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A COMPARATIVE STUDY OF BUCKMINSTERFULLERENE AND HIGHER FULLERENE SEPARATIONS BY HPLC

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ABSTRACT

Several HPLC columns that have been recommended for the separation of fullerenes were compared. Each column (i.e., stationary phase) had a different optimum mobile phase. In general, mobile phases consisted of binary mixtures of a "good" fullerene solvent and a "poorer" fullerene solvent. Reversed phase stationary phases with alkyl (aliphatic) substituents produced superior analytical separations for all fullerenes. However, stationary phases with aromatic substituents were better for preparative separations. Increasing the proportion of the "poor" fullerene solvent in the mobile phase generally increased resolution and retention but decreased the mass load. Injecting too high a concentration of fullerenes in any column caused peak splitting to occur. The fullerene retention order was the same on all columns and with all mobile phases. The fullerene separation ability of all columns tended to deteriorate with time. Overloading the column or doing preparative separations greatly accelerated the deterioration process. It is believed that irreversible adsorption of fullerenes or fullerene by-products is responsible for the degradation of column performance.

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INTRODUCTION

The number of high performance liquid chromatography (HPLC) stationary phases for the separation and purification of fullerenes and their derivatives has increased tremendously over the past few years. The first fullerene separations were reported on neutral alumina and silica gel.[1-5] Subsequently several commercially available alkyl and aromatic HPLC stationary phases were examined.[6-17] Also, several stationary phases (some of which are very costly) were developed solely for the purpose of separating fullerenes.[18-22] Thus far, over fifteen liquid chromatographic stationary phases have been used in the separation of fullerenes. Duplication, overlap, and discrepancies exist when comparing the claims and selectivities of these columns. Most of the available stationary phases give a more than adequate separation of C_{60} and C_{70} fullerenes provided the optimum mobile phase is used in each case. However, separation problems can arise in separating the higher fullerenes, specific isomers, fullerene derivatives, and fullerene-metal complexes. The recent demand for larger quantities of fullerenes has led to an additional challenge of separating fullerenes on a preparative basis.[23-25]

It has been suggested in the literature that some columns are better than others in regard to the aforementioned separations. However, to our knowledge, no one has compared different stationary phases regarding efficiency, loading, stability, and higher fullerene separation ability. A greater understanding of these separations will allow rational selection to be made based on specific needs.

Buckminsterfullerene and higher fullerenes (sometimes known as buckyballs) are known to be produced in appreciable quantities via graphite

vaporization with a laser, or by resistive heating of graphite, and in toluene or carbon disulfide soxlet extracts of carbon soot materials.[1,3,23-24,26-28] Approximately 85% of the fullerene material produced is C_{60} and 10% is C_{70}. Higher fullerenes are only 3-5 % by weight of the mixture. This percentage may vary significantly with different fullerene production and extraction methods.

Fullerenes are known to be most soluble in chlorinated benzenes, carbon disulfide, toluene, methylene chloride, and chloroform. Only 0.043 mg/ml of C_{60} can be dissolved in n-hexane and 0.00 mg/ml in methanol and most other polar solvents.[29] These solvents are frequently used as mobile phases in HPLC, therefore, the solubility limitation is a problem. Even toluene can solublize only 2.8 mg/ml.[29] Ruoff et. al. has determined the room temperature solubility of C_{60} as a function of solvent properties (i.e., refractive index, dielectric constant, Hildebrand solubility parameter, molecular size, and hydrogen bonding strength) in over 46 solvents using calibrated HPLC.[29] Although both polar and non-polar stationary phases have been used to separate fullerenes, the mobile phases are fairly limited to the less polar solvents because of the solubility properties of fullerenes. So, even though a reversed phase stationary phase may be used for a separation, typical reversed phase mobile phases (hydro-organic solvents) cannot be used.

Numerous chromatographic techniques for the purification of fullerenes as well as several different stationary phases have been evaluated. The first chromatographic fractionations were achieved by classic column chromatography utilizing neutral alumina and silica gel, respectively.[1-5] A simple method incorporating soxlet extraction and liquid chromatography was evaluated, however, it was found to be impractical

since the recovery yields were quite low.[30-31] Other stationary phases utilized without much success were the graphite, polystyrene gel, and other gel permeation stationary phases.[32-34]

The first aromatic stationary phase used to separate fullerenes was the 3,5-dinitrobenzoylphenylglycine (DNBPG) column. Hawkins and co-workers believed the support's π-acidic dinitrobenzamide groups would interact with the π-basic groups of the aromatic "soccerball-like" structures.[10] Up to 0.5 mg of C_{60} and C_{70} fullerenes were baseline resolved in under 30 minutes on this semi-preparative sized chiral stationary phase. This was the first report of a stationary phase that could purify milligram quantities as opposed to the lower levels reported previously. As a result, it was suggested that the aromatic phases were more efficient at separating fullerenes. This assumption remained unchallenged while other charge-transfer phases were utilized for these separations.[11] In 1991, Cox et. al. studied the retention mechanism of C_{60} and C_{70} using dinitroanilinopropyl (DNAP) silica.[12] They found that the retention of C_{60} and C_{70} resembled that of flat planar polyaromatic hydrocarbons (PAH's) such as tyriphenylene and benzo[a]pyrene, respectively. The successful use of the phenylglycine based stationary phase as well as the DNAP stationary phase led Jinno and co-workers to synthesize a "multi-legged" phenyl phase bonded to silica gel.[18] Isolation of C_{60} and C_{70} was achieved using capillary liquid chromatography, however, this method is not suitable for large scale purifications.[18]

In 1991, Diederich et. al. were the first to isolate higher fullerenes using column chromatography to fractionate and collect C_{60}, C_{70}, and a higher fullerene sample. The higher fullerene fraction was then reinjected onto a silica-gel semi-preparative support to further separate the higher

fullerene mixture by HPLC utilizing a mass spectrometer for detection.[4] Information on C_{76}, C_{84}, C_{90}, C_{94}, and a stable oxide of D_5h-C_{70} was found. Later that year, Diederich was the first to chromatographically isolate an additional isomer of C_{78} on a Vydac C_{18} phase.[6] The direct injection of fullerene soot on semi-preparative and eventually analytical C_{18} phases followed.[7-8,15] Baseline resolution of C_{60}, C_{70}, and a few higher fullerenes was observed.

A preparative recycling method was proposed by Kikuchi et. al. which isolated higher fullerenes previously reported as well as two additional members of the fullerene family.[35] Small portions of C_{82} and C_{96} were found utilizing preparative HPLC with CS_2 as a mobile phase. This repetitive method took 6 cycles to partially resolve C_{60} and C_{70} and 30 cycles for the partial separation of the higher fullerenes.[35] This technique was very tedious and time consuming and therefore not applicable to large scale purification.

Welch and Pirkle, in 1992, investigated a few commercially available π-basic stationary phases and synthesized several other π-acidic phases for fullerene as well as PAH recognition.[19] Altogether, ten phases were analyzed. The tripodal ligand phase, known as the Buckyclutcher I stationary phase, provided the highest selectivity compared to any of the other phases analyzed.

Recently, other charge-transfer phases were investigated. A semi-preparative tetrachlorophthalimidopropyl-modified silica column (TCPP) separated a 1 mg mixture of fullerenes, baseline resolved a few higher fullerenes, and provided an isomeric separation of C_{78}.[17] Diack, Compton, and Guiochon reported that the 2-(2,4,5,7-tetranitro-9-fluorenylideneamino-oxy)propionic acid (TAPA) chiral stationary phase was a stronger electron acceptor than the DNBPG phase.[14] C_{60} and C_{70} were baseline resolved and

these studies indicated the PAHs were stronger electron donors than the fullerenes. Furthermore, a dynamic temperature study involving van't Hoff plots proved that increasing the temperature of the TAPA column resulted in an increase in retention.[14] Therefore, fullerene adsorption on this stationary phase is entropy driven and endothermic. This trend is unusual in classical chromatography.[14] These results were in agreement with those of Pirkle and Welch regarding their DNBPG phase.[12] In 1993, Kibbey and co-workers, synthesized several metalated and unmetalated tetraphenyl-porphyrin- (TPP-) stationary phases for the separation of C_{60} and C_{70} fullerenes.[9] Highly selective separations were acheived using neat toluene as the mobile phase.

Another chiral stationary phase used in the separation of fullerenes is the γ-cyclodextrin (γ-CD) chemically bonded to silica. γ-cyclodextrin is made up of eight D-glucose molecules and is a cyclic oligosaccharide composed of glucopyranose units bonded through α-(1,4)-linkages. The hydrophobic interior cavity is approximately 10 angstroms in diameter and is known to form inclusion complexes with various structural compounds. C_{60} is known to be approximately 12 angstroms and cannot be totally included inside the cyclodextrin cavity. Although we were unable to separate any fullerenes on the native γ-cyclodextrin, Cabrera et. al. reported the isolation of C_{60} and C_{70} in neat hexane and in hexane/toluene.[16] It was claimed that partial inclusion and various interactions at the mouth of the cavity provided retention and isolation of C_{60} and C_{70}. We were able to obtain extremely efficient fullerene separations using chiral and non-chiral derivatized cyclodextrin bonded stationary phases. The multi-modal (R)- and (S)-naphthylethylcarbamate-β-cyclodextrin (RN-β-CD and SN-β-CD) and the 3,5-dimethylphenyl-β-cyclodextrin stationary phase baseline resolved C_{60}, C_{70}, (and higher

fullerenes) C_{76}, C_{78}, and C_{84}.[13] The RN-β-CD phase was eliminated from our study because of the high cost of chiral phases and the wide variety of other successful buckyball stationary phases available. Most of the chiral stationary phases previously mentioned (i.e., γ-CD, RN-β-CD, DNBPG, TAPA) offer a high degree of selectivity, however, none of them are considered effective preparative purification methods because of the high cost and small conversion yield.

Many of the aforementioned stationary phases were not significantly, if any, better than the separations achieved on various conventional C_{18} supports. It is well known that many variations exist among octadecylsilica (ODS) phases (i.e., monomeric, polymeric, encapped, etc.). The earlier success of these stationary phases led to a comparison study involving several C_{18} stationary phases. Two groups simultaneously studied the differences between monomeric and polymeric ODS solid supports.[7-8] Jinno's results were in agreement with those of Anacleto and Quilliam. They determined that the polymeric ODS phases were better stationary phases for distinguishing between geometric isomers while the monomeric phases were better in distinguishing differences in fullerenes based upon carbon number or molecular weight. Jinno also showed that one C_{78} isomer eluted before C_{76} and in turn described a molecular shape dependence.[8] This was the only report concerning the elution of a higher molecular weight fullerene prior to a smaller one.

Recently, we evaluated a variety of different, organic, biphasic solvent systems to be used in conjunction with centrifugal partition chromatography (CPC) for the preparative fractionation of fullerenes.[36] This method was found to purify approximately 100 times the amount of fullerenes (per batch) as compared to previously reported HPLC methods. Preparative separations of fullerenes are obviously important. Presently,

fullerenes are being used as lubricants and some are known to have superconducting properties at 18-28 ^{o} K when complexed with certain metals.[37-40] Various research groups have demonstrated that C_{60} may also have possible biological activity.[41-42] Computer modeling has shown that the water soluble derivative of C_{60} may have use in blocking the active site of the open-ended cylinder of the HIV protease.[41-42]

In this study, we compare the selectivity, efficiency, resolution, loadability, column deterioration, and higher fullerene separation ability of various commercially available aliphatic and aromatic stationary phases. Fullerene samples were obtained from various sources and include both toluene and carbon disulfide extracts of fullerene soot materials. In addition to comparing the various fullerene separation methods, we also evaluated commercially available fullerene samples for purity.

EXPERIMENTAL

Chemicals. All solvents used were of HPLC grade and obtained from Fisher Scientific (St. Louis, MO). Pure fullerene standards of C_{60}, C_{70}, and C_{84} were purchased from either Polygon Enterprise (Waco, TX), MER Corporation (Tuscon, AZ), Fluka (Ronkonkoma, NY), or Texas Fullerene (Houston, TX). All other fullerene samples were provided by IBM at the Almaden Research Center (San Jose, CA).

Instrumentation. The chromatographic studies were performed using the following Shimadzu (Columbia, MD) equipment: a LC-6A pump, a SCL-6B system controller, a SPD-6AV UV/VIS variable wavelength spectrophotometric detector, and a CR601 chromatopac integrator. The system also included a Rheodyne (model 7125) injector.

Stationary Phases. A total of nine chromatographic stationary phases were evaluated and include the following: native γ-cyclodextrin (γ-CD), (R)-naphthylethylcarbamate-β-cyclodextrin (RN-β-CD), (S)-naphthylethylcarbamate-β-cyclodextrin (SN-β-CD), high loading (3,5-dimethylphenyl)-β-cyclodextrin (DMP-β-CD), low loading (DMP-β-CD), reverse-phase C_{18}, Buckyclutcher I, Buckysep-RP, and ChromSpher Fullerene. All stationary phases consist of 5 μm diameter particles while column dimensions were 250 x 4.6 mm. The high and low loading (DMP-β-CD), the (RN-β-CD), the (SN-β-CD), and the Astec C_{18} (monomeric-encapped ODS) were donated by Advanced Separations Technology Inc. (Whippany, NJ). Typical eluents for these columns mainly consisted of acetonitrile and added interchangeably as modifiers were methylene chloride, chloroform, and toluene. The Buckyclutcher I stationary phase was purchased from Regis Chemical Company (Morton Grove, IL) and evaluated with the recommended hexane/toluene mixture as a mobile phase. A detailed description of the structure and preparation procedures of the aromatic Buckyclutcher I stationary phase is described elsewhere.[19] Phenomenex (Torrance, CA) supplied the Buckysep-RP alkyl column and recommended an acetonitrile and toluene mixture as the mobile phase. The aromatic ChromSpher Fullerene packing was purchased from Chrompak (Raritan, NJ) and separation was achieved using the recommended combination of isooctane and toluene. Analysis on all columns occurred at 1 ml/min with the exception of the ChromSpher packing which was used at 3 ml/min as recommended by the company.

The C_{18} stationary phase as well as the Buckysep-RP column consisted of an alkyl chain bonded stationary phase while the packing of all other columns contained bonded aromatic functional groups. All fullerene

samples were dissolved in toluene. The solution concentrations of fullerenes used for analytical separations were 0.10 mg/ml, while preparative separation studies used solutions of 5 mg/ml. Between 2 and 500 µl of the analyte was directly injected to the column depending on the type of study being done. Because the mobile phase composition plays a vital role in the retention of fullerenes and different mobile phases were used with each stationary phase, all columns were compared by appropriately adjusting the eluents so that comparable k' values were obtained. All other chromatographic parameters (i.e., absorbance, attenuation, chart speed, etc.) were held constant.

RESULTS AND DISCUSSION

The solvent systems used to separate fullerenes on an analytical scale are generally very different from those that are used to separate fullerenes on a preparative basis.[9,13-14,17] Fullerenes are most soluble in carbon disulfide and in chlorinated benzenes, however, the odor, volatility, viscosity, and toxicity limits the use of these solvents in HPLC. Two component solvent systems used as mobile phases in the separation of fullerenes usually combine a "good" and/or "moderate" fullerene solvent with a "poor" fullerene solvent. C_{60}, for example, is not soluble in "poor" solvents such as acetonitrile, methanol, ethanol, isopropanol, etc.[29] Examples of "good" fullerene solvents are toluene and chloroform which dissolves 2.8 mg/ml and 0.16 mg/ml, respectively.[29] Moderate fullerene solvents such as hexane (dissolving 0.043 mg/ml C_{60}) and isooctane have intermediate solubility properties.[29] While the distinction (i.e., the dividing line) between "good" and "moderate" fullerene solvents is somewhat subjective, there is little question as to the negligible solublizing properties

of "poor" fullerene solvents. Interestingly, this approach of using "good/poor" solvent combinations is somewhat analogous to a technique developed several years ago for the LC and TLC fractionation of polymers by molecular weight.[43-47] Varying the amount of the "poor" fullerene solvent in the system enables one to control retention and resolution (see Figure 1). As the proportion of the "poor" fullerene solvent in the mobile phase increases, C_{60} and C_{70} are retained longer and the resolution increases. Our results are in good agreement with those reported by Kibbey, Guiochon, and Herren on other stationary phases.[9,13-14,17] When too much of a "poor" fullerene solvent is used or too high a concentration of fullerenes is injected, some fullerenes come out of solution or phase separate until enough solvent passes through to re-elute them. When this phenomenon occurs, double peaks are formed. This "peak doubling" effect occurred with every column tested when the fullerene solubility limit in the mobile phase was reached. The peak doubling effect is shown in Figure 2.

By in large, fullerene separations appear to be more affected by the solvent composition of the mobile phase than by stationary phase chemistry. Regardless of which stationary phase (i.e., non-polar, aliphatic reversed phase, and aromatic reversed phase) or mobile phase is used the same retention order for fullerenes is obtained. However, every stationary phase seems to have a different optimum mobile phase composition. The solvent compositions of the Astec C_{18} and the Buckysep-RP stationary phases were the most similar. Both stationary phases used similar mobile phase ratios (within 5 %) of acetonitrile mixed with toluene.

The HPLC separation of C_{60} and C_{70} has become by far the most widely studied and reported fullerene analytical methodology. Most stationary phases baseline resolve C_{60} and C_{70} provided the optimum mobile

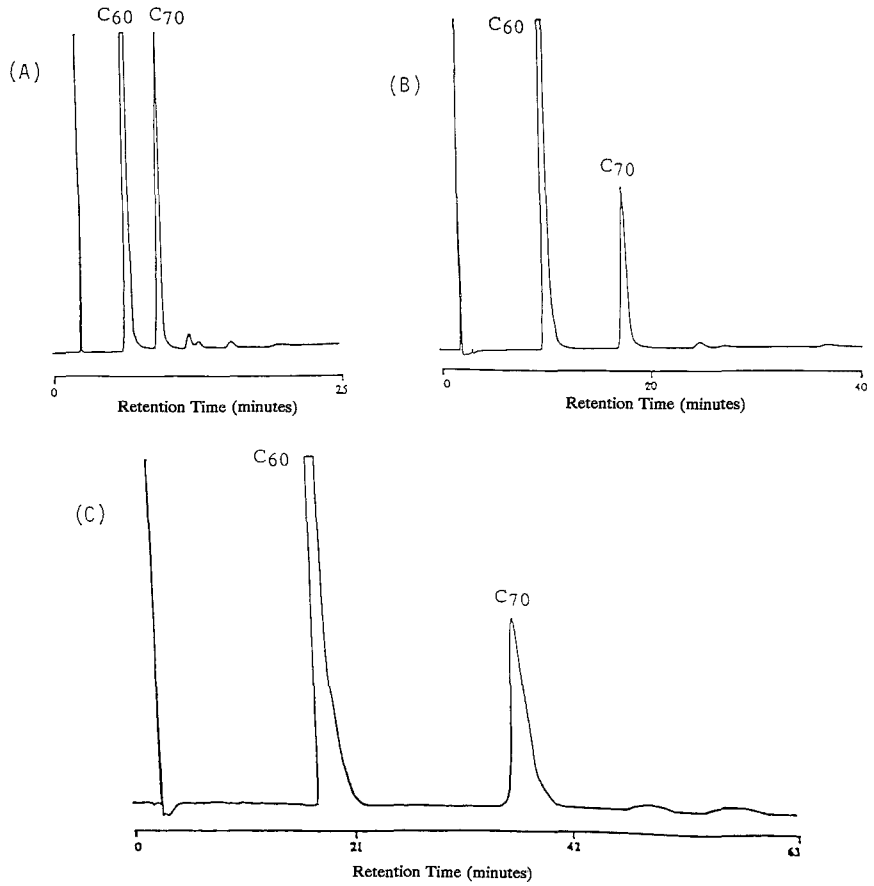


Figure 1. Chromatograms showing how the retention and selectivity can be controlled by altering the composition of the "poor" fullerene solvent. All three separations were performed on a high loading DMP-β-CD column and eluted with a acetonitrile/toluene mixture. Chromatogram (A) used a 60:40 mixture, chromatogram (B) a 70:30 mixture, and chromatogram (C) was eluted with a 80:20 ratio of acetonitrile/toluene (v/v). Separations were carried out at 1 ml/min and a 310 nm.

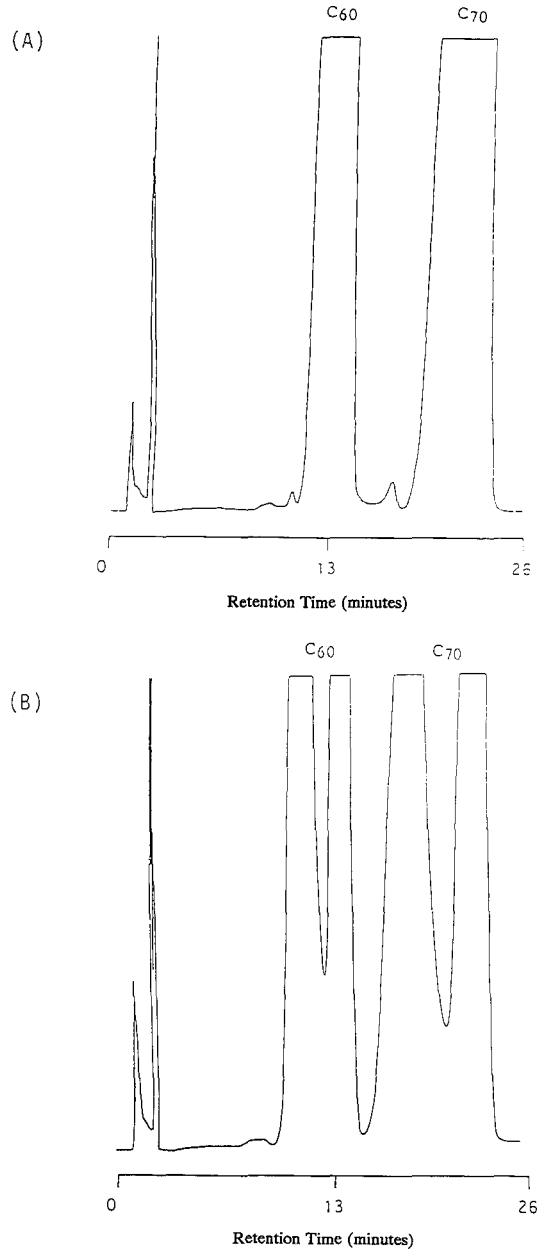


Figure 2. Representative chromatograms showing the "peak doubling" phenomena on an aliphatic C_{18} stationary phase. 0.30 mg of C_{60} and C_{70} was separated in chromatogram (A) and 0.40 mg was isolated in (B). Separations were achieved with a 45:55 ratio of acetonitrile/toluene (v/v) at 1 ml/min and at 310 nm.

phase is used. Without proper analytical methodology, the ability to ascertain the exact composition and purity of the fullerene soot starting material would be limited. The determination and quantitation of higher fullerenes (i.e. C_{76}, C_{78}, C_{84}, etc.) would be difficult and the exact purity would have to be determined by other instrumental techniques. Figure 3 shows the analytical separation of commercially available "pure" samples of C_{70} and C_{84}. In chromatogram A, small portions of C_{60} are found in the "pure" C_{70} sample. The sample in chromatogram B ("pure" C_{84}) shows significant levels of C_{60}, C_{70}, C_{76}, and C_{78}. To stress the importance of the analytical methodology, Table 1 shows the exact purity obtained in a few commercial fullerene standards. MER Corporation produced the purest C_{60} sample (99.9 %) while the Polygon Enterprise C_{70} sample was only 94.9 % pure. At the time of this study, Polygon Enterprise was the only company producing C_{84}.

Columns that are good for analytical separations are not necessarily good on a preparative basis. Although several other stationary phases were examined (See Experimental), Tables 2 and 3 show the results of loading studies for the two best analytical stationary phases and the two best preparative stationary phases, respectively. The optimum analytical stationary phases were the n-alkyl Astec C_{18} and the Buckysep-RP. Note that both of these stationary phases have surface bonded alkyl chains. It is evident from the data in Table 2 that good efficiency, selectivity, and resolution was observed with both of these phases when up to 0.30 mg mixtures of C_{60} and C_{70} fullerenes was injected. When the concentration of fullerenes injected exceeded 0.3 mg on the C_{18} column, "peak doubling" occurred (see Figure 2) as a result the solubility limitation of fullerenes in these solvent systems. The same results occurred on all of the other alkyl chain stationary phases evaluated.

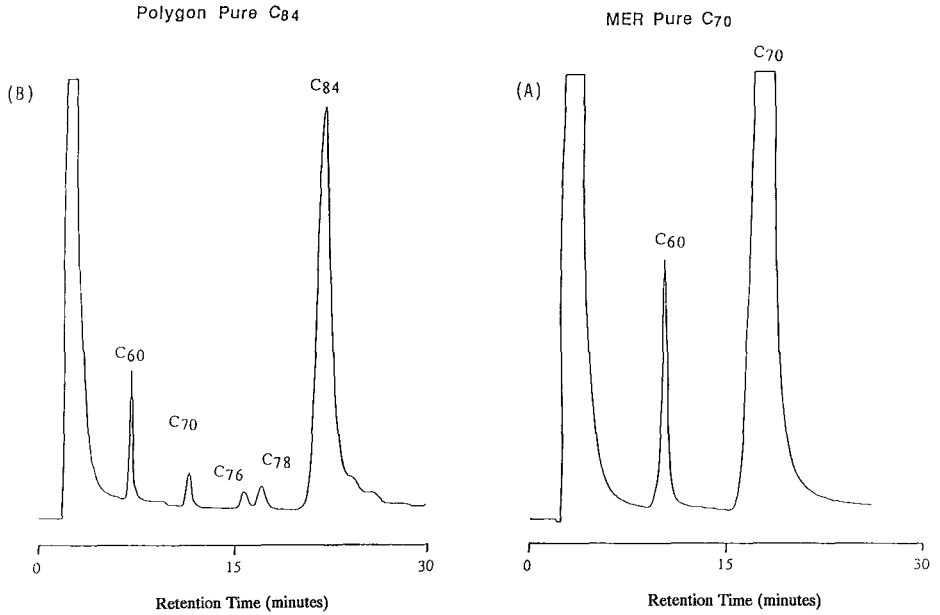


Figure 3. Liquid chromatographic separations of pure fullerene standards obtained from two commercial companies. A high loading DMP-β-CD column was used in (A) and a low loading DMP-β-CD column in (B). The mobile phase in (A) consisted of 70:30 acetonitrile/chloroform mixture (v/v). A 60:40 combination of acetonitrile/chloroform (v/v) was used as the eluent for chromatogram (B). Both separations were performed at a flowrate of 1 ml/min and at 254 nm. Chromatogram (A) (MER Corporation pure C_{70}) has a detectable level of C_{60}. The pure sample of C_{84} (Polygon Enterprise) has disclosed levels of C_{60}, C_{70}, C_{76}, and C_{78}.

Table 1. % Purity of Commercial Fullerene Standards [a,b]

Company	C_{60}	C_{70}	C_{84}
MER Corporation	99.9[c]	98.6[d]	-
Polygon Enterprise	98.9[c]	94.9[d]	97.8[e]
Texas Fullerene	99.3[c]	97.2[d]	-
Fluka	99.7[c]	98.4[d]	-

[a] The pure fullerene standards may differ slightly on a batch to batch basis
[b] The percent purity was quantitated by determining C_{60}, C_{70}, and C_{84} extinction coefficients at 254 nm and 310 nm using a UV/Vis Spectrometer and Beer's law
[c] The chief contaminating homologue is C_{70}
[d] The chief contaminating homologue is C_{60}
[e] The chief contaminating homologues are C_{60}, C_{70}, C_{76}, and C_{78}

As can be seen by the data in Table 3, the aromatic ChromSpher Fullerene and Buckyclutcher I stationary phases were the two best columns for separating fullerenes on a preparative basis. Approximately 2.5 mg of C_{60} and C_{70} was baseline resolved on both stationary phases. Unlike conventional HPLC, the efficiency of the ChromSpher column appeared to increase when greater sample quantities were injected. The chromatogram in Figure 4 shows the difference in the analytical and preparative separation of C_{60} and C_{70} on the ChromSpher column. Although the efficiency of a small 0.1 mg injection was fairly poor compared to the best analytical separations (Table 2 and Figure 1), there was little or no decrease in the efficiency and resolution when a larger

Table 2. Optimum Analytical Separation of C_{60} and C_{70}

Column: Astec C_{18}	Mobile Phase Conditions: 55% Toluene / 45% MeCN			
amount injected (mg)	k_1'	α	Rs	n
0.01	3.80	1.68	10.07	8464
0.05	3.81	1.63	7.82	4975
0.08	3.82	1.64	6.72	3857
0.10	3.83	1.64	6.00	2820
0.20	3.41	1.69	4.04	1244
0.30	3.43	1.72	3.47	872

Column: Buckysep-RP	Mobile Phase Conditions: 60% Toluene / 40% MeCN			
amount injected (mg)	k_1'	α	Rs	n
0.01	3.10	1.73	8.86	6099
0.05	3.25	1.71	7.86	3686
0.08	3.28	1.73	7.68	3257
0.10	3.44	1.72	7.54	2869
0.20	3.50	1.75	6.44	2244
0.30	3.95	1.72	2.27	644

sample (1 mg) was injected. Nevertheless, 3 mg of C_{60} and C_{70} fullerenes caused the peaks to begin to split on the Buckyclutcher I phase and 3.5 mg caused "peak doubling" on the ChromSpher packing. For all columns, it appears that the loading ability is mainly a function of how much C_{60} and C_{70} fullerenes can be dissolved in the mobile phase. The ChromSpher fullerene column was the "best" column examined for these separations.

Table 3. Preparative Separations of C_{60} and C_{70}

Column: Buckyclutcher I Mobile Phase Conditions: 65% Hexane / 35 % Toluene				
amount injected (mg)	k_1'	α	Rs	n
0.05	3.85	2.69	5.30	990
0.10	3.63	2.64	4.85	812
0.20	3.67	2.67	4.54	580
0.30	3.97	2.89	4.21	576
0.40	3.71	2.67	3.93	355
0.50	3.17	2.60	3.36	204
1.0	1.78	2.52	3.09	118
2.5	1.47	1.60	2.71	21

Column: ChromSpher Fullerene Mobile Phase Composition: 95% Isooctane / 5% IPA				
amount injected (mg)	k_1'	α	Rs	n
0.05	4.70	3.20	3.72	297
0.10	4.51	3.14	3.70	276
0.20	4.45	3.02	3.77	269
0.30	4.35	2.97	3.80	256
0.40	4.21	3.01	3.76	180
0.50	4.04	2.88	3.78	112
1.0	3.87	2.75	3.75	107
2.5	3.23	2.42	3.70	89

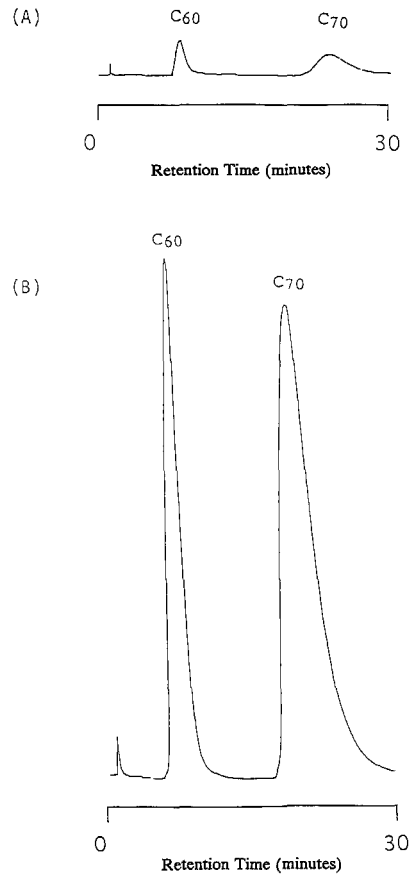
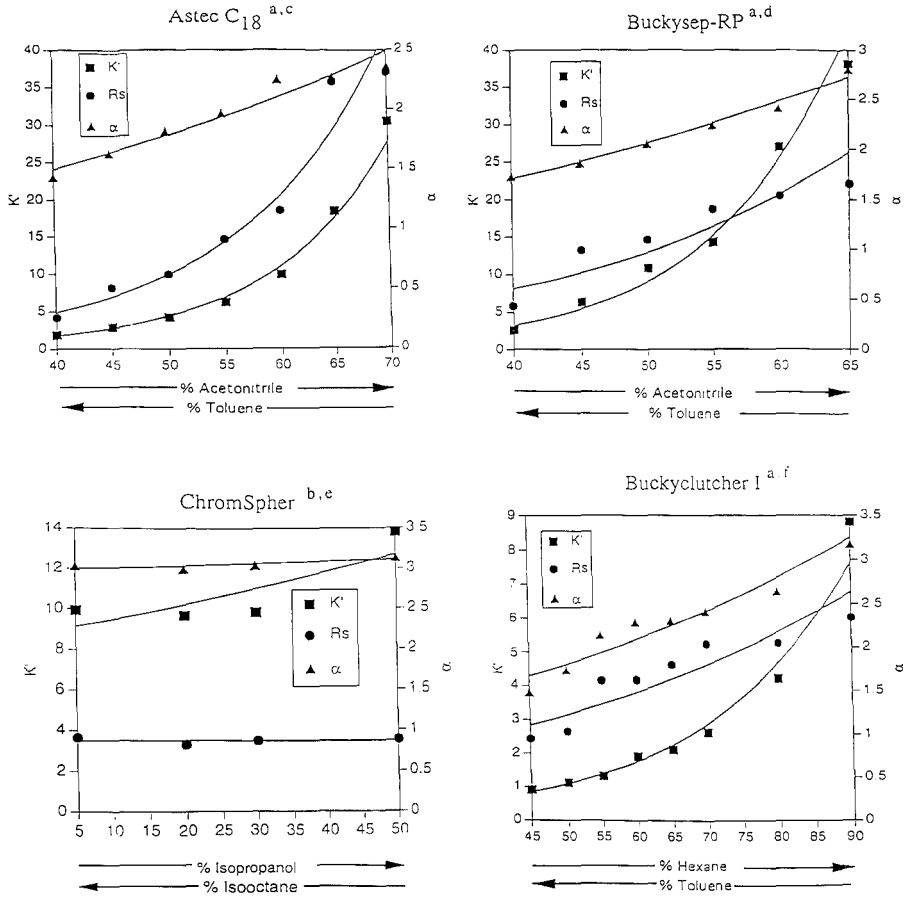


Figure 4. Chromatograms showing how the efficiency and resolution of was only slightly effected when the concentration of the fullerenes sample increased on the ChromSpher fullerene stationary phase. In (A), 0.1 mg of C_{60} and C_{70} was separated in under 30 minutes. In chromatogram (B), the concentration was increased and 1 mg of C_{60} and C_{70} was separated. Separations were carried out at 3.0 ml/min with a 95:5 isooctane/ isopropanol eluent (v/v). Detection was set at 310 nm.

The mobile phase used was a 95:5 (v/v) mixture of isooctane/isopropanol. Neither of these solvents alone would be categorized as "good" fullerene solvents. It is possible that more fullerenes can be dissolved in the two component isooctane/isopropanol solvent mixture than can be dissolved in either neat solvent.

Figure 5 shows that the retention time, selectivity, and resolution of fullerenes usually can be manipulated by controlling the mobile phase composition.[13-14] The ChromSpher column was unusual in that it produced the least variation in these parameters when the mobile phase composition was changed. Even the resolution and α value remained relatively constant at all solvent compositions (Figure 5). The retention, resolution, and selectivity increased with increasing proportions of the "poor" fullerene solvent for all other columns. Clearly the mobile phase composition affects retention, selectivity, and analyte mass load (via solubility). Unfortunately, there is usually a trade-off between selectivity and loadability in these systems. Higher proportions of the "poorer" fullerene solvent tend to increase the peak to peak distances often producing exceptional analytical separations on high efficiency columns (Figure 5). However, these mobile phase mixtures cannot solubilize enough of the fullerenes to make production scale separations practical. Columns that use better fullerenes solvents (or solvent ratios) often produce poorer analytical separation because of lower efficiency and selectivity. However, the analyte mass on these columns can be increased substantially before peak splitting occurs and with much less deterioration in the efficiency (Figure 5). Hence they tend to be superior for preparative separations. It should be noted that the optimum fullerene mobile phase for one type of column does not usually produce the best results on another column. This



[a] Separations were carried out at 1.0 ml/min flowrate.
[b] Separations were carried out at 3.0 ml/min flowrate.
[c] The recommended mobile phase compostions were between 60:40 acetonitrile/toluene (v/v) and 40:60 acetonitrile/toluene (v/v).
[d] The recommended mobile phase compostion was 50:50 acetonitrile/toluene (v/v)
[e] The recommended mobile phase composition was 95:5 isooctane/isopropanol (v/v)
[f] The recommended mobile phase composition was 50:50 hexane/toluene (v/v)

Figure 5. Multiple graphs showing how the change in mobile phase composition altered the selectivity (α), capacity factor (k'), and the resolution (Rs) on the C_{18}, Buckysep-RP, ChromSpher Fullerene, and Buckyclutcher I stationary phases. Mobile phase solvents used in this study were recommended by the respective companies. An exponential curve fitting program was used on all graphs according to the following equation: f(x) = 9.284512E-2*exp(4.897805E-2*x).

is particularly true when going from an aliphatic-type bonded stationary phase to an aromatic-type and vice versa.

Another problem in comparing fullerene separations reported in the literature is that the authors use different fullerene preparations and different detection methods. It is possible that some reports of certain columns being superior to others (because of their ability to separate one or another small fullerene component and/or isomers) may in fact be the result of the sample composition and/or detection sensitivity. The problem of sample composition and consistency is illustrated in Figure 6. The first chromatogram shows the separation of a toluene extract of fullerenes, the second is a carbon disulfide extract of the same starting material, and the third chromatogram shows the separation of a sample of scandium complexed metallofullerenes. Note that there are different minor peak components in these chromatograms. Clearly both the presence and proportion of many peaks are dependent on the manufacturing and/or extraction process (Figure 6). It is also apparent that there are many additional smaller peaks (Figure 6 C) that can be seen if sensitive detection methods are used. Hence the ability to find many of the minor components and later eluting "higher fullerenes" may be as much of a detection problem as a separation problem.

Some recent papers have focused on higher fullerenes. Peters and Jansen reported a new method of synthesis which produces greater concentrations of higher fullerenes than the contact arc method.[48] Figure 7 shows the higher fullerene separation obtained with our higher fullerene sample on two aliphatic and two aromatic stationary phases. Clearly the Buckysep-RP and the Astec C_{18} phases baseline resolved C_{76}, C_{78}, and C_{84}. The poor resolution of higher fullerenes on the aromatic phases limits the possibility of using these stationary phases to the purify large quantities of

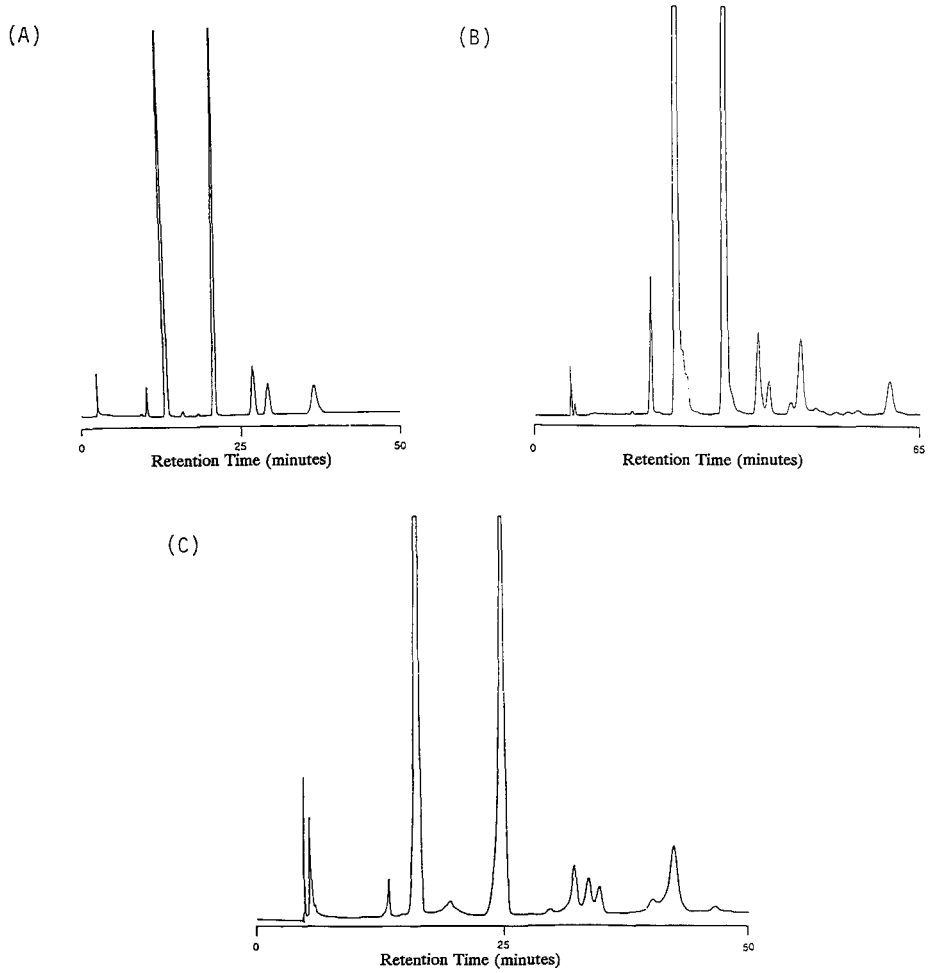


Figure 6. Multiple chromatograms showing the changes in the higher fullerene (C_{76} and greater) ratio produced when different production and extraction method are used. All fullerene samples were analyzed on the Astec C_{18} stationary phase. Chromatogram (A) is a toluene extract evaluated with a 50:50 acetonitrile/toluene mixture (v/v) while chromatogram (B) is a carbon disulfide extract of fullerenes examined with a 55:45 ratio of acetonitrile/toluene (v/v). Chromatogram (C) is a scandium complexed metallo-fullerene sample analyzed with a 50:50 acetonitrile/toluene mixture (v/v).

