratic mode with heptane-chloroform-methanol 70-10-20. Right : Gradient mode starting with solvent A heptane-chloroform-methanol 75-10-15, and adding solvent B chloroform-methanol 50-50 according to a linear gradient from 0 to 10 % B over 15 minutes.

indicates that the separation is somewhat better than in the isocratic mode, with a better definition and resolution of the peaks in the whole EON range.

However the difference is not that striking and the high EON oligomers are still retained too long a time. It is found that an increase in solvent B polarity or proportion does not improve the performance of the method. It is thus concluded that a less polar column should be

selected, in particular if surfactants with EON higher than 15 are to be analyzed.

ISOCRATIC HPLC ON A NH_2 COLUMN

As in the previous case, the NH_2 column is tested first in isocratic mode in order to optimize the solvent mixture. Since the purpose is to separate higher oligomers, a NP20 commercial surfactant with an EON distribution from 10 to 30 is tested.

Since the column is less polar than the previous one, the appropriate solvent mixture is probably less polar. Figure 2 shows the chromatograms produced with three heptane-chloroform-methanol mixtures, respectively: (a) 80-10-10, (b) 85-5-10, (c) 90-5-5.

Solvent mixture (a) exhibits a quick elution and a poor separation. It is obviously too polar. In mixture (c), half the methanol and half the chloroform have been replaced by heptane, to make up a much less polar solvent. The separation is better than in the previous case (a), but the retention times are much longer. Mixture (b) exhibits an intermediate polarity, and the corresponding chromatogram displays a good compromise between separation and retention time; however it is worth noting that the very low EON species (not present in NP20) might not be well separated. This method is thus satisfactory for ethoxylated nonylphenol with EON ranging from 12 to 28.

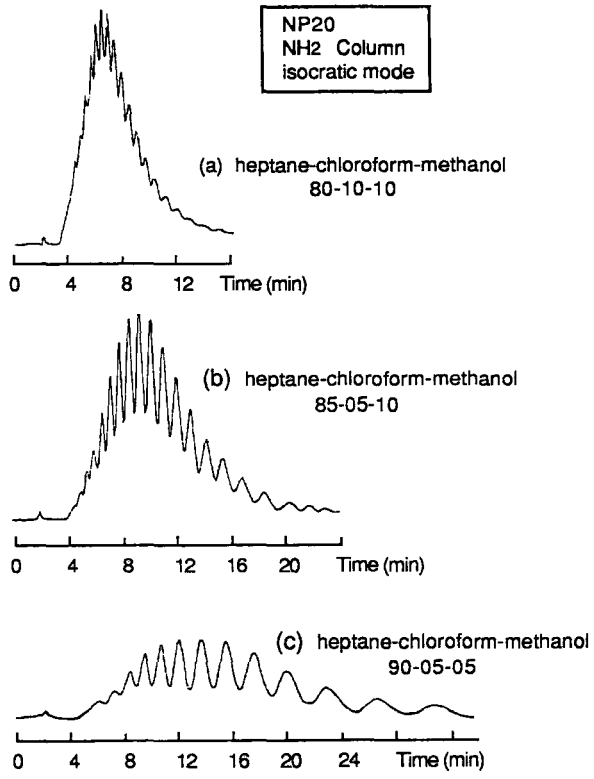


FIGURE 2 : Chromatograms from the analysis of NP20 on a NH_2 column. Isocratic mode with different solvent mixtures.

SOLVENT GRADIENT HPLC ON A NH_2 COLUMN

A wider EON range can be reached by using a solvent gradient. In order to separate the very low EON species first, a slightly less polar solvent is taken as base solvent A (heptane-chloroform-methanol 90-5-5), then it is mixed with a more polar solvent B (chloroform-methanol 50-50) according to a linear program from 0 to 20% of B in 15 minutes.

Figure 3 shows the separation of an equimolar mixture of NP4 + NP10 + NP20 surfactants with an extremely wide range of EON (1 to 28), in the above mentioned conditions. It is seen that the separation is fairly satisfactory over the whole range, altogether with a reasonable retention time. The chromatogram displays the three modes resulting from the superposition of three Poisson distributions, with maxima located at EON = 3, 9 and 18 respectively

APPLICATION TO THE ANALYSIS OF ETHOXYLATED NONYLPHENOL MIXTURES IN MICROEMULSIONS

Such wide range EON distributions are encountered in surfactant fractionation, although the limitations of the GC analytical technique have restricted the studies to low EON mixtures up to now (5-6). Real nonionic surfactant formulations can fairly well contain mixtures of very different products such as NP4 and NP20. These mixtures can exhibit a differential rather than collective behavior at interface; as a consequence, the different oligomers can fractionate between the two or three phases at equilibrium, as discussed elsewhere (46).

The phase behavior of a NPX mixture/water/heptane system is scanned as in previous work (44, 46), but this time by mixing NP4 and NP20, i. e., a very lipophilic surfactant and a very hydrophilic one. As discussed elsewhere (46) there is no three phase microemulsion at optimum formulation, which is located by the minimum in interfacial tension at 91 mol.% NP4 and 9 mol.% NP20. It has been shown that

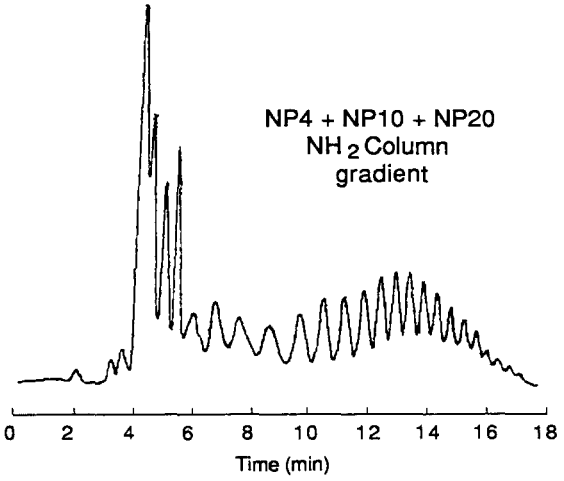


FIGURE 3 : Chromatograms from the analysis of equimolar mixtures of NP4, NP10 and NP20 on a NH_2 column. Gradient mode starting with solvent A heptane-chloroform-methanol 90-5-5, and adding solvent B chloroform-methanol 50-50 according to a linear gradient from 0 to 20 % B over 15 minutes.

adding some amount of NP10 fills the gap between the two distributions and contributes to the formation of a three phase system at optimum : a microemulsion with excess oil and water phases (46). The three phase behavior is found to occur at the following overall composition : 86 mol.% NP4 + 6.2 mol.% NP10 + 7.8 mol.% NP20. The microemulsion, oil and water phases are analyzed according to the previously described procedure (NH_2 column and solvent gradient). Figure 4 shows the resulting chromatograms corresponding to similar surfactant concentrations. Actually 78 mol.% of the total surfactant is

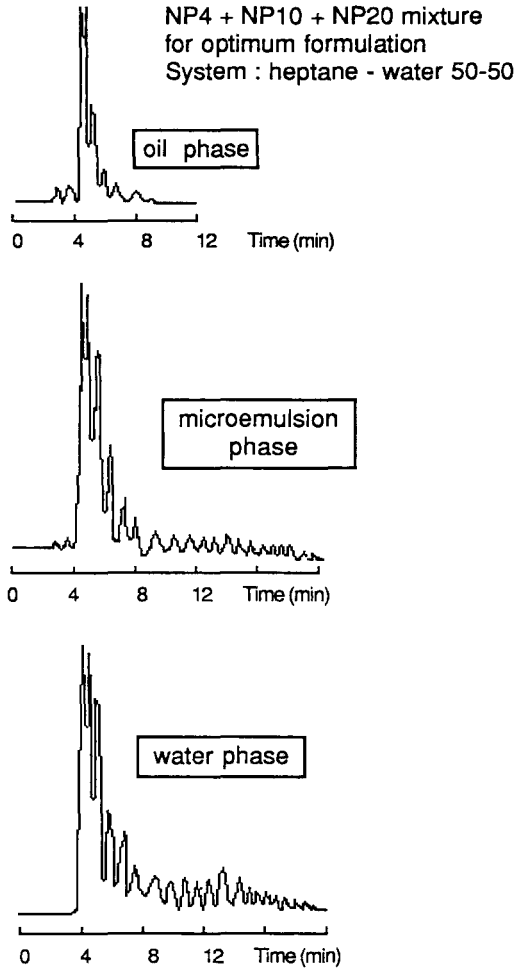


FIGURE 4 : Chromatograms from the analysis of NPX surfactants in the oil, microemulsion and water phases of an optimum three-phase system. Same experimental conditions as in Figure 3.

found in the microemulsion phase, 20 mol.% in the oil phase and only a residual 2 mol.% in the water phase. The data indicates that the oil phase extracts the low EON species, while there are more high EON species in the water phase than in the microemulsion phase, an evidence of the severe fractionation which occurs in such systems.

APPLICATION TO THE ANALYSIS OF ETHOXYLATED TRIBUTIL-PHENOL COMMERCIAL SURFACTANTS.

The gradient technique on NH_2 column is applied to commercial ethoxylated tributyl phenol (TBP) surfactants. Solvent A (heptane-chloroform-methanol 90-5-5) and B (chloroform-methanol 50-50) are the same than previously, but the trial and error optimization leads to a slightly modified program to produce a more polar mixture at the end : linear gradient from 0 to 30% of B in 20 minutes.