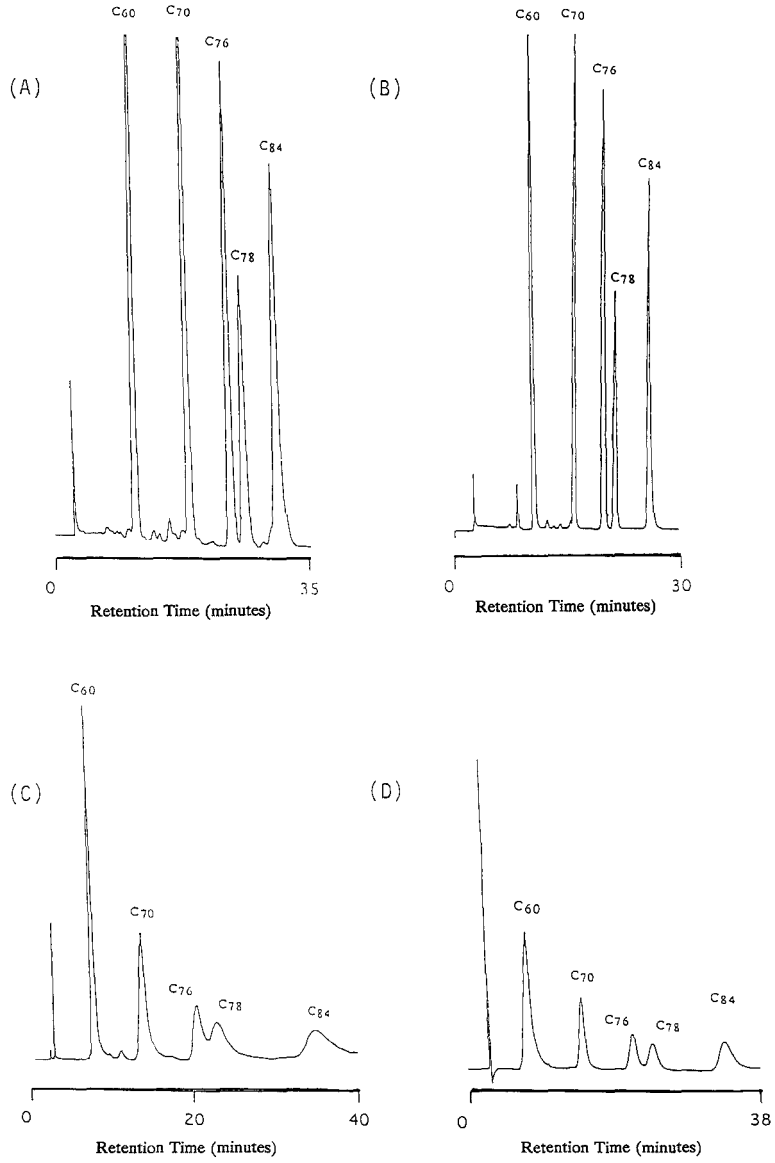


Figure 7. Chromatograms showing the higher fullerene separation on (A) Buckysep-RP, (B) Astec C_{18}, (C) Buckyclutcher I, and (D) 3,5-dimethylphenyl-β-cyclodextrin stationary phases. Clearly, the alkyl phases baseline resolved the higher fullerenes. Different solvent ratios were used to obtain similar k' values. All separations were carried out a 1 ml/min and at 310 nm.

higher fullerenes. The ChromSpher column is not featured in this study because after pumping the recommended 95:5 isooctane/isopropanol (v/v) at 3.0 ml/min, the higher fullerenes were irreversibly retained making this stationary phase a poor choice for the isolation of higher fullerenes. Possibly a solvent system other than the one recommended by Chrompak would elute fullerenes in reasonable time span. Another alternative is to increase the column temperature. Chrompak has shown the partial resolution of some higher fullerenes at 40 °C.[20] In this case, the fullerene adsorption appears to be similar to that of more conventional chromatographic separations which are enthalpy driven and exothermic.

Several isomeric forms of some of the higher fullerene allotropes have been reported.[2] In 1991, Diederich and coworkers reported the first chromatographic isomeric separation of C_{78}.[4,6] With the higher fullerene sample discussed above, no isomeric separation was obtained on any of the columns analyzed. The Buckyclutcher I phase has been said to separate specific isomers in a previous publication[22], however, in our hands, no isomeric separations were found. Of course it is not known if any isomers were present in this sample to begin with or whether they were at high enough levels for our detection method.

As yet there have been no significant published discussions on the deterioration or changes in HPLC columns used for the separation of fullerenes over a period of time. After several months of daily use, all columns investigated in this study deteriorated. It should be noted that for any fullerene separations with any stationary and mobile phase combination, there always seems to be irreversible adsorption to the column. Consequently, retention characteristics of all columns used in fullerene separation change with time. We have found that the retention, selectivity, efficiency, and loadability of every column tested in this study

became worse with time. When doing analytical separations, these parameters decrease slowly (after 100-500 injections). However, when doing loading studies, larger amounts of fullerenes are injected and the separation properties degraded after relatively few injections. It is not known whether the fullerenes themselves, their degradation products, coextracted contaminants or some combination of these three things are irreversibly binding to the columns and degrading the separation. However this does occur on all columns and with all mobile phases tested.

CONCLUSIONS

In general, aliphatic stationary phases separated C_{60} and C_{70} fullerenes better on an analytical scale while the aromatic stationary phases were more effective at purifying larger quantities of these fullerenes. The optimum solvent conditions for one column are not the same for another. The optimum mobile phases used with the aromatic stationary phases tended to solubilize greater quantities of fullerenes while the optimum mobile phases for the alkyl stationary phases did not solubilize a great quantity of fullerenes. The aliphatic-type bonded stationary phases produced analytical fullerene separations of greater efficiency, shorter retention times, better sensitivity, and greater resolution for a larger range of fullerenes. Higher molecular weight fullerenes were baseline resolved on the alkyl stationary phases but not always on the aromatic phases. Therefore, a good technique to purify large quantities of higher fullerenes is still needed. Increasing the percentage of "poor" fullerene solvent in the mobile phase tends to enhance retention and selectivity. However, it decreases the amount of fullerenes that can be dissolved in the mobile phase which detracts from preparative separations. The retention order of

the fullerenes is similar on all columns and with all mobile phases. The separation performance of all columns degrade with time due to irreversible adsorption of fullerenes and/or their associated contaminants.

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IDENTIFICATION OF HEMORPHINS FROM BOVINE HEMOGLOBIN HYDROLYSATE: APPLICATION OF UV SECOND ORDER DERIVATIVE SPECTROSCOPY

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ABSTRACT

Aromatic amino acids have very informative second order derivative spectra. Whereas they exhibit overlapping maxima between 250 and 300nm in the zero order spectra, thin minima are obtained in their second order derivative spectra. This feature allowed to develop a method to identify aromatic amino acids, but also to calculate the ratio between these amino acids in peptides and proteins. This method has been used successfully for the detection of hemorphins in a peptic bovine hemoglobin hydrolysate. The constant ratios between aromatic amino acids are an important characteristic of lots of bioactive peptides; the advantage of this spectral method is to be non-destructive for the identification of these amino acids espacially for tryptophan.

INTRODUCTION

HPLC combined with sensitive and selective detection has become a widely used technique for both purification and identification of compounds [1, 2]. The advent of ultraviolet-visible (UV-VIS) diode-array detection system in HPLC has made it possible not only to perform on-the-fly real-time scanning but also to

obtain their derivative spectra at the same time [3, 4]. The UV spectra of proteins and peptides are greatly dependent on their amino acids contents [5, 6]. Amino acids have very uninformative spectra except for the aromatics. Only phenylalanine, tyrosine and tryptophan exhibit absorption maxima between 250 and 300 nm. Nevertheless their UV-spectra are broad and they overlap. As a consequence, trying to use the unenhanced spectrum of a protein to determine the aromatic amino acids contents becomes very difficult. To estimate the relative ratio of these aromatic amino acids becomes almost impossible. The derivative spectroscopy, firstly developped by Savitzky and Goloy [7], offers the advantage of sharper spectral features when compared to conventional absorbance spectroscopy. For instance, a peak shoulder present in a zero order derivative spectrum can be transformed into a peak minimum when the second order derivative spectrum is obtained. Furthermore, overlapping bands can be transformed into resolved bands. Second order derivatives are usually performed instead of higher orders derivatives since they represent a compromise between selectivity (intensity of absorption minima and maxima increase with each derivative) and interference from false absorption (due to noise factors) [5]. So the derivative spectroscopy has become a revival as an analytical tool to identify and quantify aromatic amino acids in proteins and peptides [8, 9, 10].

We have previously reported isolation of two opioid peptides, LVV-hemorphin-7 and VV-hemorphin-7 from a very complex bovine hemoglobin peptic hydrolysate [11]. These peptides and those obtained either from bovine or human beta-chain of hemoglobin [12] contain important amounts of aromatic amino acids. The constant ratios between aromatic amino acids are an important characteristic of hemorphins. So these peptides provided us defined patterns to investigate second derivative spectra, and led us to develop a simple method for qualitative and quantitative determination of aromatic amino acids. This determination was efficient to improve the identification of hemorphins from a bovine hemoglobin peptic hydrolysate by UV-spectra comparison [13] and should be easily applied in peptide and protein hydrolysate research.

EXPERIMENTAL

MATERIALS AND CHEMICALS

All common chemicals and solvents were of analytical grade from commercial sources. Tryptophan, tyrosine, phenylalanine, pepsin and rabbit lung

angiotensin converting enzyme (ACE) was purchased from Sigma Chemicals. Tuna myoglobin, cutinase and peptide GI were kindly provided by the colleagues of our laboratory. Hemorphin-7 was synthesized by C.Guillon, Laboratoire de Technologie Enzymatique, University of Compiègne, Compiègne, France

Bovine hemoglobin Hydrolysate, LVV-hemorphin-7 and VV-hemorphin-7

Bovine hemoglobin hydrolysate was obtained at pilot-plant scale by peptic proteolysis in an ultrafiltration reactor as previously described [14]. Active fraction FVII was prepared by gel permeation HPLC using TSK G2000 SWG column (19mm i.d. X 600 mm) and analyzed by reversed phase (RP) HPLC in order to obtain LVV-hemorphin-7 and VV-hemorphin-7 [11].

LVV-hemorphin-5, VV-hemorphin-5 and dipeptide Arg-Phe

According to LANTZ [15], 1 mg of either VV-hemorphin-7 or LVV-hemorphin-7 were dissolved in 1 ml 0.05 M Tris-HCl buffer pH 7.4 and incubated at 37 °C with angiotensin converting enzyme (ACE, 7.5 mU) for 7 h. The reaction mixture was resolved on a Nova-Pak C-18 column (3.9 mm i. d. X 150 mm). LVV-hemorphin-5, VV-hemorphin-5 and dipeptide Arg-Phe were identified by mass spectrometry.

HPLC system

The liquid chromatographic system consisted of a Waters 600 automated gradient controller-pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 hard disc using a NEC image 466 computer. Millennium software was used to plot, acquire and treat chromatographic data.

METHODS

Mobile phase for Delta Pak C-18 column (19 mm i.d.X 300 mm)

The mobile phase was composed of 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow rate was 12 ml/min. Samples were dissolved in buffer A, filtered through 0.22 µm filters before injection. The gradient applied was 0-40% B in 80 min.

Mobile phase for Nova-Pak C-18 column (3.9 mm i.d. X 150mm)

The mobile phase comprised: 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. A linear gradient (15-30% B in 15 minutes) was applied. The flow rate was 1.5 ml/min.

Mobile phase for TSK G2000 SWG column (7.6 mm i.d. X 600mm)

The elutions were performed with 5mM ammonium acetate buffer pH 6.0 . The flow rate was 0.9ml/min..

Procedure

Hemorphin-7, VV-hemorphin-7 and LVV-hemorphin-7 were chromatographed at room temperature on a Delta Pak C-18 and a Nova Pak C-18 columns respectively under conditions described above. Total hydrolysate was also injected on Delta Pak C-18 column under the same conditions. Tyrosine, phenylalanine, tryptophan and peptides were injected on the Nova Pak C-18 column and pepsin, tuna myoglobin and cutinase were loaded successively on the TSK G2000 SWG column. On-line instantaneous UV absorbance spectral scan was performed between 190 nm and 350 nm with a rate of one spectrum/second; spectral resolution was 1.2 nm. Chromatographic data were processed using Millennium software. Spectrum matching results (comparison between spectra of each peaks in the chromatographic profile with the library spectra of the hemorphins) [13] and second derivative spectra were obtained by Waters Millenium system.

Amino acid analysis

Amino acids were analyzed using a Waters Picotag Work Station. Peptide hydrolysis was achieved with constant-boiling HCl containing 1% phenol, for 24h. at 109°C. Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids on a Waters RP-Picotag column (150mm x 3.9mm i.d.) were performed according to Bidlingmeyer et al. [16]. The detection wavelength was 254 nm and the flow rate 1 ml/min.

Mass spectrometry analysis

Mass spectra, generated from Fast Atom Bombardment (FAB) mass spectrometry of the peptides, were recorded on a four sector "Concept II" tandem mass spectrometer (Kratos, Manchester, UK). Ions were produced in a standard

FAB source by bombarding the sample with xenon atoms having a kinetic energy of 8 KeV and the instrument was operated at an accelerating voltage of 8 KV. The peptide was dissolved in water (1 mg/ml) and 1ml of the solution was loaded on the stainless steel tip with thioglycerol as matrix. The mass range was scanned at 10 s/decade with a mass resolution of 3000. Caesium iodide was the standard for mass calibration.

RESULTS AND DISCUSSION

SPECTRAL ANALYSIS OF AROMATIC AMINO ACIDS AND PEPTIDES

Phe, Tyr, Trp and peptides listed in Table 1 were successively injected on RP-HPLC and their absorbance spectra were monitered with Waters 996 UV-Vis Photodiode array detector and processed to give second order derivative spectra. Figure 1 illustrates the second order spectra of each aromatic amino acid. The most characteristic feature of these second derivative spectra is that they exhibit a minimum at every maximum in the zero order spectra. As shown in table 2 the most prominent minima for Phe, Tyr, and Trp were 259nm, 283.5nm and 289.5nm respectively. These values were slightly different in regards to those of Palladino [8] and Zavitsanos [5], especially the secondary minimum for

TABLE 1

Peptides, proteins and aromatic amino acids used for second order derivative spectral analyses.

Compound	Structure	Phe	Tyr	Trp
Phenylalanine		1		
Tyrosine			1	
Tryptophan				1
Dipeptide	Arg-Phe	1		
F2-8	Tyr-Gly-Ala-Glu-Ala-Leu		1	
P41	Ser-Ala-Ala-Asp-Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp			1
VV-hemorphin-5	Val-Val-Tyr-Pro-Trp-Thr-Gln		1	1
LVV-hemorphin-5	Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln		1	1
Hemorphin-7	Tyr-Pro-Trp-Thr-Gln-Arg-Phe	1	1	1
VV-hemorphin-7	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	1	1	1
LVV-hemorphin-7	Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	1	1	1
Peptide GI	Glu-Lys-Leu-Gly-Glu-Tyr-Gly-Phe-Gln	1	1	
Pepsine		14	16	5
Tuna myoglobin		6	2	1
Cutinase		8	5	1

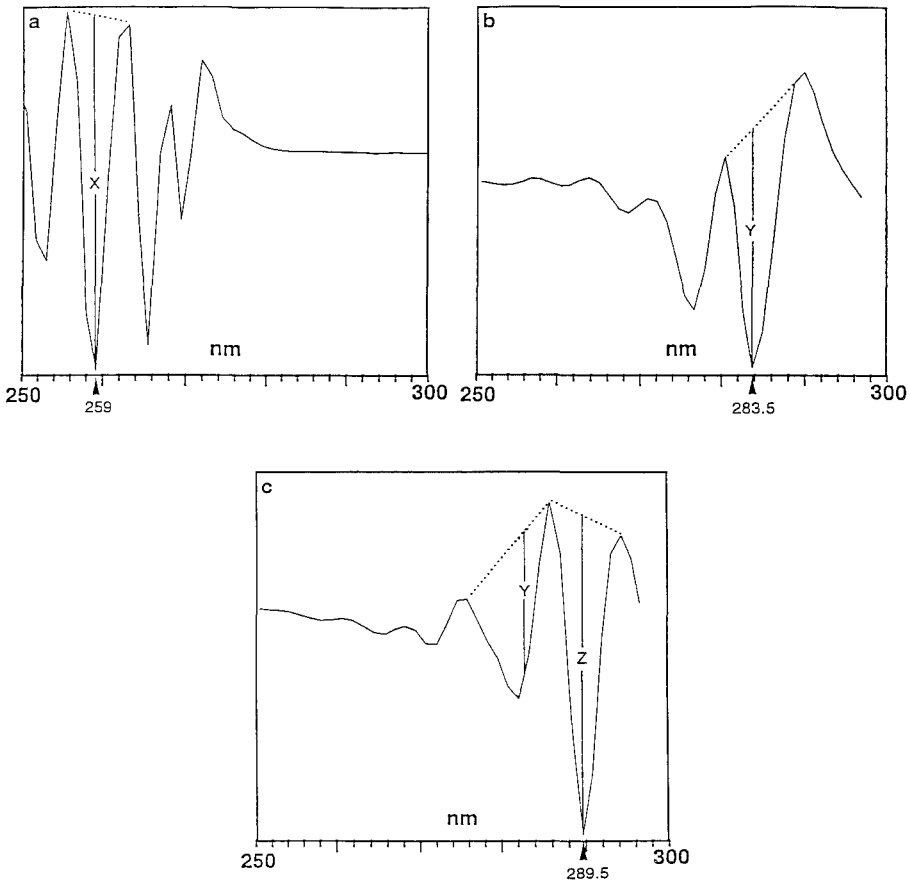

Figure 1. Second order derivative spectra of free aromatic amino acids. (a) phenylalanie, (b) tyrosine, (c) tryptophan. X, Y and Z: heights at the major minima.

TABLE 2
Major and side minima of aromatic amino acids second order derivative UV spectra.

Aromatic amino acids	Major minima	side minima
Phenylalanine	**259nm**	253nm, 264nm, 268nm
Tyrosine	**283.5nm**	276.5nm
Tryptophan	**289.5nm**	282nm

tryptophan (282nm instead of 278 nm). This may be due to the more precise spectral resolution of the 996 photodiode array detector. So the presence or absence of minima at 259, 283.5 and 289.5 nm allowed us to determine the presence or absence of Phe, Tyr and Trp. Figure 2 shows the second order derivative UV-spectra of the three known peptides Arg-Phe, FII 8, P41, containing Phe, Tyr and Trp respectively. The wavelengths of major minima were exactly the same as for free aromatic amino acids. This indicated that non-aromatic amino acids were not involved in the absorbance between 250nm and 300nm. This resulted in an easy identification of aromatic amino acids by their second order derivative spectra.

When tyrosine and tryptophan coexisted in the same peptide, the primary minimum for tyrosine (283.5nm) and the second minimum for tryptophan (282nm) overlaped. As shown by Palladino[8] and Zavitsanos [5] this overlapping made the identification of tyrosine in the presence of tryptophan difficult. The amplitude of minima should be accurately defined in order to determine the presence of tyrosine and to calculate the ratio between aromatic amino acids. Figure 1 c illustrates how the amplitude of minima for Trp were evaluated (Y at 283.5nm, Z at 289.5 nm). For tryptophan (figure 1 C), the proportion between Z and Y was 1:0.45. In the presence of tyrosine, we must consider its contribution at 283.5 nm, so if we postulated that the amplitude at 283.5 nm was simply additional, the contribution of tyrosine at 283.5 nm minima was Y - 0.45 Z .

Figure 3 show the second derivative spectra of VV-hemorphin-5 (a), hemorphin-7 (b), VV-hemorphin-7 (c) and LVV-hemorphin-7 (d), and the relative values of their amplitude at 258.5nm (X), 283.5 nm (Y) and 289.5 nm (Z) are presented in table 3. When Phe, Tyr and Trp were present in a peptide with a molar ratio 1:1:1 (as for hemorphin-7, VV-hemorphin-7 and LVV-hemorphin-7), the relative values of their amplitude were 1:7.5:11. Considering the tryptophan contribution at 283.5 nm, the contribution of tyrosine at 283.5nm was calculated as

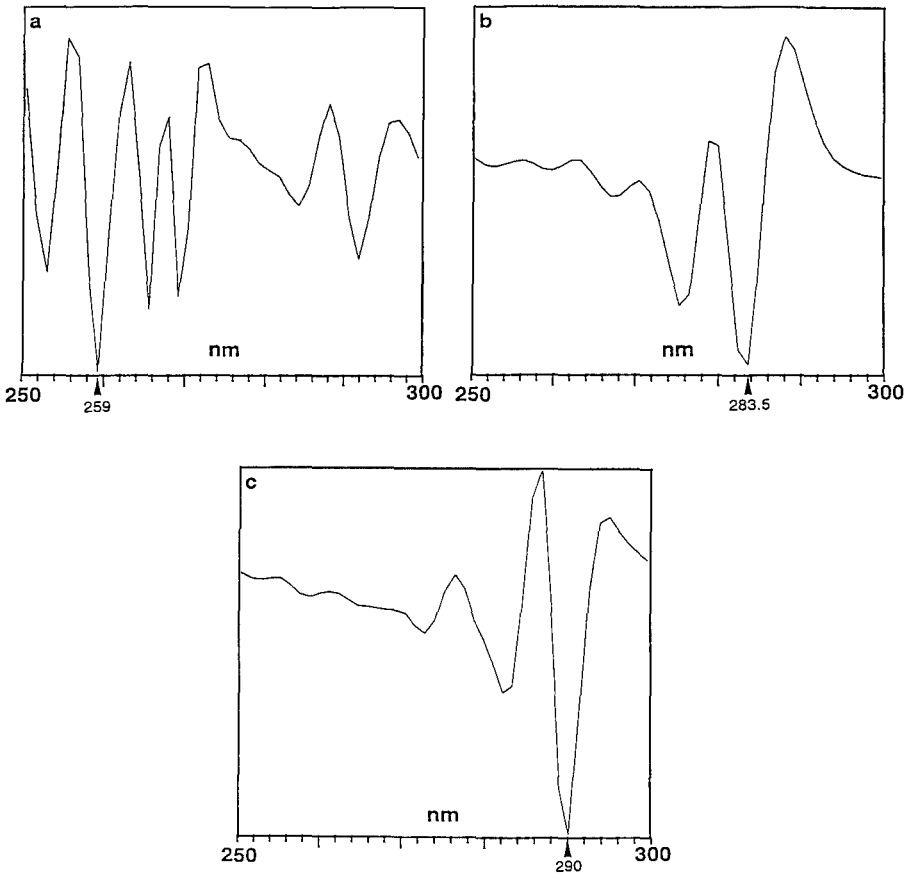

Figure 2. Characteristic minima in second order derivative UV-spectrum of small peptides indicating the presence of aromatic amino acids. (a) Arg-Phe, (b) FII 8, (c) P41. Peptide sequences: FII 8, Tyr-Gly-Ala-Glu-Ala-Leu; P41, Ser-Ala-Ala-Asp-Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp.

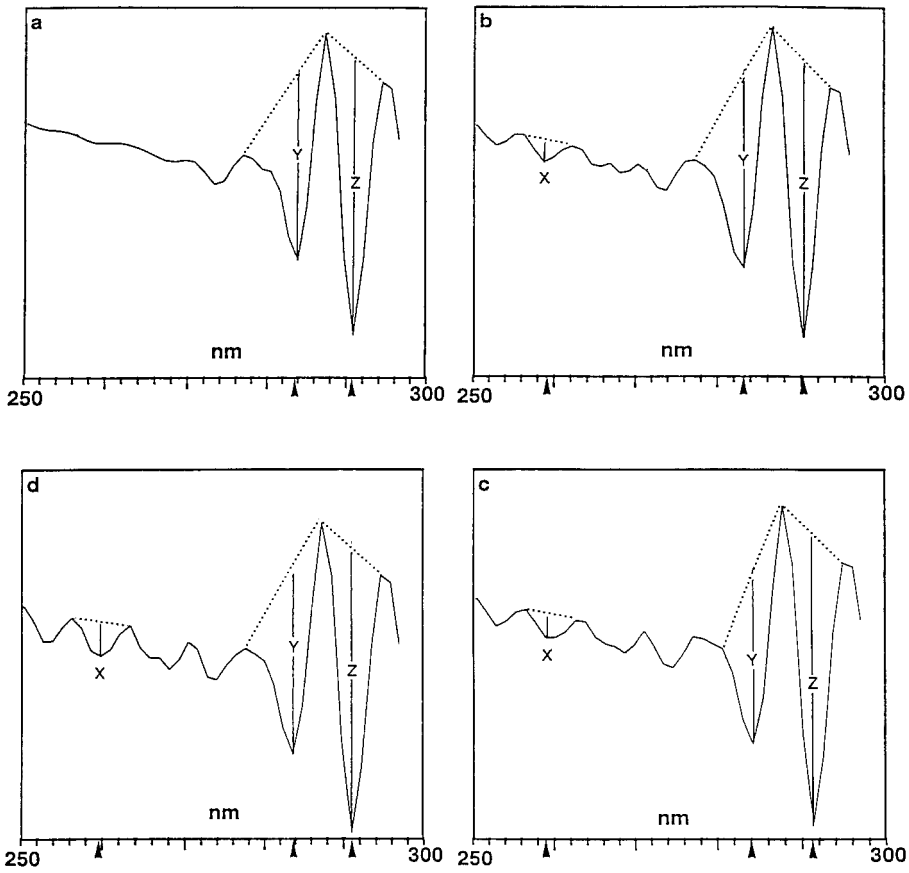

Figure 3. Characteristic minima in second order derivative spectra of some hemorphins indicating the content of aromatic amino acids. (a) VV-hemorphin-5, (b) Hemorphin-7, (c) VV-hemorphin-7, (d) LVV-hemorphin-7. X, Y and Z: as in figure 1.

TABLE 3
Second order derivative spectra of some hemorphins: relative amplitudes at 259nm, 283.5nm and 289.5nm.

Hemorphins	Amplitude at 259 nm (Phe)	Amplitude at 283.5nm (Tyr+Trp)	Amplitude at 289.5 nm (Trp)
VV-hemorphin-5		7.5	11
Hemorphin-7	1	7.5	11
VV-hemorphin-7	1	7.5	11
LVV-hemorphin-7	1	7.5	11

7.5 - 0.45 x 11 = 2.55. From here we can deduce the relation between the ratios of the three aromatic amino acids, determined by their amplitude, as follows:

$$\text{Phe} : \text{Tyr} : \text{Trp} = X/1 : (Y - 0.45Z) / 2.55 : Z/11, \quad (1)$$

When Z = 0, tryptophan was absent, $\text{Phe} : \text{Tyr} = X : Y/2.55$ (2)

When Y = 0.45Z, tyrosine was absent $\text{Phe} : \text{Trp} = X : Z/11$ (3)

When X = 0, phenylalanine was absent $\text{Tyr} : \text{Trp} = (Y - 0.45Z)/2.55 : Z/11$ (4)

VALIDATION OF THESE METHODS OF CALCULATIONS WITH SOME PEPTIDES OF KNOWN COMPOSITION

These rules were then checked with some known peptides (figure 4). In figure 4a the secondary derivative spectrum of peptide GI is presented. A zero amplitude at 289.5 nm (Z=0) indicated the absence of tryptophan. On the contrary, the presence of phenylalanine and tyrosine were demonstrated by minima at 258.5nm and 283.5 nm. The proportion between the two amplitudes was 1:2.45. So, according to equation (2) the ratio between Phe and Tyr in this peptide was 1:1. In the same way, the presence of Tyrosine and tryptophan in LVV-hemorphin-5 was verified by its second derivative spectrum (Figure 4b). According to equation (4) the ratio between tyrosine and tryptophan was also calculated as 1:1.

Figure 4c presents the second derivative spectrum of a peptide selected at random in a total bovine hemoglobin hydrolysate chromatographed on a Delta Pak C-18 column (figure 6, peak 33). The absence of tyrosine was demonstrated by Y-0.45Z = 0 and the presence of phenylalanine and tryptophan was evidenced by the minima at 258.5nm and 289.5nm. The proportion between amplitudes at 258.5nm and 289.5nm was 3.2:12. So according to equation (3), the ratio of phenylalanine and tryptophan in this peptide was 3:1. When the known amino acid

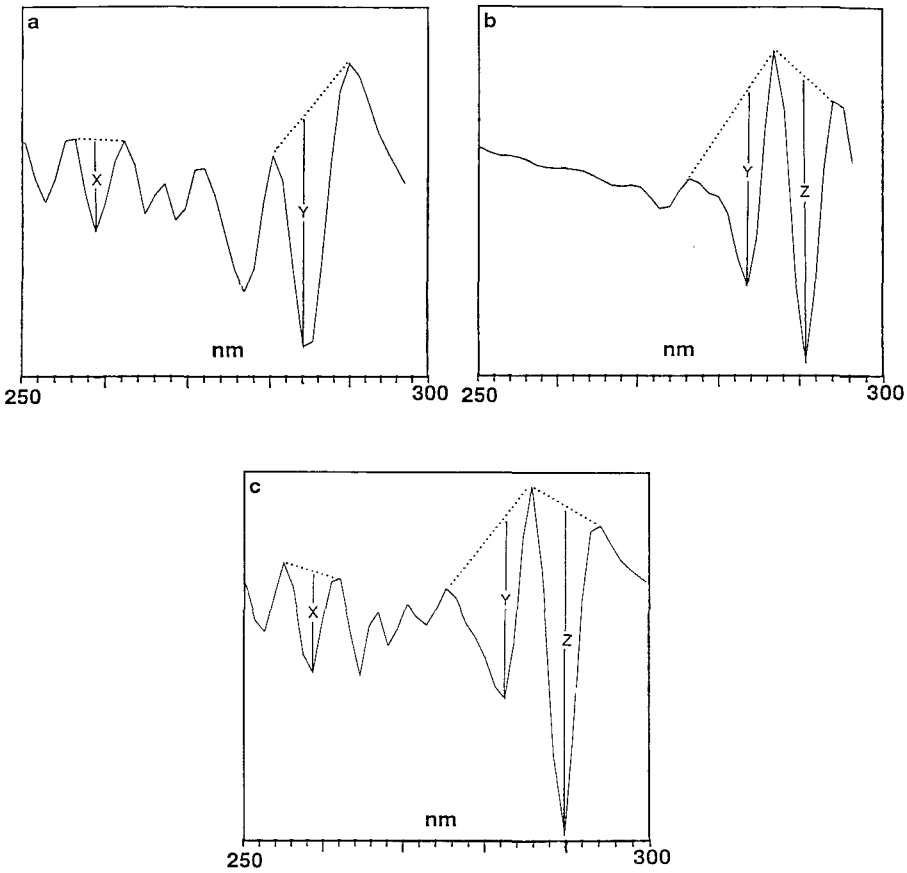

Figure 4. Second order derivative spectra of test peptides containing aromatic amino acids. The Tyr/Trp ratio is known from free amino acids and is used here to analyse spectral results. (a) peptide GI, (b) LVV-hemorphin-5, (c) peptide from bovin hemoglobin peptic hydrolysate -peak 33, see figure 6.

TABLE 4
Analysis of aromatic amino acid content of some proteins by second order derivative spectra: comparison of real and calculated content.

Proteins	Calculated content			Real content		
	Phe	Tyr	Trp	Phe	Tyr	Trp
Porcine pepsine	18	16	5	14	16	5
Tuna myoglobin	5	2	2	6	2	1
Cutinase	4	4	1	8	5	1

sequences of alpha and beta bovine globins were examined for regions containing these amino acids, it was clear that the composition found was only compatible with peptide including residues 36 to 44 of the bovine hemoglobin beta chain. The N-terminal residue of this peptide may be proline or tryptophan because of the presence of tryptophan and absence of tyrosine. So the non-destructive identification of tryptophan and the informations resulting from secondary derivative UV spectra are of great interest in peptide maps.

APPLICATION OF THESE METHODS TO SOME PROTEINS OF KNOWN COMPOSITION

Porcine pepsin, tuna myoglobin and cutinase were chromatographed by gel permeation HPLC under conditions described in experimental section and their second order derivative spectra were obtained (figure 5). The presence of the three aromatic amino acids in these proteins was clearly evidenced by the minima at 259, 284 and 290nm. The ratios between the aromatic amino acids were calculated (Table 4). It indicated that the presence of aromatic amino acids was identified qualitatively but the correct proportion was not obtained. This might be the result of the three-dimentional structure of native proteins. In fact, when the polypeptidic chain folds into a secondary or tertiary structure, aromatic amino acids can be buried within the molecule. Thus, these hidden aromatic amino acids are not involved in the protein absorbance. This observation shows that there is some limitation in the use of second derivative spectra for an accurate quantitative determination of native protein aromatic amino acid content. Further investigation should be undertaken with denatured proteins. Nevertheless, this method is of great interest for the analysis of oligopeptides.

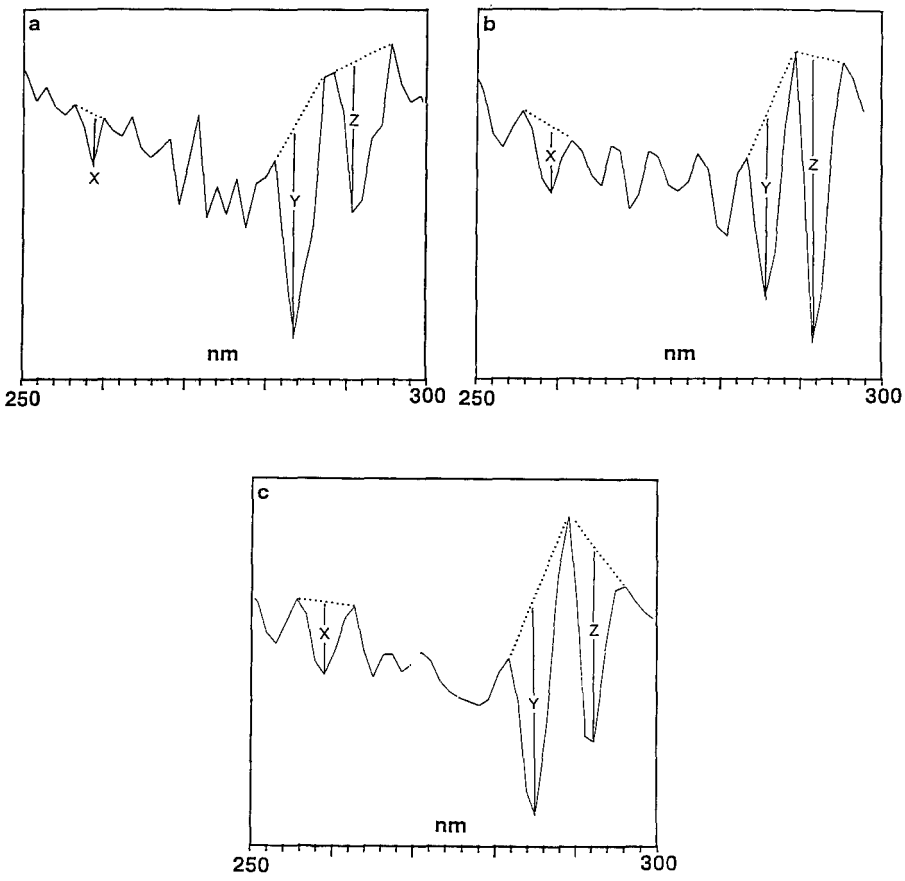

Figure 5. Second order derivative spectra of some proteins. (a) Porcine pepsin, (b) Tuna myoglobin, (c) cutinase. Estimation of their aromatic amino acid contents is carried out using the methods presented in the results and discussion section.

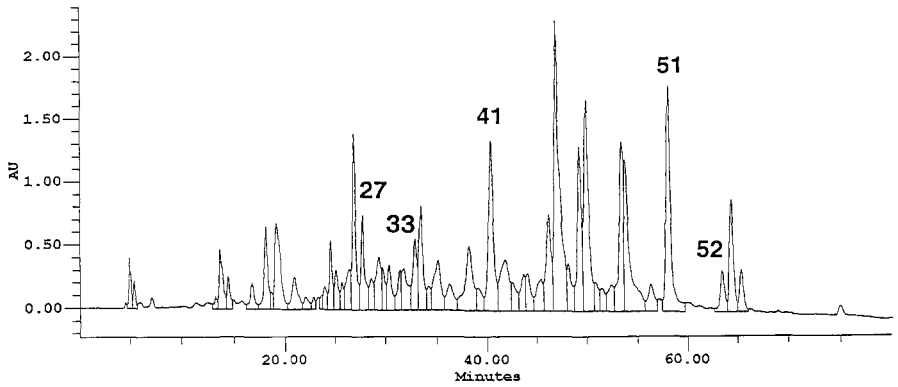

Figure 6. Reverse phase HPLC of total peptic bovin hemoglobine hydrolysate on a Delta pak C18 column. Peptides were eluted with a linear gradient of acetonitrile (eluent B) from 0% to 40% in 80min. (solvent A: 10mM ammonium acetate, pH6.0).

APPLICATION OF THIS METHOD TO THE DETECTION OF HEMORPHINS IN A TOTAL BOVIN HEMOGLOBIN HYDROLYSATE

The total bovine hemoglobin hydrolysate chromatographic profile is shown in figure 6. UV-spectra comparison performed as previously described [13] originally revealed four peaks (P27, P41, P51 and P52) as hemorphins. Analysis of their second order derivative spectra was carried out to evaluate their aromatic amino acid content. Figure 7 exhibited the second derivative spectra of peak 27 (a), 41 (b), 51 (c) and 52 (d). Figure 7a revealed the presence of tryptophan (minima at 289.5 nm) and the absence of phenylalanine and tyrosine ($X = 0$ and $Y - 0.45Z = 0$). The same results were obtained from figure 7b. This indicated that tryptophan was the only aromatic amino acid present in the peptides P27 and P41. Regarding the structure of hemorphins, these peptides could not be classified in the category of hemorphins. On the contrary, figure 7c and 7d demonstrated the presence of Phe, Tyr and Trp in peaks 51 and 52 with the ratio 1:1:1 which is representative of hemorphins. These results were verified by amino acid analysis.

Therefore, the successive application of UV-spectra comparison [13] and second order derivative spectra could be an accurate procedure to determine quickly and unambiguously the presence of hemorphins in complexe enzymatic hydrolysate.

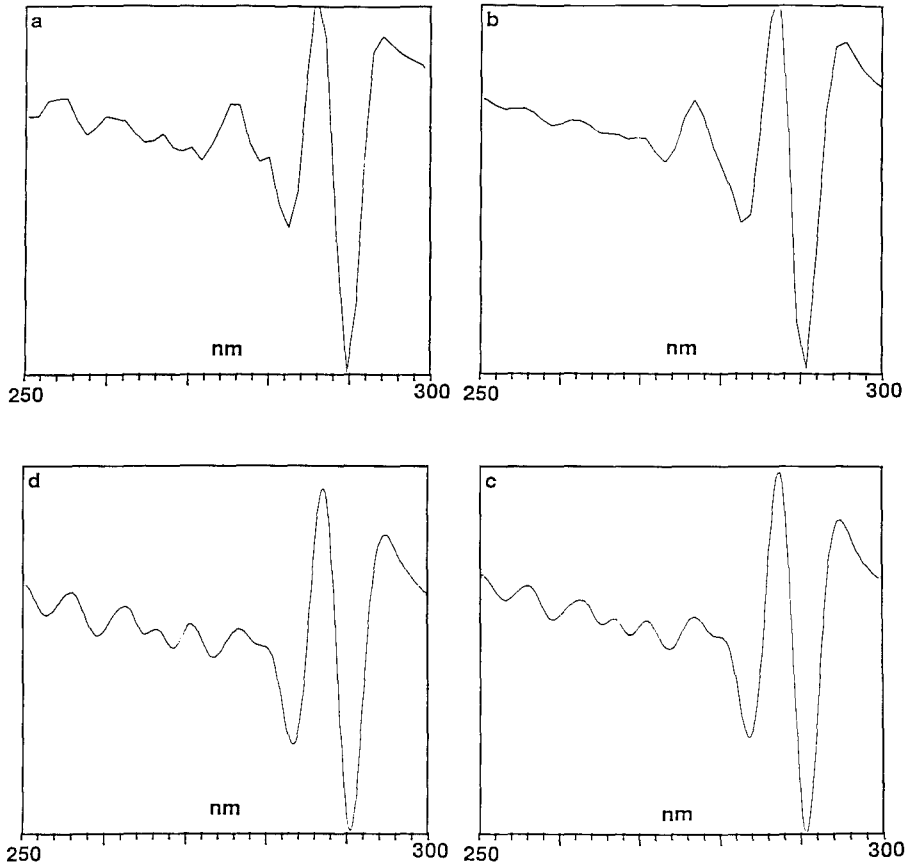

Figure 7. Second order derivative spectra of P27 (a), P41 (b), P51 (c) and P52 (d) from the total peptic bovin hemoglobine hydrolysate chromatographed on a Delta pak C18 column, selected as hemorphins by UV-spectra comparison.

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A NOVEL METHOD OF ISOLATING TAXANES FROM CELL SUSPENSION CULTURES OF YEW (*TAXUS* SPP.)

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ABSTRACT

A method is described to isolate a mixture of taxanes from cell suspension cultures of *Taxus* species. The aqueous suspension medium is pre-filtered and centrifuged to remove cellular debris, and then passed through either nylon or PVDF membranes. Contaminants are washed from the membranes and the taxanes are eluted with appropriate solvents. This method provides a rapid, efficient, and inexpensive means of extracting taxanes from cell suspension medium, as well as a significant reduction in the total volume of solvents used.

INTRODUCTION

One possible alternative to the extraction of taxol and related taxanes from bark or needles is the use of continuous, sustainable cultures of cells of *Taxus* to produce these complex diterpenoids *in vitro* (2-7). Taxol and other taxanes are excreted from the cell into the aqueous culture medium and can be isolated

without contamination by chlorophyll or other pigments and compounds associated with isolation of taxanes from needles or bark (2,3).

Most reports of the purification of taxanes from cells or bark of *Taxus* rely on the use of chlorinated hydrocarbons, such as methylene chloride or chloroform (4,6,8,9). The feasibility of isolating taxanes from suspension cell cultures with a minimal amount of solvent, and without the use of any chlorinated hydrocarbons, would have important environmental implications. The unique ability of various *Taxus* cell lines to grow in an aqueous medium, and to excrete taxanes into that medium, eliminates the need to do solvent:solvent partitioning to extract taxanes from these cultures. In this paper we describe a novel method to isolate taxanes from *Taxus* cell suspension medium using a technique developed in this laboratory.

MATERIALS

Chromatographic Conditions

Authentic samples of taxanes were very generously provided by Dr. David T. Bailey, Hauser Chemical Research, Inc., Boulder, CO, and by Dr. Kenneth M. Snader, NCI, Bethesda, MD. All presumptive taxanes in suspension cultures of *Taxus cuspidata* cell line P991 were identified and quantified with HPLC analysis by comparison to retention times and absorption spectra with a standard mixture consisting of 10-deacetyl baccatin III [**1**], 7-epi-10-deacetyl baccatin III [**2**], baccatin III [**3**], 10-deacetyl-7-xylosyl taxol C [**4**], 10-deacetyl-7-xylosyl taxol [**5**], 10-deacetyl-7-xylosyl taxol B [**6**], 10-deacetyl taxol [**7**], cephalomannine [**8**], 7-epi-10-deacetyl taxol [**9**], taxol [**10**], taxol C [**11**] (not quantified), and 7-epi taxol [**12**].

Taxane concentrations were determined by comparison to an external standard curve over the range of 5 to 50 μg/mL. A method for rapid isocratic analysis of taxanes has been developed in our laboratory (7). Crude taxane

mixtures in methanol were separated on a Phenomenex 4 μm, 250 x 4.6 mm, Curosil G column with an Upchurch ODS disposable guard cartridge (1). Mobile phase was CH_3CN:H_2O (52.5:47.5), 1 mL/min flow rate, with UV detection at 228 nm and diode array scans of each peak from 200 to 300 nm. All solvents and samples were filtered through 0.2 μm Nylon 66 or PVDF filters (Gelman) prior to use.

Instrumentation consisted of a Beckman Model 126 binary pump and gradient mixer, Beckman Model 168 Diode Array Detector, and Beckman Model 507 autosampler. Data acquisition, processing, integration, and instrument control was with a Gateway 2000 computer with 80486 microprocessor running Beckman System Gold v. 6.07 software.

Tissue Culture Reagents

All chemicals used for tissue culture of *Taxus* plant cells were obtained from Sigma Chemical Company (St. Louis, MO, USA). Pre-packaged media mixes were not used.

METHODS

Experimental Design and Statistical Analysis

All treatments within an experiment were triplicated. Data is presented as the mean of the triplicates and error bars represent the standard error of the mean. Because amounts of taxanes varied between cell suspension cultures, only data from one randomly selected set of experiments is presented, although all experiments were done at least three times.

Statistical analysis, where appropriate, was performed with Minitab v. 8 software (Addison-Wesley). Treatments were analyzed with a one-way ANOVA

and differences between means tested with a Tukey multiple comparison test with a 0.05 family error rate.

Cell Line Initiation and Maintenance

Cell line P991 is a *Taxus cuspidata* cell line that has been growing in suspension cell culture since it was first isolated and established in our laboratory in September 1991. Every two weeks it was subcultured by aliquot transfer of 10 mL of cells and suspension media into 40 mL of fresh B5NB medium (Gamborg B5 liquid medium with 2% sucrose, 2.7 μM NAA, 0.01 μM BA, 0.25 mM ascorbic acid, 2 mM glutamine, pH 5.50). Cultures were grown in 125 mL Erlenmeyer flasks capped with Bellco ™ silicone/foam caps, in the dark at 25 °C and 125 rpm. For each experiment, medium was used from a single flask of P991 cells that were harvested after 21 days of growth. The culture was filtered through a single layer of Miracloth (Calbiochem) and the filtrate was used for subsequent extraction experiments.

Analysis of Taxanes in Suspension Medium

Taxanes were routinely analyzed from aqueous suspension medium by taking 1.0 mL samples of the cell-free suspension medium, and evaporating to 100 μbar dryness on a Savant Speed-Vac (Savant Instruments, Farmingdale, NY). The dried pellet was extracted with 0.2 mL acidified methanol (0.01 % glacial acetic acid added for stabilization of taxanes), agitated in an ultrasonic bath for 60 min, centrifuged at 16,000 x g for 15 min, and filtered through a 0.2 μm PVDF syringe filter (Gelman Sciences). This 5X concentrated methanol extract was then analyzed via HPLC against an external standard curve containing a mixture of 5,10, 25, and 50 μg/mL taxanes. All peaks in the suspension extracts were scanned with a diode array, and scans were compared to those of authentic taxane standards.

Modifications to this method have included extraction in 0.1 mL acidified methanol for a 10X concentration, or 0.5 mL of suspension extracted with 0.1 mL for samples where suspension medium volumes are limited.

Centrifugation Studies on the Precipitation of Taxanes in Aqueous Solution

Suspension medium was filtered through Miracloth, and 1.5 mL samples were transferred to 1.5 mL microcentrifuge tubes. Samples were centrifuged at 1000, 2000, 4000, 8000, or 16000 x g for 30 min. After centrifugation, 0.75 mL was removed from the upper portion of the medium, and transferred to a separate centrifuge tube. The separate upper and lower fractions of each tube that had been centrifuged were then prepared by drying and extracting in 0.2 mL of acidified methanol (3.75X concentration), as outlined above.

Taxane Adsorption and Selective Elution Experiments

Suspension medium was prefiltered through Miracloth. A 1.0 mL aliquot of this medium was filtered through a 0.2 μm nylon or PVDF, 13 mm syringe filter. The filtrate was dried in the same manner as the control, and residue extracted in 0.2 mL acidified methanol (5X concentration). The filter was then washed with 0.2 mL of acidified methanol (5X concentration). Samples that were prepared by adsorption to membrane filters were compared to unfiltered controls prepared in the conventional manner (see above). All samples were then analyzed via HPLC.

For selective elution experiments, 1.0 mL of suspension medium was filtered through a 0.2 μm nylon or PVDF filter. Filter was then washed with 0.2 mL of solvents of decreasing polarity starting with 10% solvent in water, and increasing the solvent concentration by 10% until reaching 100% solvent. Solvents that were tested were methanol, ethanol, isopropyl alcohol, and acetone.

Samples were dried, redissolved in 0.2 mL acidified methanol, and filtered through a 0.2 μm filter prior to HPLC analysis.

RESULTS AND DISCUSSION

Cell Line Initiation and Maintenance

A *T. cuspidata* cell line, P991, has been in culture for nearly three years and continues to produce taxol [**10**] by excretion into the culture medium. While taxol [**10**] concentrations vary considerably, these cultures have produced as much as 25 mg/L taxol [**10**] with typical production of 10-15 mg/L by day 28 of culture. For these experiments, cells were harvested at day 21 to minimize the presence of any competing or interfering compounds from older cells, such as phenolics, xanthophylls, and metabolic degradation products.

Cells were viable at the time of harvest and had a light beige to white coloration. The cell-free suspension medium was colorless with a very slight shimmering or opalescent appearance.

Analysis of Taxanes in Suspension Medium

Our method for analyzing taxanes from suspension medium was developed to give the most complete and accurate quantification of taxanes with the minimum amount of interfering compounds, and minimum loss of sample during preparation. Drying samples and then extracting the residue in acidified methanol gave us higher yields for taxol and other taxanes than solvent:solvent partitioning methods that we tried with methylene chloride, chloroform, or ethyl acetate. This method also eliminates any methanol-insoluble compounds from contaminating samples to be analyzed. Cell line P991 typically produces a specific taxane profile consisting of taxanes in roughly equivalent proportion, regardless of actual concentration

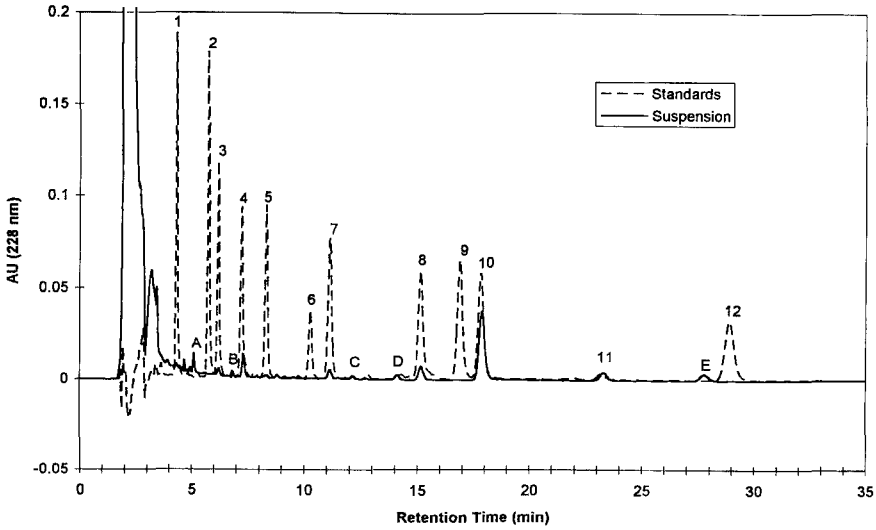

FIGURE 1. Comparison of a mixture of taxane standards, each at a concentration of 50 µg/mL, to a methanol extract of *T. cuspidata* suspension media. The suspension extract is a 5X concentration of a 1mL dried aqueous sample, redissolved in 0.2 mL acidified methanol. For each chromatogram, 10 µL samples were injected. **1** = 10-deacetyl baccatin III; **2** = 7-epi-10-deacetyl baccatin III; **3** = baccatin III; **4** = 10-deacetyl-7-xylosyl taxol C; **5** = 10-deacetyl-7-xylosyl taxol; **6** = 10-deacetyl-7-xylosyl taxol B; **7** = 10-deacetyl taxol; **8** = cephalomannine; **9** = 7-epi-10-deacetyl taxol; **10** = taxol; **11** = taxol C (not quantified); **12** = 7-epi taxol. Peaks A-D are unidentified taxanes, based on their absorption spectra. Peak E is not a taxane or taxinine.

(Fig. 1). In this 21-day old culture, the medium contained 10-deacetyl baccatin III [**2**] (0.004 mg/L), baccatin III [**3**] (0.19 mg/L), 10-deacetyl -7-xylosyl taxol [**5**] (1.11 mg/L); 10-deacetyl taxol [**7**] (0.38 mg/L), cephalomannine [**8**] (1.01 mg/L), taxol [**10**] (6.6 mg/L), and taxol c [**11**]. There are 4 additional unidentified taxanes that are produced by the cell lines, according to their UV absorption spectra. These unknown taxanes are not found in bark or needle samples of *Taxus brevifolia* (J. Yeggie, Hauser Chemical, personal communication). At least one of

the unknown taxanes, "A," is often the second most abundant taxane in some of the P991 cultures, next to taxol [**10**].

Filtration and Adsorption of Taxanes to Membrane Filters

Early experiments to analyze taxanes directly from suspension media resulted in failure to detect any taxanes, even though other methods to extract taxanes from the suspension media had demonstrated that the cultures were producing taxanes. An experiment was designed to see if taxanes were being filtered out of solution by a 0.2 μm filter when the suspensions were prepared for HPLC analysis (Fig. 2). Indeed, all of the 10-deacetyl taxol [**7**], significant amounts of cephalomannine [**8**], and virtually all of the taxol [**10**] was missing from the filtrate after suspension media was passed through a nylon membrane filter. The missing taxanes were then recovered from the nylon filter after washing with 0.2 mL methanol. One of the interesting features of the experiment was that not all of the taxanes were retained by the membrane filter. One of the possible explanations of the selective nature of the filter is that taxanes exist in the suspension medium as insoluble aggregates, either smaller or larger than the 0.2 μm pore size of the membrane. Another possible explanation is that the membrane itself was selectively retaining taxanes.

When the experiment was repeated with a 0.2 μm PVDF filter membrane, all of the taxanes were retained by the filter (Fig. 2). Thus, differences in retention of taxanes is not a result of the 0.2 μm pore size but, more likely, a selective affinity for some of the taxanes to the nylon membrane. That affinity also does not appear likely to be based solely on the polarity of individual taxanes. While taxanes of similar polarity (10-deacetyl taxol [**7**], cephalomannine [**8**], and taxol [**10**]) are preferentially retained by the nylon membrane in comparison to more polar taxanes (baccatin III [**3**], and 10-deacetyl-7-xylosyl taxol [**5**]) the less polar

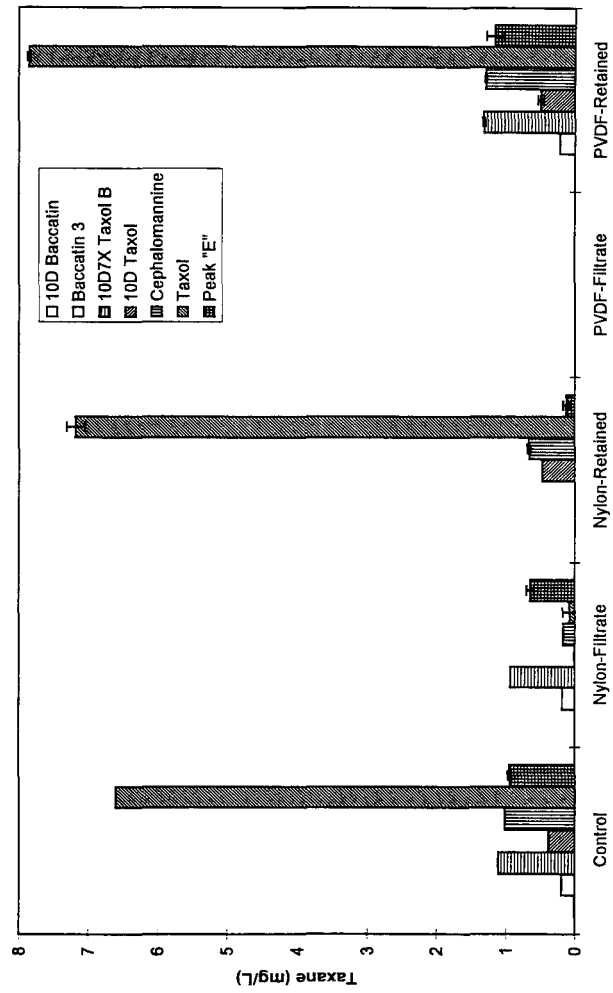

FIGURE 2. Comparison of retention of taxanes on membrane filters with 0.2 µm pore size. Control was a 1 mL suspension sample, dried and redissolved in 0.2 mL acidified methanol. Filtrates were prepared the same way as the control. Retained taxanes were washed from the filters with 0.2 mL acidified methanol. Taxane values are actual concentrations present in the suspension medium.

peak "E" is only partially retained by the membrane. These experiments demonstrated that taxanes in the medium must be dissolved in a less polar solvent than water prior to filtration. Also, the results from these experiments suggested that it might be possible to exploit filtration as a selective method for isolating certain taxanes.

Centrifugation of Taxanes in Aqueous Solution

If the taxanes present in the aqueous medium were present as small, insoluble particles, or as an insoluble suspension, it might be possible to centrifuge them out of solution (Fig. 3). When comparisons are made between different speeds of centrifugation on the amount of taxol in the upper or lower fractions of the centrifuged suspension medium, there are almost no significant differences. The only significant difference in taxol [**10**] concentration between upper and lower fractions of the centrifuged suspension media was in the 4000 x g treatment, with 9% more taxol found in the lower phase. Differences between the control (no centrifugation) and the centrifugation treatments were significant in all lower fractions except the 16,000 x g treatment. These results may be more of a reflection of differences between the analysis of a 1.0 mL sample at a 5X concentration (control) and a 0.75 mL at a 3.75X concentration (centrifuged samples), rather than real differences. Thus, it is possible to centrifuge the aqueous media at 16,000 x g for 30 minutes without causing a pelleting of taxanes. This means that relatively high speed centrifugation can be used as a simple and efficient means of removing cells, cell wall material, and other insoluble debris from the medium prior to extraction of taxanes.

Taxane Adsorption and Selective Elution Experiments

Attempts were made to see if taxanes retained by either nylon or PVDF membrane filters could be selectively eluted from the filter with solvents of

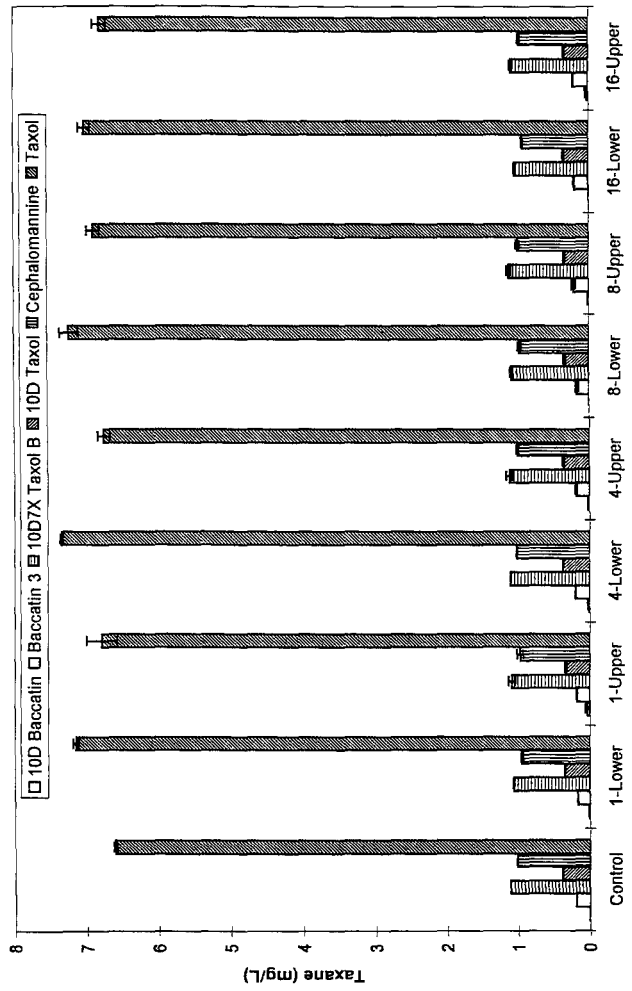

FIGURE 3. Effect of centrifugation on pelleting of taxanes in suspension medium produced by P991 cultures. Control consisted of 1 mL suspension medium dried, and extracted with 0.2 mL acidified methanol. A 1.5 mL sample of suspension medium was centrifuged for 30 minutes, split into separate 0.75 mL samples, dried, and each fraction extracted with 0.2 mL acidified methanol. Taxane values are actual concentrations present in the suspension medium.

gradually decreasing polarity (Fig. 4). When retained by a nylon membrane, no taxanes were eluted by washing with 10% or 20% aqueous methanol (Fig. 4a). The majority of taxol **[10]** comes off in three different eluates: 30%, 40%, and 60% methanol with lesser amounts coming off in the 50% fraction.

A similar pattern of elution occurs when ethanol is used instead of methanol (Fig 4b). Taxol **[10]** begins to elute or wash off of the filter with 10% aqueous ethanol with a peak at 20%, slightly less at 30%, and another peak of taxol **[10]** coming off in the 40% and 50% washes. Few additional taxanes remain on the filter after washing with 60% or greater aqueous ethanol. The reason for the reproducible occurrence of taxol **[10]** in two fractions, in aqueous methanol or ethanol, is not known, but may be related to the existence of two taxol **[10]** isomers or conformational variants. Magee (10) has suggested that the binding of aromatic hydrocarbons to nylon is highly sensitive to structural change, and thus the apparent elution of taxol in two predominate pools may be a result of the partial resolution of two structurally distinct forms of taxol.

This fractionation of taxol **[10]** into two separate pools that can be washed from a nylon membrane with different aqueous solvent fractions does not occur when the taxol **[10]** is bound to a PVDF membrane (Fig 5). Taxol **[10]** and all other taxanes were washed off of the PVDF filter in a fairly narrow range of polarities of aqueous solvents. Not surprisingly, the less polar the solvent, the lower the concentration of solvent in water was required to elute the taxanes. For methanol, the majority of taxanes were washed off in the 40% to 60% eluates (Fig. 5a). For ethanol, taxanes were washed off in the 30% to 50% eluates (Fig. 5b). For 2-propanol, some of the early eluting taxanes came out in the 10% and 20% washes, with cephalomannine **[8]**, taxol **[10]**, and taxol c **[11]** coming out almost exclusively in the 30% wash, and the remainder coming out in the 40% wash (Fig. 5c). Washes with aqueous acetone resulted in a fairly broad elution of taxanes from the PVDF filter, with taxanes coming off of the filter in the 10% to 50% eluates (Fig. 5d).

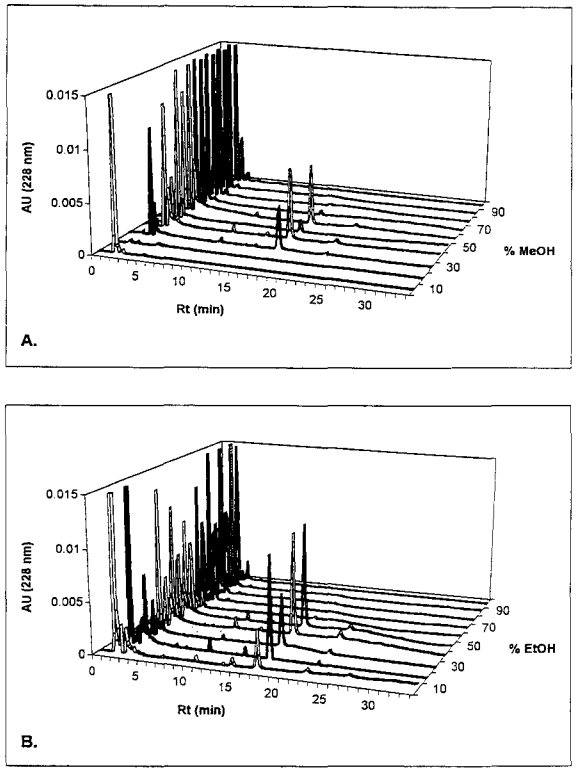

FIGURE 4. Effects of gradual elution of taxanes retained by nylon membrane filter with 0.2 μm pore size. A 1 mL sample of suspension medium was eluted with a discontinuous gradient of **A**) methanol or **B**) ethanol. Samples were analyzed directly after elution. The largest peaks are taxol; other peak identities are the same as in Fig. 1 and have been omitted for clarity.

Selective elution of taxanes from the filters was not attained, in these experiments, although it seems that it should be theoretically possible (10). Chemical modification of nylon membranes may allow the technique to be refined to more selectively elute taxol from a mixture of taxanes. Attempts to selectively elute taxol did, however, give rise to a technique that can be used to further purify the taxane fraction prior to extraction (Fig. 6). When taxanes that are bound to

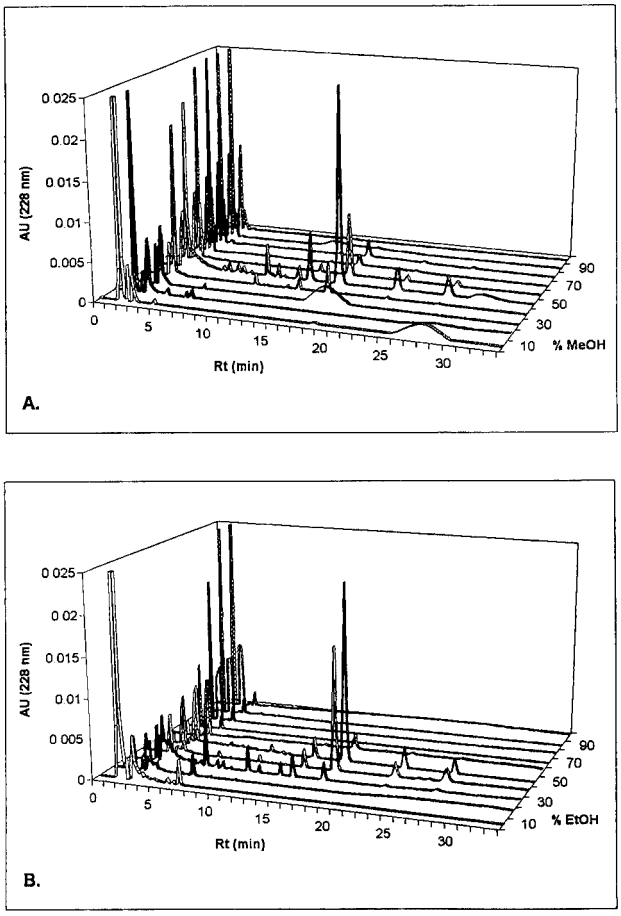

FIGURE 5. Effects of gradual elution of taxanes retained by PVDF membrane filter with 0.2 µm pore size. A 1 mL sample of suspension medium was eluted with a discontinuous gradient of solvent. Methanol and ethanol samples were analyzed directly after filtration; 2-propanol and acetone samples were first dried, then redissolved in 0.2 mL acidified methanol. **A**) methanol; **B**) ethanol; **C**) 2-propanol; **D**) acetone. The largest peaks are taxol; other peak identities are the same as in Fig. 1 and have been omitted for clarity.

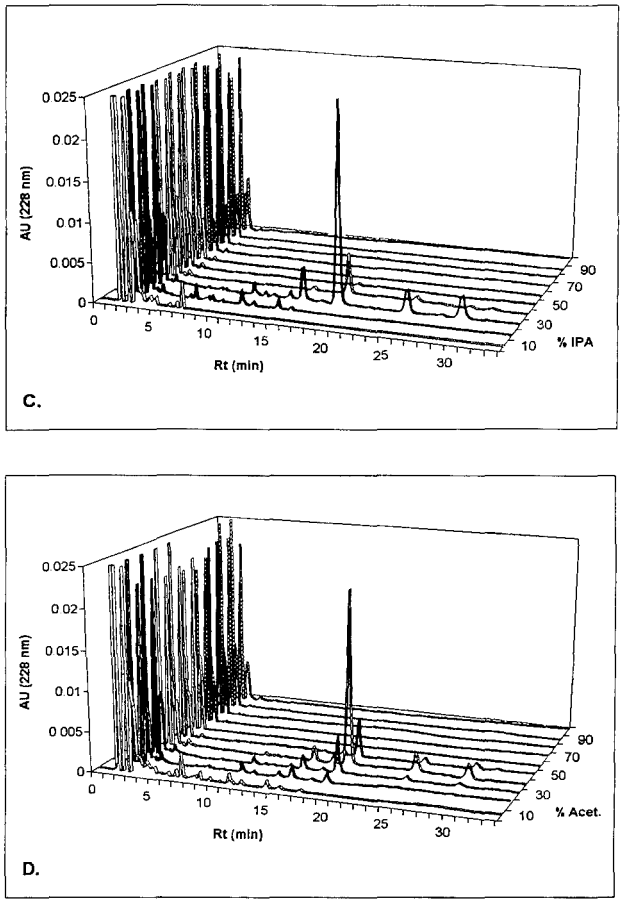

FIGURE 5 (continued).

either nylon or PVDF are eluted with 100% acidified methanol, a large number of polar, non-taxane, compounds elute with the solvent front on HPLC (Fig. 6a and 6b). When the filter is first washed with 20% aqueous methanol, which was found to be too polar to elute any taxanes from the filters (Fig. 4 and 5), the compounds eluting with the solvent front are significantly reduced. Further purification of the

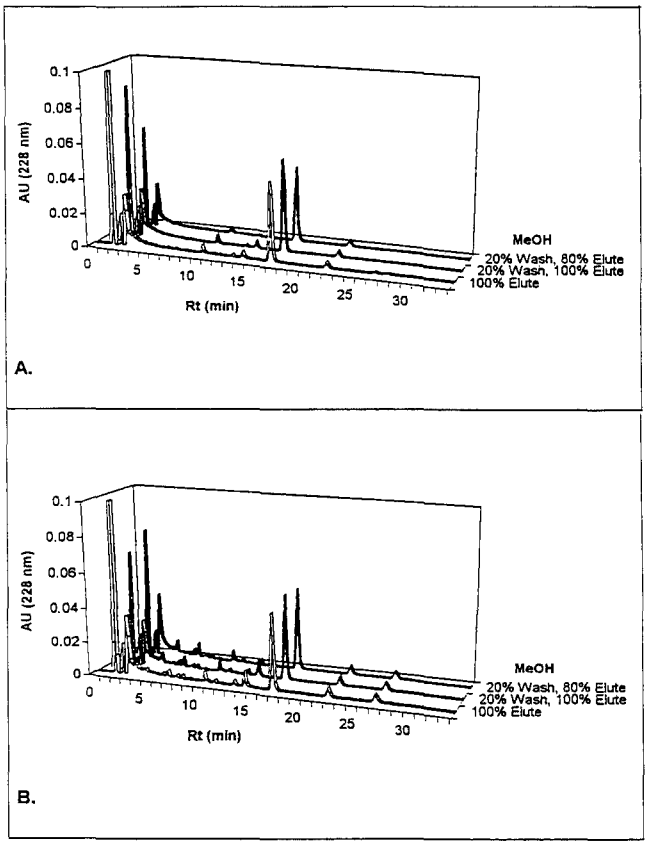

FIGURE 6. Washing of filters prior to elution of taxanes retained by **A**) nylon or **B**) PVDF membrane filters. The largest peaks are taxol; other peak identities are the same as in Fig. 1 and have been omitted for clarity.

samples is achieved by following the 20% methanol wash with elution with 80% aqueous methanol, which was found to be adequate for elution of all taxanes from either membrane (Fig. 4 and 5). This washing procedure produces no significant reduction in the number or quantity of taxanes recovered regardless of whether a nylon or PVDF membrane is used (Fig 6). It is significant that this procedure of

washing taxanes from membrane filters is remarkably similar to a procedure described by Wickremesinhe and Arteca (4) for purification of taxanes on C_{18} solid phase extraction (SPE) columns..

In our experience, we have found that with pre-filtration through a single layer of Miracloth, it is possible to filter 3 mL of suspension medium containing a concentration of 20 mg/L taxol through a 13 mm Gelman nylon syringe filter having a filter area of 0.8 cm^2. It should, therefore, be possible with prefiltration and centrifugation, to filter 7.5 L of suspension medium through readily available commercial filtration capsules that contain 2000 cm^2 0.2 μm nylon membranes. This would give a yield of 187.5 mg of taxol **[10]**, in addition to other taxanes, from a 7.5 L suspension culture of one of our higher production cell lines (25 mg/L). Additional investigations on pore size may reveal that much larger volumes of media can be passed through filters without affecting ability of the membranes to retain the taxanes. Also, customization of membrane filters would undoubtedly enable this method to be scaled to handle volumes of media that would be necessary for commercial production of taxol **[10]** from cell suspension cultures.

We have described a relatively inexpensive and efficient means of separating a mixture of taxanes from aqueous cell suspension media. The method relies on the inability to pellet taxanes produced by *T. cuspidata* cell cultures, from their culture broth, by centrifugal force of 16,000 x g for 30 min. When the cells are filtered through 0.2 μm nylon or PVDF membranes, the taxanes are retained by the filters. These taxanes can then be washed and eluted to produce a relatively clean mixture of taxanes. This technique eliminates the need for a solvent partitioning step to extract the crude taxane mixture, as well as eliminating the need for chlorinated hydrocarbons typically used in these extractions. A relatively small volume of methanol or ethanol is all the solvent that is needed to elute the taxanes from the filter membranes, greatly reducing the environmental impact of this method.

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GLUCURONIC ACID-SILICA, A NOVEL SUPPORT FOR HIGH-PERFORMANCE CATION-EXCHANGE CHROMATOGRAPHY

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ABSTRACT

Glucuronic acid was covalently coupled to aminopropyl derivatized silica by reducing the Schiff base with $NaCNBH_3$. This glucuronic acid-silica has a functional pKa in the pH 3-4 range and binds 202 mg hemoglobin per gram material at pH 5. The support is a high-performance cation-exchanger and exhibited only minor hydrophobic interaction with applied samples. It also gave reasonably constant retention of the proteins tested.

INTRODUCTION

Ion-exchange chromatography has been a powerful technique in purifying proteins based on their charge characteristics. Low-pressure ion-exchangers such as carboxymethyl-Sephadex have been widely used. Low-pressure ion-exchange chromatography, however, has limited resolution and, because of pressure limitations, can be quite slow (1, 2).

In 1976, Chang et al. (3) introduced high-pressure ion-exchange chromatography (HPIEC) of proteins using microparticulate spherical silica. Silica supports are rigid and capable of withstanding high pressure. However, the silica surface must be chemically

modified for HPIEC to minimize non-specific interactions between mobile solutes (e.g., proteins) and the stationary phase matrix.

Mixed-mode behavior occurs in HPIEC when, in addition to the primary interaction (i.e. ion-exchange), a secondary separation mechanism (e.g. hydrophobic) is found (4). Although a mixed-mode column as such may offer unique selectivities for protein separations, its major problems are poor protein recovery, tendency to denature protein, and less predictable chromatographic behavior (2, 5, 6). Most high-performance liquid chromatography (HPLC) columns available today for protein separation show various degrees of mixed-mode behavior1. The most common mixed-mode in HPIEC reported is the reversed phase (hydrophobic) mode (4, 5, 7, 8).

Hydrophobic mixed-mode behavior can be minimized by adding organic modifier to mobile phase (e.g., 10% isopropanol); maximizing the hydrophilicity of the stationary phase provides an alternative approach. Here, we report the coupling of glucuronic acid, a hydrophilic ionic molecule, to aminopropyl derivatized silica. Potential uses of this glucuronic acid-silica as a cation-exchange HPLC support were investigated.

METHODS

Chromatography: The Chromatograph was a Rainin Rabbit-HP solvent delivery system outfitted with a Knauer variable wavelength detector, a Macintosh SE computer, and Rainin Dynamax version 1.2 software for data collection and analysis. Chromatography was at room temperature (20 ^{o}C) with a 1 ml/min flow-rate throughout.

Preparation of Glucuronic Acid-silica: Glucuronic acid-silica was prepared by a modification of the procedure used to couple glucose to silica (9). Five milliliters (1 mmol) 0.2 M D-glucuronic acid (Aldrich Chemical Company, Inc., Milwaukee, Wis.), 0.4 M sodium phosphate, pH 6.8, and 126 mg (2 mmol) $NaCNBH_3$ was reacted per gram of 7-µm aminopropyl-silica (300 Å pore) at 60 ^{o}C for 5 h. The reaction was carried out twice in order to obtain product which was consistently Cd-ninhydrin10 negative at room temperature. Glucuronic acid-silica after the first reaction was washed with water only and after the second reaction was first washed with acetone and then with water. The product was then dried from acetone at 60 ^{o}C.

Titration of Glucuronic Acid-silica: Glucuronic acid (100 mg) was titrated in 10 ml 1 M NaCl. Enough 0.1 M HCl was initially added to bring the pH to 2. Then 50 µl portions of 0.1 M NaOH were added and the pH recorded until it reached 12. pH was monitored

using a Corning pH Meter Model 220 and Orion Combination pH Electrode (Model 91-02).

Hemoglobin (Hb) Binding Capacity Determination: Glucuronic acid-silica and glucose-silica (50 mg each) were equilibrated in 20 mM acetic acid-NaOH, pH 5. The supports were then mixed with 1 ml of 15 mg/ml Hb for 15 minutes. The amount of Hb bound was determined spectrophotometrically by measuring the absorption of the supernatant at 410 nm, based on one absorbance unit = ~0.158 mg/ml Hb (11). Glucose-silica, which does not bind Hb, was used as control.

Elemental Analysis: Glucuronic acid-silica and aminopropyl-silica were sent to Galbraith Laboratories, Inc. (Knoxville, TN) for elemental (C, N, and H) analysis.

Column Packing: Glucuronic acid-silica was packed into 100 x 4.6 mm I.D. columns using a stainless steel reservoir and a Rainin Chromatography pump. A slurry containing 2 grams glucuronic acid-silica and 4 ml water was prepared to fill the column and reservoir. Using water as the solvent, the flow-rate was initially at 0 ml/min at time zero and increased linearly to 5 ml/min in five minutes, remained at 5 ml/min for next ten minutes, and returned back to 0 ml/min by 20 minutes.

Column Testing: Columns were tested as described in figure legends. All proteins and biochemicals used for column testing were from Sigma Chemical Co. (St. Louis, MO). Columns were stored at 4 ^{0}C in 10 mM sodium azide, 10 mM acetic acid-NaOH, pH 5.

Hydrophobicity Test: Concentrated lysine and lysyl-leucine (> 10 mg/ml) were prepared in aqueous solution. Each was injected in triplicate on the column. Both separations used a 10 minute linear gradient from 0 M NaCl to 0.1 M NaCl in 10 mM acetic acid-NaOH, pH 5. Detection was at 230 nm.

RESULTS

The titration of glucuronic acid-silica is shown in Fig. 1. The only titratable groups observed have a pKa in the pH 3-4 range. The carboxylic acid at carbon six of a series of glucuronides also has a pKa in this range (12, 13). This low pKa should allow the support to be used for chromatography over a larger pH range than the carboxymethyl type cation-exchangers (pKa ~4.7).

The protein binding capacity of glucuronic acid-silica was quite high. Glucuronic acid-silica bound 202 mg-hemoglobin per gram at pH 5. In agreement with a pKa in the

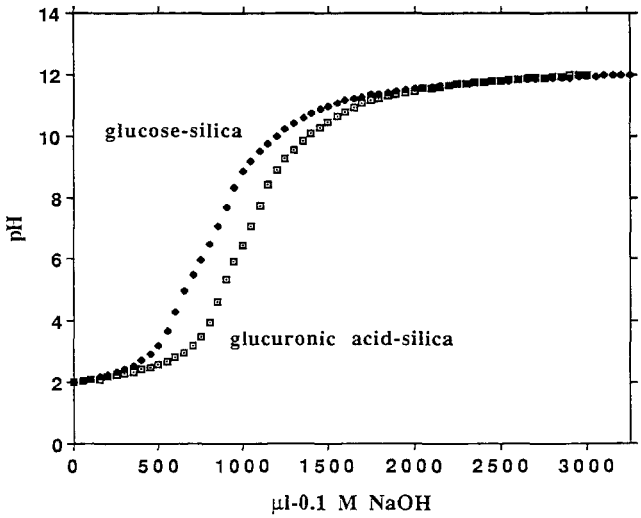

FIGURE 1. Titration curves of glucuronic acid-silica and glucose-silica.

pH 3-4 range, about 10% as much hemoglobin bound at pH 3 and almost none bound at pH 2.5. The shift in the titration curve for glucuronic acid-silica relative to a glucose-silica control (Fig. 1) gave a measure of 0.2 milliequivalents per gram for this particular batch of glucuronic acid-silica. On a different batch of material, we found by elemental analysis that the support contained 0.11 mmol glucuronic acid per gram silica. Thus, capacities in the range of 0.1-0.2 milliequivalents per gram were found.

All of the glucuronic acid-silica prepared as described gave no detectable reaction with the Cd-ninhydrin reagent at room temperature suggesting that no unreacted aminopropyl groups remain. However, elemental analysis revealed this is not the case. Elemental analysis of a typical batch of support gave 0.11 mmol glucuronic acid and 0.38 mmol aminopropyl per gram silica showing that reaction was only about 30% complete. Although the reaction used to synthesize glucuronic acid-silica was carried out twice, steric hindrance and charge repulsions between adjacent glucuronic acids might account for this observed low coupling efficiency. Why Cd-ninhydrin reaction failed to detect remaining aminopropyl groups is unclear but may suggest that the reagent is sterically excluded from the aminopropyl layer. However, the pH dependency

of hemoglobin binding already discussed suggests that glucuronic acid-silica is a high capacity cation-exchanger which was next characterized chromatographically.

In Fig. 2, four proteins (carbonic anhydrase, myoglobin, cytochrome c, and lysozyme) were found to elute in the order predicted from their pI values (5.9, 6.9, 9.4, and 11.0, respectively). The unlabeled peak eluting near six minutes is a contaminant found in the cytochrome c used. Proteins with basic isoelectric pH (pI) values such as lysozyme and cytochrome c should bind tighter to the column and be eluted later than proteins with more neutral pI values such as carbonic anhydrase and myoglobin. Although this relationship was demonstrated by the separation of the proteins selected, injection with bovine pancreas ribonuclease A (pI 8.7) (14) disobeyed the relationship. It eluted before myoglobin while it should elute after based upon pI (data not shown). Kopaciewicz et al. (15) have shown that the chromatographic behavior of proteins is not entirely predictable based upon pI value alone and have suggested this is due to the complexity of protein structure. Alternatively, our results with ribonuclease A may be due to mixed chromatographic modes due to residual, unreacted propylamines.

Protein recovery was high as shown in Fig. 3. Five injections of myoglobin were eluted with an average recovery of 95%. The fluctuation seen was probably due to combined errors in injections and protein concentration determinations. In general, high dynamic protein mass recovery is indicative of a hydrophilic column since hydrophobic mixed-mode columns generally give poor protein mass recovery (5, 6). Hydroxyl groups provided by glucuronic acid on the silica surface should contribute significantly to this hydrophilicity.

A hydrophobicity test was designed to investigate more directly if glucuronic acid-silica also had hydrophobic interactions with applied samples. Two compounds of equal charge but different hydrophobicity were used to assess hydrophobic interaction with the stationary phase. The compounds were lysine (lys) and lysyl-leucine (lys-leu) with the latter being the more hydrophobic. Results summarized in Table I show lys-leu was retained significantly longer than lys by the column under the same conditions. A column without any hydrophobic interaction should give the same retention times for both compounds. The results suggest that glucuronic acid-silica also has some hydrophobic interaction with these samples though the effect is relatively minor.

Fig. 4 shows the results of a dynamic stability test performed on glucuronic acid-silica. Lysozyme, myoglobin, benzylamine, and phenylacetic acid were examined for five 8 h. days of rigorous chromatography. The results show that retention times of both proteins were slightly decreased throughout. The biggest change, however, occurred

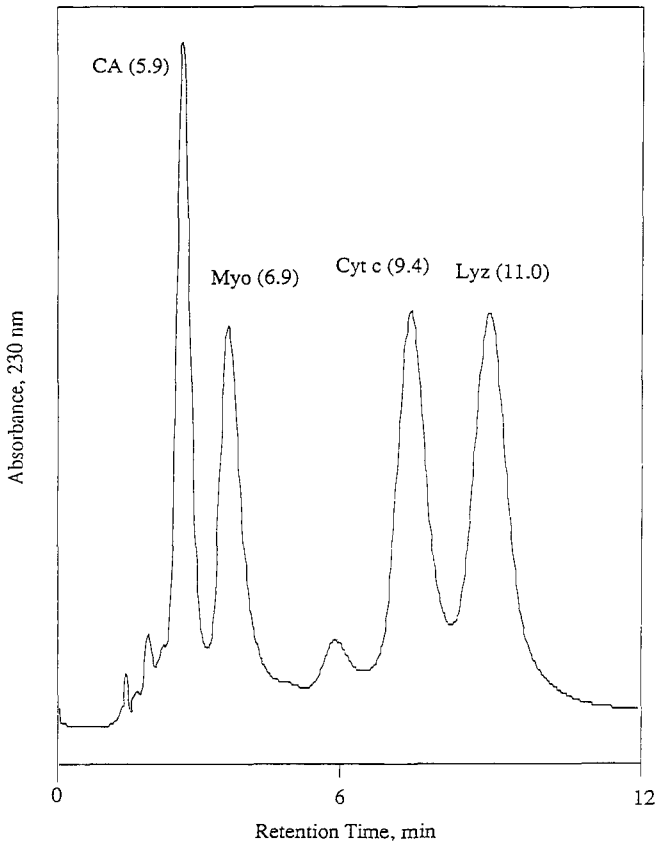

FIGURE 2. The cation exchange chromatography separation of four proteins is shown. A protein mixture containing bovine carbonic anhydrase (CA), horse heart myoglobin (Myo), horse heart cytochrome c (Cyt c), and chicken egg white lysozyme (Lyz) were separated in order of their isoelectric pH values shown (on the figure in parentheses) (14, 15). Buffer A (20 mM acetic-NaOH, pH 5) and buffer B (0.5 M NaCl in buffer A) were used in a 10 minutes linear gradient from 30% to 40% buffer B. Injection was 10 µl of ca. 4 mg/ml protein mixture. Each protein was also injected individually onto the column to confirm the identity of each peak in the mixture.

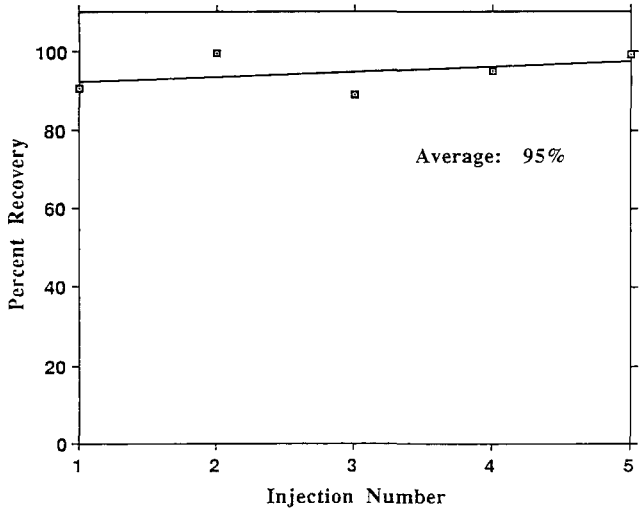

FIGURE 3. Recovery of myoglobin injected onto a glucuronic acid-silica column. After two test runs, the glucuronic acid-silica column was used to study the dynamics of protein recovery. Each injection was 10 µl of 10 mg/ml myoglobin. The bound myoglobin was eluted from the column using a 10 minute linear gradient from buffer A (20 mM acetic acid-NaOH, pH 5) to buffer B (20 mM acetic acid-NaOH, 1 M NaCl, pH 5). Peak fraction was collected and the myoglobin concentration was determined16 using bovine serum albumin as standard. 100% recovery is that found when a zero dead volume union was used in place of the column.