The chromatograms of both surfactants shown in figure 5 display an excellent separation of the oligomers. The average EON is found to be respectively 13 and 18, in accordance with the manufacturer data. However, it is worth noting that this sample of TBP13 exhibits a strongly bimodal EON distribution, an indication that it is probably a mixture of substances; if it is assumed that it is composed of two mixtures, each of them with a Poisson EON distribution, a straightforward calculation indicates that these mixtures should contain an average EON of 7 and 14 respectively.

Since the tributylphenol group possesses twelve alkyl carbon atoms, while the nonylphenol has only nine, it might be thought that

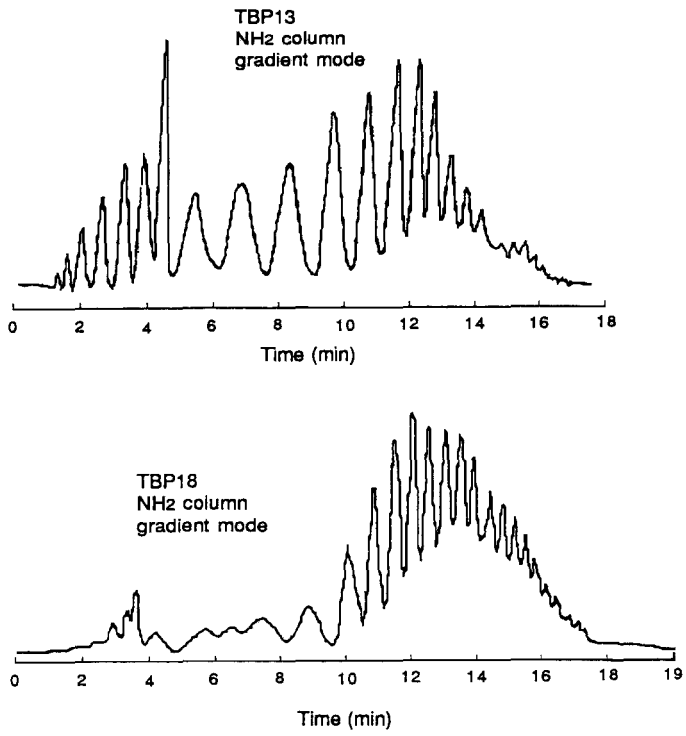


FIGURE 5 : Chromatograms from the analysis of TBP13 and TBP18 on a NH_2 column. Gradient mode starting with solvent A heptane-chloroform-methanol 90-5-5, and adding solvent B chloroform-methanol 50-50 according to a linear gradient from 0 to 30 % B over 20 minutes.

each tributylphenol ethoxylate would be as a whole less polar than its nonylphenol counterpart (with same EON). Figure 5 data indicates the contrary. In effect, the retention time of oligomers with EON = 18 is 14 minutes in both cases, but the solvent mixture is more polar in the TBP18 case than in the NP18 case.

On another hand, if the same solvent gradient is used for both types, the tributylphenol ethoxylate exhibits a longer retention time than the nonylphenol ethoxylate counterpart; this means also that the tributylphenol group is more polar or less apolar than the nonylphenol group, a result which may be attributed to the branching.

The relative polarity of nonionic surfactants is usually measured through their Hydrophilic-Lipophilic Balance, so-called HLB number, which ranges from a few units for hydrophobic substances to 12 and more for hydrophilic ones. HLB was introduced as an empirical parameter by Griffin (47, 48), and later on was correlated with many surfactant properties, as reviewed recently by Becher (49). For monoalkylphenol ethoxylates the HLB number is generally estimated as 1/5 of the weight percentage of ethylene oxide in the molecule (48). Since the tributylphenol group has been found to be less hydrophobic than the nonylphenol group, a coefficient substancially greater than 1/5 is expected to apply in the computation of the HLB of TBPX surfactants. However physico-chemical studies would have to be carried out to know the exact value of this coefficient.

ANALYSIS WITH SILICA AND NH_2 COLUMNS IN SERIES

The previous discussion has shown that the silica column exhibits a good separation of low EON oligomers, while the NH_2 column is well suited for the high EON ones. If they are combined in series it might be possible to take advantage of both features. Of course

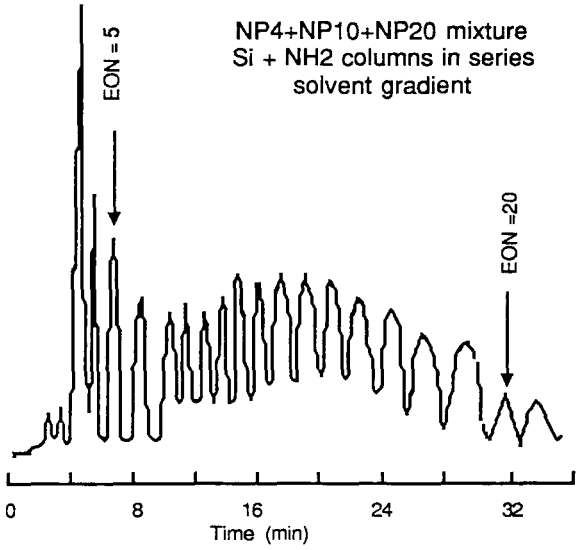


FIGURE 6 : Chromatograms from the analysis of equimolar mixtures of NP4, NP10 and NP20 on Silica-NH_2 columns in series. Gradient mode starting with solvent A heptane-chloroform-methanol 70-15-15, and adding solvent B chloroform-methanol 25-75 according to a linear gradient from 0 to 40 % B over 25 minutes.

the solvent would have to be adapted to each column separation through an appropriate program.

The silica and NH_2 columns are placed in series, and several solvent mixtures are tested to separate an equimolar NP4 + NP10 + NP20 mixture. A polar solvent made of chloroform and methanol (from 25-75 to 75-25) is tested first, with poor results. Then a ternary solvent (heptane-chloroform-methanol) is tested, with the better separation reached with 70-20-10 and 70-15-15 mixtures, but with very

long retention times. In order to reduce the retention time, solvent B should be selected as more polar than in previous experiments.

Gradient elution is optimized by varying solvents A and B and manipulating their mixing program. The best separation is attained in the following conditions : solvent A heptane-chloroform-methanol 70-15-15; solvent B chloroform-methanol 25-75; gradient program from 0% to 40% B in 25 minutes.

Figure 6 shows the separation of the wide EON range mixture in these conditions. The chromatogram displays an excellent separation of the peaks, either at low or high EON, which makes the technique an attractive candidate for preparative separation purposes.

It is worth noting that an even better separation can be attained by mixing a lesser amount of solvent B, although it requires a retention time beyond 45 minutes, which is considered excessive for practical purpose.

CONCLUSIONS

Low EON oligomers (EON < 8) of ethoxylated alkyl phenol surfactants are easily separated on a silica column with an appropriate mixed solvent; the use of solvent gradient programming allows to increase the limit up to EON = 15 on a silica column.

Higher oligomers are separated better by using a NH_2 column, either by isocratic elution or solvent gradient programming. The technique can be applied to the analysis of wide EON distribution (1 to

28) in complex systems such as microemulsions. It also allows the separation of the ethoxylated tributyl phenol oligomers in a commercial surfactant.

Finally, by using two columns (Si and NH_2) in series, with a proper solvent gradient programming, an even better separation method is attained, which may be the choice method for preparative separation.

ACKNOWLEDGMENTS

The leave of absence of one of us (N. M.) from Universidad del Zulia has been made possible by a grant from CEPET, a subsidiary of Petroleos de Venezuela, and a grant from FUNDAYACUCHO scholarship program. Some of this work has been financially backed by the Universidad del Zulia Research Council CONDES. The Lab. FIRP research program at Universidad de Los Andes is backed by the University Research Council (CDCHT), and the National Research Council (CONICIT), and sponsored by the following companies : CORIMON, CORPOVEN, HOECHST de Venezuela and INTEVEP.

REFERENCES

1. Briceño M. I., Chirinos M. L., Layrisse I., Martínez G., Nuñez G., Padrón A., Quintero L., and Rivas H., *Rev. Técn. INTEVEP*, **10**, 5 (1990)

2. Hayes M., El-Emary M., Bourrel M., Schechter R. S., and Wade W., paper SPE 7581, 53rd Annual Fall Techn. Conf., Houston, TX oct. 1978

3. Hayes M., El-Emary M., Schechter R. S., and Wade W., *J. Colloid Interface Sci.*, **68**, 591 (1979)

4. Schick M.J., Ed., *Nonionic surfactants*, Marcel Dekker, Inc., New York (1967)

5. Graciaa A., Lachaise J., Sayous J. G., Grenier P., Yiv S., Schechter R.S., and Wade W. H., *J. Colloid Interface Sci.*, **93**, 474 (1983)

6. Graciaa A., Lachaise J., Bourrel M., Osborne-Lee I., Schechter R. S., and Wade W. H., *SPE Reservoir Eng.*, **2**, 305 (1987)

7. Salager J. L., Loaiza-Maldonado I., Miñana-Pérez M., and Silva F., *J. Dispersion Sci. Technol.*, **3**, 279 (1982)

8. Salager J. L., Miñana-Pérez M., Pérez-Sánchez M., Ramírez-Gouveia M., and Rojas C., *J. Dispersion Sci. Technol.*, **4**, 313 (1983)

9. Miñana-Pérez M., Jarry P., Pérez-Sánchez M., Ramírez-Gouveia M., and Salager J. L., *J. Dispersion Sci. Technol.*, **7**, 331 (1986)

10. Rabagliati F., Falcón A., González D., Martín C., Antón R. E., and Salager J. L., *J. Dispersion Sci. Technol.*, **7**, 245 (1986)

11. Lachaise J., Breul T., Graciaa A., Marion G., Monsalve A., and Salager J. L., *J. Dispersion Sci. Technol.*, **11**, 443 (1990)

12. Hayano S., Nihongi T., and Asahara T., *Tenside*, **5**, 80 (1968).

13. Patterson S. J., Scott C., and Tucker K. B., *J. Am. Oil Chem. Soc.*, **45**, 528 (1968).

14. Ludwig F. J., Sr., *Anal. Chem.*, **40**, 1620 (1968).

15. Selden G. L., and Benedict J. L., *J. Am. Oil Chem. Soc.*, **45**, 652 (1968).

16. Konishi K., and Yamagushi S., *Anal. Chem.*, **38**, 1755 (1966)

17. Favretto L., and Stancher B., *J. Chromatogr.*, **108**, 183 (1975).

18. Farkas L., Morgos J., Sallay P., Rosznan I., Bartha B, and Veres G., *J. Am. Oil Chem. Soc.*, **58**, 650 (1981).

19. Nadeau H. G., Oaks D. M., Nichols A. W., and Carr L. P., *Anal. Chem.*, **36**, 1914 (1964).

20. Glindenberg L., and Trowbridge J. R., *J. Am. Oil Chem. Soc.*, **42**, 69 (1965).

21. Tornquist J., *Acta Chem. Scand*, **20**, 283 (1966).

22. Huber J. F. K., Kolder F. F. M., and Miller J. M., *Anal. Chem.*, **44**, 105 (1972).

23. Van der Maeden F. P., Biemond M., and Janssen P., *J. Chromatogr.*, **149**, 539 (1978).

24. Ahel M., and Giger W., *Anal. Chem.*, **57**, 1577 (1985).

25. Nagami K., and Chiba M., *Shikizai Kyokaishi*, **50**, 262 (1977).

26. Alexander J. N., McNally M. E., and Rogers L. B., *J. Chromatogr.*, **318**, 289 (1985).

27. Takeuchi T., Watanabe S., Kondo N., Goto M. and Ishii D., *Chromatographia*, **25**, 523 (1988).

28. Varughese P., Gangoda M. E., and Gilpin R. K., *J. Chromatogr.*, **499**, 469 (1990).

29. Cassidy R. M., *J. Liq. Chromatogr.*, **1**, 241 (1978).

30. Jandera P., *J. Chromatogr.*, **449**, 361 (1988).

31. Escott R. E., Brinkworth S. J., and Steedman T. A., *J. Chromatogr.*, **282**, 655 (1983).

32. Jandera P., *Chromatographia*, **26**, 417 (1988).

33. Yoshimura K., *J. Am. Oil Chem. Soc.*, **63**, 1590 (1986).

34. Pilc J. A., and Sermon P. A., *J. Chromatogr.*, **398**, 375 (1987).

35. Levsen K., Wagner-Redeker W., Schafer K. H., and Dobberstein P., *J. Chromatogr.*, **323**, 135 (1985).

36. Screuder R. H., and Martin A., *J. Chromatogr.*, **435**, 73 (1988).

37. Rothman A. M., *J. Chromatogr.*, **253**, 283 (1982).

38. Holt M. S., McKerrel E. H., Perry J., and Watkinson R. J., *J. Chromatogr.*, **362**, 419 (1986).

39. Kunket E., *Tenside Deterg.*, **18**, 301 (1981).

40. Jandera P., Urbanek J., Prokes B., and Churacek J., *J. Chromatogr.*, **504**, 297 (1990).

41. Zhou C., Bahr A., and Schwedt G., *Analytica Chimica Acta*, **236**, 273 (1990).

42. Zeman I., *J. Chromatogr.*, **509**, 201 (1990).

43. Zeman I., *J. Chromatogr.*, **363**, 223 (1986).

44. Márquez N., Antón R. E., Usubillaga A., and Salager J. L., *Separation Sci. & Technol.*, **28**, 1769 (1993)

45. Scheuder R. H., and Martijin A., *J. Chromatrogr.*, **435**, 73 (1988)

46. Márquez N., Antón R. E., Usubillaga A., and Salager J. L., *Separation Sci. Technol.*, **28**, 2387 (1993)

47. Griffin W. C., J. Soc. Cosm. Chem., **1**, 311 (1949)

48. Griffin W. C., J. Soc. Cosm. Chem., **5**, 249 (1949)

49. Becher P., Encyclopedia of Emulsion Technology, 3 vol., Dekker, New York (1984-88)

Received: June 6, 1993
Accepted: September 28, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1171-1182 (1994)

MICROANALYSIS OF GINSENG SAPONINS BY ION CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION

MAN KI PARK, JEONG HILL PARK, MI YOUNG LEE, SU JIN KIM, AND IN JEONG PARK
College of Pharmacy
Seoul National University
Seoul 151-742, South Korea

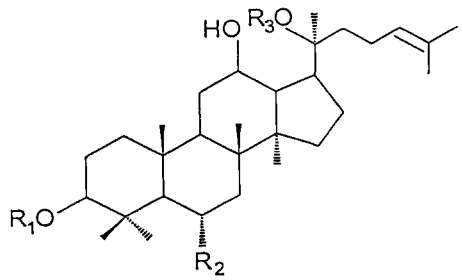
ABSTRACT

Analysis of ginseng saponins by ion chromatographic separation with pulsed amperometric detection(IC/PAD) was examined. Ginseng saponins were separated on Carbopac PA1 or AS4A anion exchange column with 1M NaOH as mobile phase. The measuring potential(E1), oxidation potential(E2), and reduction potential(E3) were 0.0V, +0.6V, and -0.8V, respectively. The dynamic linear range was over three orders and the limit of detection of ginsenoside Re was 0.8 pmol(S/N=2).

INTRODUCTION

Ginseng is an important herbal medicine which has been widely used in the Orient as a tonic, sedative, anti-fatigue, or anti-gastric ulcer drug[1]. Ginseng saponins(TABLE 1) were known as the major components of ginseng and intensive studies on the activity and analysis of them have been undertaken[2-4].

TABLE 1. Structure of ginseng saponins.



R_1=R_2=R_3=H 20(S)-protopaxanadiol
R_1=R_3=H R_2=OH 20(S)-protopaxanatriol

	R_1	R_2	R_3
Ginsenoside Rg_1	H	Glc-O-	Glc-
Ginsenoside Rg_2	H	Rha-Glc-O-	H
Ginsenoside Re	H	Rha-Glc-O-	Glc-
Ginsenoside Rf	H	Glc-Glc-O-	H
Ginsenoside Rh_1	H	Glc-O-	H
Ginsenoside Rb_1	Glc-Glc-	H	Glc-Glc-
Ginsenoside Rb_2	Glc-Glc-	H	Ara(p)-Glc-
Ginsenoside Rc	Glc-Glc-	H	Ara(f)-Glc-
Ginsenoside Rd	Glc-Glc-	H	Glc-
Ginsenoside Rg_3	Glc-Glc-	H	H

The techniques developed so far for the analysis of ginseng saponins include colorimetry[5], TLC-densitometry[6], GC[7,8], HPLC[9], and radioimmunoassay methods[10,11]. Among these, HPLC method is widely adapted[12-14]. However, UV detection at short wavelength, typically at 207nm[15], or refractive index(RI) detection method[16] in HPLC did not provide enough sensitivity for the microscale analysis of ginseng saponins.

Amperometric detector, one of the most sensitive detectors for HPLC, can be used for the microscale detection of the compounds having the functional

groups that can undergo the oxidation-reduction reaction. However, as the oxidation product of analyte accumulates on the surface of the electrode, the detection sensitivity and reproducibility decrease as time goes. Pulsed amperometric detection(PAD) was developed to overcome this drawback by applying oxidation potential(E2) and reduction potential(E3) after the measuring potential(E1) as the cleaning process of the electrode. Detection sensitivity and reproducibility were greatly enhanced by this pulse sequence. Hughes *et al.* reported microscale analysis of sugar alcohol, sugar, aldehyde, or formic acid using amperometric detector with Pt electrode,[17),18)] and Rocklin *et al.* reported the microanalysis of carbohydrate using PAD with Au electrode[19)].

This paper describes the analysis of ginseng saponins by ion chromatographic(IC) separation with PAD.

EXPERIMENTAL

Reagents and Chemicals

Nine kinds of ginseng saponin standards were generous gift from Korean Tobacco and Ginseng Research Institute and 6-year old white ginseng was purchased from the local botanical market in Seoul. Distilled deionized water was prepared with Barnstead ultrapure water system(U.S.A.). Acetonitrile and methanol were HPLC grade and other chemicals were all reagent grade.

Chromatography

Ion chromatograph used was Dionex system 4500i Ion Chromatograph (Dionex, U.S.A.) with 50μl injection loop. Young In 910 pump(Young In Sci. Co., Korea) with Rheodyne Model 7125 injector was used for HPLC.