TABLE 1.

Elution* (Column Volumes)		
Lysine	Lysyl-leucine	% Difference
1.506 ± 0.055	1.662 ± 0.020	9.4 ± 4.4

*Retention times are expressed as the mean ± the standard deviation with n = 4.

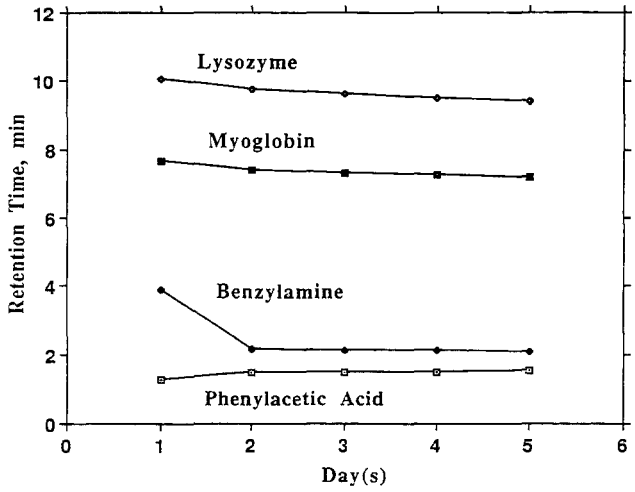

FIGURE 4. Dynamic stability of glucuronic acid-silica. A glucuronic acid-silica column was subjected to continuous repetitions of a chromatographic cycle (15 minute linear gradient from 0 M to 0.5 M NaCl in 20 mM acetic acid-NaOH, pH 5, followed by a 10 minute reequilibration with the zero salt buffer, then repeat) for 8 hours each day except the first day. The first day was devoted to working out the method, obtaining the initial chromatograms, and about four hours of repetitive chromatography. Subsequent chromatograms were obtained each day in the morning and evening and the retention times for these two runs were averaged. Average retention times for each day are plotted.

with benzylamine between day 1 and day 2. The results suggest that the anionic characteristic of glucuronic acid-silica diminished during the initial day of chromatography but remained fairly constant afterward. Behavior of oppositely charged phenylacetic acid whose retention time was increased by the second day also agrees with this conclusion. One likely explanation for the phenomenon is that some portion of the glucuronic acids is attached noncovalently and washed away during initial hours of chromatography. Despite the behavior observed with benzylamine and phenylacetic acid, the column gives reasonably constant retention of the proteins and gave quite consistent performance for both proteins and small molecules after an initial one day washing period.

The long term stability of glucuronic acid-silica was also investigated over a period of two years and nine months. When not in use, the column was stored at 4^{0} in buffer A containing 10 mM NaN_3. At the end of the period, the separation in Fig. 2 was repeated and again all four proteins were well separated and eluted in the same order although column performance was somewhat degraded (data not shown).

DISCUSSION

A novel and simple way of making HPLC cation-exchange silica-based support is described. The support has a lower pKa than the conventional carboxymethyl type and high Hb static binding capacity at pH 5. Despite the presence of residual cationic propylamine moiety, four basic proteins were easily separable under the conditions used and overall our study suggests that glucuronic acid-silica is a high-performance cation-exchanger. It also exhibited relatively minor hydrophobic interactions. The columns also gave high dynamic protein mass recovery and appeared to be fairly stable for protein separations during the time tested. In short, glucuronic acid-silica represents a novel hydrophilic support suitable for high-pressure cation-exchange chromatography of proteins.

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STRUCTURE-ACTIVITY RELATIONSHIPS OF 2-CHLORO-2'-*ARABINO*-FLUORO-2'-DEOXY-ADENOSINE AND RELATED ANALOGUES: PROTEIN BINDING, LIPOPHILICITY, AND RETENTION IN REVERSED-PHASE LC

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ABSTRACT

Plasma protein binding of 2-chloro-2'-*arabino*-fluoro-2'-deoxyadenosine (CAFDA), 2-chloro-2'-deoxyadenosine (CdA), 2-fluoro-1-β-D-arabinofuranosyladenine (F-araA) and structurally related analogues 2-chloro-adenosine (2-Cl-Ado), 5'-chloro-5'-deoxyadenosine (5'-Cl-5'-dAdo), as well as parent nucleosides 2'-deoxyadenosine (dAdo), 1-β-D-arabinofuranosyladenine (araA) and adenosine (Ado) was determined and correlated with lipophilicity expressed as the logarithm of partition coefficient in n-octanol/water system (log $P_{O/W}$). Drug binding to human serum albumin (HSA) was utilized since it is considered to exemplify nonspecific binding of small molecules to other macromolecules.

Percentage of drugs bound to HSA increased from 3.5 % to 27 % following the order of increase in lipophilicity (log $P_{O/W}$

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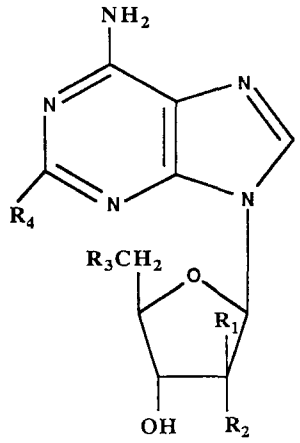
increased from -0.970 to 0.498). A similar correlation was observed when protein binding was correlated with retention in reversed-phase liquid chromatography (RP-LC) (capacity ratios, k', increased from 0.36 to 1.15), but the elution order of some compounds did not follow the parallel increase in either protein binding or lipophilicity.

The introduction of a fluorine at the 2'-arabino position of CdA not only increased the acid stability of CAFDA, but also resulted in a higher binding to HSA (27.0% for CAFDA versus 24.3% for CdA) and much higher lipophilicity (log P of 0.498 for CAFDA compared to 0.025 for CdA).

INTRODUCTION

2-Chloro-2'-*arabino*-fluoro- 2'-deoxyadenosine (CAFDA) (Fig. 1) is an acid-stable 2'-*arabino*-fluoro-derivative of CdA (Cladribine, Leustatin), a newly developed anticancer drug currently in phase II trials in the treatment of lymphoprolipherative disorders. CdA is acid-labile and has only approximately 50 % oral bioavailability (1). The synthesis of CAFDA was based on the rationale that the introduction of a fluorine at 2'-arabino (up) position of 2',3'-dideoxyadenosine increased the chemical stability of the glycosidic bond of the compound to acidic or enzymatic hydrolysis (2, 3). This was confirmed by the study of Carson et al (4), where the in vitro antilymphocytic activity of CAFDA was demonstrated. The results suggested that CAFDA might substitute for CdA as an effective oral drug.

The importance of physico-chemical properties including ionization constants, pK_a, and lipophilicity for drug absorption has been widely recognized. The interaction of drugs with plasma proteins (i.e. human serum albumin (HSA), α_1-acid -glycoprotein (AGP),...) may have important pharmacokinetic implications as regards drug disposition and action. Though it has long been thought that only the free drug in plasma was available for diffusion into tissues, recent studies have shown that a part of the drug bound in plasma can be dissociated in capillaries and thus becomes available for transfer (5).



Compound	R_1	R_2	R_3	R_4
Ado	H	OH	OH	H
2-Cl-Ado	H	OH	OH	Cl
5'-Cl-5'-dAdo	H	OH	Cl	H
dAdo	H	H	OH	H
CdA	H	H	OH	Cl
CAFDA	F	H	OH	Cl
ara A	OH	H	OH	H
F-ara A	OH	H	OH	F

FIGURE 1. Structural formulae of studied compounds.

The goal of our study was to assess the relationship between protein binding and lipophilicity, as a prerequisite for drug absorption, disposition and also penetration into CNS, of CAFDA, clinically used CdA and F-araA, and structurally related analogues 2-Cl-Ado, 5'-Cl-5'-dAdo, as well as parent nucleosides dAdo, araA and Ado. The binding to HSA was correlated with lipophilicity expressed as the logarithm of partition coefficient (log $P_{O/W}$) in n-octanol/water system. The use of capacity ratios (k') in the reversed-phase LC to predict hydrophobicity of studied compounds was assessed and the correlation between retention behaviour and plasma protein binding was compared with the above mentioned.

EXPERIMENTAL

Materials

CdA was synthesized by Dr. Zygmunt Kazimierczuk (Foundation for the Development of Diagnostics and Therapy, Warsaw, Poland). CAFDA was a gift from Dr. Howard Cottam (University of California, San Diego, CA, USA) and F-araA was a gift from Dr. Ze've Shaked (Berlex, Alameda, CA, USA). The purity of CdA, CAFDA and F-araA was more than 99 % as checked by HPLC and mass spectrometry. The nucleosides Ado, dAdo, 2-Cl-Ado, araA and 5'-Cl-5'-dAdo, human serum albumin (99% purity) and 1-octanol were obtained from Sigma (St. Louis, MO, USA). Methanol was of HPLC grade (J.T.Baker, Deventer, Netherlands). Analytical-reagent grade potassium dihydrogen phosphate and potassium hydroxide were purchased from Merck (Darmstadt, Germany).

Determination of protein binding

In vitro protein binding of the studied compounds was measured in triplicate by equilibrium dialysis, at 37°C, in the dark under continuous agitation for 6 hours. The apparatus consisted of Lucite cells using SPECTRA/POR molecular porous membranes (Spectrum Medical Industries Inc., LA, U.S.A.) (m.w. cut off 3500). Two hundred µl of 1 µmol/L solutions of drugs dissolved in either human serum albumin (40 g/L) or in human plasma (pooled from 6 healthy volunteers) were introduced on one side of the dialysis membrane and an equal volume of saline solution (pH 7.4) to the other side. To prevent the deamination during dialysis, the adenosine deaminase inhibitor, deoxycoformycin (dCF), was incubated with HSA or blank plasma samples for 30 min at 37°C in the concentration of 5 µmol/L before solutions of Ado, dAdo and araA

were added. At equilibrium, the volumes of both compartments were measured to assess the possible volume shift and the concentrations of studied compounds were determined from 100 µl in both compartments by reversed-phase LC. Samples from the buffer compartment were injected directly and those from the protein compartment were diluted 10 times before the analysis. Nonspecific binding to the filter was assessed by comparing the concentrations of the drugs in a saline buffer alone, before and after dialysis, and was consistently less than 2%. The bound fraction was calculated as $(C_1 - C_2)/C_1$, where C_1 is the concentration in the protein compartment and C_2 the concentration in the buffer compartment at the end of the dialysis.

High performance liquid chromatography

The chromatographic system consisted of a Shimadzu LC-9A pump (Shimadzu Corp., Kyoto, Japan), a CMA-240 Carnegie autosampler (Carnegie Medicine, Stockholm, Sweden) and a Milton Roy variable wavelength detector (Milton Roy, LDC Division, USA). A Macintosh Classic computer (Apple Inc., Chicago, IL, USA) equipped with Chromac 3.1. software (Drew Ltd, London, UK) was used for collecting the HPLC data. The capacity factors k' were determined isocratically on a high speed C_{18} column (80 x 4.6 mm, 3 mm, Perkin-Elmer, Norwalk, CT, USA) at 265 nm and 22°C. An aqueous mobile phase of 0.01 M KH_2PO_4 with 20 % of methanol, pH 7 at the flow-rate of 1 ml/min was used. The pH was adjusted with a few drops of potassium hydroxide before methanol was added using a PHM 62 standard pH meter (Radiometer, Copenhagen, Denmark). The k' values were calculated as $(t_r-t_o)/t_o$, where t_r is the retention time of an individual compound and t_o, the retention time of an unretained compound determined as the time from injection to the first distortion of the baseline.

Determination of pK_a and lipophilicity (log P)

pK_a was determined spectrophotometrically and lipophilicity by shake-flask method as described in our previous study (6).

RESULTS AND DISCUSSION

The binding of the studied compounds to HSA and plasma proteins is reported in Table 1. The percentage of drugs bound to HSA and plasma proteins was in the range of 3.5% to 27% and 14% to 47.1%, respectively. A good correlation (polynomial equation, $y = 24.3 + 5.2*x - 5.2*x^2 + 10.8*x^3$, $r = 0.989$) was observed between binding to HSA and lipophilicity expressed as log P (Fig. 2), while no correlation was found between log P and the total plasma protein binding (Table 1). The spectrophotometrically determined pK_a of CAFDA was higher than that of CdA (1.75 for CAFDA versus 1.28 for CdA).

We have recently reported on the effects of structural changes in the molecules of Ado, dAdo and araA on ionization constants (pK_a), lipophilicity (log P) and retention in RP-LC (log k') (6). It was confirmed that the introduction of a halogen atom (chlorine, fluorine) into a molecule increases the lipophilic character of the compound, with CdA as the most lipophilic in the studied group. There was a good linear correlation observed between log P and log k' and, in agreement with other studies, it was concluded that lipophilicity of new nucleoside analogues could be predicted from their retention in RP-LC. Thus, CAFDA, a new derivative of CdA, which was the most retained in RP-LC in the present study (Fig. 3), was supposed to be the most lipophilic from all studied structurally related purine analogues. This was confirmed by the log P value determined in n-octanol/water system (log P of 0.498).

The binding of CAFDA to HSA was higher than that of CdA (27.0% for CAFDA versus 24.3% for CdA) and it was the highest in the group of studied compounds. While binding to HSA followed the

TABLE 1

Lipophilicity, Protein Binding and Retention in RP-LC
of CAFDA and Related Nucleoside Analogues

Compound 1 μmol/L	Log P	% B ± SD HSA	% B ± SD Plasma	Log k'
Ado + 5 μmol/L dCF	-0.970*	3.5 ± 0.1	22.4 ± 1.2	0.48
ara A + 5 μmol/L dCF	-0.955*	6.6 ± 0.0	-	0.36
F-ara A	-0.801*	9.1 ± 0.8	14.0 ± 3.5	0.52
dAdo + 5 μmol/L dCF	-0.611*	18.9 ± 0.1	22.4 ± 3.2	0.58
2-ClAdo	-0.368*	20.2 ± 6.0	30.1 ± 3.3	0.96
5'-Cl-5'-dAdo	-0.055*	24.4 ± 3.1	22.4 ± 9.5	1.10
CdA	0.025*	24.3 ± 1.2	21.1 ± 8.7	1.01
CAFDA	0.498	27.0 ± 2.0	47.1 ± 7.6	1.15

*Log P values were taken from Reichelova et al (Ref. 6), protein binding was determined using equilibrium dialysis and drug concentrations were analyzed on HPLC using C_{18} column (80 x 4.6 mm, 3 μm), mobile phase, 0.01 M KH_2PO_4 with 20% MeOH pH=7.0, flow rate 1 ml/min, detection 265 nm
Results for % bound drugs are the mean (±SD) of 3 experiments

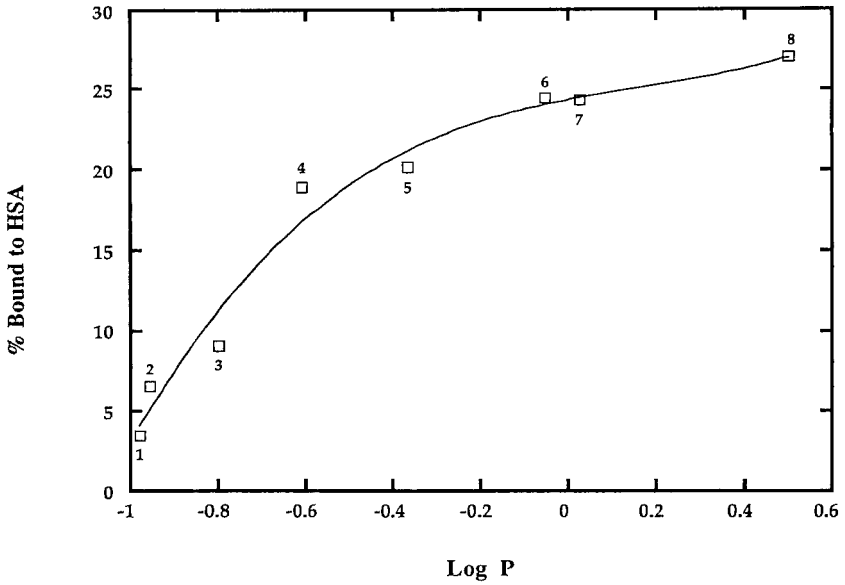


FIGURE 2. Correlation between percentage of drugs bound to HSA and logarithm of partition coefficients (log P) in n-octanol/water system. (1) Ado, (2) ara A, (3) F-ara A, (4) dAdo, (5) 2-ClAdo, (6) 5′-Cl-5′-dAdo, (7) CdA and (8) CAFDA.

order of increase in lipophilicity expressed as log P, it did not follow the elution order (increase in log k') so rigidly (Fig. 4). The explanation can be found in the differences of interactions of protein binding, partitioning and retention in RP-LC. Binding to HSA and liquid-liquid partitioning are both nonspecific processes, while retention in RP-LC is of a mixed character involving both partitioning and adsorptive interactions (7). Though HPLC has often been used for determination of the lipophilicity of drugs our results show that log P determined by a traditional liquid-liquid partitioning predicts the binding to HSA better than log k'.

In spite of a better correlation between the binding to HSA and the log P we would like to emphasize the importance of using

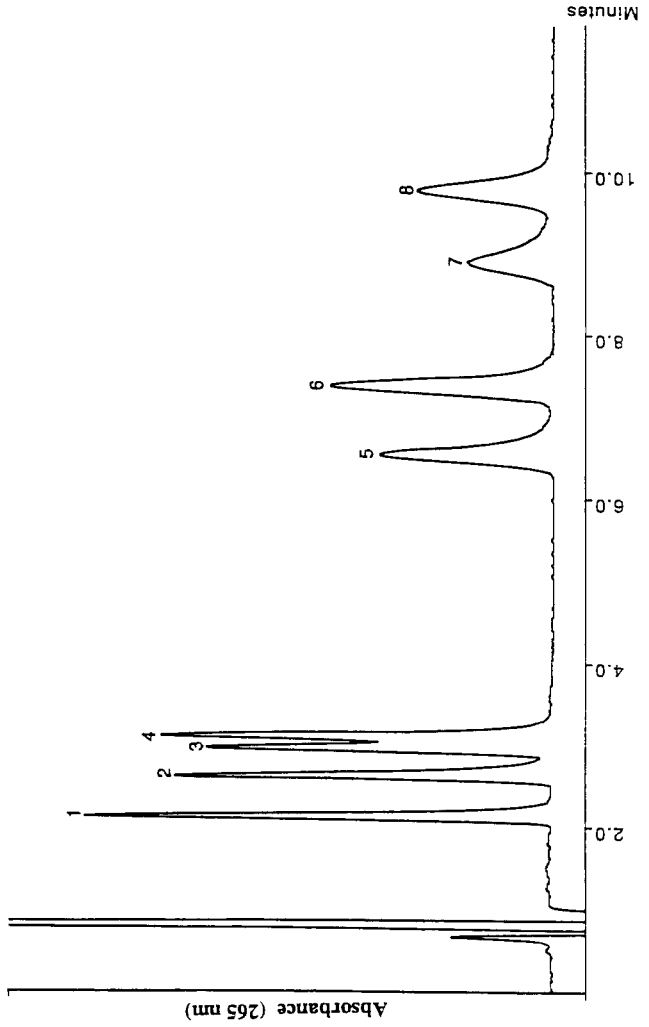


FIGURE 3. Reversed-phase chromatographic separation of (1) ara A, (2) Ado, (3) F-ara A, (4) dAdo, (5) 2-ClAdo, (6) CdA, (7) 5′Cl-5′-dAdo, and (8) CAFDA. Column, high-speed C18 (3 μm) (80 x 4.6 mm I. D.); mobile phase, 0.01 M KH2POH4 (pH 7.0) with 20% of methanol; flow-rate, 1 ml/min; detection 265 nm.

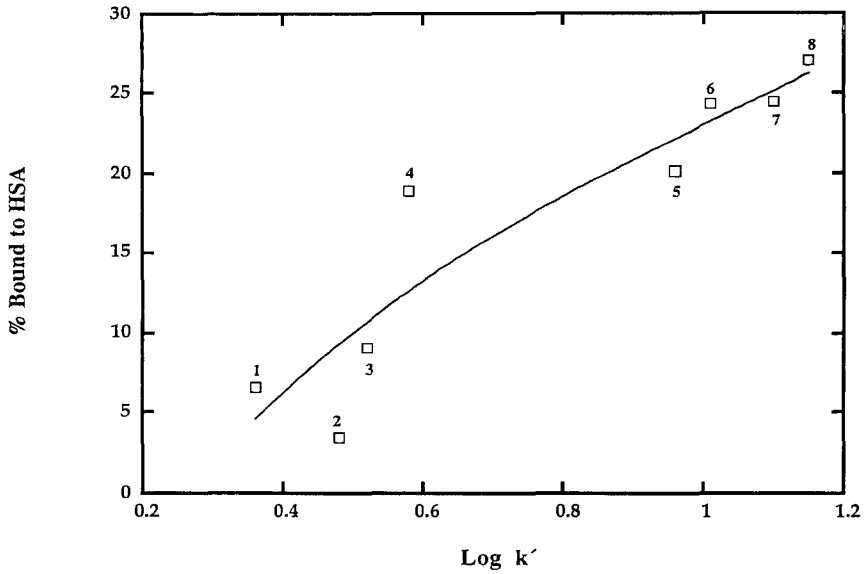


FIGURE 4. Correlation between percentage of drugs bound to HSA and logarithm of capacity factors in reversed-phase LC (log k') of (1) ara A, (2) Ado, (3) F-ara A, (4) dAdo, (5) 2-ClAdo, (6) CdA, (7) 5′Cl-5′-dAdo, and (8) CAFDA.

retention characteristics of drugs in RP-LC in order to predict the binding of metabolites and analogues. Our results lend support to the conclusion of a similar study of correlation between retention in RP-LC and plasma protein binding of betaxolol and its analogues (8). Although the retention behaviour may not always quantitatively predict the binding in plasma it can give a prediction of potential binding interactions. In this view, the reversed-phase LC is considered to be a useful tool for the estimation of both lipophilicity (log P) and protein binding. It is simple and easy to use and only small amounts of drugs are required compared to equilibrium dialysis and shake-flask method.

No correlation was observed between log P and total plasma protein binding. The composition of plasma and the binding

mechanisms may explain this lack of correlation. Human plasma contains over 60 proteins with HSA and AGP as the most abundant and most studied. While the hydrophobic interaction governs the binding to HSA, the involvement of electrostatic interactions in the binding to AGP must be considered (9).

The importance of plasma protein binding and lipophilicity as determinants of transport across the blood-brain barrier was confirmed by a study of brain uptake of benzodiazepines (10). The relationship between lipophilicity and protein binding, as well as its effects on anti-HIV activity was recently reported in a series of anti-HIV agents (11). Again, lipophilicity was regarded an important factor which may affect the entry of the compounds into the CNS. In this respect, the higher lipophilicity of CAFDA compared to CdA makes us assume a good penetration of CAFDA into CNS since CdA itself was shown to penetrate the blood-brain barrier (12, 13).

Recently, in addition to deoxycytidine kinase (dCK), another enzyme, deoxyguanosine kinase (dGK), was observed to contribute to CdA phosphorylation in crude extracts of malignant human brain tissue (14). In the view of a similar metabolism of CAFDA and CdA our results are of a clinical importance not only for the oral administration, but also in the case if the therapeutic role of CdA in the treatment of brain malignancies is proved.

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DETERMINATION OF RATE CONSTANTS OF CONSECUTIVE FIRST ORDER REACTIONS OCCURRING ON CHROMATOGRAPHIC COLUMNS

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ABSTRACT

Approximate analytical equations were developed for the products of consecutive reactions occurring on a chromatographic column. The equations were tested both by numerically evaluated chromatograms and the acid catalyzed hydrolysis of phthalic dichloride in a liquid chromatographic reactor.

INTRODUCTION

The determination of rate constants from chromatograms of on-column reacting substances has been reported

several times by different authors /1 /, /2 /.
Mostly, however, the papers are focused on simple first order reactions or do not pay attention to the possibility of the occurrence of complex reactions.
The present paper deals with first order consecutive reactions: $A_1 \xrightarrow{k_1} A_2 \xrightarrow{k_2} A_3$.
It is characteristic for a chromatographic reactor that the separation of reactants and products is incomplete, since the reactions occur from the beginning until the end of the columns, and there is no possibility of a separation of the products formed near the end of the column. Therefore, in consecutive reactions always a mixture of the intermediate product and the final product is observed, and the evaluation of peak shape equations for both substances becomes necessary.

PULSE SHAPE EQUATIONS

Mathematical derivation and semi-analytical solution

The mathematical derivation of the pulse shape equations will be based on the following idea:
While passing a cross-section of the chromatographic column the reactant produces a differential product pulse having the initial shape of the reactant pulse at

this cross-section and an amount of substance due to the kinetics of its formation. This differential product pulse is then eluted in a normal chromatographic way, i. e. the moments of the pulse shape equation are altered in the same way as for an injected substance.

The product pulse at the end of the column is then derived by adding (integrating) all the concentration elements of all differential product pulses, which pass simultaneously at the end of the column. However, in case of an intermediate product of a consecutive reaction one must consider the loss of substance due to its transformation to the final product. Therefore, the amount of a differential pulse from the intermediate product at the end of the column is given by the product of the amount produced at x, which is $m_{10}k_{1a}\ \exp(-k_{1a}\ x/l)\ dx$, and a factor for the part not converted to the final product, which is $\exp(-\ k_{2a}(l-x)/l)$:

$$dm_2(l) = m_{10}k_{1a}e^{-k_{1a}\frac{x}{l}}e^{-k_{2a}\frac{(l-x)}{l}}dx \qquad (1)$$

Hence, a concentration element of the product pulse is given by multiplication of the amount of the product pulse and a pulse shape equation (for a summary of suitable pulse shape equations see /3/):

$$dc_2(x,l,t) = dm_2(x,l)\,\psi(\mu_i,t)\,dx \qquad (2)$$

The intermediate product pulse shape equation was found by transformation of Eqn.(2) to time coordinates using the equation

$$\frac{x}{l} = \frac{(\mu - \mu_2)}{(\mu_1 - \mu_2)} \tag{3}$$

and integration:

$$c_2 = m_{10} k_1' e^{-k_2'(\mu_1 - \mu_2)} e^{-(k_1' - k_2')(t - \mu_2)} \int_{\mu_1}^{\mu_2} e^{-(k_1' - k_2')(\mu - t)} \psi \, d\mu \tag{4}$$

The derivation of a pulse shape equation for the final product is still more complicated:
First, a differential amount of the final product pulse is considered, which is formed by a differential intermediate product pulse at position y in the column. The differential intermediate product itself was formed before at the position x (with x < y):

$$\begin{aligned} ddm_3 &= k_{2a} dm_{20}(x, x) e^{-k_{2a}\left(\frac{y}{l} - \frac{x}{l}\right)} dy \\ &= m_{10} k_{1a} k_{2a} e^{-(k_{1a} - k_{2a})\frac{x}{l}} e^{-k_{2a}\frac{y}{l}} \end{aligned} \tag{5}$$

Then, multiplication with the pulse shape equation, transformation to time coordinates and integration leads to the final product pulse shape equation:

$$c_3 = m_{10} k_1' k_2'' e^{-\Delta k(t-\mu_2)} e^{-k_2''(t-\mu_3)} \int_{\mu_2}^{\mu_1} e^{-\Delta k(\mu-t)} \int_{\mu_3}^{\mu} e^{-k_2''(\mu-t)} \psi \, d\mu \, d\mu \qquad (6)$$

Even in case of the probably simplest single pulse shape equation, the Gaussian, a numerical solution of both the product pulse equations (Eqn. 4 and Eqn. 6)is necessary, since the standard deviation of the differential pulse shapes depends on μ and μ ', respectively.

However, if the standard deviations of the pulses do not differ in order of magnitudes one can apply approximate equations for the standard deviations, in which these do not depend on the μ or μ', but on the time. In the evaluation of the standard deviation of the final product its dependence on the standard deviation of the intermediate product is completely neglected.

$$\sigma^2 = \sigma^2_i + \frac{t-\mu_i}{\mu_1-\mu_i}(\sigma^2_1 - \sigma^2_i) \qquad (7)$$

intermediate product: $i=2$

final product: $i=3$

Moreover, the approximate formulae developed here lead to slight "losses" or "gains" of mass due to an additional skewness of the product peaks, which is produced by the approximation.

Then, some partial integrations with the final product functions (Eqn.6) are possible, leading to:

$$
\begin{aligned}
c_3 = m_{10} \frac{k'_1 k''_2}{\Delta k} & e^{-\Delta k(t-\mu_2)} e^{-k''_2(t-\mu_3)} \\
& [PI(\mu_2, \mu_1, (\Delta k + k''_2)) \\
& + e^{-\Delta k(\mu_2 - t)} (PI(\mu_3, \mu_2, (k''_2)) \\
& - e^{-\Delta k(\mu_2 - t)} e^{-\Delta k \frac{\Delta\mu_{12}}{\Delta\mu_{13}}(t-\mu_3)} PI(\mu_3, \mu_1, (\Delta k \frac{\Delta\mu_{12}}{\Delta\mu_{13}} + k''_2))]
\end{aligned}
\tag{8}
$$

Similar, Eqn. 3 for the intermediate product can be rewritten:

$$
\begin{aligned}
c_2 = m_{10} k'_1 & e^{(-k'_2(\mu_1 - \mu_2)} \\
& e^{-k'_2(t-\mu_2)} PI(\mu_1, \mu_2, (k'_1 - k'_2))
\end{aligned}
\tag{9}
$$

The product integral PI in Eqn. 8 and Eqn. 9 is defined by:

$$
\begin{aligned}
PI(\mu_A, \mu_B, k, \sigma) = & e^{\frac{k}{2}\sigma^2} \\
& \left(\int_{-\infty}^{\mu_B} \frac{1}{\sqrt{2\pi}\sigma} e^{-(\frac{t-\mu}{2\sigma})^2} d\mu - \int_{-\infty}^{\mu_A} \frac{1}{\sqrt{2\pi}\sigma} e^{-(\frac{t-\mu}{2\sigma})^2} d\mu \right)
\end{aligned}
\tag{10}
$$

The main advantage of the approximation is that it becomes possible to evaluate approximate product pulses in very short time (using polynomial approximations for the Gaussian integrals /6/), which is favorable for curve fitting procedures - in our opinion the only one possibility to calculate rate constants from reaction chromatograms of complex reactions.

Discussion of the product pulse shapes

First of all it should be pointed out, that the PI-functions in the product pulse equations become one or at least constant between the moments forming the boundaries of the integral.

Therefore, similar to the product pulse in a simple irreversible reaction the intermediate product pulse is an exponential function between μ_1 and μ_2.However, while in a simple reaction there is always a decay from μ_1 to μ_2, the intermediate pulse can increase, decrease or even become constant, depending on the sign of the difference between the rate constants (Fig. 1). If there was a possibility to observe the pure intermediate, the difference of the rate constants could be evaluated by the so-called product curve method /4/,/5/.

For the final product pulse there are two different parts (Fig.2). First, the part between the retention time of the final product and the retention time of the intermediate, in which the final product curve has a convex curved shape:

$$c_3 = m_{10} \frac{k'_1 k''_2}{\Delta k} e^{-k''_2 (t-\mu_3)} \left(1 - e^{\Delta k \frac{\Delta \mu_{12}}{\Delta \mu_{13}} (t-\mu_3)}\right) \quad (11)$$

Second, the part between the retention time of the intermediate and the retention time of the reactant, in

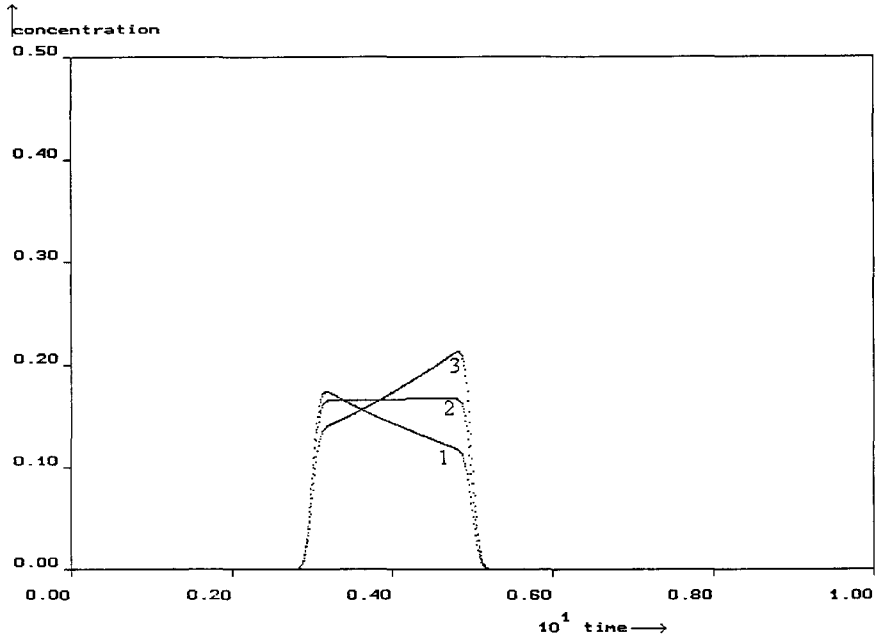


FIGURE 1: INTERMEDIATE PRODUCT PULSE OF A CONSECUTIVE REACTION
(1: $k_1'-k_2'<0$, 2: $k_1'-k_2'=0$, 3: $k_1'-k_2'>0$)

which the final product curve has a concave (almost exponentially decaying) curved shape:

$$c_3 = m_{10}\frac{k_1' k''_2}{\Delta k} e^{-k''(\mu_2-\mu_3)} e^{-(k_1'-k_2')(t-\mu_2)} \left(1-e^{-\Delta k(\mu_2-\mu_3)} e^{\Delta k \frac{\Delta\mu_{23}}{\Delta\mu_{13}}(t-\mu_3)}\right) \quad (12)$$

Also, from these equations it can be concluded that the concentration of the final product disappears at μ_1 and

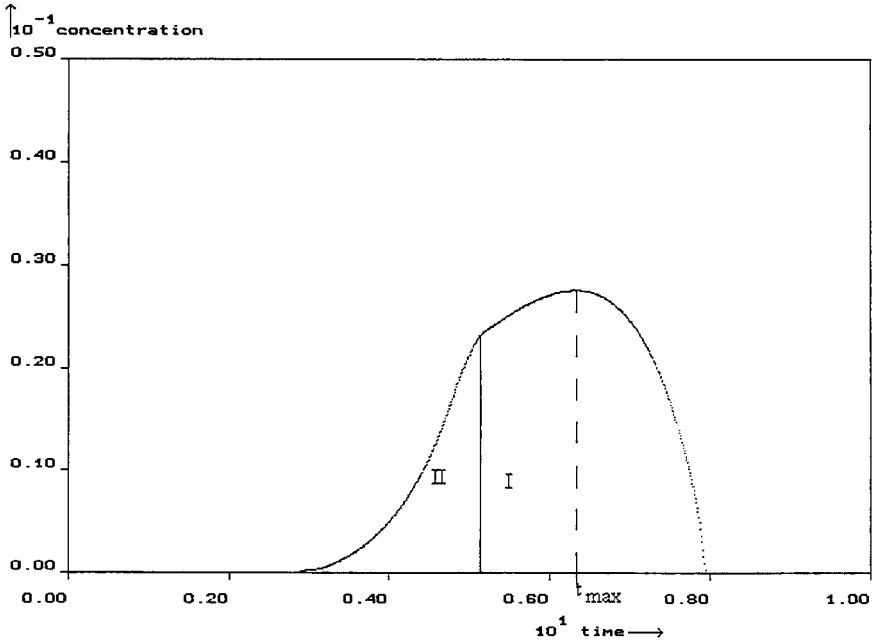


FIGURE 2: FINAL PRODUCT PULSE OF A CONSECUTIVE REACTION (I: CONVEX PART, II: CONCAVE PART)

μ_3, which is completely in contrast to the intermediate product.

There is a local maximum between μ_3 and μ_2 for the final product. The maximum concentration can be evaluated (diffusion neglected):

$$c_3(t_{max}) = m_{10} k_1' \frac{\Delta\mu_{12}}{\Delta\mu_{13}} e^{-\Delta k \frac{\Delta\mu_{12}}{\Delta\mu_{13}}(t_{max}-\mu_3)} \qquad (13)$$

It can be seen from Fig.1 and 2 that there is a marked difference between the shape of the final product pulse

and the shape of the intermediate product pulse or the shape of a simple product pulse. As was indicated in a previous publication /5/ there is no marked difference in the shapes of product pulses of parallel reactions, moreover, their shapes are similar to the shape of a simple product pulse. Therefore, the investigation of product pulse shapes might be helpful in investigations of reaction mechanisms.

Results of numerical evaluations

In order to test the possibility to obtain rate constants from reaction chromatograms by fitting the sum of Equations (8) and (9) to the corresponding part of the chromatogram a reaction chromatogram was calculated by an explicit difference method.

Since there are eight parameters in the fitting equation leading to numerous side maxima, the initial values have to be chosen carefully. Fortunately, first approximations for the moments and the standard deviations can be taken from the chromatogram (Fig.3): For the retention times for the reactant and the products from the chromatogram at the maximum of the reactant, the steep flank of the intermediate and the breakthrough point of the final product.

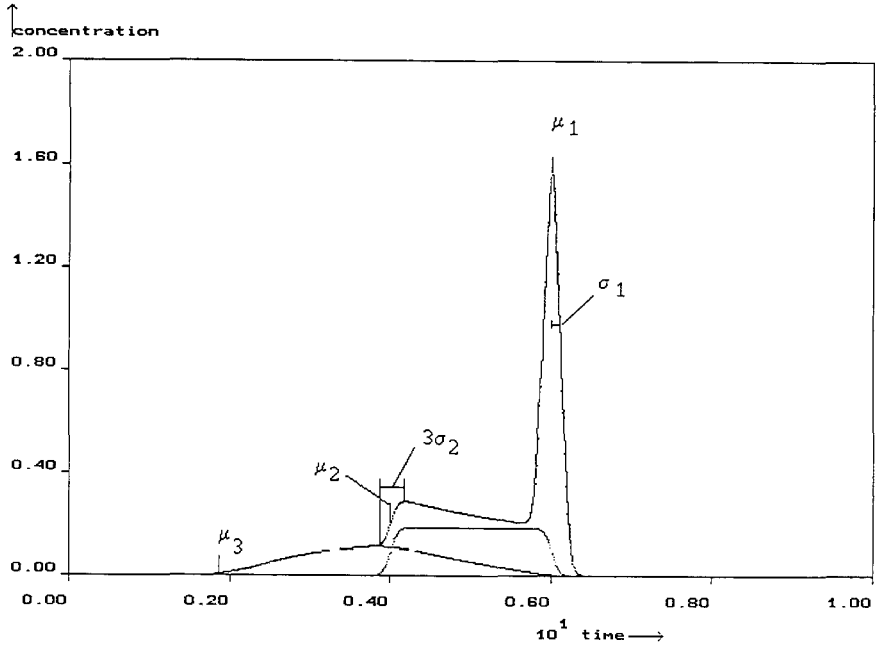


FIGURE 3: INITIAL VALUES FOR THE FITTING CALCULATIONS IN A TYPICAL REACTION CHROMATOGRAM FOR A CONSECUTIVE REACTION

The initial values for the rate constants were established by their systematic variation at the beginning of the fitting procedure.

Fig. 4 shows the excellent agreement between best fit and numerical chromatogram.

Also ,as can be seen from Table 1, the parameters from the fitting are in accordance with the parameters given for the numerical calculation of the chromatogram.

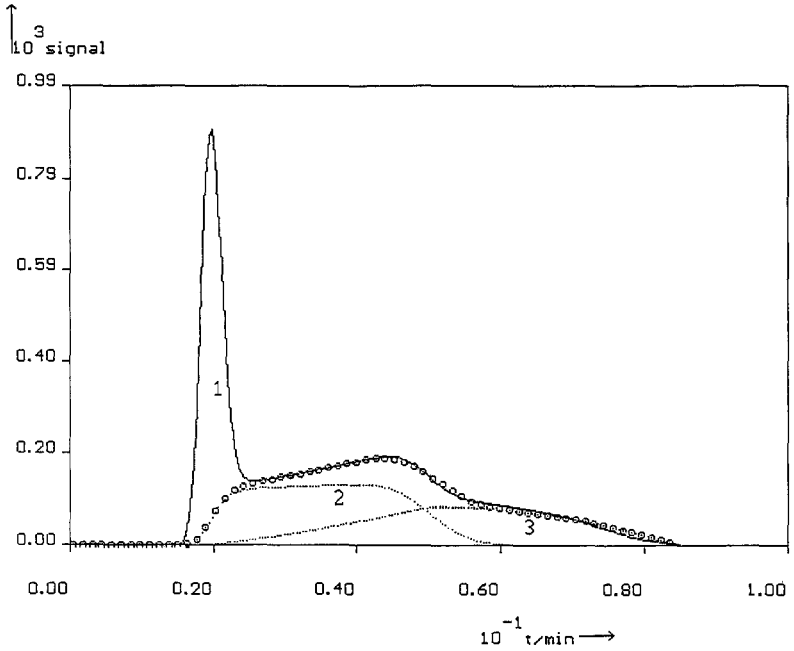


FIGURE 4: PRODUCT CURVE FUNCTIONS (EQN. 8 and EQN. 9) FITTED TO A NUMERICALLY EVALUATED CHROMATOGRAM
(LINE: NUMERICAL EVALUATION, CIRCLES: SUM OF EQN. 8 and EQN. 9 ; 1=REACTANT, 2=INTERMEDIATE, 3=PRODUCT)

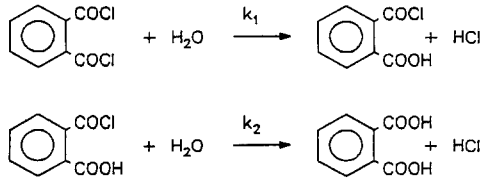
TABLE 1

Results of the Recalculation of the Parameters of a Numerical Chromatogram

	k_1	k_2	μ_1	μ_2	μ_3
num.	1.20	1.20	3.00	5.00	8.00
fitting	1.20	1.17	2.98	4.98	8.02

EXPERIMENTAL

The acid-catalyzed hydrolysis of phthalic dichloride



was investigated using the following liquid chromatographic equipment: A modular HPLC system from Gamma Analysentechnik Berlin, Germany (Kortec K35D HPLC pump, GAT-PHD 601 rapid scan UV-VIS detector with computer interface) with an EnCaPharm 100 RP 18 column from Molnar-Institut Berlin, Germany, which is persistent to high and low pH-ranges /6/ .

Acetonitrile/water (50:50) was the eluent, containing 10^{-2} mol/l HCl as acidic catalyst.

Experiments were carried out at 25°C with flow rates of 1.0 ml/min, 0.5 ml/min, 0.25 ml/min and 0.12 ml/min.

The chromatograms were recorded in the rapid scanning mode of the detector from 200 nm to 320 nm in steps of 1 nm. 0.5 μl of a 0.07 mol/l solution from phthalic dichloride in acetonitrile were injected.