Carbopac PA1 or HPIC-AS4A column(25cm x 4mm i.d., Dionex, U.S.A.) was used for ion chromatographic separation of ginseng saponins. Zorbax ODS column(8cm x 4mm i.d., 5μm, DuPont, U.S.A.) or Lichrosorb RP-18(25cm x 4mm i.d., 10μm, Merck, Germany) column was used for reverse-phase HPLC. Shimadzu CTO-6A column oven(Japan) was used to control column temperature. For the detection of ginseng saponins, pulsed amperometric detector(Dionex, U.S.A.) with gold-electrode, Hitachi L-4200 UV/Vis detector(Hitachi, Japan) and RID-6A RI detector(Shimadzu, Japan) were used. The chromatogram was recorded using IBM/PC compatible computer with homemade software.

RESULT AND DISCUSSION

Optimization of the condition of IC/PAD

The S/N ratio of ginsenoside Rg_1 and ginsenoside Rf were observed as the three potentials for PAD were changed. When E1 was changed from -0.6 V to 0.0 V, the S/N ratio increased with the increment of potential and showed maximum at 0.0 V. The S/N ratio dropped drastically after 0.0 V and kept constant from 0.2 V to 0.6 V(FIGURE 1). Only a small change in S/N ratio was observed when E2 and E3 were changed. Therefore, the condition used for carbohydrate analysis was adapted for E2 and E3, i.e. +0.6V and -0.8V, respectively[19].

The concentration of NaOH in the mobile phase was changed from 0.1M to 1.0M and ginseng saponin standard solution was injected. In HPIC-AS4A column, the retention time of panaxatriol type saponins increased, while that of panaxadiol type saponins decreased as the concentration of NaOH increased(FIGURE 2). Ginsenoside Rf and ginsenoside Rh_1 eluted at the same time when 0.7M NaOH was used as mobile phase, and the elution

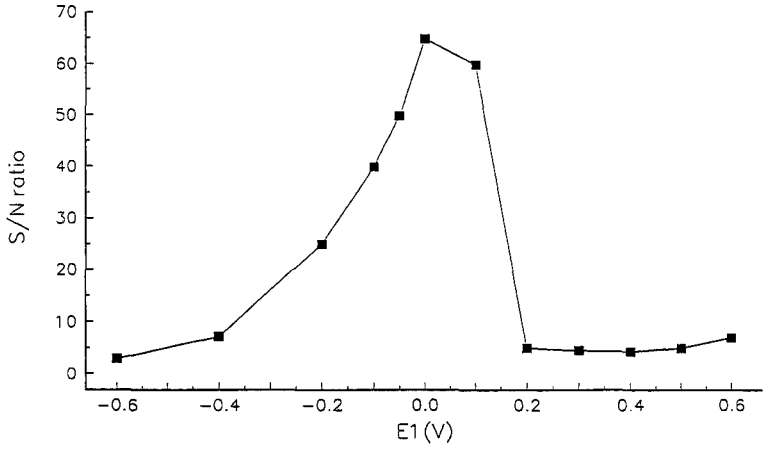


FIGURE 1. Effect of E1 to the S/N ratio of ginsenoside Rg_1. HPIC-AS4A column, 1M NaOH, 1ml/min, PAD : T1 720ms, T2 120ms (E2 +0.6V), T3 360ms (E3 -0.8V)

order was reversed before and after this concentration. In Carbopac PA1 column, only ginsenoside Rg_1 and ginsenoside Re were eluted(FIGURE 3), and the retention time of these two saponins increased as the concentration of NaOH increased(FIGURE 4). The resolution of ginsenoside Rg_1 and ginsenoside Re showed the maximum at the mobile phase of 1.0M NaOH in both columns.

FIGURE 5 shows the effect of column temperature to the capacity factors of ginseng saponins. The capacity factors increased as the column temperature rises. Ginsenoside Rb_1 and Rb_2 appeared as a single peak over 45°C while ginsenoside Rc and Rd were newly separated. The selectivity factors between each saponins were the greatest at 35°C, but the analysis was carried out at room temperature because the resolution of ginsenoside Rb_1 and Rb_2 got worse at 35°C.

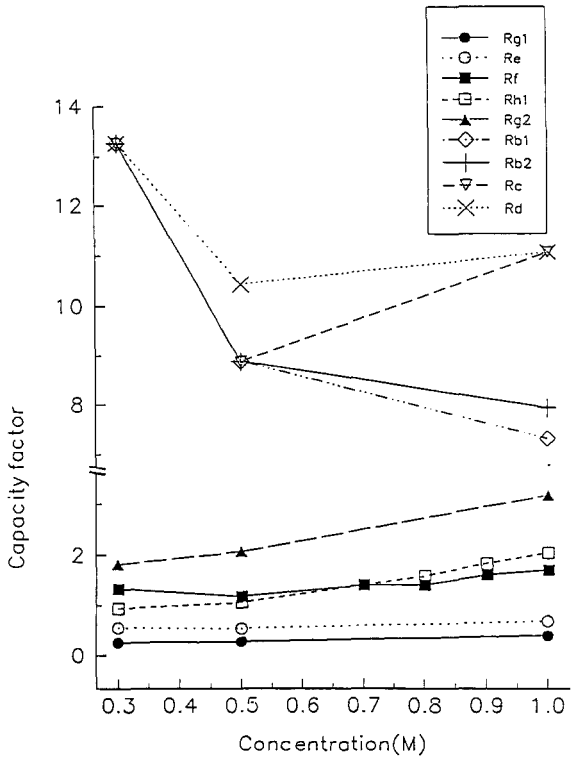


FIGURE 2. Effect of the concentration of NaOH to the capacity factor of ginseng saponins. HPIC-AS4A column, 1ml/min, PAD : 720ms (0.0V), 120ms (+0.6V), 360ms (-0.8V)

Determination of ginseng saponin

Five panaxatriol type saponins were separated well with HPIC-AS4A column, but panaxadiol type saponins were poorly separated (FIGURE 6).

The calibration curves for panaxatriol type saponins showed good linearity in the range of 10ng - 10μg, with the correlation coefficient of 0.996 - 0.999, which suggests the dynamic linear range is over three orders(FIGURE 7).

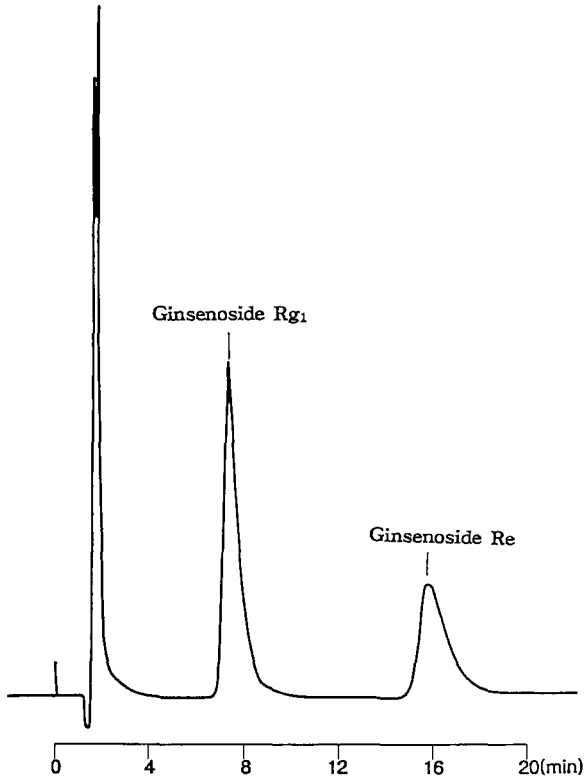


FIGURE 3. Chromatogram of mixture of ginsenoside Rg_1 and Re. Carbopac PA1 column, 1M NaOH, 1ml/min, PAD : 720ms (0.0V), 120ms (+0.6V), 360ms (-0.8V)

Ginsenoside Rg_1 and ginsenoside Re were analyzed by HPLC/UV and HPLC/RI detection methods using ODS column to compare the detection limit with that of IC/PAD method. The detection limits(S/N=2) were 400ng with UV detection method(207nm), and 8μg with RI detection method, which were somewhat higher than the reported data(300ng at 207nm with UV detection)[15].

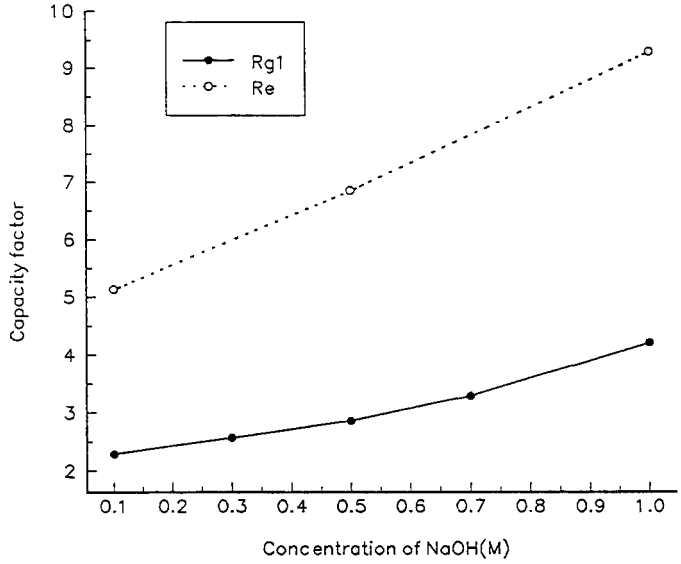


FIGURE 4. Effect of the concentration of NaOH to the capacity factor of ginsenoside Rg_1 and Re.
(Conditions are as in FIGURE 3)

The detection limit by IC/PAD was 0.8ng for ginsenoside Re and 1.0ng for ginsenoside Rg_1. The detection limits of other ginseng saponins are as follows : 1.6ng for ginsenoside Rf, 4.6ng for ginsenoside Rg_2, 2.7ng for ginsenoside Rh_1, 43.3ng for ginsenoside Rb_1, 29.6ng for ginsenoside Rb_2, 50.0ng for ginsenoside Rc, and 97.4ng for ginsenoside Rd. The detection limits of panaxadiol type saponins were greater than those of panaxatriol type saponins because the retention times of the panaxadiol type saponins were longer, which resulted in broad peak shape and low peak height.