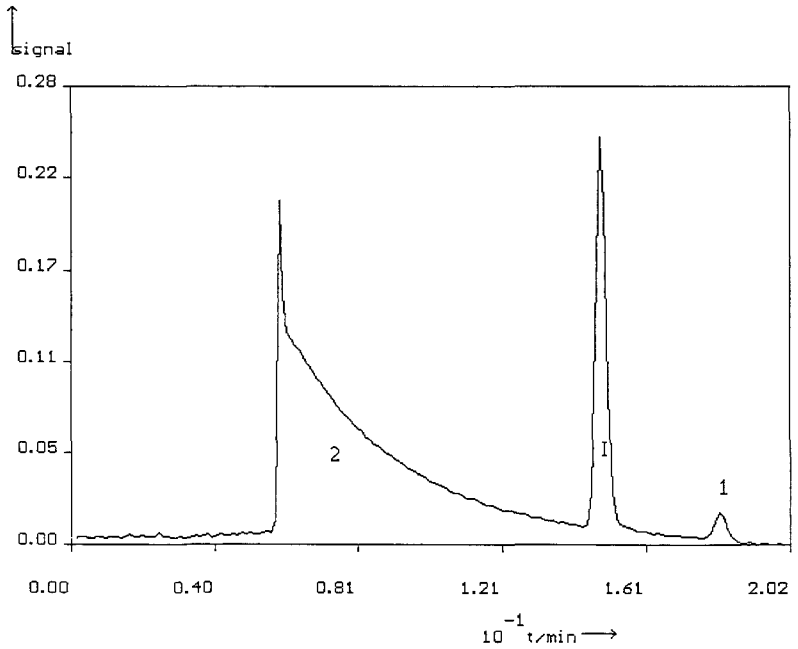


FIGURE 5: REACTION CHROMATOGRAM OF THE HYDROLYSIS OF PHTHALIC DICHLORIDE AT A FLOW RATE OF 1ml/min (1: PHTHALIC DICHLORIDE, 2: PHTHALIC CHLORIDE, I: IMPURITY)

RESULTS AND DISCUSSION

The experimental product curves were fitted by the functions (8) and (9) as shown in Fig. 5 - Fig. 8, considering a different molar detector response:

$$D=f*(c_3+f_{23}c_2) \qquad \textbf{(14)}$$

The detector response ratio of the products f_{23} was ex-

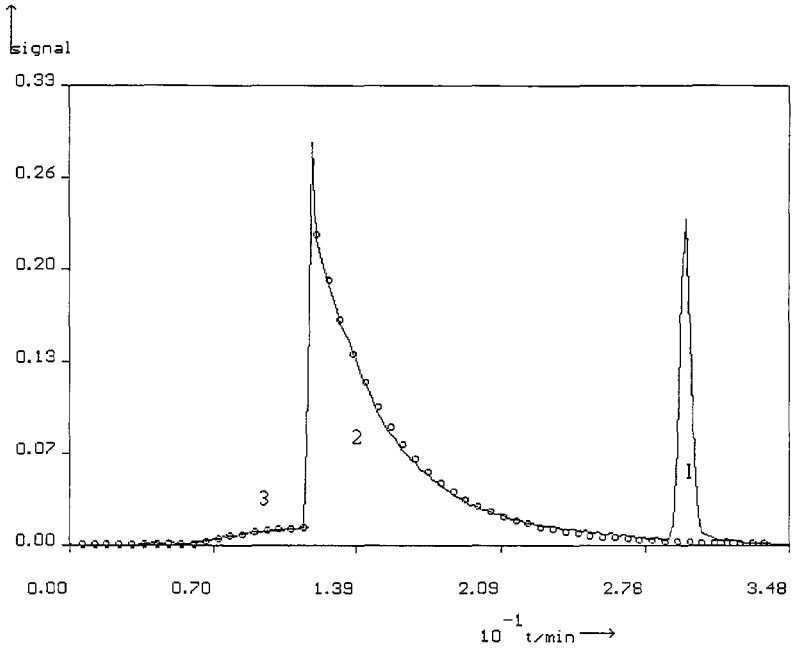


FIGURE 6: BEST FIT OF EQN. 14 TO A REACTION CHROMATOGRAM OF THE HYDROLYSIS OF PHTHALIC DICHLORIDE AT A FLOW RATE OF 0.5ml/min (LINE: EXPERIMENT, CIRCLES: EQN. 14, 2:PHTHALIC CHLORIDE, 3: PHTHALIC ACID , I: IMPURITY)

perimentally determined. In the investigations discussed here there was the problem that conversion for the reactant was already nearly complete, while there was still little final product formed. However, the phthalic chloride contained an inert impurity, the peak of which could be used to establish the reaction duration.

As can be seen from Figures 5-8 there is a good agree-

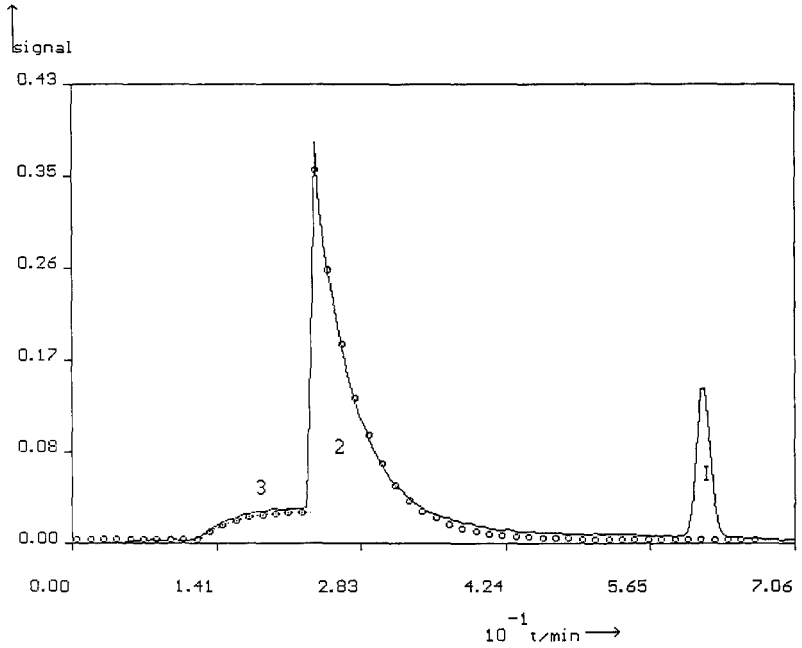


FIGURE 7: BEST FIT OF EQN. 14 TO A REACTION CHROMATOGRAM OF THE HYDROLYSIS OF PHTHALIC DICHLORIDE AT A FLOW RATE OF 0.25ml/min (LINE: EXPERIMENT, CIRCLES: EQN. 14, 2:PHTHALIC CHLORIDE, 3: PHTHALIC ACID , I: IMPURITY)

ment between the experimental results and the product curves calculated from Eqn. (14).

Apparent rate constants were calculated with respect to the retention time of the reactant. These are shown in Table 2.

There is a sufficient agreement between the rate constants of the different runs.

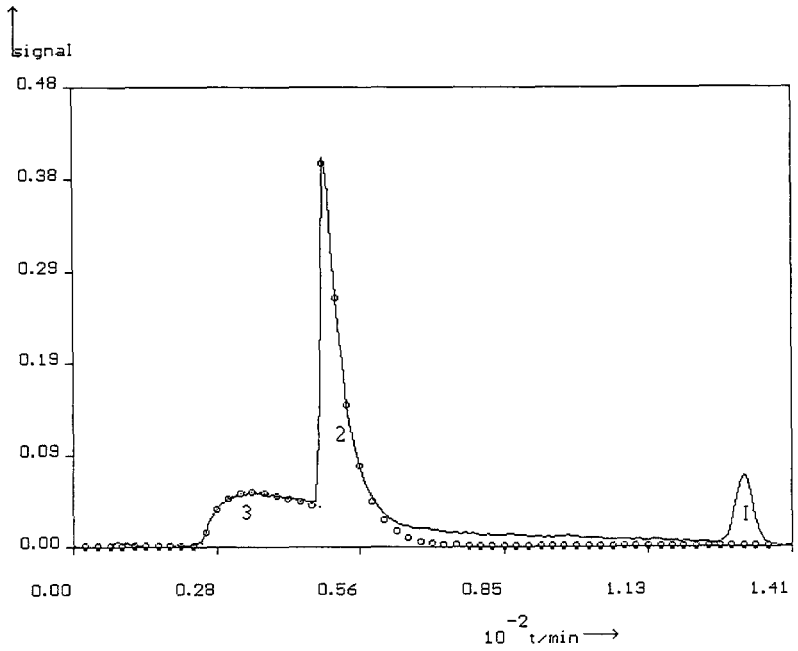


FIGURE 8: BEST FIT OF EQN. 14 TO A REACTION CHROMATOGRAM OF THE HYDROLYSIS OF PHTHALIC DICHLORIDE AT A FLOW RATE OF 0.12ml/min (LINE: EXPERIMENT, CIRCLES: EQN. 14, 2:PHTHALIC CHLORIDE, 3: PHTHALIC ACID , I: IMPURITY)

TABLE 2

Apparent Rate Constants for the Hydrolysis of Phthalic Dichloride as Evaluated from Fitting of Eqn. 14 to Experimental Chromatograms

flow rate	0.5 ml/min	0.25ml/min	0.12ml/min
k_1	0.19/min	0.18/min	0.18/min
k_2	0.0057/min	0.0054/min	0.0053/min

CONCLUSIONS

Especially Equations (8) and (9) show that the intermediate and the final product are eluted together during a considerable time.

The product pulse shape of the intermediate is closely related to the pulse shape of a simple product pulse (exponential decay function). The product pulse shape of the final product consists of a concave and a convex part, and no useful linearization can be found for these parts. Therefore, the determination of rate constants from those reaction chromatograms requires a fitting procedure.

It was found that the sum of equations (8) and (9) can be fitted to numerical chromatograms as well as to experimental chromatograms, and both rate constants were established by this procedure.

SYMBOLS

D :detector signal

f :response factor

f_{23} :detector response ratio of intermediate and final product

c :concentration

k_1' :virtual rate constant, defined by $k_1'=k_{1a}\ t_0/(\mu_1-\mu_2)$

k_2' :virtual rate constant, defined by $k_2'=k_{1a}\ t_0/(\mu_1-\mu_2)$

k_2'' :virtual rate constant,defined by $k_2''=k_{2a}\ t_0/(\mu_2-\mu_3)$

k_{ia} :apparent rate constant $k_{ia}=k_{i\ mobile} + q_i*k_{i\ stationary}$

l :length of the column

m_i :zeroth moment

m_{10} :initial zeroth moment of first reactant

q_i :retention capacity

t :time

t_0 :dead time

x :length coordinate

y :length coordinate

Δk :$k_1'-k_2'-k_2''$

$\Delta\mu_{ij}$:$\mu_i-\mu_j$

μ_1 :retention time of the reactant

μ_2 :retention time of the intermediate

μ_3 :retention time of the final product

μ_E :$\mu_3+(\mu-\mu_2)(\mu_1-\mu_3)/(\mu_1-\mu_2)$

μ :integration variable in Eqn. 4

μ' :integration variable in Eqn. 6

σ_i :standard deviation

ψ :pulse shape equation (i.e. Gaussian distribution)

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ANALYSIS OF ETHOXYLATED NONYLPHENOL SURFACTANTS BY HIGH PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY (HPSEC)

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ABSTRACT

Since Size Exclusion Chromatography does not provide a complete oligomer resolution, it has not been generally considered as a choice method for analyzing ethoxylated surfactant.

However, this situation might be changing. In effect, the use of new column technology together with non aqueous carrier solvent, and some operational optimization, can result in fairly good distribution data on the number of ethylene oxide groups per molecule (EON).

It is shown that HPSEC provides an excellent EON average estimate over a wider and higher EON range than the the up-to-date HPLC techniques.

INTRODUCTION

Size Exclusion Chromatography (SEC) has been proposed in several instances in the past 30 years as a method to characterize nonoionic surfactants [1-3]. However it seems that all trials ended in a failure to attain reliable data, and that the results were good only to provide a qualitative comparison between two substances.

The main problem in analyzing surfactants by SEC seems to come from the formation of micelles in water and other solvents. A micelle is a kind of aggregation polymer that occurs as soon as a certain concentration, so-called Critical Micellar Concentration (CMC) is reached. As a matter of fact some authors have proposed to use a early version of SEC, so-called Gel Permeation Chromatography (GPC) to estimate the extent of micelle formation [4]. Naguschi reported extensive studies on the separation of nonionic surfactant species on polyvinyl alcohol columns with acetonitrile/water mixture as the mobile phase [5]. He found that the lower the degree of ethoxylation was, the higher was the percentage of acetonitrile required to elute the compound; additionally, there was some evidence that the separation was not due entirely to size exclusion effects.

Recent technological advances have placed on the market new column packings made of very small spherical particles in the 3-5 μm range versus the 30-50 μm size available a few years ago. It is now possible to use SEC to analyze compounds with molecular weight in the range of a few hundred daltons [6]. Moreover, the increased sensitivity of the new detection devices allows to dilute the solutions below the CMC, or to detect all molecules associated into micelles whenever aggregation occurs [7-10].

Recent publications on HPLC analysis have reported that the ethoxylated alkylphenol surfactant oligomers can be readily detected by UV absorbance or fluorescence techniques [11-12]. Although it is generally less sensitive, the refractive index detection can be quite useful too, since it has be found [13] to correlate with both the surfactant concentration and the number of ethylene oxide groups per molecule so-called EON. It is worth noting that it can be used advantageously with compounds that do not absorb in the near UV or Vis spectra.

The EON distribution of ethoxylated nonionic surfactants has been extensively reported in the literature. As mentionned by Shick [2] more

than 25 years ago, the EON distribution is usually of the Poisson type, according to the polycondensation mechanism of the reaction of ethylene oxide addition in alkaline medium. Unless they are mixed, very few commercial products with short EO chain depart significantly from the Poisson distribution [14]; as will be seen later on, it is not the case of long EO chain surfactants.

Because of their widespread Poisson distribution, commercial ethoxylated nonionic surfactants can often contain substances that are very oil-soluble, as well as others that are very water-soluble. If an oil-water two-phase system is available, then the different surfactant species can behave independently from one another, and the result can be a severe fractionnation, in which the actually active surfactant mixture at interface is considerably different from the overall surfactant composition. Fractionnation has been recently investigated, and a satisfactory modeling has been proposed [15-16]. The model makes use of fractionnation data, which require in turn the analysis of every oligomer in all phases. Column chromatography and thin layer chromatography techniques have been reported [17-19]; however, they were not found appropriate for routines work, nor for analyzing nonionic surfactants containing long EO chains.

Very recently, HPLC techniques to analyze ethoxylated nonylphenol mixtures were enhanced to the point, where oligomers from 1 to 40 EON could be separated in a single run [20-22]. This was quite an improvement; nevertheless, even higher EON oligomers are often required in wetting, detergency and foaming applications [23]. Since surfactants with EON in the 50-100 range can be considered as small macromolecules, a HPSEC approach was selected in the present paper to address this problem.

FACTORS INFLUENCING THE HPSEC

Three factors should be considered in order to master the SEC technique: (1) the solvent nature and effect, (2) the efficiency of the column or column set, (3) the calibration and resolution of the column.

The selection of the eluting fluid in SEC is not critical, provided that it is a strong enough solvent, so that no other mechanism can happen, but size exclusion. As far as ethoxylated nonylphenols are concerned, the are

soluble in short chain alcohol (< C5), chloroform and tetrahydrofurane (THF). Short alcohols cannot be used in gel columns since they result in swelling and degradation, whereas chloroform is more viscous and has a lower volatility than THF, so that this latter is the selected solvent. When used as the mobil phase in SEC, THF is known to exhibit in most cases the so-called "differential solvation" (6) effect.

The two other factors, i.e., efficiency (N) and resolution (R), which are traditionally handled in other chromatographic techniques by a test with monomeric substances, cannot be dealt with the same way in SEC.

Yau and collaborators (24) introduced recently the "specific resolution" (Rsp) to free the resolution concept from the influence of the calibration sample nature in the linear range of response.

$$Rsp = \frac{0.576}{(\sigma\ D_2)} \qquad [1]$$

D_2 is the slope of the linear response range of the calibration curve, σ is the standard deviation, which is related to the number of theoretical plates in the case of a given substance, and can be estimated easily by (25):

$$\sigma = \frac{W}{(2\sqrt{2\pi})} \qquad [2]$$

where W = the peak width at the base.

In order to compare columns, equation [1] should be referred to a standard column length (L):

$$Rsp^* = \frac{0.576}{(\sigma\ D_2\sqrt{L})} \qquad [3]$$

Yau and col. established that the relative error between the true and experimentally determined molecular weights, i.e., a critical parameter for evaluating the method, is given by:

$$M^* = e^{\frac{1}{2}(\sigma D2)} - 1 \qquad [4]$$

From these expressions, it is seen that the resolution is maximized, and the error minimized, whenever the σD_2 group is minimum. As a consequence both parameters must be evaluated in the column selection process.

APPARATUS AND EXPERIMENTAL PROCEDURES

Several Perkin-Elmer HPLC modules were used in the equipment setting: a LC-250 pump, a LC-OVEN 101 thermostated enclosure and a LC-30 refractive index detector. The injector was a Rheodyne 7125 model with a 10 µl loop. The data was handled through a DTK personal computer with a PE Nelson 900 series interface and a SEC software version 5.1.

In preliminary trials a ZORBAX PSM 60S column was tested with no satisfactory results. Two other columns were used : (1) A PLgel 5 µ mixed-C column (30 cm x 7.5 mm) from Polymer Laboratories with a MW range from 10^3 to 10^7. This column is referred to as the linear column in the following text because it is filled by linear mixed beads of differents pores sizes (10^3 - 10^6 Å). (2) A Ultrastyragel 100 Å column from Waters, made from crosslinked styrene-divynil benzene gels with a low MW range from 100 to 1000 (30 cm x 7.8. mm I.D.). This column is referred to as 100 Å column in the following text.

Tetrahydrofurane (THF) HPLC grade from Baker Chemicals was used as the carrier solvent. Pretreatment of the solvent included: (1) filtration on a non-aqueous 0.45 µm Millipore filter, and (2) air scavenging by ultrasonic stirring and helium bubbling. Reference as well as measurement solutions are prepared typically at a 0.2 wt% concentration. Injected aliquot is 10 µl unless otherwise stated.

Two polystyrene standard sets from Polymer Laboratories were used: (1) a medium weight P/N 2010-0100 sample with MW ranging from 580 to 3.15 10^6, and (2) a low molecular weight P/N 2010-0101 sample with MW ranging from 162 to 22000. Each standard set contains 10 calibration samples and a verification sample. Verification samples exhibit MW of 580 and 1700 daltons respectively. A pure non-ethoxylated nonylphenol from Aldrich was also used as a reference.

Commercially ethoxylated nonylphenol surfactants from various manufacturers were tested: Makon M series from Stepan Chemicals, Igepal

CO series from Gaf Chemicals, Siponic NP series from Alcolac, Carsonon N series from Carson Chemicals, Alkasurf NP series from Alkaryl Chemicals, Emulgen E9XX series from Kao Atlas Japan, and Arkopal NP from Hoechst GmbH. In the following text, these susbtances are referred to as NPX or Trade-NameX, where X stands for the average number of ethylene oxide groups (EON) per nonylphenol molecule.

A UV detector was tested at the wavelengths corresponding to both absorbance maxima in THF (233 nm and 275 nm), but the response was not found satisfactory, probably because of the low concentration level (0.2 wt.%) required to avoid the formation of micelles. Thus, a refractive index detector was used instead, this time with reliable results.

HPSEC experiments were carried out in the following conditions, unless otherwise stated: temperature 40 °C, flow rate 1 ml/min and refractive index at 8x. The efficiency and resolution were computed according to equations [2] to [4].

RESULTS AND DISCUSSION - MW CALIBRATION RUNS

First, the linear column was used alone, and calibration runs were carried out with the low MW polystyrene standards. Fig. 1-A indicates the relationship between the standards' molecualar weight and the elution time. The variation of the logarithm of the molecular weight versus the elution time is obviously linear, with an excellent correlation coefficient. The two black points indicate the position of the verification standards (at 1700 and 580 daltons). The higher molecular weight standard is exactly in line, whereas the lower molecular weight one is slightly shifted below the line, an indication that the efficiency decreases in the lower MW range. This is no surprise since the column specification minimum MW is 1000 daltons.

Different commercial nonylphenol ethoxylates from the Igepal CO and Arkopal NP families are analyzed according to the same procedure. Since these substances are mixtures, the reported molecular weight is actually the molecular weight calculated from the average number of ethylene oxide groups per molecule according to the manufacturer. Fig 1-A shows the molecular weight vs elution time graph for nonylphenol ethoxylates ranging from EON= 9 to EON = 85. It is worth remarking that

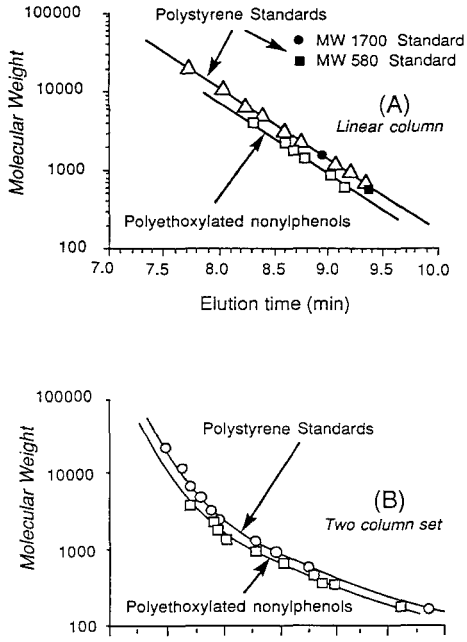

FIGURE 1. Calibration curves. (A) PLgel linear column. (B) Two column (linear +100 Å) set.

the log MW versus time graph exhibits an excellent linearity, a feature suitable for a calibration curve. Moreover, the line slope is exactly the same as in the polystyrene standards calibration graph. This shift is surely due to differences in the relationship between the molecular weight and the hydrodynamic volume. In effect, the polystyrene macromolecule has probably a larger gyration radius (and also larger hydrodynamic volume) than the corresponding ethoxylated nonylphenol molecule, because the polyethylene oxide chain is known to fold on itself to form a ball. Extra molecular weight is also conferred by the presence of oxygen atoms.

The parallelism of the two lines in a logarithmic scale, means that there is a constant factor between the two results. Thus for a given elution time, the ethoxylated nonylphenol molecular weight can be deduced readily

from the corresponding polystyrene molecular weight by dividing by 1.50, all over the range.

It is known that for each Å in length, a polystyrene molecule weights 41 daltons in average [26]; as aconsequence the Q-factor for the NPX substances is readily calculated as:

$$\text{Q-factor}_{NPX} = 41/1.5 = 27 \quad [5]$$

This value allows the calculation of the average MW of a NPX sample from the MW of the polystyrene specie with the same elution time.

It is of course even easier to use the NPX straight line in Fig. 1-A as a calibration curve for this type of substance.

As a way to evaluate better the performance of this column, the different efficiency parameters (σ, σD_2, Rsp* y M*) are calculated from the chromatograms of the Igepal CO family. The results are shown in Table 1. Whenever Rsp* is larger than 1, there is a good resolution between substances with molecular weights differing by less than MW/10. According to Table 1 results, this is occuring for oligomers with molecular weight greater than 750, i.e. for EON > 12. This is consistent with the previous remark on the lower limit of the linear column according to the polystyrene data.

In order to enhance the resolution in the low molecular range, a 100Å column is added in series to the linear column. Lower MW samples are tested, i.e., MW=162 polystyrene and NP6, NP4 surfactants. Fig. 1-B shows the resulting data in the same fashion as in Fig. 1-A. As previously there is a correlation (as a multiplicative factor) between the MW of the polysterene and the ethoxylated surfactant, which is here 1.55, a value very similar to the previous one.

This means again that polystyrene can be safely used as a standard for the determination of the MW of the surfactants, provided that the multiplicative factor is applied for correction purposes.

Table 1 indicates the performance data for the two-column set. The lower limit ($Rsp^* \geq 1$) is shifted down to EON=8 or MW=500. Since MW/10 is 50 daltons and since an ethylene oxide unit weights 44 daltons, it may be stated that the resolution at the low EON limit is almost 1 EON unit, a performance that almost matches HPLC separation. As far as the error (M*) is concerned, it is clear that the two-column set enhances the accuracy on the MW estimate, particularly at the low EON limit.

TABLE 1. Efficiency parameters calculated for the linear column and the two column (linear + 100Å) set.

Sample	EON average	(σD_2)		(Rsp^*)		(M^*)	
		Linear Column	Two column set	Linear Column	Two column set	Linear Column	Two column set
Igepal 990	85.5	0.071	0.049	1.48	1.52	0.3	0.1
Igepal 970	45.5	0.080	0.051	1.31	1.46	0.3	0.1
Igepal 890	36.4	0.089	0.051	1.18	1.46	0.4	0.1
Igepal 880	27.6	0.106	0.054	0.99	1.38	0.6	0.2
Igepal 850	18.0	0.106	0.063	0.99	1.18	0.6	0.2
Igepal 730	13.5	0.106	0.063	0.99	1.18	0.6	0.2
Igepal 720	11.0	0.124	0.073	0.85	1.02	0.8	0.3
Igepal 660	8.7	0.124	0.073	0.85	1.02	0.8	0.3
Arkopal NP6	6.0	---	0.102	---	0.73	---	0.5
Igepal 430	4.0	---	0.122	---	0.61	---	0.8
Nonylphenol	0.0	---	0.141	---	0.53	---	1.0

RESULTS AND DISCUSSION - EON CALIBRATION RUNS

In most cases the result of interest is more the average number of ethylene oxide groups per surfactant molecule, than the molecular weight. Then, the calibration curves are plotted as the logarithm of EON versus the elution time. Fig. 2 indicates an excellent correlation from EON = 10 to 85 with the linear column, and from EON = 4 to 85 with the two columns in series. In the first case the plot is linear, which is not surprising since the MW and EON varies essentially in the same fashion.

Note that in these data, the elution time is the average time for the chromatogram peak to get out and the EON is the average value for the commercial sample. The EON average value ($\overline{EON}$) is calculated by means of a linear mixing rule based on the mole fractions [14,22]:

$$\overline{EON} = \sum_i \text{mole fraction } i^{th} \text{ oligomer} \times i \qquad [6]$$

It is known that this average matches the physicochemical average. In any case it is very close to the distribution mode, particulary for high EON values.

Fig 3-A shows the chromatograms resulting from the HPSEC analysis of a NP4 sample with the two columns in series. The black dots indicate the actual RI detector output, i.e., the experimental HPSEC

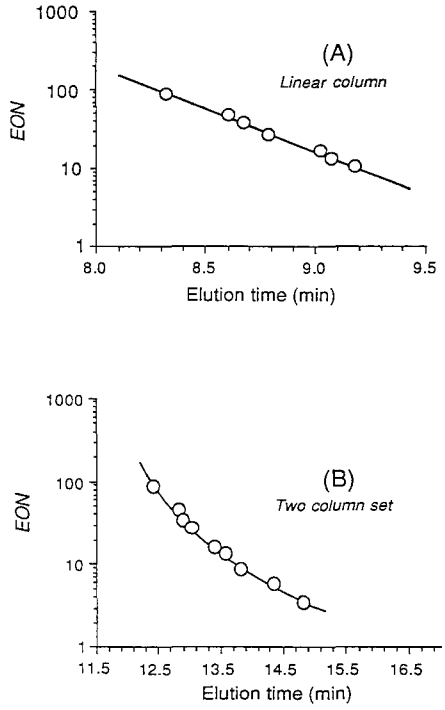

FIG. 2 : Calibration curves EON vs. Elution time.
(A) Plgel linear column. (B) Two column (linear plus 100 Å) set.

distribution, while the white dots indicates the Poisson distribution with the same EON average, and the solid line refers to the HPLC data according to a method reported elsewhere [14].

It is seen that the NP4 chromatograms are in good agreement, and that the Poisson distribution model is quite satisfactory for this case. It can be said that HPSEC gives an excellent estimate of both the distribution and the average value. Note also that the maxima are in good coincidence. The slight difference between the HPSEC and HPLC chromatograms is probably due to the fact that the HPLC method does not detect higher oligomers as well as low EON ones. As a consequence the fraction of low EON oligomers is often overestimated.

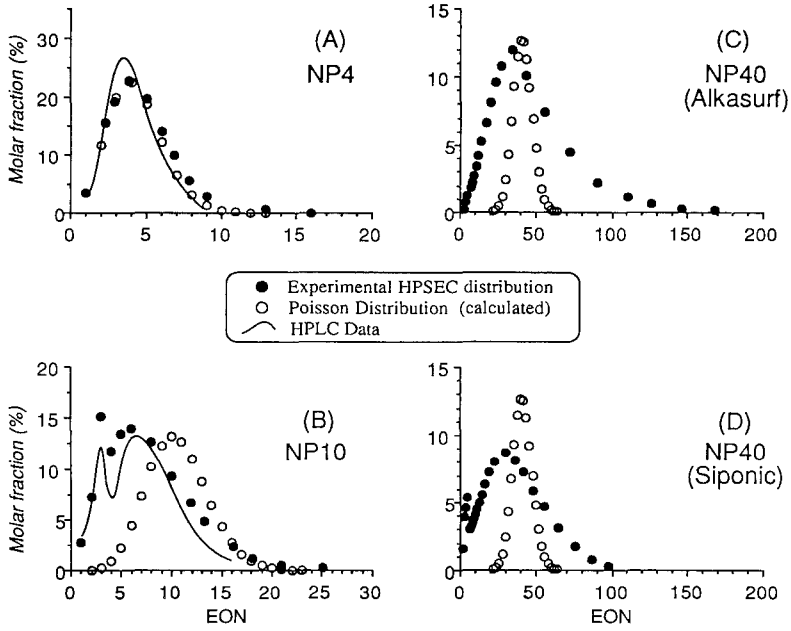

FIG. 3 : Comparison of calculated and experimental EON distributions for samples: (A) Makon 4, (B) Makon 10, (C) Alkasurf 40 and (D) Siponic 40. Conditions: as mentioned with two column set.

The NP10 data (Fig. 3-B) indicate that the HPSEC ouput and HPLC ouput are in agreement. On the contrary, it is clear that the Poisson distribution is not a satisfactory model, as pointed out by Nadeau [27].

Fig. 3 C and D indicate the HPSEC and Poisson distribution models for two NP40 samples. In both cases the HPSEC data allow to calculate the correct average EON, i.e. EON= 38.8 and 32.6 respectively, which is the important feature, as far as the performance of the method is concerned. On the other hand, it is evident that the experimental chromatograms exhibit a much wider distribution than the Poisson model. Note that the Alkasurf 40 sample is even more widely distributed then the Siponic 40 sample, although this latter presents a secondary peak at low EON, a characteristic already seen in Makon 10 (Fig. 3-B), that reduces the current

TABLE 2. HPSEC analysis of commercials samples and comparison with HPLC results.

Sample		EON average		Mn	Mw	$D = \frac{Mw}{Mn}$
		HPLC	HPSEC			
NP4 MW=396	Makon 4	4.0	4.1	359	383	1.07
	Carsonon4	-	4.9	388	413	1.06
	Alkasurf 4	-	4.1	355	378	1.06
NP6 MW=484	Makon 6	5.2	5.1	376	415	1.10
	Alkasurf 6	-	5.8	404	452	1.12
NP8 MW=572	Makon 8	7.0	6.9	441	500	1.13
	Carsonon 8	-	7.2	453	516	1.14
	Alkasurf 8	-	6.6	431	492	1.14
NP9 MW = 616	Siponic 9	-	7.5	453	529	1.17
NP10 MW = 660	Makon 10	7.3	7.2	447	517	1.16
NP30 MW=1540	Carsonon 30	-	25.3	795	1317	1.66
NP35 MW=1760	Emulgen 935	-	32.0	1212	1622	1.34
NP40 MW=1980	Siponic 40	-	32.6	967	1650	1.71
	Alkasurf 40	39.2	38.8	1323	1927	1.46

EON average by several units. As a matter of fact this multimode distribution is probably an evidence of some mixing in the manufacturing process.

EVALUATION OF THE HPSEC METHOD

The previous sections indicate that the HPSEC method with two columns in series leads to satisfactory estimates of the average EON of nonylphenol ethoxylates. The method was applied to a variety of samples produced by different manufacturers, and the results were gathered in Table 2. The average EON value computed from HPSEC data matches pretty well the manufacturer estimate and the available HPLC data[14,20,21]. The calculated molecular weights are also in good agreement with other reliable informations, say within a 10% error in most cases. However, discrepancies up to 20% are observed with products exhibiting a bimodal distribution (Siponic 9 & 40, Makon 10, and Carsonon 30 samples). Table 2 also reports the data dispersion, i. e., the ratio of the weight and number means (D = Mw/Mn). The computed values ranges from 1.0 to 1.4, as expected from the synthesis mechanism. Exceptions are found for the products that exhibit a bimodal distribution and are obviously the result of some mixing.

At the light of recent reports on the HPLC analysis of nonylphenol ethoxylates [14,20,21], HPSEC is found to exhibit interesting features. Although it does not match the single oligomer separation attained by gradient mode HPLC up to EON=25, it has several advantages over HPLC techniques. First, it requires less expensive equipments, particularly because of the isocratic mode and RI detector; then, it gives fairly good results as far as the average EON is concerned; finally, it provides the best estimate for highly ethoxylated substances, with no theoretical limit but the solubility in THF, whereas currently available HPLC methods do not reach beyond EON=25.

CONCLUSIONS

The reported results show that HPSEC techniques are worthwhile to analyze etholylated nonylphenol surfactants. Provided that two columns are used in series, a good average EON estimate can be attained over an extremely wide range of molecular weight. For surfactants with more than 30 ethylene oxide groups per molecule, HPSEC seems to be the choice method, since it is both more performant and less expensive than the competing HPLC techniques.

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ANALYSIS OF NON-IONIC SURFACTANTS BY HPLC USING EVAPORATIVE LIGHT-SCATTERING DETECTOR

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ABSTRACT

The analysis of surfactants, the basis of numerous surface wetting agents, detergents, emulsifiers, cosmetic products... is frequently carried out by liquid chromatography. Unfortunately, the analyses are very complex because of the use of gradients and the lack of suitable chromophore.
The object of this study by HPLC using an evaporative light-scattering detector was to select chromatographic conditions that characterize the majority of the different non-ionic surfactants (ethoxylated, oxypropylated, propoethoxylated, ethopropoxylated), according to their hydrophobic and hydrophilic parts.

INTRODUCTION

Surfactants are widely used for a variety of purposes, including surface wetting agents, detergents, emulsifiers, cosmetic products. Adducts of ethylene oxide or

propylene oxide and fatty alcohols or acids are important nonionic surfactants commercially used for many years.

Several publications and litterature reviews are available that describe techniques developed for surfactant analysis. Difficulties are often encountered in many analytical methods due to the complex nature of the mixture and the lack of adequate detection capabilities. HPLC methods have been developed for the separation of non-ionic surfactants according to :

- their alkyl chain lengths using a refractometer in isocratic conditions (1),
- and the distribution of ethylene oxide or propylene oxide in adducts of ethylene oxide or propylene oxide with fatty alcohol (2)(3), with alkylphenol (4 - 7) or with fatty acid (8 - 10).

Lack of suitable chromophores in the most usual surfactants molecules limits the use of UV detectors. For POE or many POE adducts, derivatization is required for sensitive LC detection (11 - 15), but the sample preparation is time-consuming and can lead to sample losses and imprecision. "Universal detectors", such as the refractive index detector (RI) and the evaporative light-scattering detector (ELSD), are most commonly used in HPLC analysis of surfactants but RI precludes the use of gradients. So ELSD has rapidly gained popularity among surfactant analysts, one of its advantages being the possibility of using complex gradients.

The theory of the ELSD has been discussed in several papers (16 - 26). In the light-scattering detector the chromatographic solvent is first nebulized by a gas stream, and the vapor enters a heated tunnel, where the solvent evaporates. The remaining analyte particles pass through a narrow light beam, and the scattered light is collected by a photomultiplier. The response of the ELSD depends on the number and size of the analyte particles. It is a suitable detector for all types of compounds that are relatively non-volatile, like waxes, sugars, lipids, glycerides (27 - 33) and surfactants (34-40).

In our study, the following aliphatic surfactants, synthesized in our research center, were examined :

(a) non-ionic ethoxylated alcohols
(b) non-ionic ethoxylated acids
(c) non-ionic propoxylated alcohols
(d) non-ionic ethopropoxylated alcohols
(e) non-ionic propoethoxylated alcohols.

The chromatographic conditions, optimized to characterize the different surfactants rapidly (<30 mn), are also described.

MATERIAL

Instrumentation :

The HPLC system was composed of an HP series 1051 HPLC pump equipped with the light-scattering detector : Cunow DDL21. The gas used in the ELSD was nitrogen passed through a filter before entering the detector. The detector temperature was 40°C and the nitrogen pressure 2 bars. The photomultiplier sensitivity was adjusted to the value (450 m Volts) of the photomultiplier gain area (400-800 m Volts). The Penelson 2600 program (Perkin Elmer) on a Prolinea 4/33 Compaq was used for data compilation and processing.

Solvents

The mobile phase was an HPLC grade hexane, chloroform and methanol. Water for use in HPLC was purified with a MilliQ reagent water system from Millipore Waters.
All solvents were filtered through a 0.45 µm filter (Millex HV13 Millipore Waters).

Columns

Various stationary phases and columns were used :
- 5 µm Spherisorb NH2 (250mmx4.6mm I.D) (Prolabo)
- 5 µm Intersphere ODS2 (150mmx4.6mm I.D) (Interchim)
- 5 µm Lichrospher 60RP select B (125mmx4mm I.D) (Merck)

RESULTS AND DISCUSSION

Ethoxylated alcohols

The general formula for these alcohol surfactants is $RO(CH_2CH_2O)_nH$ where R is $C_{10}H_{21}$, $C_{12}H_{25}$ or $C_{14}H_{29}$ and the average value for n is 2, 4, 9, 12, 16.

The non-ionic ethoxylates were separated according to the number of ethylene oxide (EO) groups (n) using normal phase chromatography. After trying different types of columns (Lichrosorb Diol, Spherisorb CN et NH2), gradient shapes and various combinations of solvents, the separation was performed with a gradient of hexane/chloroform/methanol in 30 mn (table 1).
The flow rate varied between 1 and 1,2 ml/mn depending on n.

Fig 1/2 shows the HPLC profiles for the ethylene oxide condensate of an alcohol and a fatty alcohol. Using this technique, it is possible to separate components of each product according to n.(fig3a : $n<10$ and fig3b : $n>10$). Within each group, components are further separated according to the length of the alkyl chain, i.e C12 and C14. When the alkyl chain is complex (mixture, presence of ramification...), the chromatogram is more complicated (fig4). We obtained the same results as Bear (34) using HPLC/ELSD, but in 30 mn instead of 50 mn. The EO distribution is shown to be between 1 and 30 for $n = 16$.

Normalized peak areas were used to calculate the average number (n) of the distribution of the oligomers : $n = \Sigma\ ni\ Ai\ /\ \Sigma\ Ai$, we observed good repeatability between three injections of the same product (fig5).

Studies have been also carried out with reversed phase chromatography to obtain the simplest chromatographic fingerprint, but showing different alkyl chain lengths present in these polyethoxylated alcohol mixtures. The separation was performed with a Lichrospher 60 RP Select B column in methanol - water (80/20) without gradient elution. The flow rate was 1 ml/mn. Such systems allowed, first, the elution of PEGs, then surfactants according to their alkyl chain length (fig6).