This result shows that ginseng saponins could be several hundred times more sensitively analyzed using IC/PAD method than conventional

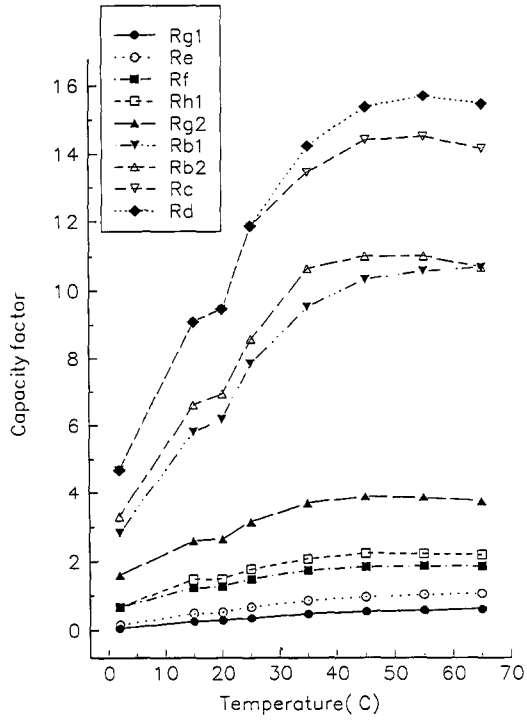


FIGURE 5. Effect of the column temperature to the capacity factor of ginseng saponins. HPIC-AS4A column, 1.0M NaOH, 1ml/min, PAD : 720ms (0.0V), 120ms (+0.6V), 360ms (-0.8V)

HPLC/UV detection method. With the development of a new column that can accomplish effective separation of all ginseng saponins, improvement in detection sensitivity as well as resolution is expected.

CONCLUSION

Ginseng saponins were analyzed by IC/PAD method. The conditions for the analysis of ginseng saponins were optimized with the change of column,

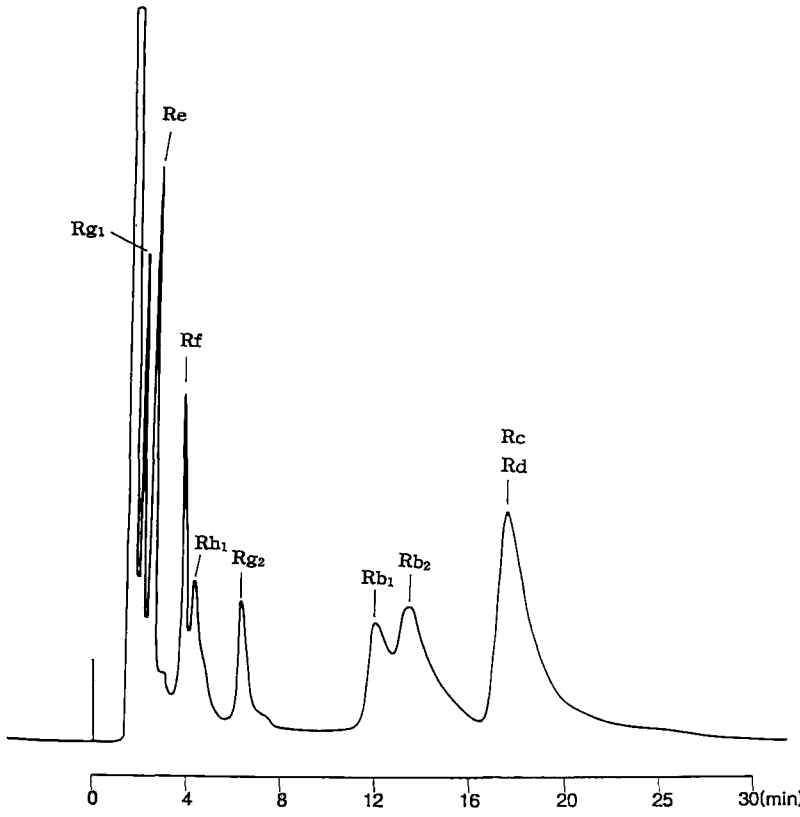


FIGURE 6. Chromatogram of mixture of ginseng saponins.
(Conditions are as in FIGURE 5)

column temperature, the concentration of NaOH in the mobile phase, and potential of E1, E2, E3.

Five panaxatriol type saponins, ginsenoside Re, Rf, Rg_1, Rg_2, Rh_1, were separated on HPIC-AS4A column and detected with PAD. The detection limit of ginsenoside Re was 0.8ng and the dynamic linear range was over 10^3. The correlation coefficient of the calibration curve was better than 0.99 in the range of 10ng - 10μg.

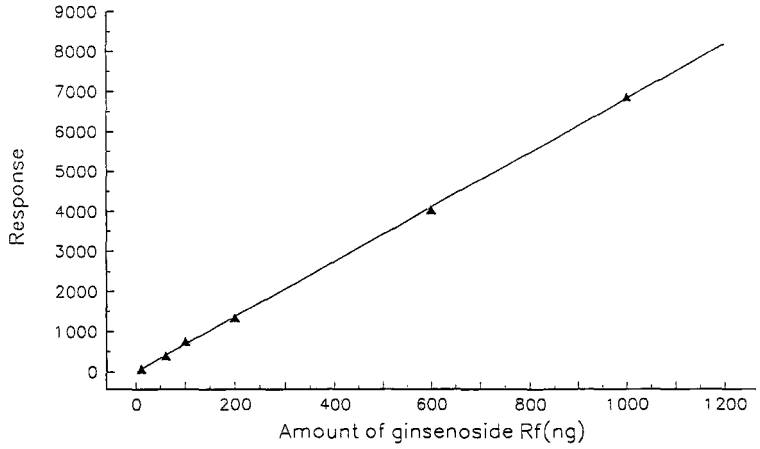


FIGURE 7. Calibration curve of ginsenoside Rf.
(Conditions are as in FIGURE 5)

So far, the analysis of ginseng saponins by IC/PAD has the drawback that the resolution of panaxadiol type saponins is unsatisfactory. However, it is expected that IC/PAD could be a method of choice for the microscale analysis of ginseng saponins premising the development of suitable column which can separate all saponins.

REFERENCE

1. D. S. Han, Pharmacognosy, Dong Myoung Sa, Seoul, p.254, 1988.

2. B. H. Han, Kor. J. Ginseng Sci., 15(1): 74-78 (1991)

3. C. N. Joo, Kor. J. Ginseng Sci., 16(1): 53-63 (1992)

4. Y. N. Han, S. Y. Kim, H. J. Lee, W. I. Whang and B. H. Han, Kor. J. Ginseng Res., 16(3): 217-222 (1992)

5. L. K. Woo, B. H. Han, D. W. Baik, and D. S. Park, J. Pharm. Soc. Korea, 17: 129-136 (1973)

6. M. Vanhaelen and R. Vanhaelen-Fastre, J. Chromatogr., 312: 497-503 (1984)

7. M. K. Park, J. H. Park, J. S. Kang, M. Y. Lee, Y. I. Park, S. J. Yu and B. H. Han, Kor. J. Ginseng Sci., 17(1): 35-38 (1993)

8. E. Bombardelli, A. Bonat, B. Gabetta, and E. M. Martinelli, J. Chromatogr., 196: 121-132 (1980)

9. H. Besso, Y. Saruwatari, K. Futamura, K. Kunihiro, T. Fuwa, and O. Tanaka, Planta medica, 37: 226-233 (1979)

10. B. H. Han and Y. N. Han, Yakhak Hoeji, 25: 43-47 (1981)

11. B. H. Han and I. M. Chang, J. Ginseng Sci., 2(1): 17-33 (1977)

12. S. R. Ko, K. J. Choi, S. C. Kim and M. W. Kim, Kor. J. Pharmacogn., 20(3): 170-174 (1989)

13. K. J. Choi, S. R. Ko, B. S. Jeon and H. S. Sung, Kor. J. Pharmacogn., 20(3): 175-179 (1989)

14. F. Soldati and O. Sticher, Planta medica, 38: 348-357 (1980)

15. O. Sticher and F. Soldati, Planta medica, 36: 30-42 (1979)

16. S. K. Hong, E. K. Park, C. Y. Lee and M. U. Kim, Yakhak Hoeji, 23: 181-186 (1979)

17. S. Hughes, P. L. Meschi and D. C. Johnson, Anal. Chim. Acta, 132: 1-10 (1981)

18. S. Hughes and D. C. Johnson, J. Agric. Food Chem., 30: 712-714 (1982)

19. R. D. Rocklin and C. A. Pohl, J. Liq. Chromatogr., 6: 1577-1590 (1983)

Received: July 14, 1993
Accepted: September 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1183-1194 (1994)

METHOD FOR DETERMINATION OF PARTITION COEFFICIENTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: APPLICATION TO O-HYDROXYLBENZENESULFONANILIDES

XIAOLIN SUN, MIN XIN, AND JUN ZHAO
Department of Chemistry
Inner Mongolia University
Huhehot, Inner Mongolia 010021
The People's Republic of China

ABSTRACT

The capacity factors (log k′) for 26 compounds of widely varying structural types were determined by high-performance liquid chromatography method using methanol—water as mobile phase and ODS column as stationary phase in which free silanol groups were fully suppressed. These determined log k′ values are correlated with known partition coefficient (log P_{OCT}) values of 26 compounds and the regression parameters show that accordingly there are two sets of correlation lines when 26 compounds are divided into two data sets depending on their hydrogen-bonding ability. The factors which caused different partitioning mechanisms of the compounds are discussed.

As an application, the partition coefficients of a series of o-hydroxylbenzenesulfonanilides (HBSA) were determined by using obtained relative correlation line and then correlated with their anthelmintic activities.

INTRODUCTION

A variety of theoretical and experimental works [1—3] have shown that the properties of drugs such as absorbability and transportation are closely correlated with their hydrophobicity, which is a critical factor affecting activities of drugs in some cases. As a result, the partition coefficients (log P_{OCT}) which present hydrophobicity of drugs are commonly used in studies on QSAR (quantitative structure—activity relationships). Through studing the anthelmintic activities, we discovered that o-hydroxyl benzenesulfonanilides (HBSA) were some very potent fasciolicides. Our initial studies[4,5] revealed that one of the most important conditions for HBSA to possess potent activities was their stability in lipophilic phase of biomembranes. For this reason, analysis of the partition coefficients of HBSA is necessary for us to thoroughly investigate anthelmintic mechanism of these compounds and search for more potent drugs.

We had previously tried to determine partition coefficients of HBSA in an n-octanol—water partitioning system by the shake-flask technique and failed because of emulsions forming between n-octanol and water. It is well known that high-performance liquid chromatography has been successfully used as a convenient method for determination of the partition coefficient, so we used the octadecylsilane (ODS) column as a stationary phase in reversed-phase HPLC, on the basis of similarities in the hydrophobic partitioning processes occurring in an octanol—water system and in a reversed-phase HPLC system with an aqueous mobile phase, to determine partition coefficients of a series of HBSA compounds. In this studing, we tried to correlate the determined partition coefficients

of HBSA to their anthelmintic activities, and we also tried to clarify the factors which govern the value of k′ (capacity factor) in terms of chemical structure and partition behaviour in chromatography on an ODS column.

EXPERIMENTAL

Materials:

All the chemicals used were analytical or chemical grade. Water for solvents were glass redistilled and methanol was spectral grade. HBSA compounds were synthesized in our laboratory and purified by recrystallization method. All samples were dissolved in methanol at 1 mg/ml, adequate amount of samples was injected on to the column according to their UV detector responses.

Apparatus:

HPLC was carried out at 30℃ with a Shimadzu LC-6A system equipped with a SPD-6AV ultraviolet detector (wavelength range 195—700nm) and LC-6A high pressure pump. Chromatographic data were recorded and processed on C-R3A data system.

The stationary phase was an ODS column (Shim-Pack CLC-ODS, 5 μ particle size, 150mm × 6mm i. d.). These silica packings are fully end-capped to suppress residual silanol group influences.

The mobile phase was a mixture of water and methanol. In order to obtain acceptable retention time, the volume fraction of methanol in mobile phase was set at 0. 7, the flow rate of mobile phase was constantly 1. 4 ml/min.

Procedures:

The correlation between the partition coefficient of samples and their chromatographic capacity factor k′ (log k′) can be expressed as follows :

$$\log P_{OCT} = a + b \log k'$$

capacity factor, $k' = \frac{t_R - t_0}{t_0}$

where t_R is the sample's retention time and t_0 is the retention time of an unretained substance, determined by formamide.

We had chosen 26 compounds of widely varying functionality and structure types for the determination of k′ values and established correlation described above. All values of log P_{OCT} of these compounds were obtained from the reference [6]. The pH of the mobile phase was adjusted to 3. 0 with acetic acid to avoid the effect of acid dissociation of the samples and HBSA compounds.

In another experiment, ammonium chloride (0. 035M in mobile phase) was added to mobile phase as a masking agent [7] to examine the extent to which the free silanol groups existed in the stationary phase. Experimental values of log k′ of the 26 compounds is listed in Table 1.

RESULTS AND DISCUSSION

At first, we determined the effect of free silanol groups in the CLC-ODS column used in our experiments. By comparing the log k′ and log k'_N (log k′ of the compounds when NH_4Cl was added into mobile phase) values listed in Table 1, it is clear that almost all the values of log k'_N of the compounds are little less than that of log k′ of the compounds, including the compounds such as benzene, toluene etc. which have minimal interactions with

Table 1

Experimental Capacity Factors and Literature Octanol—Water Partition Coefficients

No.	Compound	log P_{OCT}	log k′	log k_N' *
1	Benzyl alcohol	1.10	−0.580	−0.587
2	o-Amino benzoic acid	1.21	−0.559	−0.581
3	Cyclohexanol	1.23	−0.557	−0.545
4	Phenylacetic acid	1.41	−0.376	−0.379
5	Phenol	1.46	−0.451	−0.465
6	Benzoic acid	1.87	−0.301	−0.324
7	m-Nitrophenol	2.00	−0.267	−0.276
8	m-Methoxylbenzoic acid	2.02	−0.237	−0.260
9	Salicylic acid	2.26	0.017	−0.002
10	p-Chlorophenol	2.39	−0.036	−0.055
11	1-Naphthol	2.98	0.128	0.110
12	p-Iodobenzoic acid	3.02	0.258	0.234
13	2,4,6-Trichlorophenol	3.72	0.588	0.587
14	Anisole	2.11	0.173	0.170
15	Benzene	2.13	0.174	0.171
16	p-Nitrotoluene	2.37	0.202	0.180
17	m-Nitrochlorobenzene	2.41	0.310	0.300
18	p-Nitrochlorobenzene	2.41	0.230	0.220
19	m-Nitrobromobenzene	2.64	0.369	0.340
20	Toluene	2.69	0.452	0.441
21	Chlorobenzene	2.84	0.452	0.445
22	p-Dichlorobenzene	3.39	0.662	0.652
23	Azobenzene	3.82	0.978	0.978
24	Diphenyl	4.04	0.883	0.859
25	Phenyl ether	4.21	1.002	0.998
26	Anthracene	4.45	1.123	1.125

* $\log k_N' = \log k'$ of the compounds when NH_4Cl was added into mobile phase

silanol groups. This indicates that the diminished values of log k'_N may not be caused by the effect of silanol group but by changes in mobile phase ionic strength, for mobile phase ionic strength is also a factor that can affect partitioning processes for compounds in an HPLC system[8]. We believe that residual silanol groups in the ODS column have fully been suppressed.

Figure 1 shows a plot of the log P_{OCT} values of these 26 compounds versus their values of log k′ determined from the experiment. Linear regression parameters indicate that a linear relationship between these log P_{OCT} and log k′, and further, after comparing the chemical structure types of 26 compounds, it is found that these compounds can be divided into two classes with respect to hydrogen-bonding ability[6,9]: (i) HB(hydrogen-bonding), such as phenolic and carboxyl acid compounds etc. and (ii) NHB (non-hydrogen bonding), such as alkylbenzene, halogenbenzene and nitrobenzene compounds etc.. As shown in Figure 1, the HB (compound **1**～**13**, see Table 1)and NHB (compound **14**～**26**)have good correlations between log P_{OCT} and log k′, respectively. This again verified the view that partitioning of a compound between water and octanol is governed not only by its hydrophobicity, but also by the extent to which the compound can hydrogen-bond to octanol.

Table 2 lists the log P_{OCT} vs log k′ linear regression parameters obtained for this HPLC system when the 26 compounds are considered altogether as well as are divided into one set containing the HB and one containing the NHB. Except that correlation coefficients and variances of the fit for the two divided data sets are much better than that for the overall data set, the regression parame-

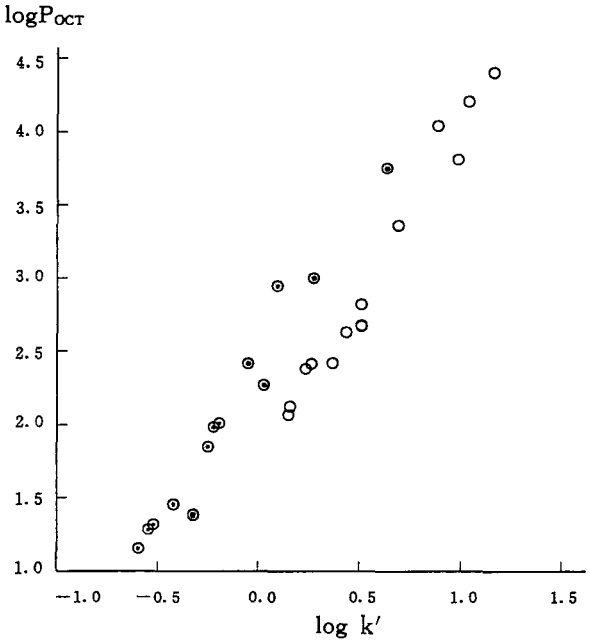


FIGURE 1. Plot of the log P_{OCT} versus the experimental log k′ for 26 compounds discussed

◉ HB compound

○ NHB compound

ters of the correlation lines for HB and NHB are significantly different from each other, especially the intercepts of the correlation lines. As demonstrated in previous studies [9—11] the differences in the intercepts of the correlation lines appears to be directly related to the differences in hydrogen-bonding ability between HB and NHB.