TABLE 1

Gradient Elution Program for Normal Phase HPLC of Non-Ionic Ethoxylated Alcohols

Time (min)	Hexane (%)	Chloroform (%)	Methanol (%)
0	76	19	5
10	72	18	10
20	64	16	20
30	56	14	30

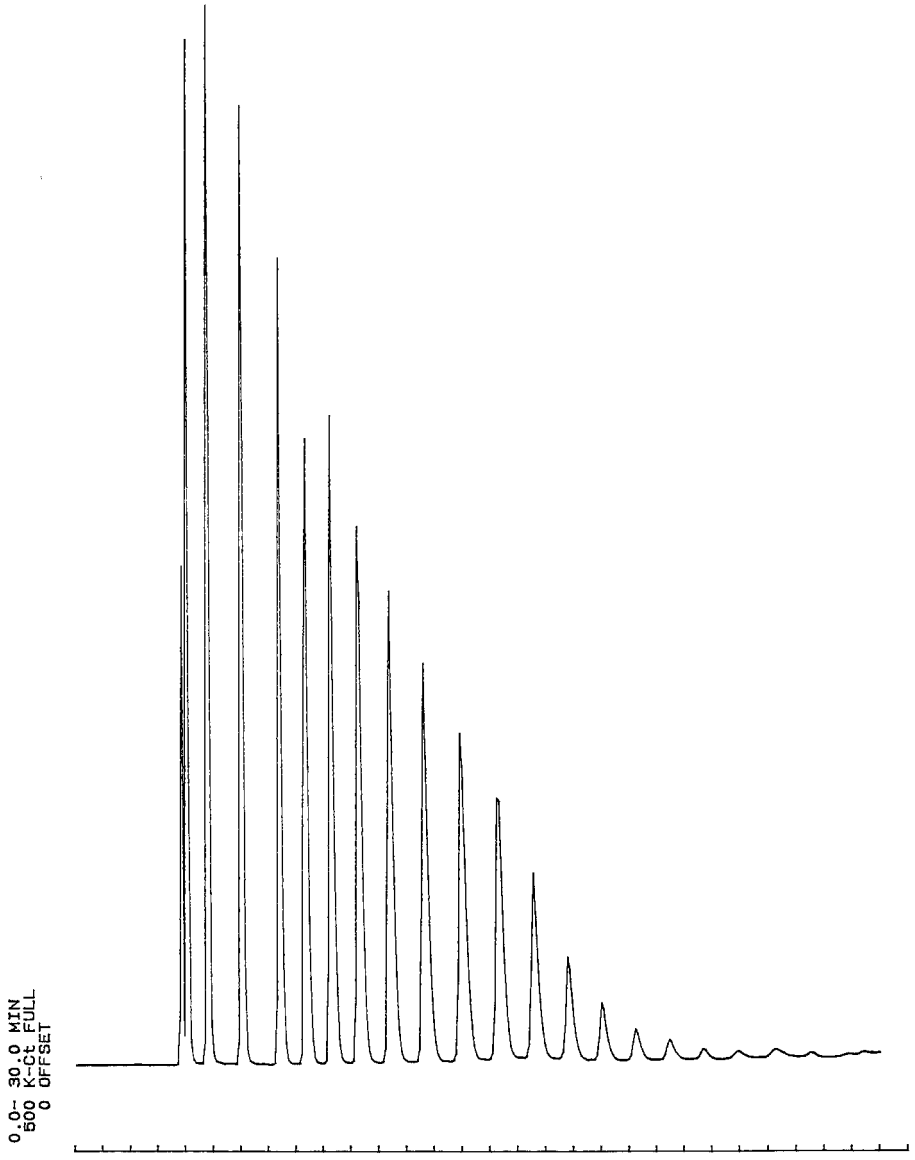

FIGURE 1 : HPLC profile for the ethylene oxide condensate of an alcohol

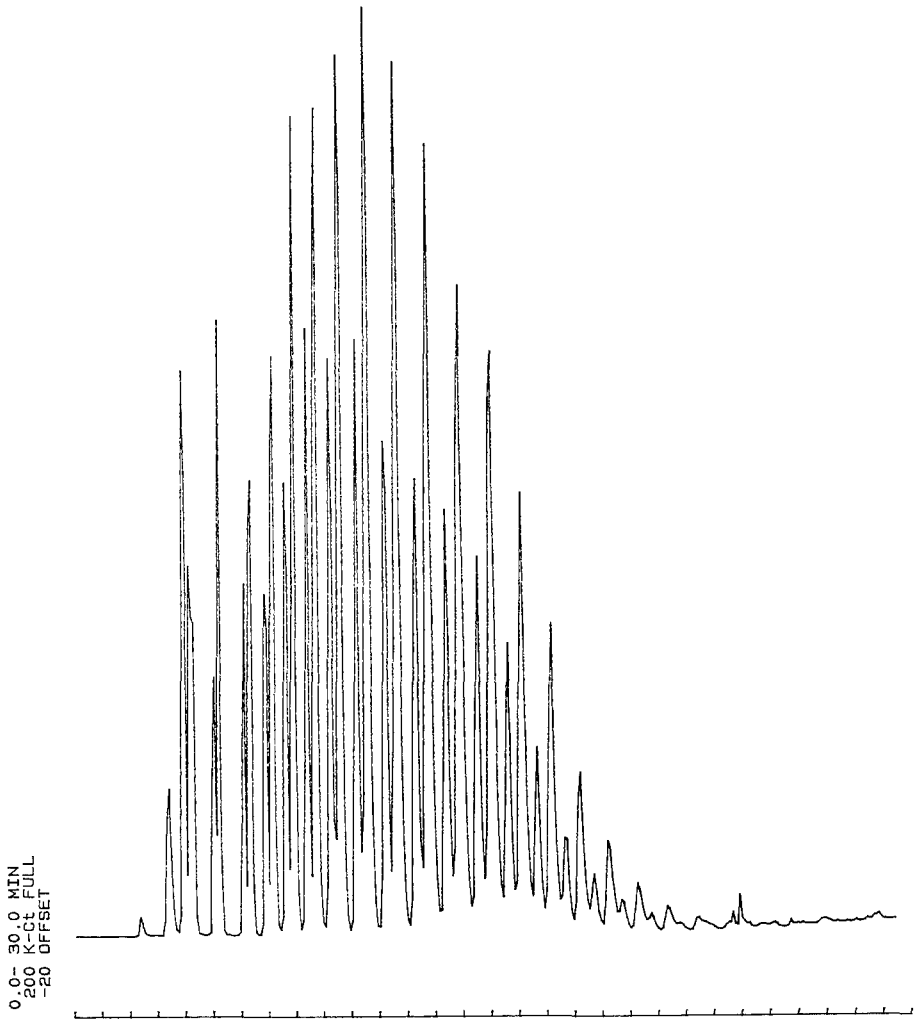

FIGURE 2 : HPLC profile for the ethylene oxide condensate of a fatty alcohol

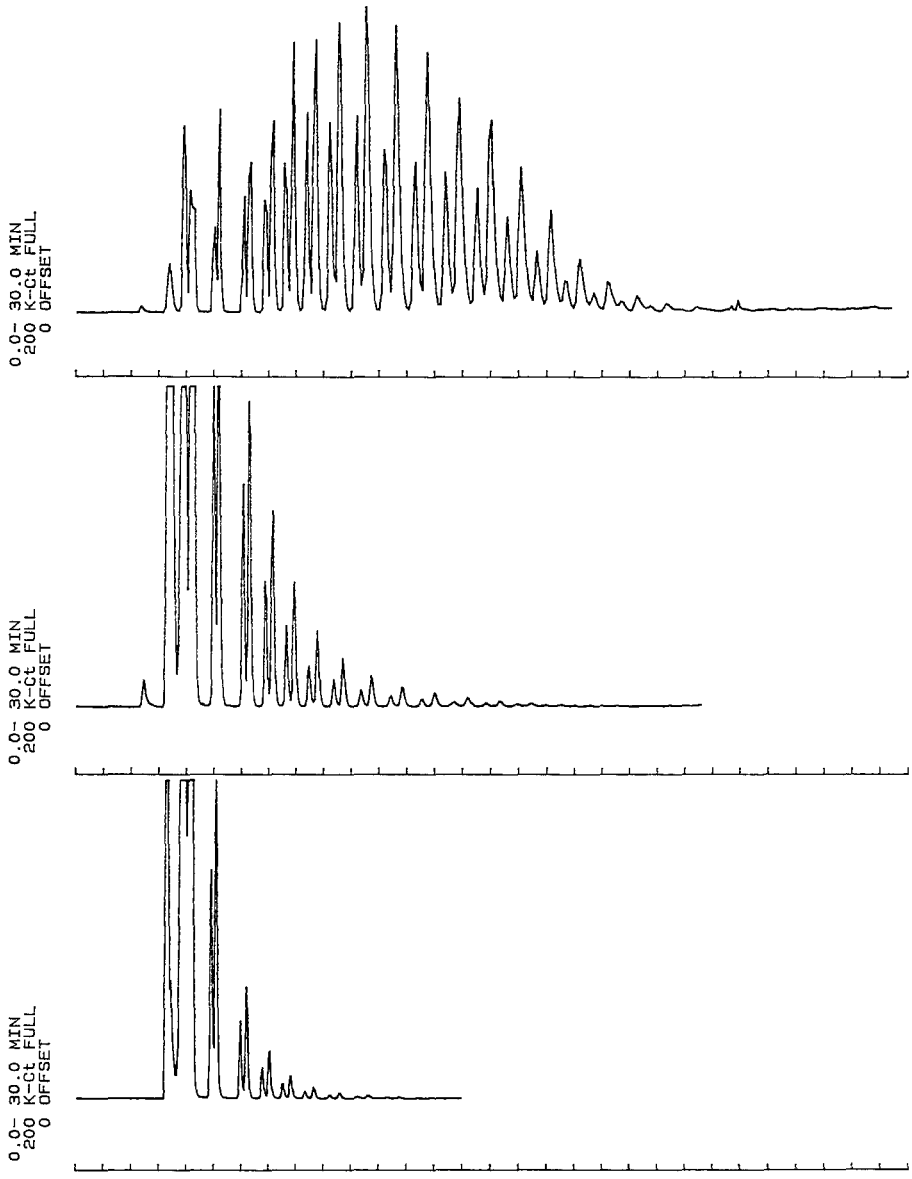

FIGURE 3a : HPLC profiles for the ethylene oxide condensate of fatty alcohols (n<10)

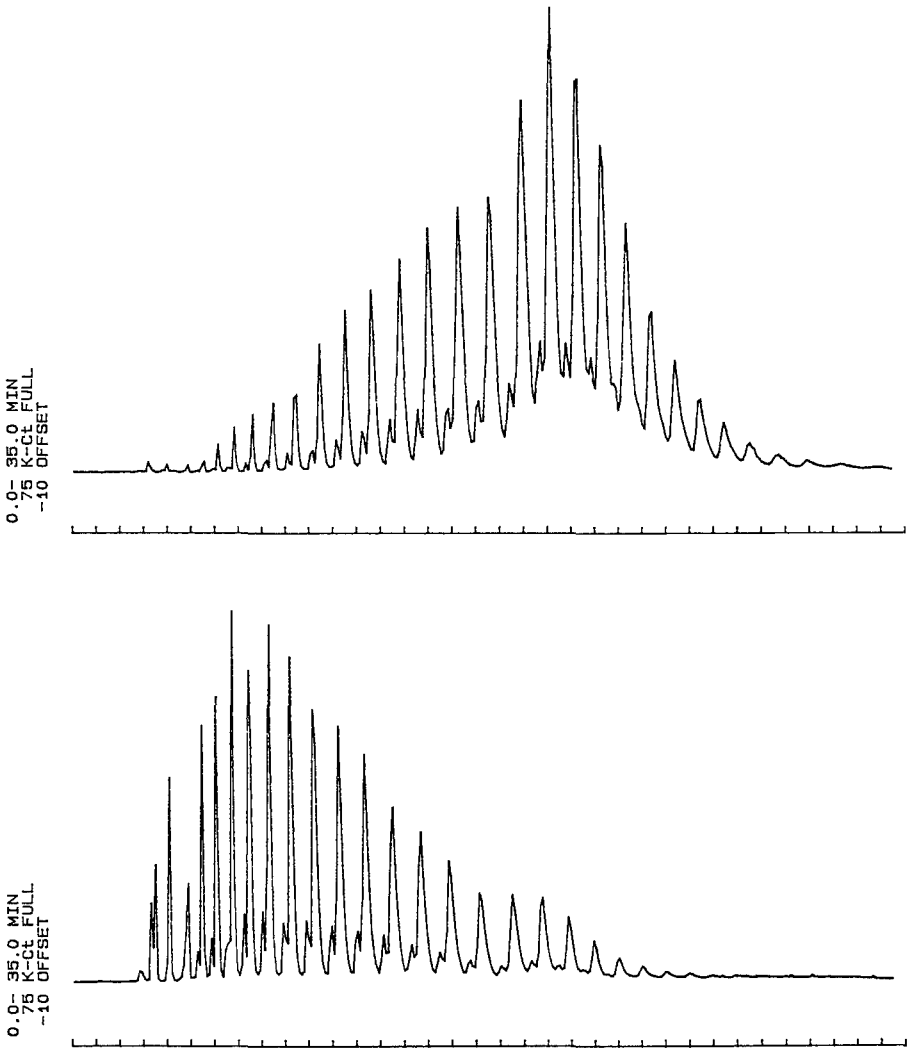

FIGURE 3b : HPLC profiles for the ethylene oxide condensate of fatty alcohol (n>10)

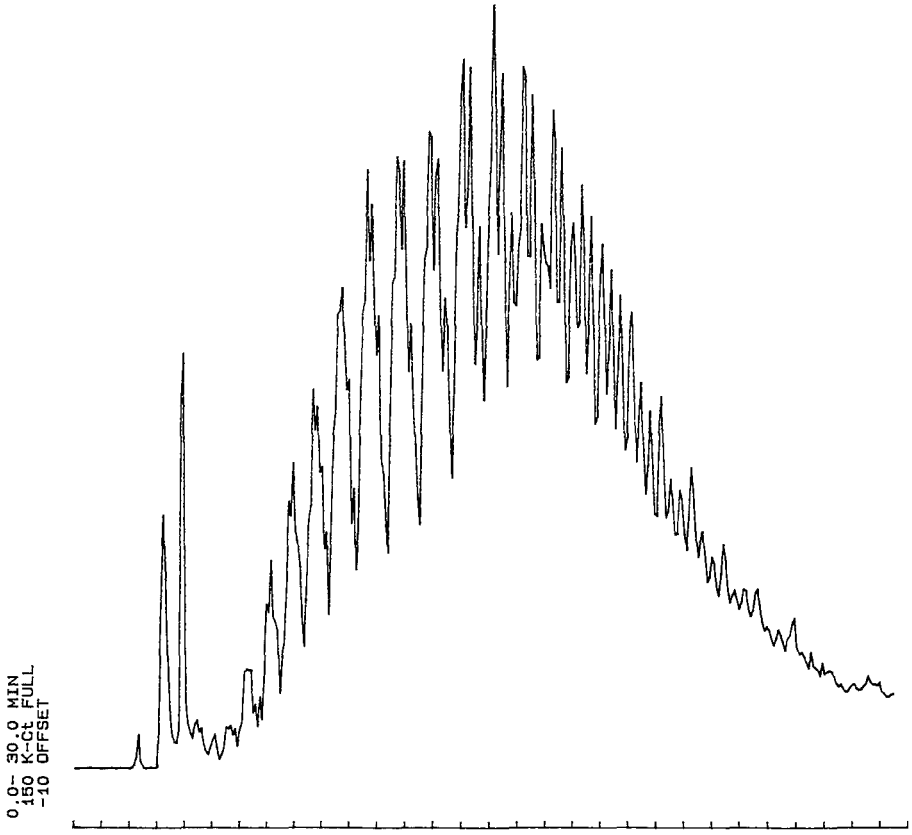

FIGURE 4 : HPLC profile for the ethylene oxide condensate of a fatty alcohol with a complex alkyl chain

Ethoxylated acids

The complete analysis of products from the reactions of fatty acids (FA) with ethylene oxide was even more complicated than for other analogous surfactants. In the reaction mixture not only the presence of main reaction products is to be expected (i.e monoesters (MES) and diesters (DES) with ethylene glycol oligomers), but also the presence of free PEG and fatty acids as by-products.

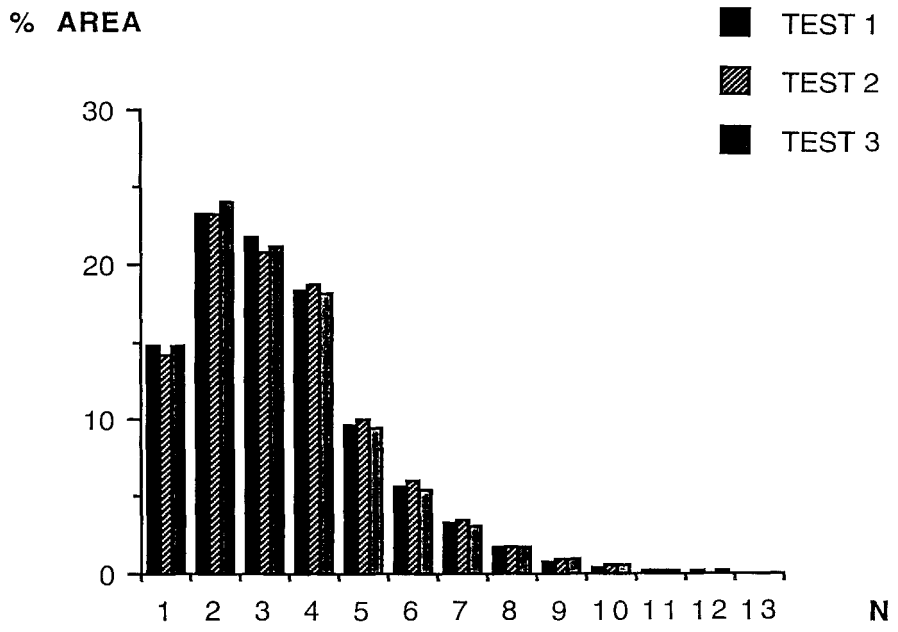

FIGURE 5 : Study of the repeatability of the distribution of the oligomers of an ethoxylated alcohol

Using an NH2 column with a Hexane/chloroform/methanol gradient quite different from that of ethoxylated alcohol (table2), the complete composition of FA ethoxylation products can be determined in 30 mn. Different examples of the complete separation of fatty acid (oleic) ethoxylate of different ethoxylation degrees (n = 6, 9, 15) are shown in fig7. Diester, monoester and PEG are found, just as Zeman observed using a refractometer (9)(10). Diester adduct oligomers remained unresolved in a single peak, whereas the monoester adduct and PEG oligomers are separated into individual oligomers.

Normalized peak areas were used to calculate the percent composition of each di, monoester and PEG. We observed good repeatability between 4 injections, and the percent of DES decreased when n rose (table 3), as Zeman observed (9).

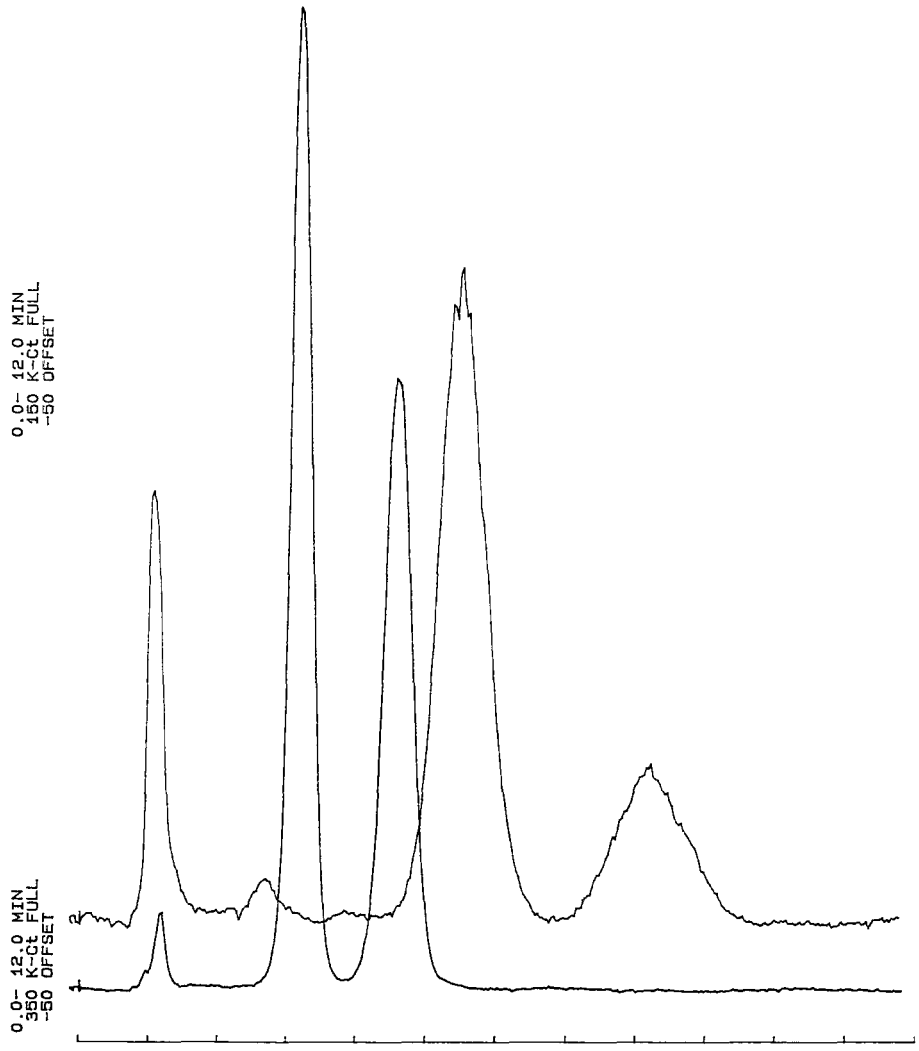

FIGURE 6 : Study of surfactants according to their alkyl chain length

TABLE 2

Gradient Elution Program for Normal Phase HPLC of Non-Ionic Ethoxylated Acids (Area %)

Time (min.)	Hexane (%)	Chloroform (%)	Methanol (%)
0	75	20	5
10	64	16	20
20	50	12.5	37.5
30	50	12.5	37.5

Oxypropylated alcohol and Propoethoxylated or ethopropoxylated alcohols

A sample of an oxypropylated alcohol was analyzed by means of HPLC on an NH2 column and by reversed phase HPLC on a C18 column. With the NH2 column, we observed only one peak.

The distribution of oligomer PO adducts was determined on an Interspher ODS2 C18 column (fig8), using a methanol/water gradient.

Furthermore, the complete analysis of products from the adduct of propylene oxide and ethoxylated alcohol is even more complicated because of the complexity of this mixture. In fact, the propylene oxide reacts partially with the ethoxylated alcohol and with the free PEG. Using the reversed phase chromatography described earlier, it is possible to separate the copolymer PEG/PPG, the free ethoxylated alcohol and the distribution of the propoethoxylated alcohol in 30 mn (fig9). We were able to observe how the distribution of the oligomers between two alcohols (C10 7OE/3OP and 7OE/5OP) evolved (fig10).

In contrast, the adduct of ethoxylene oxide and propoxylated alcohol was studied using the normal phase chromatography described earlier for ethoxylated alcohols. In this case it is possible to separate the copolymer PPG/PEG and the distribution of the ethopropoxylated alcohol. We observed the evolution of the distribution of the oligomers between 4 ethopropoxylated alcohols (C10 2OP/3,5,8,10OE) (fig11). In fact, using the reversed phase chromatography described earlier for the propoxylated alcohols and by comparing the result with that obtained from the injection of an ethoxylated alcohol (fig12), we also saw the formation of free ethoxylated alcohol, a by-product of the adduct of ethylene oxide and free alcohol .

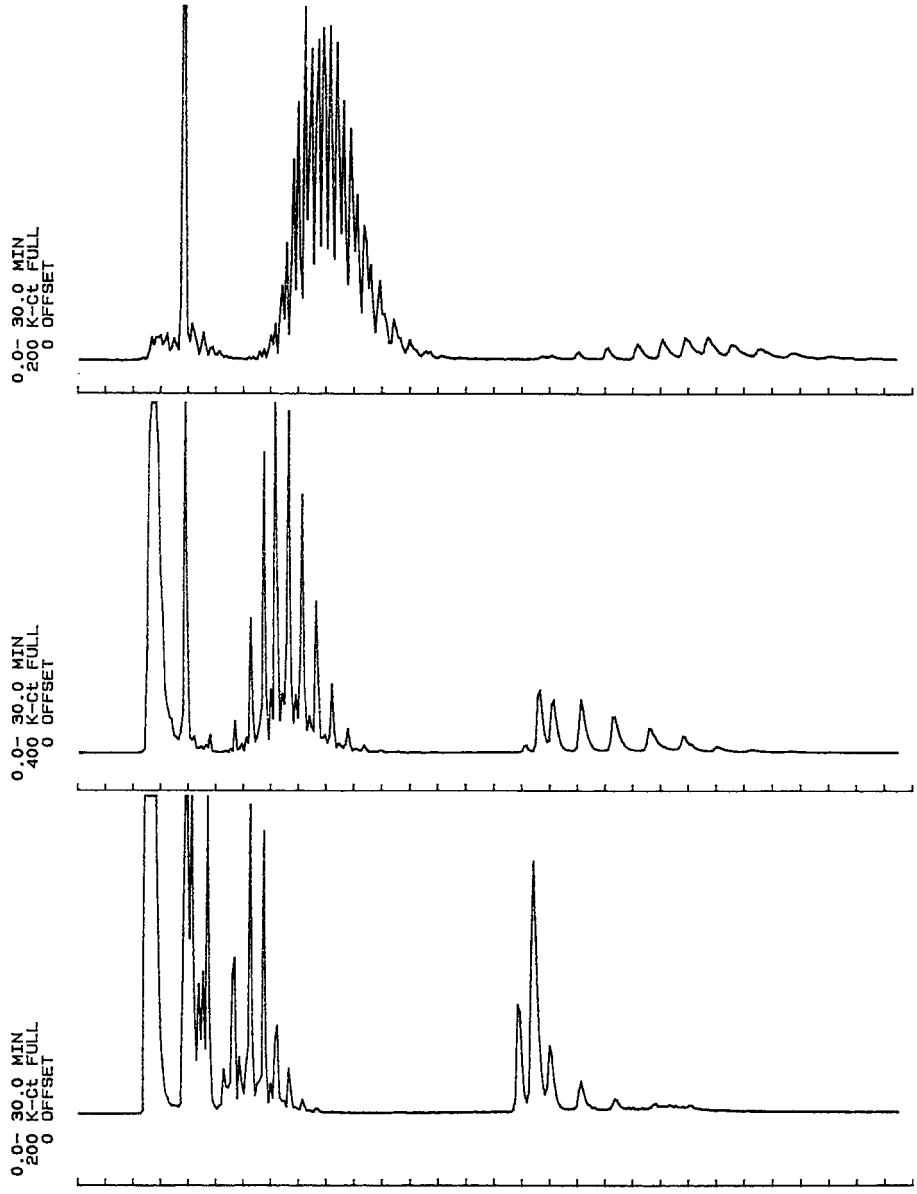

FIGURE 7 : HPLC profiles for the ethylene oxide condensate of fatty acids

TABLE 3

Composition of Ethoxylated Fatty Acids (Area %)

EO moles	% DES	% MES	% PEG
6	48	43	10
6	48	41	11
6	49	40	11
6	50	39	11
9	33	52	15
15	2	91	7

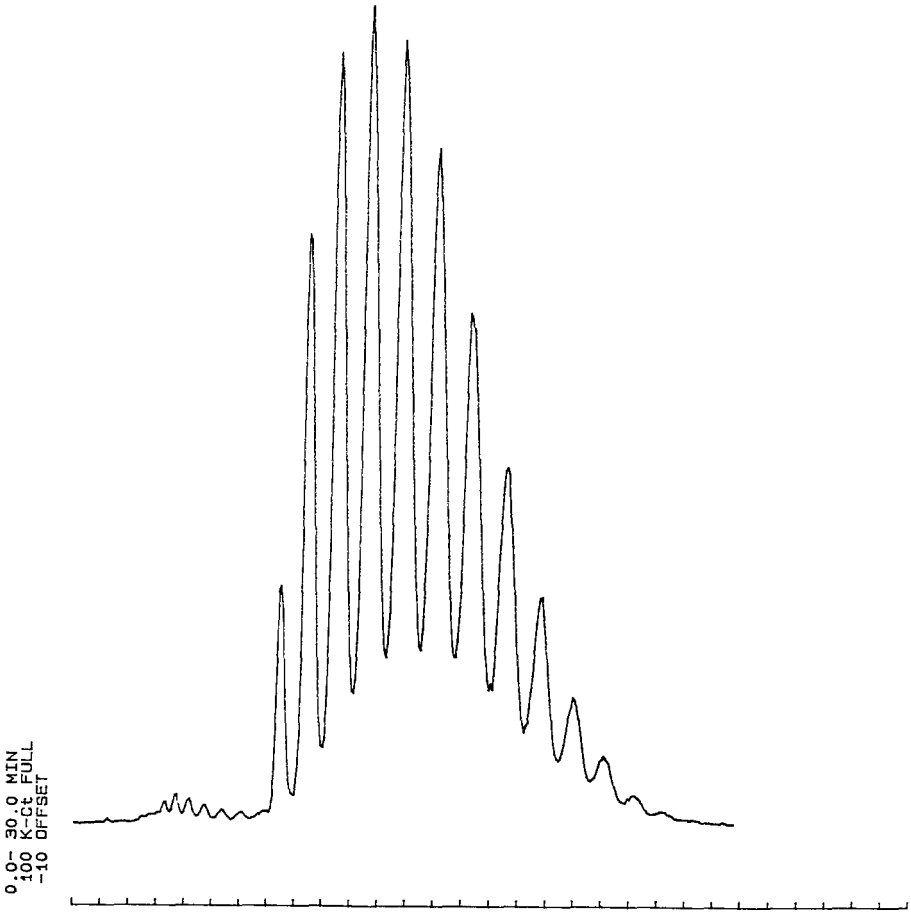

FIGURE 8 : HPLC profile for the propylene oxide condensate of an alcohol

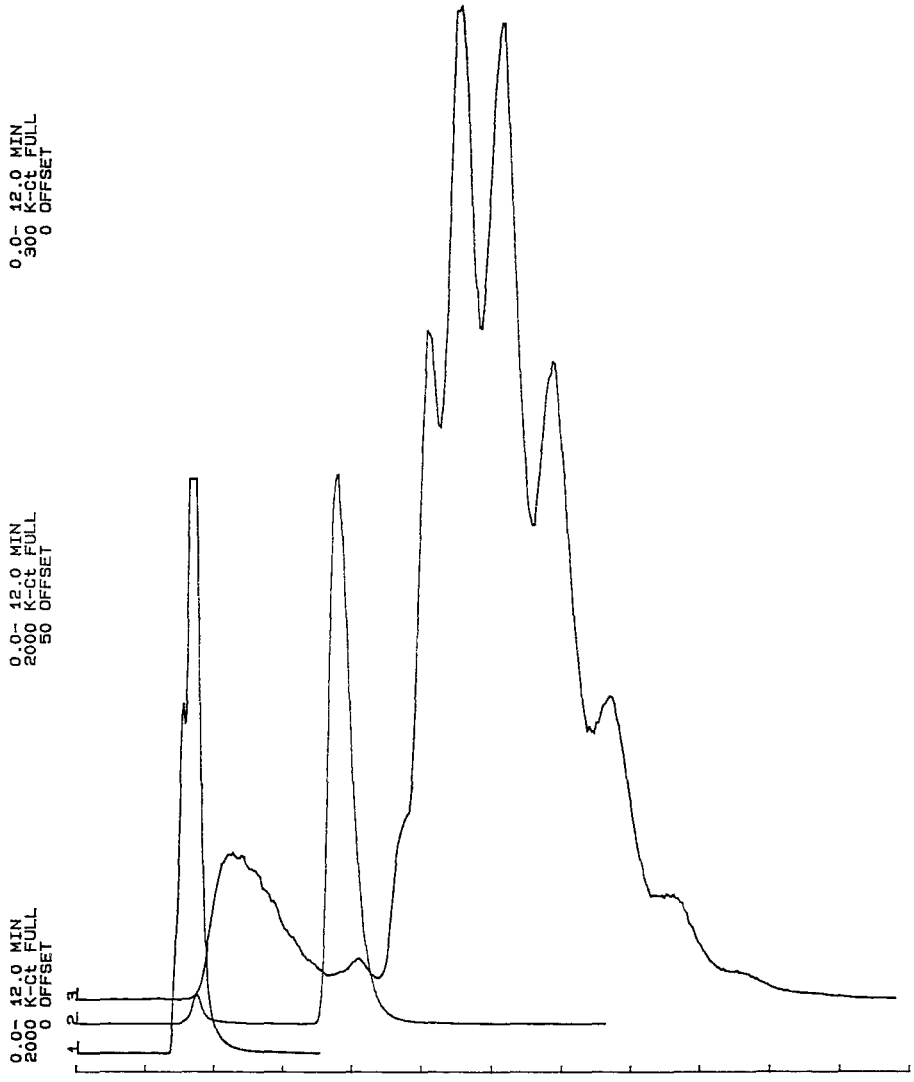

FIGURE 9 : HPLC profile for the propoethoxylated alcohol

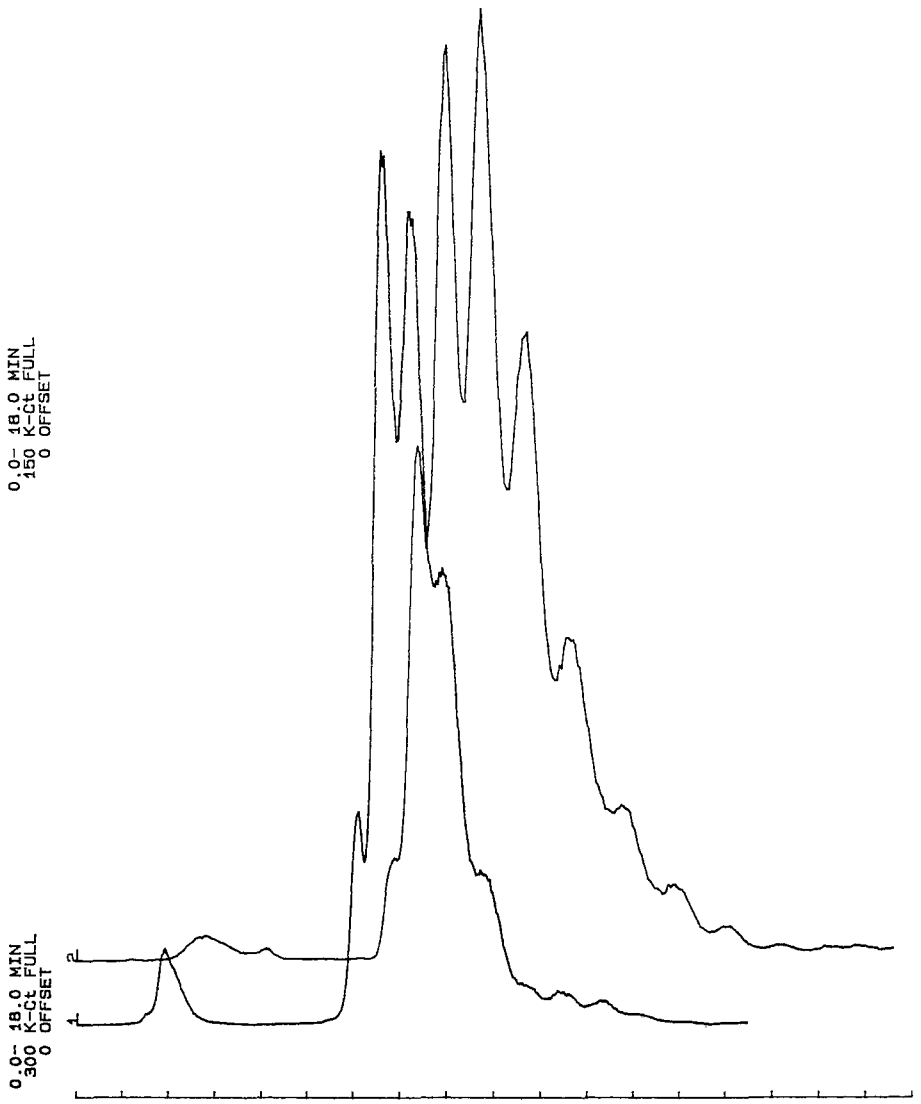

FIGURE 10 : Evolution of the distribution of the oligomers between two alcohols C10 7OE/30P et 70E/5OP

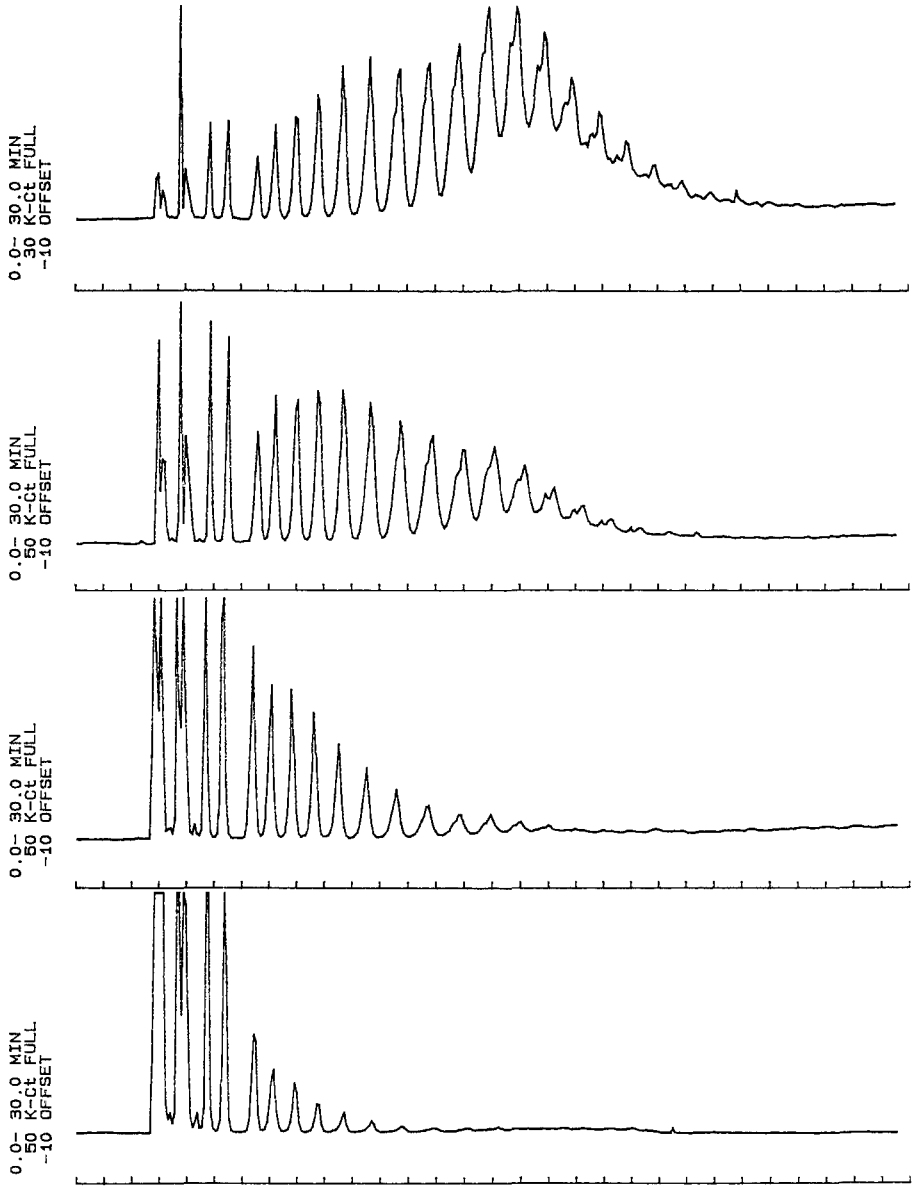

FIGURE 11 : HPLC profiles for ethopropoxylated alcohols C10 2OP/3.5-8-10 OE

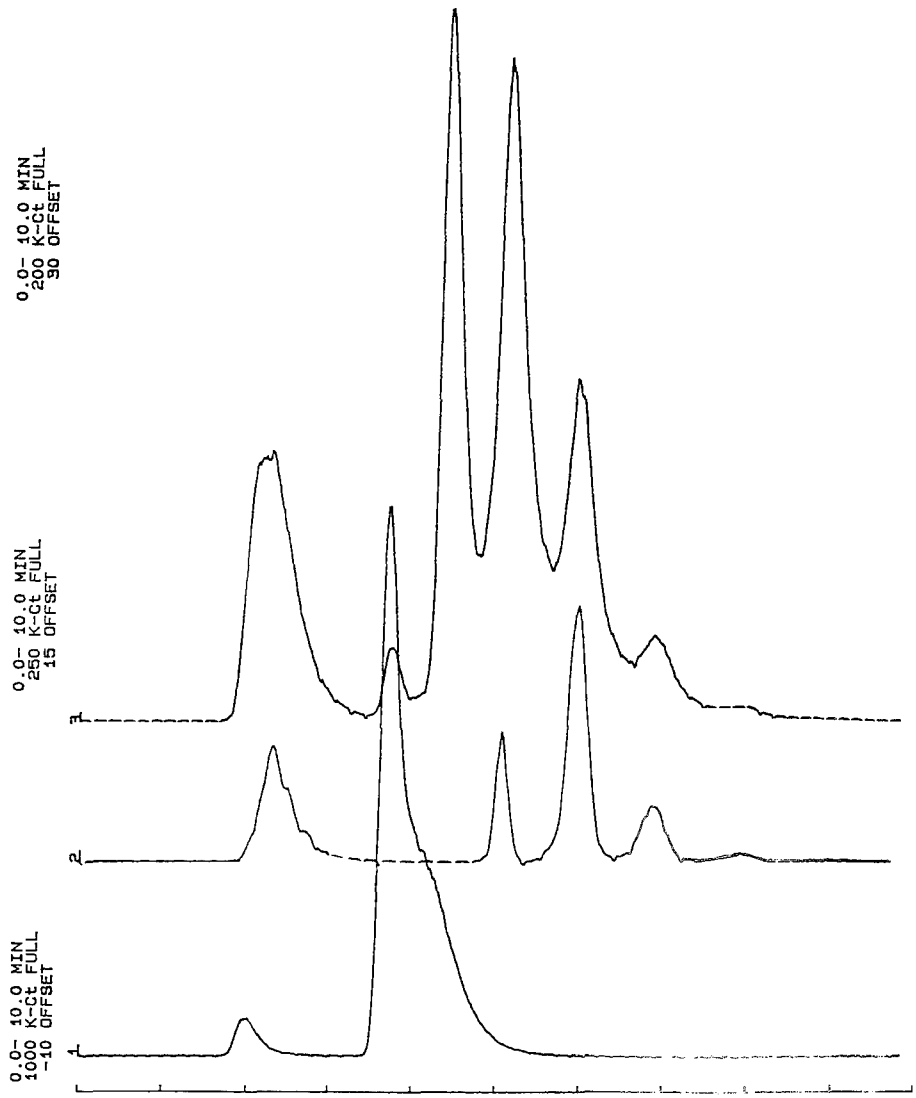

FIGURE 12 : Study of the formation of free ethoxylated alcohol in a ethopropoxylated alcohol

TABLE 4

Gradient Elution Program for Reversed Phase HPLC of Oxypropylated Alcohol

Time (min)	Methanol (Vol %)	Water (Vol %)
0	85	15
20	5	95
30	5	95

Owing to the characteristic chromatographic fingerprints in normal or reversed phase HPLC, several block oligomer POE/PPG or PPG/POE alcohols can be identified.

CONCLUSIONS

The HPLC/ELSD procedures presented here provide separations for a wide range of non-ionic aliphatic surfactants. For routine characterization, normal phase (NH2 column) and reversed phase (C18 column) HPLC are effective for the separation of ethoxylated or propoxylated oligomers.

To our knowledge, chromatography of PPG, POE/PPG or PPG/POE alcohols or POE acids had not been previously performed with HPLC/ELSD for all products and in a short time (<30 mn). HPLC/ELSD methods are sampler and faster than HPLC/RI.

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COLLABORATIVE STUDY OF THE ANALYSIS OF TYLOSIN BY LIQUID CHROMATOGRAPHY ON WIDE-PORE POLY(STYRENE-DIVINYLBENZENE)

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ABSTRACT

A previously developed method for analysis of tylosin A and related substances by liquid chromatography using a wide-pore poly(styrene-divinylbenzene) stationary phase was examined in a multicentre study involving 7 laboratories and 3 samples. The main component and the impurities were determined. An analysis of variance showed absence of consistent laboratory bias and significant laboratory-sample interaction at the 1 % level. Estimates for the repeatability and reproducibility of the method, expressed as standard deviation (SD) of the result of the determination of tylosin A, were calculated to be 1.3 % and 1.6 % respectively.

INTRODUCTION

Tylosin is a mixture of 16-membered ring macrolide antibiotics produced by fermentation of *Streptomyces* species. Tylosin A (TA) is the main component in this mixture. During fermentation several related substances can be formed: desmycosin or tylosin B (TB), macrocin or tylosin C (TC), relomycin or tylosin D (TD), demycinosyltylosin A (DMT) and 5-O-mycarosyltylonolide A (OMT) [1]. In neutral and alkaline medium tylosin A aldol (TAD) is formed [2,3]. Under the influence of light tylosin A in solution is partially converted into isotylosin (isoTA) [2]. Structures of these compounds were shown elsewhere [2,4].

A liquid chromatographic (LC) method for analysis of tylosin A and related substances on poly(styrene-divinylbenzene) (1000 Å) has been described previously [5]. The suitability of this method for general application was examined in this study, which was carried out in seven laboratories, using three samples.

EXPERIMENTAL

Apparatus and Columns

The protocol prescribed the use of a pump for isocratic delivery of the mobile phase at a flow rate of 1.0 ml/min. The equipment further consisted of a fixed loop injector with a loop of 20 µl, a column heating device, allowing continuous heating of the column at 60 °C (a water bath was preferred), a UV detector set at 280 nm and an integrator allowing peak area measurements. The participating laboratories were asked to use 25 x 0.46 cm i.d. columns, packed with poly(styrene-divinylbenzene) (PSDVB), 5 to 10 µm, 1000 Å. Laboratory packed columns were packed with PLRP-S, 8 µm,

1000 Å from Polymer Laboratories, Church Stretton, Shropshire, UK. Prepacked columns were purchased from different distributors of Polymer Laboratories.

Mobile phase

Liquid chromatographic grade or distilled tetrahydrofuran (200 ml) was added to a mixture of 50 ml of 0.2 M potassium phosphate buffer pH 9.0 and 750 ml of distilled water. The mobile phase was degassed by ultrasonication or by another suitable method. The collaborators were asked to adjust, if necessary, the tetrahydrofuran content in the mobile phase in order to reach the requirements for resolution.