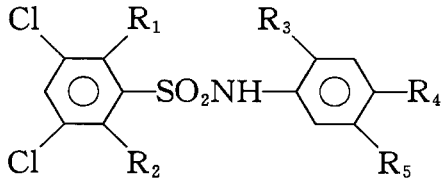
Some papers[12,13], which reported the similar experimental phenomena, attributed the causes of the phe-

Table 2
Log P_{OCT} vs. Log k′ Linear Regression Parameters for 26 Compounds Data Set

Parameter	All Data	NHB	HB
slope	1.806	2.371	2.241
intercept	2.223	1.761	2.461
correlation coefficient	0.960	0.990	0.987
variance of fit	0.074	0.016	0.017

nomena to hydrogen bonding of HB to residual silanol sites on the ODS column. However, as described above, silanol groups on the ODS column used in our experiments were fully suppressed to minimize interactions between HB and silanol groups here. Haky and Vemulapalli [14] had used ODA (Octadecyl-Bonded Alumina) as a stationary phase in HPLC system to determine the log k′ for compounds of various chemical classes. In contrast to results obtained with other columns, HB did not need to be treated as a separate data set on the ODA column to obtain good correlations between log k′ and log P_{OCT}. This clearly indicates that the HPLC system which used ODA as a stationary phase is more similar to n-octanol—water system than the ODS—HPLC system. Therefore, these data suggest that different partitioning behavior between HB and NHB in the ODS—HPLC system may be caused by differences other than those caused by analyte interactions with silanol groups.

As an application of the HPLC method, we employed correlation lines deduced above to estimate the log P_{OCT} of some HBSA compounds with different substituents. The general structural formula of these compounds can be represented as follows:



compound:

Ⅰ. R_1=OH, R_2=R_3=R_4=R_5=Cl
Ⅱ. R_1=OH, R_2=H, R_3=R_4=R_5=Cl
Ⅲ. R_1=OH, R_2=Cl, R_3=CH_3 R_4=R_5=H
Ⅳ. R_1=OH, R_2=R_3=Cl, R_4=R_5=H
Ⅴ. R_1=OH, R_2=Cl, R_3=R_4=R_5=H
Ⅵ. R_1=OH, R_2=R_3=R_5=H, R_4=Cl
Ⅶ. R_1=H, R_2=Cl, R_4=OH, R_3=R_5=H

Acorrding to result from our previous studies[5,15], the HBSA compounds shown above (except Ⅶ) are considered non—hydrogen bonding molecules due to intra—molecular hydrogen bonding, estimated log P_{OCT} values of the HBSA compounds are shown in Table 3.

In concordance with positive value of π_x (hydrophobic constant for halogen atoms), the values of determined log P_{OCT} of HBSA compounds increased with the total number of halogen atoms substituted in the benzene rings of the compounds, but obviously they did not follow the additive-constitutive property of Hansch's hydrophobic constants. This will call our attention to the special conformations of these compounds in solution.

Table 3
The Determined Log P_{OCT} and Anthelmintic Activities of HBSA Compounds

Compound	log k′	log P_{OCT}	Activity*
I	1.096	4.360	++++
II	1.078	4.317	+++
III	0.951	4.016	
IV	0.930	3.966	
V	0.834	3.738	++
VI	0.802	3.663	+
VII	0.766	4.178	−

* The activities of HBSA compounds were examined in sheep which were experimentally infected. Activities of compound III and IV were not yet obtained.

It was revealed in our earlier paper[5]that the phenolic-OH group in the HBSA molecule was the most important active-group and the anthelmintic activity of these compounds is strengthened with increasing acidity of the phenolic-OH group in their molecules. By correlating log P_{OCT} of HBSA compounds with their anthelmintic activities, we are informed that the greater log P_{OCT} (or more hydrophobic) of HBSA compounds correlate with the higher anthelmintic activities of the compounds.

In addition, although the log P_{OCT} determined for HBSA compound VII demonstrates that the compound is hydrophobic, the acid dissociation constant PKa determined [16]for this compound told us that phenolic-OH group in molecule of HBSA compound VII had extremely weak acidity. For this reason, it is not surprising that HBSA com-

pound Ⅶ possesses no anthelmintic activity. In summary, the mechanism of biological activity for HBSA compounds is influenced by a variety of factors such as acidity, hydrophobicity and even molecular conformations of HBSA compounds in solution.

ACKNOWLEDGEMENT

This work was supported by National and Inner Mongolia's Natural Science Foundation of China.

REFERENCES

1. Ariens, E. J., Drug Design, Vol. 5, P81, Academic Press, New York (1975).
2. Hansch, C. and Leo, A., Substituent Constants for Correlation Analysis in Chemistry and Biology, wiley, New York (1979).
3. Hansch, C., in "Correlation Analysis in Chemistry and Biology", plenum, New York, N. Y. (1978).
4. Hiroshi Terada, Biochim Biophys Acta, 639: 225 (1981).
5. Sun Xiaolin, Xin Min and Lee Zhe, Proceedings of the Third National Symposium on Molecular Mechanics and Design of Drugs, Beijing, P2—34 (1991).
6. Leo. A., Hansch, C. and Elkins, D., Chem. Rev., 71:525 (1971).
7. Tsukinaka, Y., Akira Tsuji, Etsuko Miyamoto and Osamu Kubo, J. Pharm. Sci., 66:747 (1977).
8. Braumann, Th., J. Chromatogr., 373:191 (1986).
9. Keishiro Miyake, Nobuyasu Mizuno and Hiroshi Terada, Chem. Pharm. Bull., 34:4787 (1986).
10. Haky, J. E. and Yong, A. M., J. Liquid Chromatogr., 7:675 (1984).
11. Seiler, P., Eur. J. Med. Chem, 9:473 (1974).

12. Unger, S. H., Cook, J. R. and Hollenberg, J. S., J. Pharm. Sci., 67:1364 (1978).

13. Unger, S. H., and Chiang, G. H., J. Med. Chem., 24:262 (1981).

14. Haky, J. E. and Vemulapalli, S., J. Liquid Chromatogr, 13:3111 (1990).

15. (a)Xin Min, Sun Xiaolin, Lee Zhe and Lian Shouren, Chinese J. Appl. Chem, 8:87 (1991).
(b)Xin Min, Sun Xiaolin and Lee Zhe, Acta Scientiarum Naturalium Universitatis Intra mongolicac, 22:141 (1991).

16. Sun Xiaolin and Xin Min ,to be published.

Received: June 1, 1993
Accepted: September 28, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1195 (1994)

ERRATUM

corrected i Aug 94/AP

In the paper entitled, "**Use of Capillary Zone Electrophoresis for the Analysis of DNA Binding to a Peptide Derived from Amyloid P Component**," which was published in J. Liquid Chrom., 16:1923-1939 (1993), two figures were inadvertently mis-captioned.

a) The caption shown with Figure 2 belongs to Figure 3.

b) The caption shown with Figure 3 belongs to Figure 2.

We sincerely apologize for any inconvenience this error may have caused.

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 1197-1202 (1994)

LIQUID CHROMATOGRAPHY CALENDAR

1994

FEBRUARY 2 - 3: AOAC Southeast Section Meeting, Ramada Hotel & Convention Center, Atlanta, GA. Contact: Doug Hite, Technical Services, P. O. Box 40627, Melrose Station, Nashville, TN 37204, USA.

FEBRUARY 28 - MARCH 4: PittCon'94: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd, Pittsburgh, PA 15235-9962, USA

MARCH 22 - 24: PrepTech '94, A New Conference on Industrial Bioseparations, Meadowlands Hilton Hotel, S3ecaucus, New Jersey. Contact: Symposium Manager, PrepTech '94, ISC, Inc., 30 Controls Drive, Shelton, CT 06484, USA.

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

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

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

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

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

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

JUNE 5 - 7: VIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques & Applications in Chromatography and Capillary Electrophoresis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. Dr. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Dept. of Pharmaceutical Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

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

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

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

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

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

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

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

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

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

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

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

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

1995

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

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

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

JUNE 15 - 17: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

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

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

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

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

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

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

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

1996

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

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

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

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

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

1997

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

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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

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

Understand one of the fastest growing analytical techniques in the field of separation science and learn its many applications with...

Furnishes more than **1200** up-to-date references and nearly **670** helpful tables, equations, drawings, and photographs!

Capillary Electrophoresis Technology

(Chromatographic Science Series/64)

edited by **NORBERTO A. GUZMAN**
The R. W. Johnson Pharmaceutical Research Institute, Raritan, New Jersey

August, 1993 / 880 pages, illustrated / $165.00

From the Foreword...

"...[this book] is a large and impressive volume....It is a thorough treatment of the field as it exists and offers many insights into where the field is heading.... the authors...have produced a marvelous, up-to-date, and well-organized guide."