Samples, Chemicals and Solvents

The reference sample (T-R) used was a house standard, containing 90.3 % m/m tylosin A, calculated on the substance "as is", and 9 % m/m water. A reference substance of TD was available, which was used to determine the resolution between TD and TA. The resolution between TB and TA was determined using a solution containing TB and TA. TB was formed in situ from TA in a solution of T-R in 0.05 M phosphoric acid solution (1.0 mg/ml) by heating for 5 min in a water bath at 60 °C. After cooling, the pH was adjusted to 7.0 and the solution was diluted to 100 ml using 0.05 M potassium phosphate buffer pH 7.0. Reference samples for the related substances were not used. The content of related impurities was expressed in terms of TA and calculated with reference to a 1 % dilution of the T-R reference solution. The samples to be examined (T-S1, T-S2, T-S3) were of commercial origin. Chemicals complied with Ph. Eur. requirements [6].

Solutions for analysis, containing 1.0 mg of tylosin/ml , were prepared in 0.05 M potassium phosphate buffer pH 7.0. Sample solutions had to be stored protected from light.

RESULTS AND DISCUSSION

Equipment and Method Performance

Table 1 includes information regarding columns, conditions used and results of performance checks carried out by each laboratory. The numbers assigned to the

TABLE 1

General Information on Equipment and Method Performance

Laboratory	Column	% THF in mobile phase	Retention time TA (n = 5) (RSD)	Sensitivity (S/N ratio)	S	Rs	Rs	Repeatability (n=5) Peak area RSD %			Linearity r
					TA	TD-TA	TB-TA	TA	TC	TD	TA
1	PP	19	36.4 (0.5)	23.7	1.0	4.6	8.7	0.4	2.1	0.7	0.9991
2	HP	20	31.3 (2.4)	10.0	1.1	5.5	9.8	0.9	2.0	1.1	0.9999
3	PP	24	28.1 (0.8)	16.8	1.1	6.1	9.7	0.8	4.2	1.8	0.9969
4	HP	21	25.5 (0.1)	42.0	1.4	4.9	8.3	0.9	1.6	1.0	0.9991
5	PP	18	28.4 (1.3)	10.0	1.0	5.2	7.7	0.6	2.0	1.8	0.9994
6	PP	25	26.6 (16)	3.2	1.1	4.5	ND	1.3	8.3	11.8	0.9990
7	HP	20	28.4 (0.2)	13.0	1.2	4.2	7.5	0.4	0.5	0.8	0.9997

PP = prepacked; HP = home-packed; THF = tetrahydrofuran; RSD = relative standard deviation; S = symmetry factor; Rs = resolution; r = coefficient of correlation; ND = not determined.

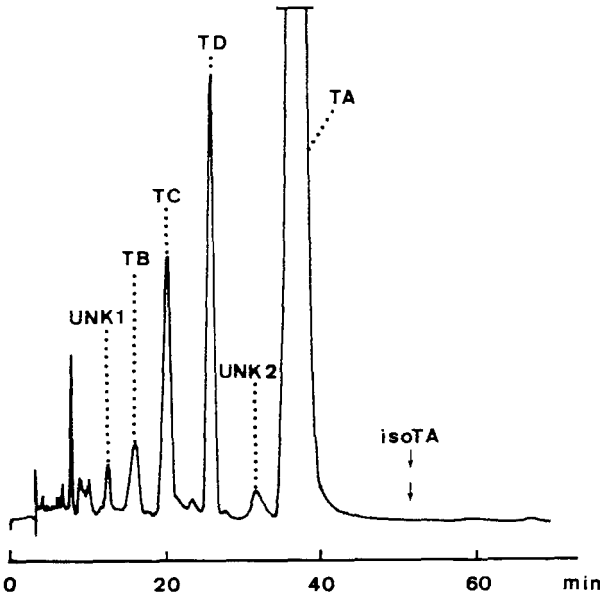

FIGURE 1: Typical chromatogram of sample T-S2, obtained in laboratory 1. TA = tylosin A, TB = tylosin B, TC = tylosin C, TD = tylosin D, isoTA = isotylosin A, UNK = substance of unknown identity.

laboratories do not correspond to the numbers assigned to the authors. A typical chromatogram is shown in Fig. 1. Three laboratories used home-packed columns, the other four used commercially purchased columns. The amount of tetrahydrofuran (THF) in the mobile phase varied between 18 % and 25 % v/v. The relative standard deviation (RSD) on the retention time of TA is not only a measure for the quality of the pumping system but also for the composition of hte mobile phase containing the volatile THF. In a first instance, laboratory 5 reported an increase of retention times during the day, due to loss of THF through evaporation. After advice was given to keep the mobile phase container well closed and not to purge continuously with helium, this problem was solved. Laboratory 6 was probably facing the same problem, but did not contact the organizing laboratory. The sensitivity of the detection system was also checked. In this test the signal-to-noise ratio was calculated of the TA peak obtained on injection of 50 ng of TA, which corresponds to 0.25 % of the amount used in sample

analysis. All laboratories, except laboratory 6, reached a ratio of at least 10. In laboratory 6 an older type of UV detector was used. However, the small amounts of impurities present in the samples were detected without any problem. Laboratory 5 used an injection volume of 10 µl instead of the prescribed 20 µl, because of overloading problems with the diode array detector used. Nevertheless, the required sensitivity was achieved.

Chromatographic characteristics were calculated according to the monograph "liquid chromatography" of the Ph. Eur. [7]. The symmetry factor S was calculated for the TA peak. The results varied between 1.0 an 1.4. Home-packed columns gave higher values. The resolution Rs was calculated first for the pair TD-TA (R_STD-TA). In the protocol a limit of 4.0 was put on this resolution. Collaborators were asked to adjust the THF content of the mobile phase in order to improve the resolution when necessary. The required resolution was reached by all participating laboratories. In a second test, the resolution between TB and TA (R_STB-TA) was determined using the conditions established for the first resolution test. Laboratory 6 did not report a result. The highest values for R_STB-TA were obtained by the collaborators who also achieved the best values for R_STD-TA. The intention was to check whether the resolution TB-TA could be used as a system suitability test instead of the resolution TD-TA. In contradistinction with TD, TB can easily be obtained by in situ decomposition of TA. The repeatability, expressed as the relative standard deviation (RSD, %) was calculated for five consecutive injections of the same solution of T-S2. This sample contained relatively high amounts (4 to 5 %) of the impurities TC and TD. The required RSD value for the peak area of TA was < 1 %. Laboratory 6 reported a slightly higher value, which might be due to the higher variation on the retention times mentioned above. This laboratory also reported the highest RSD values on the area of the impurities TC and TD.

The linearity is a measure for the quality of the detection system. The correlation coefficient r was calculated for a regression line determined over the range 16-24 µg of T-R injected, corresponding to 80 % - 120 % of the prescribed amount to be analysed. In a first instance, laboratory 4 reported a very high value for the intercept, which was probably due to overloading of the detector. The problem was solved by replacement of the detector.

Analysis of samples

Samples were analysed four times, using four separately prepared solutions. Individual results for the main compound, expressed as % m/m TA, and calculated

relative to the content of T-R, are mentioned in Table 2. Means and RSD values for TA are given in Table 3.

The protocol prescribed to calculate the mean results using the mean value of all the analyses of the reference. In a few cases this prescription was not followed. Laboratory 3 analysed the samples over a time period of four days, because an autosampler was not available. Results obtained on day 4 were systematically higher than those on the other days. Therefore, results were calculated with reference to the T-R samples analysed on that day. Laboratory 6 obtained a large variation of the results, which may be explained by the above mentioned variation of the retention times. Results for this laboratory were calculated using the nearest results obtained with the reference. The variation between replicates (RSD) was mostly less than 2 %, except in laboratories 3 and 4 (samples T-S1 and T-S2) and in laboratory 2 (sample T-S3). Laboratory 3 also obtained higher RSD values in the repeatability test. However, using Dixon's test for outlying individual results [8], no value had to be eliminated. Overall, RSD values obtained in the analysis of samples were higher than those in the repeatability test (Table 1). The means of means have RSD values lower than 2 %, which indicates that the method is robust enough to be generally applicable.

Table 4 reports means of mean values for the related substances. The content of impurities is expressed in terms of TA. Laboratory 6 did not properly integrate the peaks for UNK 1 and TB in all the samples. These results were not included in the mean values for these impurities. Laboratory 3 had a problem with the assignment of UNK 1. The reason for this is not clear, since the sensitivity test requirement (see Table 1) was met by this laboratory. Laboratory 2 reported formation of isoTA upon storage of the samples. The samples were probably not protected from light during analysis. This formation of isoTA explains the high RSD value for isoTA in sample T-S1. Although the content of impurities is rather low, the RSD values are quite small, indicating a good reproducibility due to good selectivity of the method towards the different related substances.

Analysis of variance

In order to further analyse the results obtained for the main component, a number of statistical calculations were performed following described procedures [8,9]. The results were first examined for outliers. The means were ranked for outlying laboratories [8]. The ranked mean values were also examined for outlying mean values by using Dixon's criterion [8]. No laboratory was excluded and no outlying

TABLE 2
Individual Results (% m/m) for Tylosin A

Laboratory	Sample											
	T-S1				T-S2				T-S3			
1	80.22	79.80	81.33	80.99	74.64	74.45	74.74	76.99	74.83	74.98	75.40	76.03
2	80.49	80.19	82.23	78.59	76.24	74.22	77.49	77.36	76.00	73.81	72.21	71.39
3	80.23	77.58	78.44	82.21	74.72	79.60	77.08	76.71	75.77	76.06	75.54	75.33
4	78.93	77.14	78.46	81.38	73.99	72.46	78.15	77.72	73.40	74.96	74.17	74.08
5	80.20	80.14	79.76	80.45	77.84	80.10	77.72	78.50	77.17	78.66	77.36	76.02
6	81.80	82.09	81.04	79.79	76.52	78.69	78.60	76.77	75.30	76.38	76.14	74.39
7	80.00	80.87	80.99	80.35	75.49	76.16	74.45	75.87	74.28	74.79	75.45	75.65

TABLE 3
Mean values (% m/m) for Tylosin A

Laboratory	Sample T-S1	Sample T-S2	Sample T-S3
1	80.6 (0.9)	75.2 (1.6)	75.3 (0.7)
2	80.4 (1.8)	76.3 (2.0)	73.4 (2.8)
3	79.6 (2.6)	77.0 (2.6)	75.7 (0.4)
4	79.0 (2.2)	75.6 (3.7)	74.1 (0.9)
5	80.1 (0.4)	78.5 (1.4)	77.4 (1.4)
6	81.2 (1.3)	77.6 (1.5)	75.6 (1.2)
7	80.6 (0.6)	75.5 (0.8)	75.0 (0.6)
Mean of means	80.2 (0.9)	76.5 (1.6)	75.2 (1.7)

Relative standard deviations (RSD, %) are mentioned in parentheses.

TABLE 4
Mean of mean values (%) for related substances

Sample	UNK 1[a]	TB[a]	TC	TD	UNK 2	isoTA
T-R	0.24 (16)	0.44 (15)	0.59 (17)	1.01 (15)	0.35 (29)	ND
T-S1	0.60 (15)	1.09 (14)	0.39 (23)	4.25 (12)	0.79 (26)	0.88 (68)[b]
T-S2	0.63 (15)	1.35 (13)	4.47 (15)	5.78 (14)	0.68 (19)	ND
T-S3	0.98 (19)	1.31 (14)	3.21 (14)	2.19 (14)	0.77 (22)	ND

ND = not detected; RSD (%) are given in parentheses. (a) Results for lab 6 not included. (b) Lab 2 reported formation of isoTA upon storage of the sample solution.

TABLE 5
Analysis of variance

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between labs (L)	50.43	6	8.41	L/LS = 2.77 F_{99} (6,12) = 4.82
Laboratory - sample interaction (LS)	36.47	12	3.04	LS/S = 1.71 F_{99} (12,63) < 2.50 > 2.34
Between replicates (S)	112.09	63	1.78	

mean value was found. The data were examined in order to determine whether the within-sample and within-laboratory variances may be considered as sufficiently homogeneous [9]. For both variances the limit was not exceeded.

An analysis of variance was carried out to search for consistent laboratory bias or significant laboratory sample interaction [9]. The results which are shown in Table 5 reveal that there is no significant between laboratory variance at the 1 % level, so no consistent laboratory bias exists. The laboratory-sample interaction is also not significant at the 1 % level. Estimates of the repeatability of the analytical method (intra-laboratory variance) and of the reproducibility (inter-laboratory variance) were calculated. The standard deviations thus obtained were 1.3 % and 1.6 % respectively. The method will show greater variation when carried out by different laboratories than within one laboratory, but evidently no consistent laboratory bias exists (the between-laboratory variance is not significant).

CONCLUSION

It can be concluded that the LC method shows a reproducible selectivity on PLRP-S columns of different origin and that the method is suitable for purity control and assay of tylosin.

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DETERMINATION AND ANALYSIS OF HUMAN SERUM ALPHA-1-ACID GLYCOPROTEIN BY LIQUID CHROMATOGRAPHIC METHODS

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ABSTRACT

Human serum alpha-1-acid glycoprotein (orosomucoid, AGP) with particular interest as a tumor marker was measured, separated, purified and analyzed for its carbohydrate constituents. In order to demonstrate the changes in the concentration and composition of serum AGP induced by malignant diseases an improved method of sample preparation based on solvent extraction, ion exchange and reversed phase liquid chromatographic methods were applied. In preliminary studies significantly elevated serum AGP levels and increased fucose content in the carbohydrate moiety were found in cancer patients in comparison to the healthy individuals.

INTRODUCTION

Alpha-1-acid glycoprotein (orosomucoid, AGP) is a characteristic and dominant fraction of human serum

sialoglycoproteins with a molecular mass of 40 KD, an unusually high carbohydrate content (45%), and a large number of sialyl residues (1, 2). Changes in the serum AGP concentration are considered as a marker of several metabolic disorders. Highly elevated plasma AGP levels were found in patients with inflammatory diseases and cancer (3-7). Recently, we demonstrated that sialic acid measured in a tumor marker was originated primarily from the plasma AGP content (8). It has been shown that plasma AGP is polymorph and individual molecular variants with slightly different chemical composition, electrophoretic mobility and affinity to lectins can be distinguished (5, 9-11). It has also been reported that the compositional changes in human serum AGP induced by various acute phase conditions are in coincidence with the alterations in the antennary structure of glycan chains (10, 12-14). It is of particular interest, therefore, to investigate the anomalies in the molecular heterogeneity of serum AGP induced by malignant diseases. Recent methods for measuring and preparation of human serum AGP are based on either immunological (diffusion-precipitation, affinity) or salting-out and ion exchnage chromatographic techniques (1, 3-6, 10-13). These procedures may have various difficulties in the time, expenses and purity of the product, or in the selectivity of quantitation (15-16). In the present paper we worked out a single step sample preparation and a microanalytical ion exchange chromatographic method for measuring the AGP content of human serum in cancer patients as well as in healthy individuals. In order to determine the changes in the sialic acid and sugar constituents, serum AGP was prepared and purified by ion exchange chromatography and analyzed by reversed phase HPLC methods.

MATERIALS

Chemicals and reagents used were of analytical grade from E. Merck (Darmstadt, Germany) unless stated otherwise. All solvents were of HPLC quality (LiChrosolv, Merck). Prepacked columns and Q-Sepharose Fast Flow were purchased from the producers. Bis-tris-propane, reference monosaccharides (glucose, galactose, mannose, fucose), N-acetyl neuraminic (sialic) acid, serum protein fractions including human serum alpha-1-acid glycoprotein and alpha-1-antitrypsin were obtained from Sigma Co. (St Louis, MO.).

Apparatus

The Pharmacia-LKB FPLC System composed of two P-500 pumps, an LCC-500 Liquid Chromatographic Controller and a UV-1 Monitor (at 280 nm) was applied to the ion exchange and gel chromatographic (desalting) separations using MonoQ HR 5/5 (5x0.5 cm I.D., 10 μm), Q-Sepharose Fast Flow (30x2.54 cm I.D., 45-165 μm) and Fast Desalting HR 10/10 (10x1 cm I.D.) columns (Pharmacia-LKB, Uppsala, Sweden). Measuring of sialic acid and neutral sugars was carried out on a LiChrosorb RP-18 column (25x0.46 cm I.D., 5 μm) with a Merck-Hitachi L-6200A Intelligent Pump and a D-2500 Chromato-Integrator equipped with an L-4250 UV-VIS and a Shimadzu RF-530 fluorscence detector. HPLC columns were thermostated at 30°C.

METHODS

Sample preparation

Venous blood was drawn from healthy volunteers and hospitalized cancer patients after overnight fasting. Blood was allowed to clot and serum was separated by centrifugation (1000g, 4°C, 10 min), and kept at -20°C until use. One vol of human serum was diluted with 3.5 vol of ice-cold water and extracted with 15 vol of chloroform-methanol mixture (2:1 v/v) by vigorous shaking at 0°C for 45 min. Solvent phases were separated by centrifugation (1000g, 4°C, 20 min) and the methanol-water upper phase (extract) was collected and evaporated 10-20 fold in vacuum for the preparative ion exchange chromatography.

Determination of the serum AGP content

In the microanalytical procedure 100 μl of human serum was treated according to the sample preparation with proportionally measured solvent volumes in a glass stoppered centrifuge tube (8x1.5 cm I.D.). After centrifugation 500 μl of upper phase was applied directly to the MonoQ HR 5/5 column. Separation of AGP was performed using a combined pH/NaCl gradient elution system (17) with solvents: A - 50 mM bis-tris-propane/HCl buffer (pH:7.5) containing 10% (v/v) of methanol, and

B - 50 mM bis-tris-propane/HCl buffer (pH:9.5) containing 350 mM NaCl according to the elution program: A for 2.5 ml, B 0-100% for 20 ml, B 100% for 2.5 ml and A for 5 ml (equilibration). For measuring the serum AGP content the MonoQ HR 5/5 column was calibrated with commercial AGP in a range of 10-200 µg/500 µl of sample volume. Serum AGP concentration was calculated from the linear correlation of ug AGP vs peak height (AUFS 0.1) values obtained considering that 500 µl of methanol-water extract was equivalent to 65 µl of serum. Results were expressed as mg AGP/dl of serum.

Preparative isolation of human serum AGP

In the preparative scale separation of human serum AGP 1-2 ml concentrates of the methanol-water extract were applied to a Q-Sepharose Fast Flow column (30x2.54 cm I.D.) and eluted with proportionally increased buffer volumes excepted that methanol was omitted from the buffer A. The gradient elution program was as follows: A for 75 ml, B 0-100% for 525 ml, B 100% for 150 ml, then equilibration with 300 ml of buffer A. Flow rate was 2 ml/min and 20 ml fractions were collected with detection at 280 nm. Eluates were desalted on a Fast Desalting HR 10/10 column eqilibrated with distilled water, lyophilized and analyzed by polyacrylamide gel electrophoresis as described earlier (8). Protein content was determined according to Hartree (18).

Reversed phase HPLC methods

N-Acetyl neuraminic (sialic) acid content of AGP samples was determined by the method of Hara et al (19). 1,2-Diamino-4,5-methylenedioxybenzene (DMB) derivative of sialic acid was separated by isocratic elution with methanol-acetonitrile-water (25:4:91) as mobile phase. Fluorescence detection was at excitation: 373 nm, and emission: 448 nm. For quantitative measuring calibration curve was plotted with commercial sialic acid. The major monosaccharides (galactose, mannose, fucose) were measured according to Eggert and Jones (20). Dansyl monosaccharides were separated on a LiChrosorb RP-18 column by isocratic elution with acetonitrile-water (1:4 v/v) containing 10 mM formic acid, 40 mM acetic acid and 1 mM triethylamine. Flow rate was 1.0 ml/min and detection at 254 nm. Quantitation was done by means of reference monosaccharides.

RESULTS

In the present work a representative sialoglycoprotein AGP was separated and purified partially using a single-step solvent extraction with chloroform-methanol. Chromatographic and electrophoretic analysis of the sample preparation demonstrated that under the conditions reported here solvent extraction resulted in the precipitation and bulk elimination of major plasma proteins (mostly albumin, alpha-1-antitrypsin, globulins, Figure 1), while serum AGP remained solved in the methanol-water (upper) phase, probably, due to its exceptionally acidic and soluble character (8). The relative enrichment of AGP in the methanol-water extract and an improved technique of ion-exchange chromatography allowed either the quantitative and selective measuring of serum AGP content (Figure 1-B), and/or the scaling-up for its preparative isolation (Figure 2). As regards as the analytical method studies on pooled human serum samples supplemented with AGP in a range of 10-500 mg/dl showed a recovery of 96% (3.1% C.V.). Accuracy of the method was 2.2% (C.V.) in average in a range of 10-200 mg AGP/dl of serum, and sensitivity was found to be 5 μg AGP in 500 μl of sample (AUFS 0.1). Serum concentrations and retention time of AGP could be reproduced with 95% (1.14% C.V.). Our results concerning the serum total AGP content obtained for healthy individuals as well as for cancer patients (Table I) are in accordance with the figures of others (5, 10, 22). One of the major advantage of the sample preparation is that the methanol-water extract of human sera can directly be applied to the MonoQ HR 5/5 column in a volume of 500 μl. It has been observed that using 10% (v/v) of an organic modifier (methanol, acetonitrile) in the mobile phase (buffer A) during the equilibration and gradient elution reduced the retention of some contaminants (e.g. nucleotides, peak 2 in Figure 1-B) by 15-20%, and resulted in sharper peaks and better resolution. It has to be noted, on the other hand, that the methanol content in the mobile phase suppressed the UV absorption of proteins by 30-40% and, therefore, the sensitivity of detection. Figure 2 shows the ion exchange chromatographic separation of human serum AGP on a Q-Sepharose Fast Flow column. In contrast to the polymer based MonoQ particles the methanol content of the sample should be removed when the polysaccharide Q-Sepharose packing was used. Selectivity of the preparative scale isolation was checked by polyacrylamide gel electrophoresis and serum AGP (fractions

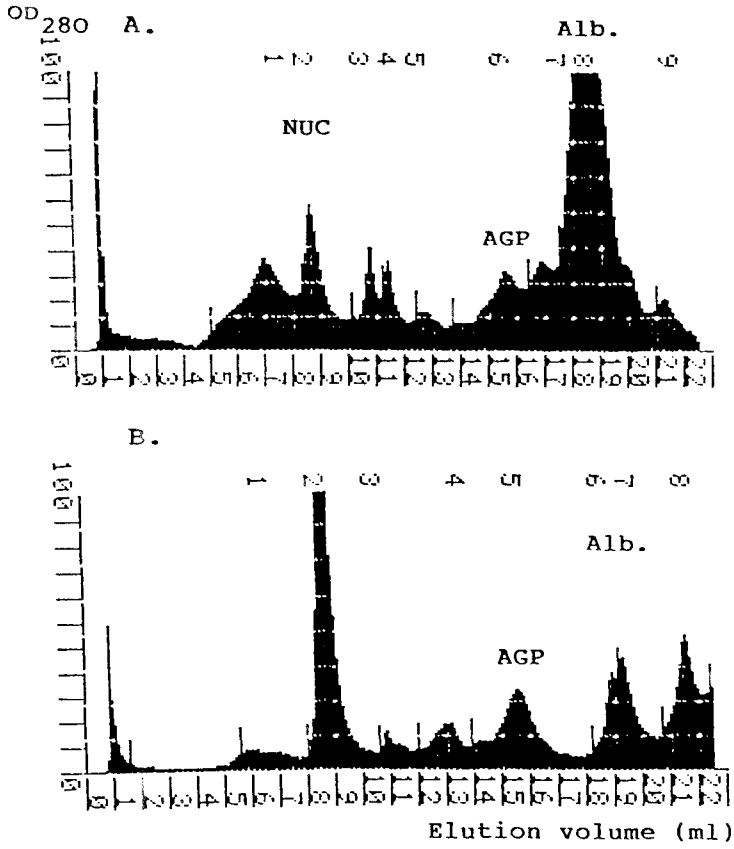

FIGURE 1.

Ion exchange chromatography of human serum proteins on a MonoQ HR 5/5 column (5x0.5 cm I.D., 10 µm) operated by the Pharmacia-LKB FPLC System. Gradient elution see Methods. Flow rate: 0.5 ml/min. Detection: 280 nm (AUFS 0.1). Abbreviations: NUC - Nucleotides, AGP - Alpha-1-acid glycoprotein, Alb - Albumin.

A - Separation of the total serum protein content, (Sample: 50 µl of diluted (1:25) human serum)

B - Separation of proteins in the methanol-water extract of human serum (Sample: 500 µl of extract).

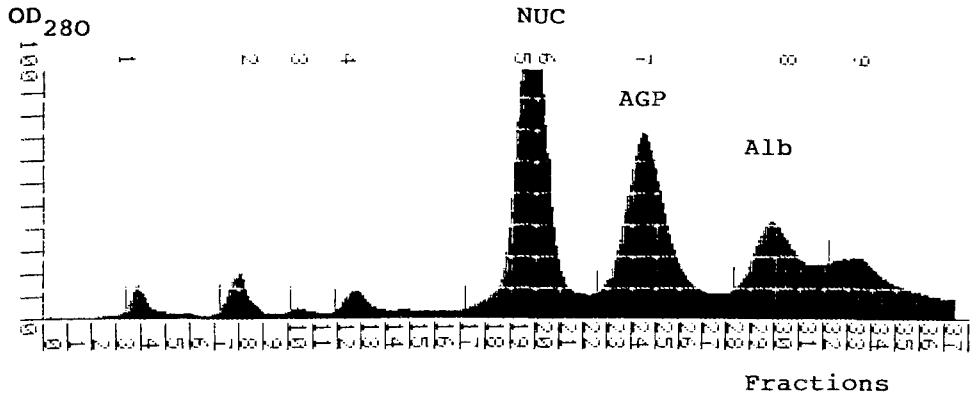

FIGURE 2.

Preparative ion exchange chromatographic separation of human serum AGP on a Q-Sepharose Fast Flow column (30x 2.54 cm I.D., 45-165 μm). Gradient elution see Methods. Flow rate: 2.0 ml/min. Detection: 280 nm (AUFS 0.2-0.5). Sample: 2.0 ml concentrated (10x) methanol-water extract of human serum. Fractions: 20 ml. Abbreviations see Figure 1.

24-25, Figure 2) could be obtained in a purity over 99%. Alpha-1-antitrypsin contamination could not be detected.

Analytical data concerning the sialic acid and monosaccharide constituents of human serum AGP are summarized in Table I. Similarly to others (3-7) we have found significantly higher total AGP levels in the sera of cancer patients (mainly in ovary, colon and breast carcinomas) in comparison to the healthy individuals. However, there was no specificity for the various forms of neoplasma. Our investigations demonstrated some characteristic compositional changes of serum AGP in the presence of advanced malignancy. Figure 3 shows that the distribution of neutral sugars in serum AGP was similar to that of reported for the healthy individuals (19). On contrary, the non-protein constituents (mainly neutral sugars) seemed to be markedly increased, and significantly higher fucose content was measured in the serum AGP of cancer patients supporting the relevance of serum fucose determination as a marker in malignant diseases (21-23).

TABLE 1
Human Serum AGP Content and Constituents in Healthy Individuals and Cancer Patients

Serum concentrations mg/dl	Healthy individuals (n=9)	Cancer patients * (n=15)
Total AGP	83.3 ± 21.5 (62-104)	255 ± 62 (193-317)
Protein content	47.0 ± 0.9	130.6 ± 3.5
Sialic acid	9.7 ± 1.7	26.4 ± 2.4
Galactose	17.6 ± 2.3	61.3 ± 6.3
Mannose	8.3 ± 1.4	32.1 ± 4.3
Fucose	0.7 ± 0.2	4.6 ± 0.5

* Ovary, colon and breast carcinomas

DISCUSSION

Sialoglycoproteins, in general, comprise a large variety of natural compounds with various molecular characteristics and biological activity, and as carrier biopolymers represent the typical structural and functional forms of carbohydrate and sialic acid transport in the circulation (1, 16). Isolation of these solutes from a complex matrix with particular respect to the preservation of their structural integrity and function requires a reliable combination of sample preparation and separation techniques. The present method of sample preparation based on solvent extraction and combined with an improved technique of ion exchange chromatography proved to be suitable either for analytical purposes in measuring the serum AGP content, or for the preparative scale purification of AGP. In comparison to other methods providing high capacity (Cohn's fractionation), or selectivity (immunoaffinity) in the isolation of AGP, the solvent extraction seems to be faster and less expensive. While the automatized techniques (RIA, Delphia) are not comparable, but in price, the selectivity and accuracy of the analytical ion exchange chromatography prevails the immunodiffusion or precipitation techniques in the serial investigation of individual serum samples. From the results presented here it can be concluded that some disadvantages of the

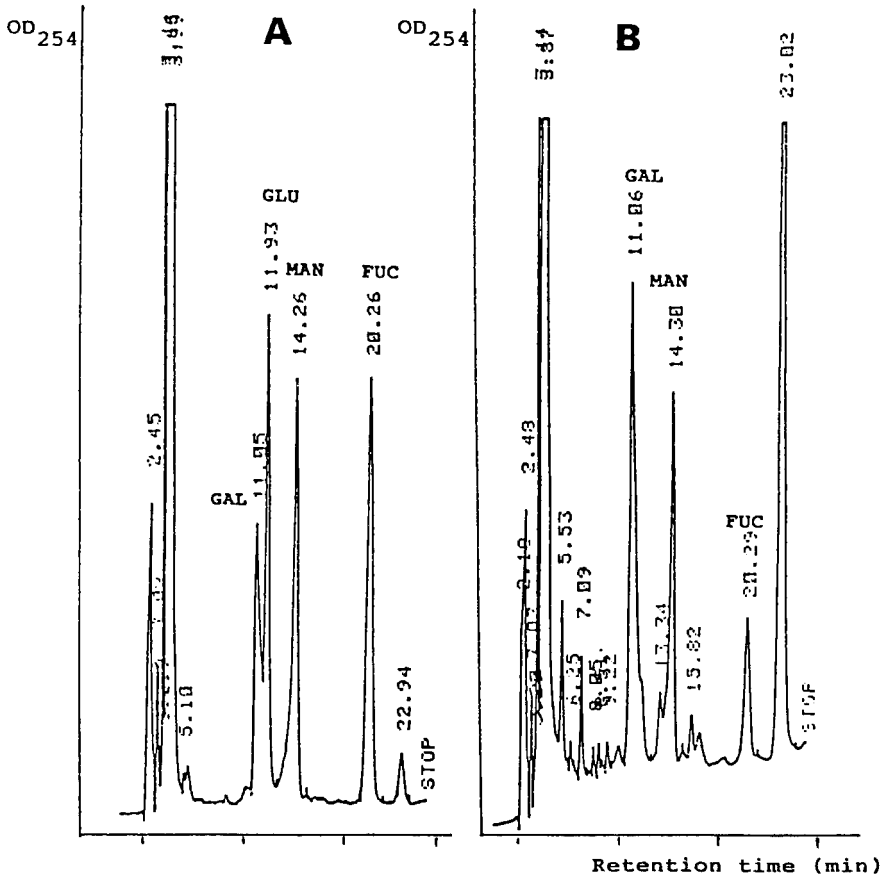

FIGURE 3.

HPLC separation of dansyl monosaccharides on a LiChrosorb RP-18 column (25xO.46 cm I.D., 5 μm).Isocratic elution with acetonitrile-water (1:4 v/v) containing lO mM formic acid, 4O mM acetic acid and 1 mM triethylamine. Flow rate: 1 ml/min. Detection: 254 nm.
A - Refernce monosaccharides: GAL - Galactose, GLU - Glucose, MAN - Mannose, FUC - Fucose.
B - Monosaccharides of human serum AGP.

former methods having difficulties in the separation and purification of AGP from alpha-1-antitrypsin (15) can be overcome by selecting a suitable sample preparation, an appropriate buffer system (bis-tris-propane) with a simultaneous pH-NaCl gradient elution program, and a macroporous anion exchanger with $-CH_2-N^+(CH_3)_3$ functional groups (MonoQ, Q-Sepharose). Hydroxylapatite proved to be useful for the elimination of the alpha-1-antitrypsin contamination (15), but without the ability of fractionating a complex protein mixture. The more favourable hydrodynamic properties and the protective effects of the polysaccharide matrix prefer to the use of dextran-based ion exchangers (Q-Sepharose).

Investigating the compositional changes in human serum AGP induced by the malignant diseases our results seem to confirm that AGP represents one of the main structural forms of serum fucose content elevated in cancer, and probably, molecular variants of AGP different from the normally existing biopolymers appear and increase in the blood of cancer patients. To clarify the molecular bases behind the changes of sugar constituents related to the malignant diseases further attempts are needed to investigate the polymorphism of AGP.

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MICELLAR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF SERUM BILIRUBIN SPECIES WITH DIRECT SAMPLE INJECTION

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ABSTRACT

A rapid reversed-phase high performance liquid chromatographic method is described using a C_8 column and a mobile phase containing sodium dodecyl sulfate (SDS) as a modifier to separate the four bilirubin species present in serum: unconjugated, monoconjugated, diconjugated and biliprotein. The results show that using SDS as a mobile phase modifier improves separation efficiency and increases sample solubility. This simple HPLC procedure allows direct sample injection and makes it possible to quantitatively determine bilirubin species in biological fluids.

INTRODUCTION

The degradation of hemoproteins in humans and most mammals leads to the formation of bilirubin (1, 2). In normal serum, bilirubin is almost completely unconjugated as it is transported from the reticuloendothelial system to the liver, where conjugation of one (mono-conjugated) or

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both (di-conjugated) propionic acid side-chains with glycones occurs. In 1966, Kuenzle, et al. (3,4) first discovered that sera from adults with jaundice contained a fourth bilirubin species firmly bound to protein which was termed biliprotein.

The measurement of bilirubin and its metabolic forms has been recently reviewed by Doumas and Wu (5). In spite of extensive research that has been published on the assay of bilirubin, there is a lack of analytical methods which are capable of accurate, fast determination and quantitation of the metabolic forms of bilirubin in serum.

Currently, most clinical laboratories utilize methods based on coupling bilirubin with a suitable diazonium salt to form colored diazo derivative for the fractionation of "direct" reacting (β- and γ-fractions) and "indirect" reacting (α-fraction) bilirubins. However, the diazo reaction method is not very selective and is prone to interference problems. The δ-fraction and approximately 10-15% of the α-fraction can be direct reacting; therefore, the "direct" diazo reaction can overestimate the level of conjugated bilirubin.

Since the early 1980s, HPLC has been utilized for quantitative fractionization of the four bilirubin species (6-8). The first HPLC method is reported by Lauff and co-workers (6), in this method, the serum is treated with saturated sodium sulfate to precipitate most globulins, but not albumin, and bilirubins are resolved on a reversed-phase column, eluting in order of decreasing polarity. The pretreatment of the serum may entail a variable loss of the biliprotein fraction.

Although high-performance liquid chromatography (HPLC) and anion-exchange chromatography (IEC) techniques have permitted the simultaneous resolution and quantitation of the bilirubin species present in the serum, the complexity of these HPLC or IEC methods have prevented their application for routine clinical use.

It is well known that reverse-phase HPLC columns are modified by the addition of alkyl sulfates commonly used as ion-pair reagents or micelle-forming agents. This modification has been shown to "protect" reverse-phase columns from the deleterious effects of repeated injection

of untreated protein-containing samples (9). Unprotected reverse-phase columns suffer severe efficiency loss, followed by plugging of the column frits or the packing material itself with proteinaceous material, usually in short order.

This effect of surface modification, along with the solubilization of serum proteins has been one of the most important aspects of micellar liquid chromatography (MLC) (10). In micellar solutions, there is an unchanging concentration of free surfactant (given by the critical micelle concentration) in the mobile phase. Since it is the free surfactant molecules which modify the reverse-phase column packing in MLC, gradients have been shown to be possible over wide surfactant concentration gradients (11). The purpose of this study is to investigate the feasibility of the using SDS micelles to enhance the selectivity of the reverse-phase packing material and improve the solubility of unconjugated bilirubin and serum proteins at physiological pH.

EXPERIMENTAL

Materials

HPLC grade methanol, propanol, sodium phosphate monobasic, and phosphoric acid were from Fisher (Springfield, NJ, USA). Human serum albumin (fraction V), normal human serum and SDS were from Sigma (St. Louis, MO, USA). SDS was recrystallized from methanol before use.

Bilirubin Standards

Unconjugated bilirubin containing 9% XIIIα; 80% IXα and 11% IIIα (12) were from Porphyrin Products (Logan, UT, USA). Diconjugated bilirubin (bilirubin ditaurite·Na) was obtained from U.S. Biochemical (Cleveland, OH, USA). Unconjugated bilirubin was used as received since its extinction coefficient was in agreement with accepted value for pure pigment and its separation using high-performance thin-layer chromatograph (HPTLC) and micellar electrokinetic chromatography showed no detectable impurity. However, diconjugated bilirubin

was purified using HPTLC according to previously described procedures (7) since the electropherogram showed approximately 5% impurity. Monoconjugated bilirubin was extracted from rabbit bile using Eberlein's method (13) and further purified on HPTLC plates. Covalent complex of bilirubin and albumin (biliprotein) was synthesized from unconjugated bilirubin and Woodward's reagent K (*N*-ethylphenylisoxazolium-3'-sulfonate) according to the method of Kuenzle *et al.* (14).

Rabbit Bile and Human Serum Samples

Fresh bile from young rabbits was purchased from PEL-Freez Biologicals (Rogers, AR, USA) and pathological serum samples were obtained from three patients in Moses Taylor Hospital (Scranton, PA, USA). Bile and serum samples were kept frozen and stored in the dark before the experiments. To prepare spiked model serum samples, appropriate amounts of a mixture of the four bilirubin standards were dissolved in 20 mM phosphate buffer (pH = 7.0) solutions containing 6% human serum albumin. Serum samples were 1 to 3-fold (v/v) diluted with the mobile phase and then directly injected onto the column.

Chromatographic Conditions

The HPLC system consisted of a Varian 5500 liquid chromatograph (Houston, TX, USA), a Model 7125 sample injector with 10 μl injection loop (Reodyne, Cotati, CA, USA), and a Spectra-100 UV-Vis detector (Spectra Physics, San Jose, CA, USA) equipped with 10 μl flow cell. The absorption wavelength was set at 450 nm for detecting bilirubin species. Chromatograms were recorded on a Hewlett-Packard Model 3390A integrator (Avondale, PA, USA). The analytical HPLC column was a 4 μm Waters Nova-Pak C_8 (3.9 x 150 mm) column (Waters Associates, Milford, MA, USA). This column was protected by a 30-mm refillable pellicular C_8 guard column (Alltech, Deerfield, IL, USA). Mobile phase A was prepared by adding appropriate amounts of SDS to 15% methanol : 85% 50 mM NaH_2PO_4, pH 7 to give 0 to 50 mM micellar solutions. Mobile phase B was 95% methanol : 5% H_2O. Solutions were degassed by sonication.

A linear gradient was applied from 100% eluent A to 30% eluent B in 5 min, followed by holding at 30% eluent B for 10 min, then returned to initial conditions in 2 min and finally re-equilibrated for 5 min. A total mobile phase flow rate of 1 ml/min was used at all times and retention times were measured from point of injection to the peak maxima on the chromatogram.

Recovery Studies

Appropriate volumes of standard solutions were added to normal human serum. These sample solutions of bilirubins in serum were then thermally equilibrated for 2 hours at 37^0C in the dark.

RESULTS AND DISCUSSION

It has been demonstrated that even in the presence of significant amounts of methanol, SDS will still allow the solubilization and elution of serum proteins with minimal damage to the chromatographic system (9). It is also possible to have gradients both with respect to SDS concentration and methanol concentration, and still allow reproducible separation of analytes of interest in untreated human serum samples (15).

Many of the interactions of analytes with micelles are based primarily on hydrophobic interactions between the analyte and the hydrophobic core of the micelle. Electrostatics also play a role, as oppositely charged analytes and micelles typically have higher degrees of association that do like-charged species. It has been shown that conjugated bilirubins have slightly more affinity for SDS micelles than free bilirubin (16). Protein-bound bilirubin would have the greatest interaction with SDS micelles due to the dissipation of the negative change of bilirubin by the protein and the solubilizing interaction of protein and SDS. The elution order of the bilirubin species as shown in Figure 1A is in agreement with these concepts. It was not observed that SDS affected the protein-bilirubin interaction, although it may prevent any further association of free-bilirubin with protein by competitive binding or slight denaturation.

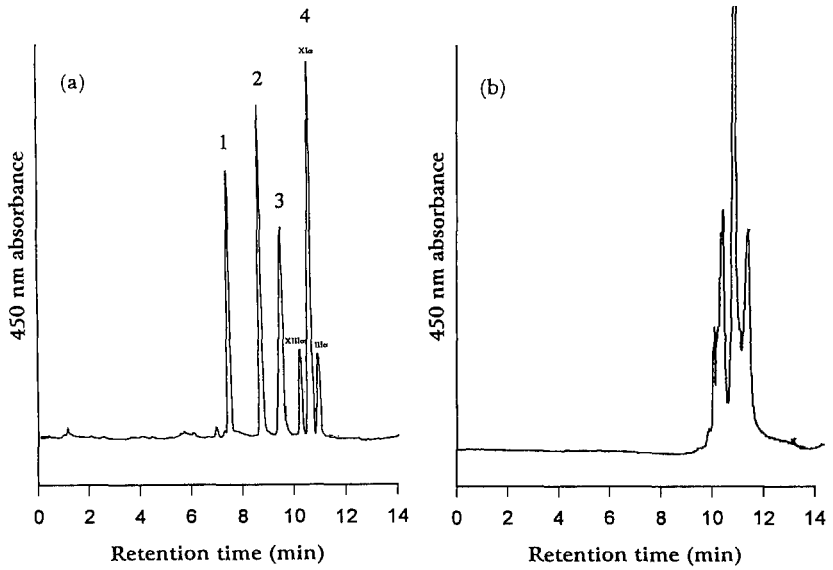

Figure 1. Chromatograms of four bilirubin standards in presence of 3% human serum albumin. Peak Idntification: 1 = 6.1μM biliprotein; 2 = 5.2 μM diconjugated bilirubin; 3 = 3.8 μM monoconjugated bilirubin and 4 = 11.3 μM unconjugated bilirubin. (a) eluent A contains 25 mM SDS and (b) 5 mM SDS.

The separation is affected by the amount of surfactant in the mobile phase. The capacity factors of each of form of bilirubin increased with decreased concentration of SDS from 25 to 10 mM as demonstrated in Figure 1B. Figure 2 shows this effect more clearly with only free bilirubin, at an SDS concentration of 2mM, resolution of the unconjugated bilirubin isomers is completely lost.

Limits of Detection (LOD) and Linearity

For each individual bilirubin species investigated, a linear relationship was found between the amount of bilirubin injected and the corresponding peak area on the chromatogram.