—James W. Jorgenson
Department of Chemistry
University of North Carolina at Chapel Hill

Tracing the sequence of new observations that has led to current understanding in the field, this **outstanding** reference presents the basic concepts, instrumentation, and applications of capillary electrophoresis—examining its many **unique** features such as high-power resolution, high-mass sensitivity, overall sensitivity, and low-sample volume requirements.

Highlights the use of capillary electrophoresis for the identification, separation, detection, and characterization of substances *on the molecular counting level!*

Illustrating the major technical maneuvers for common operations and applications, ***Capillary Electrophoresis Technology***

- outlines the theoretical concepts and mathematical expressions of capillary electrophoresis
- describes advances in instrumentation hardware and detection systems
- explains the advantages and limitations of the different variants of capillary electrophoresis
- provides **special coverage** of areas in which capillary electrophoresis has grown increasingly popular, including the identification and characterization of small molecules and macromolecules
- and much more!

Written by **over 50** experts from universities, laboratories, and industrial and research centers throughout the U.S. and abroad, ***Capillary Electrophoresis Technology*** is an essential reference for analytical and clinical chemists and biochemists, chemical engineers, biologists, pharmacists, biotechnologists, and upper-level undergraduate and graduate students in these disciplines

Contents

ISBN: 0—8247—9042—1

This book is printed on acid free paper

Marcel Dekker, Inc.
270 Madison Avenue
New York, NY 10016
(212) 696-9000

Hutgasse 4, Postfach 812
CH-4001 Basel, Switzerland
Tel 061 261 8482

Use a selective solid phase for any given bioorganic mixture effectively *with...*

Handbook of Affinity Chromatography

Contains over 200 helpful tables, figures, and equations!

(Chromatographic Science Series/63)

edited by

TONI KLINE, *Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey*

June, 1993
344 pages, illustrated
$135.00

Outlining the fundamental principles by which all interactions occur, this **practical** reference focuses on harnessing the biochemistry of bioorganic compounds in order to separate them—presenting new techniques and applications that affect the planning of research strategies.

Written by international experts in the field, the ***Handbook of Affinity Chromatography***

- discusses how to create a customized separation system without having to invent a whole new technology to support it
- details current purification methods for several classes of proteins
- introduces surface plasma resonance detection and weak affinity chromatography
- documents successful laboratory experiences relevant to future purifications
- explores research on affinity chromatography as a topic in its own right within the field of biorecognition
- and more!

Providing **more than 1200** literature citations to allow for in-depth study of specific topics, the ***Handbook of Affinity Chromatography*** is an invaluable resource for analytical and protein chemists; research biochemists; molecular biologists; immunologists; pharmacologists; separation scientists; bioprocess technologists; design, chemical/biochemical, and research and development engineers; process development managers; and upper-level undergraduate and graduate students in these disciplines.

Marcel Dekker, Inc.
270 Madison Avenue, New York, NY 10016
■ (212) 696-9000
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
■ Tel. 061-261-8482

CONTENTS

ISBN: 0-8247-8939-3

ELECTRONIC MANUSCRIPT SUBMISSION

Effective immediately, manuscripts will be accepted on computer diskettes. A printed manuscript must accompany the diskette. For approximately one year, the diskettes will be used, on an experimental basis, to produce typeset-quality papers for publication in the Journal of Liquid Chromatography. Diskettes must be in an IBM-compatible format with MS-DOS Version 3.0 or greater. The following word processing formats can be accommodated:

ASCII	DisplayWrite Native
EBCDIC	Enable 1.0, 2.0, 2.15
Framework III 1.0, 1.1	IBM Writing Assistant
Microsoft Word 3.0, 3.1, 4.0, 5.0	Multimate 3.3
Multimate Advantage 3.6	Multimate Advantage II 3.7
Navy DIF	Office Writer 4.0, 5.0, 6.0, 6.1
PeachText 5000 2.12	PFS:First Choice 1.0, 2.0
PFS:Write Ver C	Professional Write 1.0, 2.0, 2.1
Q&A Write 3.0	RapidFile (Memo Writer) 1.2
Samna Word IV & IV+ 1.0, 2.0	Total Word 1.2, 1.3
Volkswriter 3, 4	Volkswriter Deluxe 2.2
Wang PC Ver 3	WordPerfect 4.1, 4.2, 5.0, 5.1*
WordStar 3.3, 3.31, 3.45, 4.0, 5.0, 5.5, 6.0	XyWrite III XyWrite III+

* The **preferred** word processor is **WordPerfect 5.1.**

Manuscripts and diskettes should be prepared in accordance with the **Instructions for Authors** given at the back of this issue of the Journal. They should be sent to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 2180
Cherry Hill, NJ 08034

INSTRUCTIONS TO AUTHORS

Journal of Liquid Chromatography is published in the English language for the rapid communication of research in liquid chromatography and its related sciences and technologies.

Directions for Submission

One typewritten manuscript, suitable for direct reproduction, and two (2) clear copies with figures must be submitted. Since the Journal is produced by direct photography of the manuscripts, typing and format instructions must be strictly followed. Non-compliance will result in return of the manuscript to the author and will delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards before mailing.

Manuscripts may also be submitted on **computer diskettes**. A printed manuscript must also be submitted with diskettes because, at the present time, we are experimenting with manuscripts on diskettes. Diskettes must be readable with an IBM-compatible computer (Macintosh or other type not acceptable) and must be formatted with MS-DOS 3.1 or greater. Be sure to indicate the word processing software that was used to prepare the manuscript diskette.

Manuscripts and computer diskettes should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 2180
Cherry Hill, NJ 08034

Reprints

Due to the short production time for papers in this journal, it is essential to order reprints immediately upon receiving notification of acceptance of the manuscript. A reprint order form will be sent to the author with the letter of acceptance for the manuscript. Reprints are available in quantities of 100 and multiples thereof. Twenty (20) free reprints will be included with orders of 100 or more reprints.

Format of the Manuscript

1. The general format of the manuscript should be:
   Title
   Author(s)' names and full addresses
   Abstract
   Text Discussion
   References

2. **Title & Authors:** The entire title should be in capital letters and centered within the width of the typing area, located at least 2 inches (5.1 cm) from the top of the page. This should be followed by 3 lines of space, then by the names and addresses of the authors, also centered, in the following manner:

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN WATER BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

3. **Abstract:** The title **ABSTRACT** should be typed, capitalized and centered, 3 lines below the addresses. This should be followed by a **single-spaced**, concise abstract. Allow 3 lines of space below the abstract before beginning the text of the manuscript.

4. **Text Discussion:** Whenever possible, the text discussion should be divided into major sections such as

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGEMENTS
REFERENCES

These **major headings** should be separated from the text by two lines of space above and one line of space below. Each major heading should be typed in capital letters, centered and underlined.

Secondary headings, if any, should be placed flush with the left margin, underlined and have the first letter of main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. The first word of each **paragraph** within the body of the text should be indented five spaces.

6. **Acknowledgements**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

7. **References** should be numbered consecutively and placed in a separate section at the end of the manuscript. They should be typed single-spaced, with one line space between each reference. Each reference should contain names of all authors (with initials of their first and middle names); do not use *et al.* for a list of authors. Abbreviations of journal titles will follow the American Chemical Society's Chemical Abstracts List of Periodicals. The word **REFERENCES** should be capitalized and centered above the reference list.

Following are acceptable reference formats:

Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18: 1979-1998 (1985)

Book:

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

2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page.

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used.

10. Material that cannot be typed, such as Greek symbols, script letters and structural formulae, should be drawn carefully with dark black India ink. Do not use any other color ink.

Additional Typing Instructions

1. The manuscript must be prepared on good quality **white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). The typing area of the first page, including the title and authors, should be 5½ inches wide by 7 inches high (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches high (14 cm x 21.6 cm).

2. The **title, abstract, tables and references** are typed single-spaced. All other text should be typed 1½-line spaced or double line spaced.

3. It is essential to use **dark black** typewriter or printer ribbon so that clean, clear, **solid characters** are produced. Characters produced with a dot/matrix printer are not acceptable, even if they are "near letter quality" or "letter quality." Erasure marks, smudges, hand-drawn corrections and creases are not acceptable.

4. **Tables** should be typed on separate pages, one table to a page. A table may not be longer than one page. If a table is larger than one page, it should be divided into more than one table. The word **TABLE** (capitalized and followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

5. **Figures (drawings, graphs, etc.)** should be professionally drawn in **black** India ink on separate sheets of **white** paper, and should be placed at the end of the text. They should not be inserted in the body of the text. They should not be reduced to a small size. Preferred size for figures is from 5 inches x 7 inches (12.7 cm x 17.8 cm) to 8½ inches by 11 inches (21.6 cm x 27.9 cm). **Photographs** should be professionally prepared *glossy* prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn.

Captions for figures should be typed single-spaced on a separate sheet of white paper, along the full width of the type page, and should be preceded with the word FIGURE and an Arabic numeral. All figures and lettering must be of a size that will remain legible after a 20% reduction from the original size. Figure numbers, name of senior author and an arrow indicating "top" should be written in light blue pencil on the back of the figure. Indicate the approximate placement for each figure in the text with a note written with a light blue pencil in the margin of the manuscript page.

6. The **reference list** should be typed single-spaced. A single line space should be inserted after each reference. The format for references should be as given above.

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), (1994)

Contents Continued

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 17, Number 5, 1994

CONTENTS

(continued on inside back cover)

MARCEL DEKKER, INC. New York, Basel, Hong Kong
Contributions to this journal are published free of charge