Recovery

Recovery was based on a single point comparison of peak areas of bilirubin standards prepared in mobile phase with those prepared in 3% human serum albumin. Average recovery

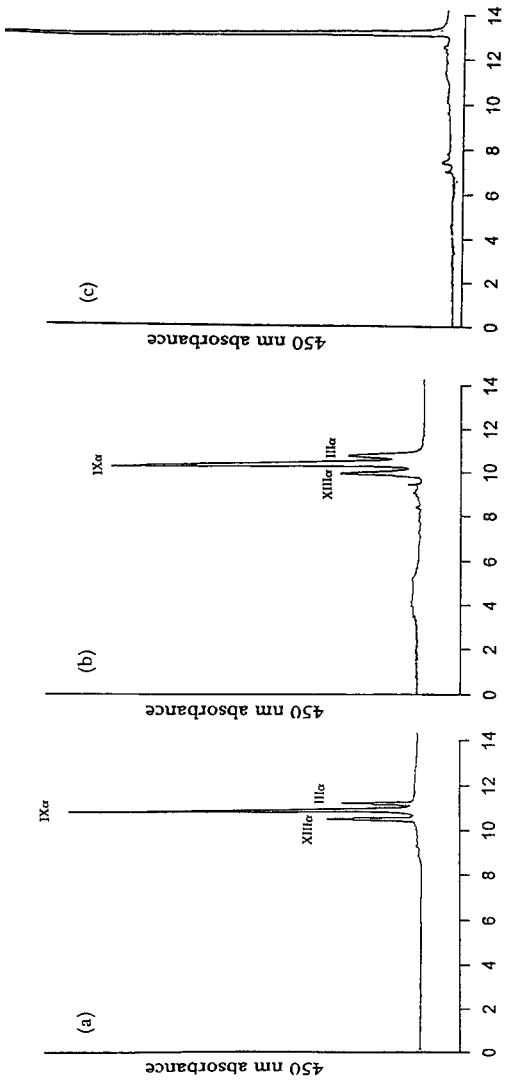

Figure 2. Chromatograms of 12 μM unconjugated bilirubin. Eluent A contains (a) 25 mM SDS, (b) 10 mM SDS and (c) 2 mM SDS.

TABLE I

Detection Limits and Linearity

Bilirubin	Detection limit (nM)	Linearity Upper limit (μM)	r
unconjugated	33	0.85	0.996
monoconjugated	35	1.32	0.995
diconjugated	39	1.41	0.998
biliprotein	43	0.83	0.993

LOD based on S/N = 3 according to peak heights.

Linear regression constants determined from LODs up to the upper limits.

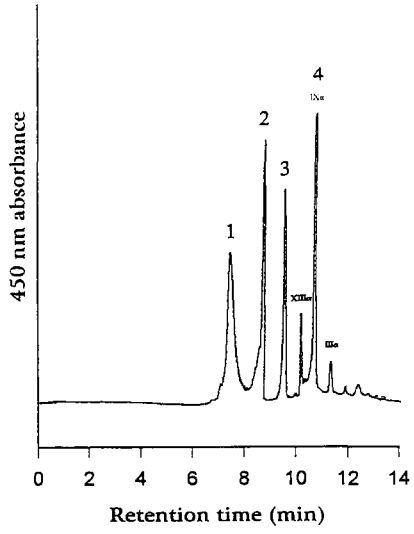

Figure 3. Chromatogram of four bilirubin standards spiked into normal human serum and after 2 hours of 37^{0}C incubation.

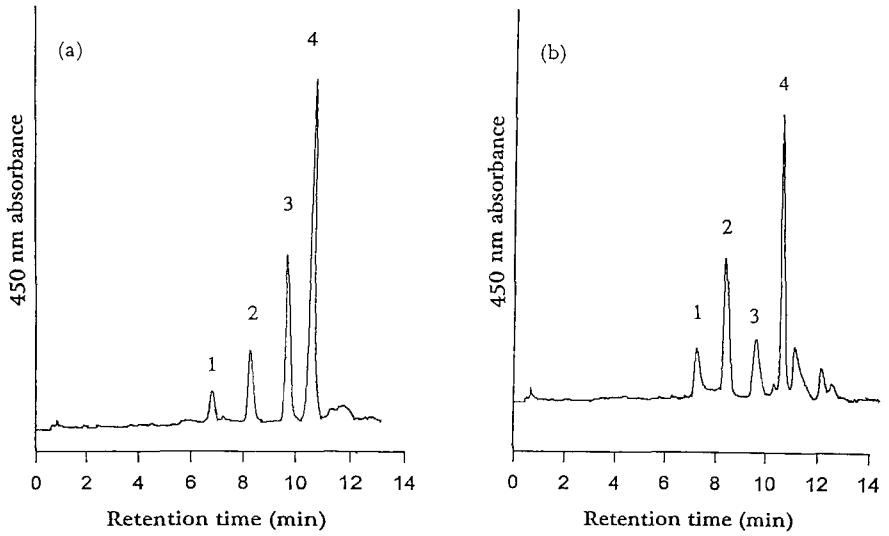

Figure 4. Chromatograms of patient serum samples from individuals with obstructive jaudice. See text for details.

values for duplicate sample preparation was 96% or the mixture of unconjugated bilirubin, 98% for monoconjugated bilirubin, 92% or diconjugated bilirubin and 89% for biliprotein.

Reproducibility

At approximately 25 μmol of total bilirubins per liter, the relative standard deviation (RSD) of peak areas were less than 3% for within 24 hours, and $< 6\%$ for day-to-day analysis over a period of 7 days. At 0.5 to 1.0 μmol total bilirubins per liter, RSD $< 5\%$ and $< 8\%$ were found for within 24 hours and day-to-day (over 7 days) analysis, respectively.

Stability

Serum samples frozen at -60^{0}C are stable for at least 1 month and bilirubin residues obtained from extracts remain stable for at least a week when stored under argon at -20^{0}C.

Using mobile phase as mentioned earlier, with eluent A containing 25 mM SDS, the separation of bilirubin standard spiked into normal human serum is shown in Figure 3. Although

there is a slight loss of efficiency, probably due to interaction of bilirubin with other serum components, peaks are still well resolved. In cases of obstructive jaundice, where there is an obstruction of the normal hepatic bile flow, serum bilirubin is known to be elevated. Serum samples from patients with this affliction were chromatographed using this system. In Figure 4, the four principle types of bilirubin are easily detected and quantitated in these samples. In Figure 4a, a total bilirubin concentration of 110 μM/L (7.4 μM biliprotein, 12 μM diconjugated bilirubin; 29.3 μM monoconjugated and 62.1 uncojugated bilirubinun) and 4b total bilirubin 102 μM/L (13.5, 26, 9 and 53.5 μM, respectively).

In conclusion, we have demonstrated a simple HPLC procedure which makes possible the quantitative determination of the major bilirubin species in serum. This method makes use of the solubilizing power of sodium dodecyl sulfate micelles to allow direct sample injection without modification of the state of conjugation of bilirubin and minimal damage to the analytical column.

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DETERMINATION OF PRAZIQUANTEL IN MEDICATED FISH FEED AND SEDIMENT BY HPLC

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ABSTRACT

A simple method for the determination of praziquantel (2-cyclohexylcarbonyl-4-oxo-1,2,3,6,7,11b-hexahydro-4H-pyrazino [2,1-a]isoquinoline-4-one) in medicated fish feed, and sediment by HPLC, is presented. The calibration curves were linear in the investigated areas, 0.5 - 5 mg/g praziquantel for fish feed and 30 - 500 ng/g for sediment, and the recovery rates were 99 to 100%, respectively.

INTRODUCTION

Praziquantel (PQ) is a drug known for its broad-spectrum activity against trematodes and cestodes (1, 2, 3, 4). The presence of tapeworm in farmed Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss), has become an increasing problem in Norwegian fish farming (5) and presumably also in other countries. PQ is often used in treatment against this parasite, being administered as medicated feed. After

administration, some of the drug will enter the sediment under the net pens, as reported for various other drugs (6 - 9).
Various assay procedures for studying PQ levels in serum and other body fluids have been reported, namely: radiometry (10), fluorimetry (11), gas chromatography (12), and biological assay (13). A HPLC method for the determination of PQ in serum (14) and tissues (15) has also been published.
However, none of the published methods appeared to be applicable to medicated fish feed and sediments.
The purpose of the present study was thus to develop a rapid and efficient HPLC method for routine analysis of PQ in fish feed and sediments.

MATERIALS AND METHODS

Materials and Reagents

Samples of fish feed and sediments free of PQ were used. The fish feed was produced by Skretting (Stavanger, Norway). The sediment was taken from an area with no fish farming activity or known effluents possibly containing antibiotics or chemotherapeutical substances.
All chemicals and solvents were of analytical or HPLC grade. PQ (Droncit vet."Bayer") was donated by Bayer Kjemi A.S. (Oslo, Norway). Stock solutions (1 mg/ml) of PQ were prepared by dissolving the compound in a small amount of acetone (6 ml), and diluting to volume with water. Working standards were prepared by dilution with water. The working standard solutions, when stored in the refrigerator are stable for five days.
Solution A was 0.02 M 1-heptane sulfonic acid sodium salt (Supelco, USA) - 0.01 M di-sodium hydrogen phosphate-2-hydrate (Ferax, Germany), made by dissolving 4.45 g/l heptane sulphonate and 1.779 g/l di-sodium hydrogen phosphate-2-hydrate in c. 750 ml of water when preparing 1 litre of solution. The pH was then adjusted to 6 with 2 M phosphoric acid and the solution made up to volume with water.
A Spin-X centrifuge filter unit with a 0.2 µm nylon membrane from Costar (Cambridge, MA, USA) was also employed.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio-solvent delivery system, an ISS 100 sampling system equipped with a Lauda RMT6 cooler (12°C) from Messgeräte Werk Lauda, (Lauda Königshafen, Germany), and a LC 235C Diode Array detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at 205 nm. The integration was carried out using the software programme Omega-2 (Perkin-Elmer), which was operated on an Olivetti M300 personal computer connected to a BJ-330 printer (Canon).
The analytical column (stainless steel, 15 cm x 4.6 mm ID) and guard column (stainless steel, 2 cm x 4.6 mm ID), were packed with 5 µm particles of Supelcosil LC-ABZ (Supelco, Bellefonte, PA, USA).
The mobile phase was a mixture of water-acetonitrile (60:40 for fish feed and 61:39 for sediments). The flow rate was 1.0 ml/min. for fish feed and 1.0 ml/min. for 3 min. followed by 0.8 ml/min. for 6 min. for sediments. The samples (10 µl for fish feed and 20 µl for sediments) were injected at intervals of 10 and 12 min., respectively.

Sample pretreatment

Fish feed. The stepwise procedure for the pretreatment of fish feed is shown in Fig. 1.
0.5 g ground feed was weighed into a 50 ml graduated centrifuge tube with screw cap (Nunc, Roskilde, Denmark), and 6 ml of acetone added. The sample was mixed for 5 s., and then left to stand with the extraction fluid for 5 min. before again being whirlimixed for 5 s. The homogenate was then made up to 50 ml volume with water-acetonitrile (60:40). The sample was blended, and then centrifuged for approximately 3 min. (3000 rpm). To 0.5 ml of the supernatant was added a 4.5 ml volume of water-acetonitrile (60:40) and the mixture blended. Approximately 0.5 ml of the water-based phase was filtered through a Spin-X centrifuge filter, by centrifugation for 3 min. at 10000 rpm. (5600 g). Aliquots of the filtrate (10 µl) were injected into the HPLC system.

Sediment. The sediment sample (2 g) was weighed into a 50 ml centrifuge tube with screw cap (Nunc). Volumes of 200 µl water (or standard) and 6 ml acetone were added.

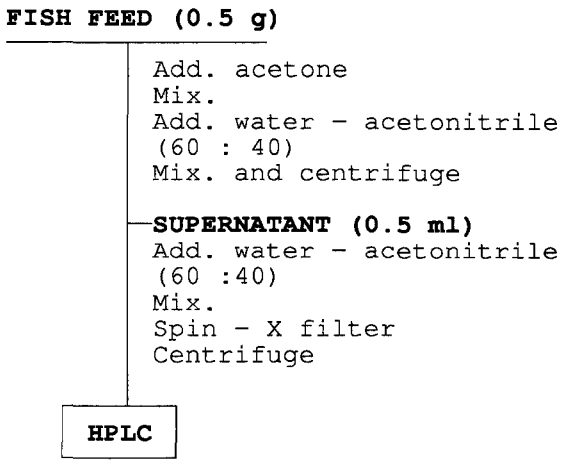

FIGURE 1

Extraction and clean-up procedure for PQ from fish feed.

The sample was mixed for 5 s., and then left to stand with the extraction fluid for 5 min. before again being whirlimixed for 5 s., and then centrifuged for 3 min. (5000 rpm).
4.1 ml of the supernatant (corresponding to 1 g sediment) were transferred into a glass-stoppered centrifuge tube, and 50 µl 1 M NaOH and 5ml diethylether-hexane (3:2) added. The sample was shaken vigorously for 5 s., and centrifuged for 3 min. at 3000 rpm. The upper layer (acetone, diethylether, hexane) was transferred to another glass-stoppered tube. The organic layer was evaporated to dryness at 60 °C under a stream of nitrogen. After adding 1 ml methanol-solution A (70:30) and 1 ml hexane to the dry residue, the sample was again whirlimixed. After centrifugation for 3 min., the hexane layer was discharged, and 2 ml of 0.01 M phosphoric acid-acetonitrile (60:40) added. The sample was again mixed and c. 0.5 ml filtered through a Spin-X centrifuge filter.
Aliquots of the filtrate (20 µl) were injected into the HPLC system.

Calibration curves and recovery studies

The calibration curves for PQ were obtained by spiking fish feed and sediment samples with standard solutions, to yield 0.5, 1.0, 1.5, 3.0, and 5.0 mg/g, and 30, 50, 75, 100, 200 and 500 ng/g of PQ for fish feed and sediment, respectively. Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked fish feed and sediment samples with those of standard solution. The linearity of the standard curves for PQ in fish feed and sediment was tested using peak-height measurements.

RESULTS AND DISCUSSION

Chromatograms of clean and spiked sediment samples, clean samples of fish feed, and a commercial sample of medicated fish feed containing PQ, are shown in Figure 2.

The standard curves were linear in the investigated areas; 0.5 - 5.0 mg/ml, and 30 - 500 ng/g, in fish feed and sediment, respectively. The linearity of the standard curves was 0.9998 and 0.9999 for fish feed and sediment, respectively, when using the external standard method of calculation. The precision and recovery rates for PQ from fish feed and sediment were also calculated, and are shown in Table 1.

The extraction procedures were validated, and showed good recovery of PQ. The recovery of PQ varied from 99 to 100 % for fish feed and sediment, respectively. The precision of these recovery studies varied from 0.37 to 1.07 % and from 0.32 to 2.04 %, for PQ in fish feed and sediment, respectively.

The limit of quantification of PQ was 30 ng/g for sediment, and 0.1 mg/g for fish feed, respectively. No interference was seen during analysis, when calibrating the curves, or when performing recovery studies.

The fish feed clean-up procedure was tried out on sediment. However the limit of quantification in sediment was only 200 ng/g, when aliquots of the filtrated extract (25 µl) were injected into the HPLC system. Unfortunately, when 30 µl or more were injected, minor residues of endogenous compounds in the sediment extracts interfered with PQ in the chromatograms.

The method was tested under practical conditions by analysing about 40 different sediment samples from a

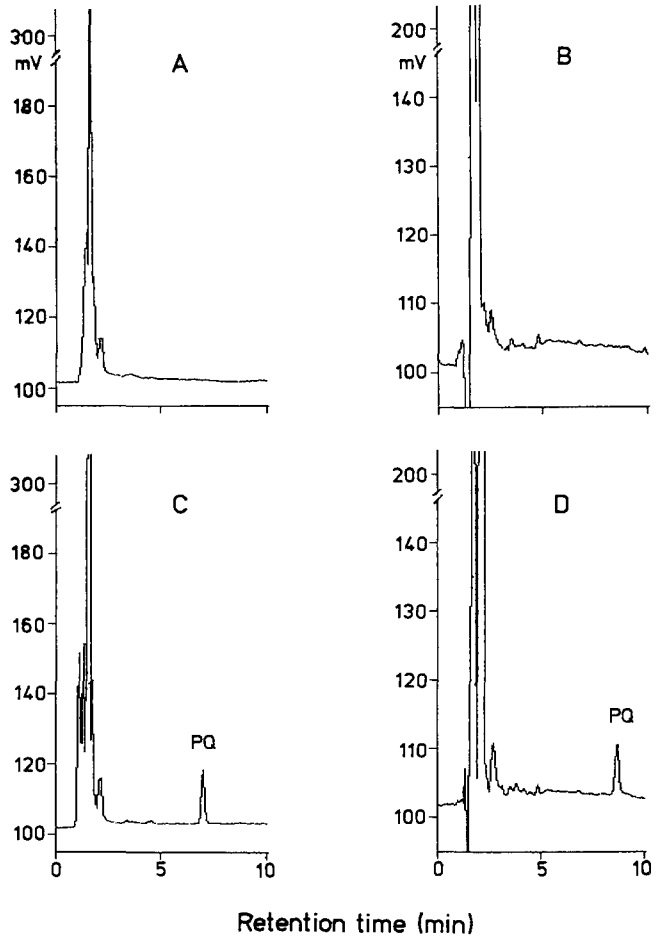

FIGURE 2

Chromatograms of extracts from fish feed and sediment. **A**: Drug-free fish feed, **B**: drug-free sediment, **C**: "real" sample of fish feed contains 1.5 mg/g PQ, **D**: Sediment spiked with PQ (500ng/g).

TABLE 1

Recovery and repeatability for PQ from spiked samples of fish feed and sediment.

Material	No. of samples	Amount in spiked samples (mg-µg/g)	Recovery % PQ Mean	S.D.
Fish feed (0.5 g)	8	0.50	99	1.07
	8	3.00	100	0.37
Sediment (2g)	8	0.05	99	2.04
	8	0.50	100	0.32

S.D.= standard deviation

field study, as well as six "real" samples of medicated fish feed produced by Skretting (Stavanger, Norway). No interfering peaks were observed in the chromatograms

CONCLUSION

This study showed that the content of the antiparasitic compound, PQ, in medicated fish feed, and residue levels in sediment, can be determined by very simple procedures. The assay shows good precision when using the external standard method. The method is robust, simple, and sufficiently sensitive, with good recovery. The quantification is linear over a wide concentration range. The amount of solvents required is minimized. The pretreatment of samples by liquid-liquid extraction combined with centrifugation filters, is preferable to solid-phase extraction columns when performing the pretreatment manually. The chromatographic system was specific with regard to PQ.

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A SENSITIVE STABILITY INDICATING ASSAY FOR THE H_2 BLOCKER RANITIDINE

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ABSTRACT

A stability indicating high performance liquid chromatography assay for ranitidine has been developed for the study of ranitidine in intravenous solutions. The assay is based on an isocratic mobile phase, a C_{18} reverse phase column, UV detection and utilizes an internal standard. The assay is shown to be accurate and precise over a range of ranitidine concentration commonly found in IV solutions. The mean inter- and intra-assay variability is low and retention times are stable. The assay separates ranitidine form ranitidine decomposition products produced at acidic and basic pH's.

INTRODUCTION

Ranitidine is a selective histamine H_2-receptor antagonist used in the treatment of peptic ulcer [1], reflux esophagitis [2] and dyspepsia [3]. Ranitidine is also prescribed in the

prophylaxis and treatment of stress ulcer [4]. For this latter clinical indication, ranitidine is administered intravenously (IV) often in combination with other drugs. Therefore, it is necessary to establish that ranitidine is stable under the conditions encountered during the coadmixing and administration of IV solutions.

High performance liquid chromatography (HPLC) is a commonly utilized assay methodology in studying the stability of ranitidine in IV solutions. Das Gupta and coworkers has published a study of the stability of ranitidine in IV admixtures [5]. The HPLC assay utilized in this study was purported to be stability-indicating although the documentation for this assertion was not adequately presented. Additional studies of the stability of ranitidine hydrochloride in intravenous infusion fluids and parenteral mixtures have been performed [6,7]. Both of these reports cited Evans et. al. [8] and the United States Pharmacopeia [9] as the basis for the HPLC assays utilized in the studies. The Pharmacopeia method cited is a TLC method which is stability indicating. Evans et. al. [8] developed an HPLC assay "for the assay of ranitidine content (i.e. strength).". Although aspects of the Evans assay resemble stability-indicating assays, the method does not establish stability-indicating capability as outlined by Trissel et.al. [10]. The ranitidine was not subjected to stresses that would potentially degrade ranitidine, therefore, the assay did not have the opportunity to separate ranitidine from potentially unknown degradation products. The only statement by Evans et. al. regarding the stability of ranitidine as related to this assay was that ranitidine was stable in the mobile phase as based on peak height ratio. In addition, Evans et. al. chose a wavelength of detection that reduced interference from related compounds but did

not provide maximum sensitivity. Therefore, several previous [6,7] studies of ranitidine stability used assays that may not have been stability-indicating.

The purpose of this study is to establish and document a stability-indicating assay for ranitidine. The assay is able to detect and separate ranitidine and ranitidine degradation products that are formed when ranitidine is subjected to stresses such as extremes in temperature and pH. The method developed is isocratic, utilizes low concentrations of organic solvents, and does not require either ion-pairing reagents or organic modifiers.

MATERIALS AND METHODS

Injectable ranitidine hydrochloride solution, analytical grade ranitidine HCl powder, and analytical grade ranitidine-S-oxide powders were supplied by Glaxo Pharmaceuticals, Inc., Research Triangle Park, NC. The internal standard was analytical grade caffeine (Sigma Chemical Co., Gaithersburg, MD). Analytical grade potassium phosphate monobasic (KH_2PO_4) (Mallinckrodt, Paris, KY) was used to for the mobile phase the pH was adjusted using phosphoric acid or sodium hydroxide. HPLC grade acetonitrile (EM Science, Gibbstown, NJ) was used as the organic component of the mobile phase. The water used in the buffer and mobile phase was glass distilled, treated by ion exchange, charcoal filtered and subsequently filtered through a 0.45 μg pore nylon filter.

Instrumentation and Chromatographic Conditions:

The HPLC system utilized for this assay consisted of a Beckman Ultrasphere-ODS C_{18} column 0.46 cm x 25 cm (Beckman Instruments Inc., San Ramon, CA), a Spectra-Physics Isochem LC pump, a Spectra-

Focus detector, a SP8880 Autosampler, and a ChromJet integrator (Spectra-Physics, San Jose, CA).

The chromatographic separation utilized in this assay was based on a mobile phase consisting of 0.01M monobasic potassium phosphate (KH_2PO_4) adjusted to pH 5.0 (using phosphoric acid and/or sodium hydroxide), and acetonitrile (92:8 v/v). The mobile phase was delivered at a constant flow rate of 2 mL/min. The column and mobile phase were maintained at ambient temperature. The deuterium lamp was lit and mobile phase was pumped through the column for 30 minutes prior to analytical assays to allow for equilibration of the lamp and column. In addition, a conditioning injection containing 50 μg each of ranitidine, ranitidine-S-oxide and caffeine was applied to the column prior to analytical assays. The detector was set to measure U.V. absorbance at 262 nm.

Preparation of Standard Solutions and Samples:

Standards for the standard curve were made from a stock solution of 5 mg/mL of ranitidine hydrochloride. The standard concentrations were 50, 100, 250, 500, 1200 μg/mL. A stock solution of caffeine 1 (mg/mL) was diluted with water to make a working standard of 200 μg/mL. One hundred microliters of the standards or unknown solutions were mixed with 50 μL of the internal standard caffeine and 850 μL of water and pipetted into duplicate, labelled conical tubes. The tubes were vortexed briefly and 50 μL was injected onto the HPLC column for analysis. All standards and unknowns were analyzed in duplicate.

A standard curve consisting of 5 standard concentrations ranging from 0.05 to 1.2 μg/mL was performed during each analytical assay. In addition to the standards, duplicate blank solutions, consisting of 950 μL H_2O and 50 μL internal standard, and spiked

samples were also analyzed, as zero and accuracy controls respectively, during every analytical investigation. The spiked samples were prepared by a third party to concentrations unknown to the HPLC operator. Solutions for the study of ranitidine stability were treated in the manner previously outlined for the standards. All standards, controls, and unknowns were analyzed in duplicate. The concentrations of spiked solutions and unknown solutions were determined by back calculating from the best fit line as established by linear regression based on x = standard concentrations and y = ranitidine/internal standard peak area ratios.

Assay Validation:

During each analysis the standard curve, blanks, and spiked samples with concentrations of 200 and 750 μg/mL were run. Intra-assay variation was estimated by calculating the mean and standard deviation of the concentration of 8 samples of each spiked standard assayed on the same day. Inter-assay variation was estimated from the concentration of spiked samples determined in 5 consecutive chromatographic assays. The results of intra- and inter-assay variation are listed in Table 3.

The accuracy and precision of the assay was determined by comparing five consecutive chromatographic assays. A comparison of calculated concentrations versus actual concentration of the standards spanning the range of the standard curve 50 through 1000 μg/mL was made to determine accuracy. The precision of the assay is demonstrated by the inter-assay coefficient of variation of the calculated concentrations of the standards.

To demonstrate that the assay was stability-indicating it was necessary to subject ranitidine to extreme conditions to cause

TABLE 1
Stability Indicating Nature of Ranitidine Assay

Treatment	Mean Peak Area	%Δ in Peak Area
Original Solution	0.74060	
Refrigerated 7 days	0.73536	- 0.7%
Freezing 7 days	0.74334	+ 0.4%
Room Temperature 24 hr	0.75041	+ 1.3%
Room Temperature 48 hr	0.74041	0.0%
Heating 75°C 24 hr	0.72822	- 1.7%
Heating 75°C 48 hr	0.68062	- 8.1%
1 M NaOH 24 h	< detection limit	> - 95%
1 M NaOH 48 hr	< detection limit	> - 95%
1 M HCl 24 hr	0.40304	- 43.6%
1 M HCl 48 hr	0.32721	- 55.1%

degradation of ranitidine. The assay must then separate the parent compound from the degradation products. The conditions consisted of subjecting ranitidine (50 μg/mL) to refrigeration, below freezing temperatures (-15°C), heat (75°C), 1M HCL (pH 2), 1M NaOH (pH 11) and room temperature with fluorescent light exposure. Table 1 lists the average temperature recorded in room air, the heating block and the freezer. A decrease in the peak area ratio of 10% was considered a significant decrease in the parent compound ranitidine (see Table 2).

RESULTS

The temperatures of the heating block, refrigerator and room temperature ranitidine were monitored hourly for ten hours per day for three days. The average room temperature was 22°C ± 1.0°C, the

TABLE 2
Intra-assay and Interassay Variability of Ranitidine Assay

Intra-assay Variation (n=8)

Spiked Concentration (μg/mL)	Mean Calculated Concentration (μg/mL)	Standard Deviation	% Coefficient of Variation
200	198	3.46	1.7%
750	758	7.31	0.97%

Interassay Variation (n=5)

Spiked Concentration (μg/mL)	Mean Calculated Concentration (μg/mL)	Standard Deviation	% Coefficient of Variation
200	194	8.89	4.6%
750	730	50.3	6.9%

TABLE 3
Precision and Accuracy of Ranitidine Assay

	Mean	%	Δ of Means
Actual Concentration (μg/mL)	Calculated Concentration (μg/mL)	Coefficient of Variation	Actual versus Calculated
50	50.9	0.0699	1.72%
100	98.3	0.0359	1.70%
250	249.7	0.0099	0.12%
500	501.9	0.0325	0.38%
1000	999.3	0.0071	0.07%

average temperature for the heating block was 75°C ± 0.5°C and the average freezer temperature was -15°C ± 0.5°C. Table 1 shows the ratio of the peak areas of ranitidine divided by the internal standard caffeine. Ranitidine proved to be very stable at room temperature, in the refrigerator and in the freezer. Heating appears to have decreased the ranitidine concentration, but it was less than a 10% change and is not considered significant. The only significant decreases in ranitidine were noted after exposure to 1M NaOH (pH 11) and 1M HCL (pH 2). After exposure to either acid or base, the concentration of ranitidine fell to less than 10% of the original concentration of ranitidine. The chromatograph of ranitidine exposed to NaOH showed the break-down products

The inter- and intra-assay variability of the assay are listed in Table 2. The intra-assay variability was calculated from concentrations of 200 and 750 ug/mL spiked samples of ranitidine using the concentrations determined for 8 samples that were analyzed during the same analysis. The mean calculated concentration was 198 μg/mL for the 200 μg/mL spiked sample and 758 μg/mL for the 750 μg/mL spiked sample. The percent coefficient of variation was 1.7% and 0.97% for the 200 and 750 μg/mL spiked sample respectively.caused by exposure to strong base (see Figure 1).

The interassay variability was calculated from the concentration of ranitidine determined during 5 assays run on consecutive days. The mean calculated concentration of 200 μg/mL and 750 μg/mL spiked samples was 194 μg/mL and 730 μg/mL, respectively. The percent coefficient of variation was 4.6% and 6.9% for the 200 μg/mL and 750 μg/mL, respectively.

The accuracy and precision of the assay are shown in Table 3. The accuracy of the assay was the greatest at the 1000 μg/mL

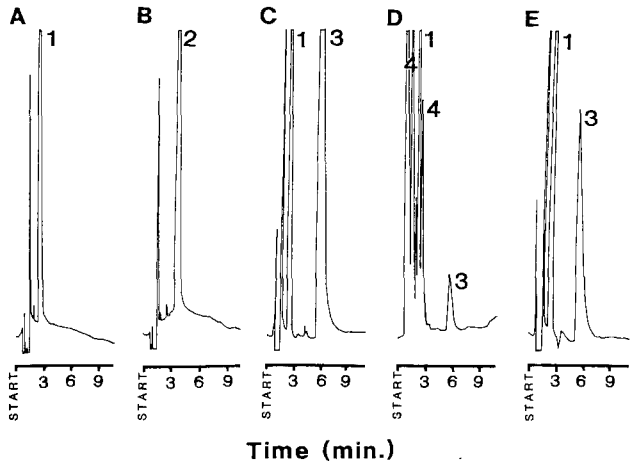

Figure 1: Chromatograms of standards and treated ranitidine solutions. The chromatograms presented represent chromatography of solutions containing; A) caffeine, B) ranitidine-S-oxide, C) internal standard and ranitidine, D) ranitidine in 2 M NaOH for 72 hrs., E) ranitidine stored frozen for 72 hrs. The numbered peaks represent; 1) caffeine, 2) ranitidine-S-oxide, 3) ranitidine, 4) unidentified ranitidine breakdown products.

concentration and least accurate at the 50 μg/mL concentration. However, the percent difference in means (actual versus calculated) concentration never exceeded 1.72%. The precision of the assay was estimated with the coefficient of variation. The greatest precision was with the 1000 μg/mL standard and the least with 50 μg/mL. This is expected when using a non-weighed linear curve.

DISCUSSION

This assay is stability-indicating by the ability to separate of the parent compound, ranitidine, from its breakdown products

produced by exposure to 1M NaOH, 1M HCL and elevated temperature. The assay shows excellent interassay variability with a percent coefficient of variability less than 2%. The intra-assay variability had a percent coefficient of variability of less than 7%. The accuracy and precision of the assay has a percent change in mean of less than 2%.

In Figure 1 ranitidine, ranitidine-S-oxide and caffeine have retention times of 5.67, 3.67 and 2.32 minutes, respectively. The peaks have good symmetry and demonstrate little tailing. Also shown in Figure 1 the degradation products caused by 1M NaOH (pH 11) and 1M HCL (pH 2) all have retention times less than 3 minutes. This assay results in separation of ranitidine from all degradation products created during this study.

Our lowest level of detection was achieved utilizing the 50 μg/mL standard. 100 μL of the standard was diluted to 10% and a 50 μL sample of the dilution was injected onto the column. The amount of ranitidine injected was 250 ng. This assay was designed to study the stability and compatability of ranitidine in IV solutions. The concentration of these solutions generally range from 0.1 to 1.0 mg/mL. Therefore, the range of the standard curve was established between 0.05 and 1.2 mg/mL. During analytical evaluations utilizing this standard curve, it was necessary to use a relatively high attenuation setting on the detector and integrator. These settings could be lowered to achieve a limit of detection of 5 ng injected on column. Based on the previous data we feel that this assay is very suitable for stability studies of ranitidine and may be suitable for quantitating patient blood concentrations of the drug.

This assay has demonstrated the accuracy, precision, and low variability desired in an HPLC assay utilized for the quantitation of unknown samples. In addition, the assay has shown the ability

to separate ranitidine degradation products from ranitidine. These findings support the use of this assay for stability studies of ranitidine. The simplicity of the chromatographic conditions, isocratic solvent delivery, low concentration of organic solvent needed in the mobile phase, and dearth of organic modifiers or ion pairing agents also make this an appealing assay for conducting stability studies involving ranitidine.

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A SELECTIVE LIQUID CHROMATOGRAPHY ASSAY FOR THE DETERMINATION OF d1-α-TOCOPHEROL ACETATE IN PLASMA SAMPLES

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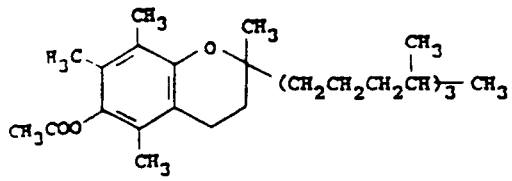
ABSTRACT

A high-performance liquid chromatographic (HPLC) method for the assay of tocopherol and tocopherol acetate in biological samples is described. Results was presented for the linearity, sensitivity and reproducibility. This HPLC method for tocopherol acetate was acceptable in terms of linearity, sensitivity and inter-day reproducibility and was convenient for the routine analysis of plasma samples.

INTRODUCTION

dl-α Tocopherol acetate (2,5,7,8, tetramethyl-2-(4',8',12',trimethyltridecyl)-6-chromanol acetate); **1** and dl-α-Tocopherol (2,5,7,8, tetramethyl-2-(4',8',12', trimethyltridecyl)-6-chromanol); **2**, figure 1, is

* Servicio de Farmacia Hospital "La Paz".



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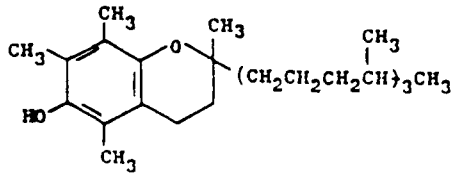

II

FIGURE 1. Chemical structures of Tocopherol acetate (I) and Tocopherol(II).

currently receiving attention concerning its efficacy in preventing or reducing the incidence of severe clinical conditions associated with adverse oxidation events in premature newborns (retolental fibroplasia, intraventricular haemorrhage, bronchopulmonary dysplasia, haemolytic anemia) and in adults (coronary, rheumatic and hypertensive heart diseases) [1,2]. When adminisnistred, the **1** is hydrolyzed to **2**, the active moiety. High plasma concentrations are obtained during three days following I.V. injections [3].In the USP XXII [4], a HPLC method have been developmen for tocopherol acetate determination

in pharmaceutical preparations. In recent years, numerous HPLC procedures have been developed for the determination of **2** in biological samples foods and pharmaceuticals [5-10]. Thus it is desirable to have an analytical method to cuantify the **1** and **2** in biological samples with both drugs, which could be readily applied to analyze several clinical samples daily.

EXPERIMENTAL

Material

dl-α-tocopherol and the dl-α tocopherol acetate were obtained from Merck (Darmstadt, Germany). HPLC grade methanol were purchased from Panreac (Spain). Distilled de-ionized water was used for the preparation of all aqueous solutions.

Instrumentation and Chromatographic Conditions

HPLC system was used, consisting of two Gilson (Middleton, WI, USA) 305 and 306 pumps, and a Gilson 231 XL automatic sampler attached to a injection valve (20μl sample loop), which was coupled to a 200 x 4.6 mm I.D. LiChrosorb RP-18 column, particle size 10 μm. The column was maintained at ambient temperature.

The mobile phase consisted of 100% methanol. The mobile phase was filtered through a Millipore HV LP

(Bedford, MA, USA) filter 0.45 μm prior to use. Total run time was 15 min. A flow-rate of 1.0 ml/min was employed. Injections were made 20 min apart to allow for complete column re-equilibration.

Detection of the analytes was accomplished using a Gilson 116 variable wavelength uv detector at 292 nm. Data were recorded on a Spectra-Physics SP4270 integrator (San Jose, CA, USA).

Animal

Male Beagle dogs (age 12 months; weight 10-12 Kg) were obtained from a recognized supplier, uniquely identified and housed individually in cages.

Sample Preparation

2 ml of methanol were added to aliquots (0.4 ml) of plasma to precipitate proteins. After vortex-mixing for 5 min, the plasma proteins were precipitated by centrifugation at 1200 g for 10 min and filtered through a 0.45 μm Millipore HV filter (Bedford, MA, USA). A 20 μl aliquot was injected onto the HPLC column.

Treatment of Analytical Data

The gradients, intercepts of the calibration curves, and linearity of each calibration graph were obtained by regression analysis. The different retentions time were

calculated. The resolution between two chromatographic peaks (R) was calculated from the equation [1] as follows [4]:

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2} \qquad (1)$$

Where t_1 and t_2 are the retention times and W_1 and W_2 the width of the peaks, measured by extrapolating the relatively straight sides to the baseline.

The interception values is calculated by the following equation:

$$a \pm t\ S_a \qquad (2)$$

Where, t is the value of t-Student for n-2 degrees of freedon and a probability of 0.05; and S_a is the variance of the interception values. If the zero value is between these limits, the proportionally condition is achieved.

The confidence intervals for the slope of the line of regression is calculated by the following ecuation:

$$b \pm t\ S_b \qquad (3)$$

Where, t is the value of t-Student for n-2 degrees of freedom and a probability of 0.05; and S_b the variance of the slope.

Lineality test. It was evaluated by the standard relative desviation for the slope according to the following ecuation:

$$S_{b\ rel\ (\%)} = \frac{S_b}{b}\ 100 \qquad (4)$$

Detection limits (D.L.) were statistically calculated from the following equation [11]:

$$D.L. = S_0^2 \frac{n-2}{n-1}^{1/2} \cdot \frac{t_p}{b} \qquad (5)$$

Where n is the number of values, t_p is the value of Student t at P= 0.05 level of significance and (n-2) degrees of freedom, b is the gradient and S_0^2 is the variance characterizing the dispersion of the points with respect to the line of regression.

The limit for experimental detection is the lower concentration that can be found.

The analytical recovery was calculated from 100 x amount found/ amount added at these concentrations.

Stability of **1** and **2** were measured in different samples after storage at -20 C and room temperature. Stored samples containing **1** and **2** were analyzed and compared to freshly prepared standards. Linear regression analysis of percent analyte found versus storage time was performed.

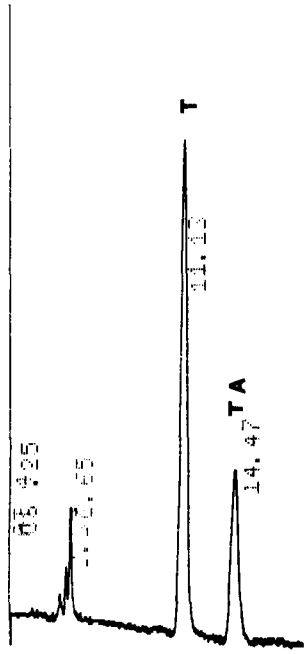

FIGURE 2. Chromatogram of Tocopherol acetate (**1**) and Tocopherol (**2**) in plasma samples.

RESULTS AND DISCUSSION

Chromatography

Figure 2 shows the HPLC chromatograms of **2** and **1** with retention times of 11.13 and 14.47 min respectively. The resolution between chromatographic peaks (R) for **1** and **2** was 1.33. This value allows good resolution without interference from **1** in analysis of **2**.

Linearity

The gradients, intercepts and linearity of each calibration graph were calculated and are summarized in table 1. The intercept values of **2** and **1** were not statistically ($P<0.05$) different from zero. Calibration curve for these methods were linear in the ranges tested, the linearity of **1** is higher than **2**.

The concentration range and detection limit are summarized in table 2. The detection limits evaluated by the statistical method were similar to those calculated according to the experimental method. The lowest detection limit calculated was obtained for **1** 0.17 $\mu g\ ml^{-1}$. The difference between the detection limits, "calculated" and "found" for **2** is probably due to the poorer linearity for this method.

Recovery

The absolute recoveries in plasma samples at these low, medium and high concentrations of **1** and **2** were evaluated. The analytical recovery were between 103.2-104.3% for **1** and between 96.7-102.6% for **2**. These results are provided in Table 3.

Specificity

Plasma was collected from 4 dog and screened for interference at the retention times of **1** and **2**. No

TABLE 1
Comparative Analytical Data for the Determination of dl-α-Tocopherol Acetate and dl-α-Tocopherol.

	dl-α-Tocopherol Acetate	dl-α-Tocopherol
Slopes $b \pm S_b$	1860.78±80.52	4879.82±440.09
Intercept $a \pm t\ S_a$	2829.10±54609.21	699.70±2696.78
Linearity $S_{b\ rel}$ (%)	1.67	3.51

TABLE 2
Concentration Range and Detection Limit for dl-α-Tocopherol Acetate and dl-α-Tocopherol.

	dl-α-Tocopherol Acetate	dl-α-Tocopherol
Concentration Range ($\mu g\ ml^{-1}$)	0-50	0.10
Detection Limit		
Calculated ($\mu g\ ml^{-1}$)	0.17	0.59
Found ($\mu g\ ml^{-1}$)	0.20	0.75

TABLE 3
Recovery of dl-α-Tocopherol Acetate and dl-α-Tocopherol from plasma samples*.

Drug	Concentration (μg/ml)	% Recovery
dl-α-Tocopherol	1.0	104.3
	5.0	103.7
	10.0	103.2
dl-α-Tocopherol Acetate	12.5	96.7
	25.0	101.5
	50.0	102.6

(*) n=4

significant interference was observed with free plasma samples.

Inter and Intra-Day Precision

Due to the large concentration range of **1** required for this assay, standard curves were constructed in order to adequately quantitate unknown samples. Plasma concentrations (n=4) of 1.0, 5.0 and 10.0 μg/ml for **2**, the method yields relative standard deviations (RSD) of 4.68, 4.27 and 4.00% respectively; similar results were obtained by Miller and Vranderick [12] in inter-day precision of carbamazepine in human plasma. Plasma concentrations of 12.5, 25 and 50 μg/ml for **1**, the method yields RSD of 1.17, 1.21 and 0.61% respectively. Under the experimental conditions described the **1** was better than **2** method in the inter-day precision.

The intra-day precision was determined by the evaluation of a typical production run consistiny at concentrations of 12.5, 25.0 and 50.0 μg/ml of **1** and concentrations of 1.0, 5.0 and 10.0 μg/ml of **2**. The RSD of these samples were within 6.23%. These result are provided in table 4.

Application to Pharmacokinetic Study

The method was successfully been applied to a pilot pharmacokinetic study in dog. Figure 3, represents the

TABLE 4
(a) Inter-Day Precision in Plasma.

Drug	Concentration (μg/ml)	n	% RSD
dl-α-Tocopherol	1.0	4	4.68
	5.0	4	4.27
	10.0	4	4.00
dl-α-Tocopherol Acetate	12.5	4	1.17
	25.0	4	1.21
	50.0	4	0.61

(b) Intra-Day Precision in Plasma.

Drug	Concentration (μg/ml)	n	% RSD
dl-α-Tocopherol	1.0	4	6.32
	5.0	4	5.78
	10.0	4	4.87
dl-α-Tocopherol Acetate	12.5	4	2.27
	25.0	4	2.07
	50.0	4	1.97

plasma **1** and **2** concentration versus time profile, by a I.V. injection at 8 mg/kg of a **1** solution to a male beagle dog. All samples were analyzed by the method presented here.

Stability

1 and **2** appear to be stable in plasma upon storage at -20 C for up to 1 year, similar findings have been reported by Constock et al [13]. Plasma samples of **2** and **1** presented retention time of 10.24 and 13.62

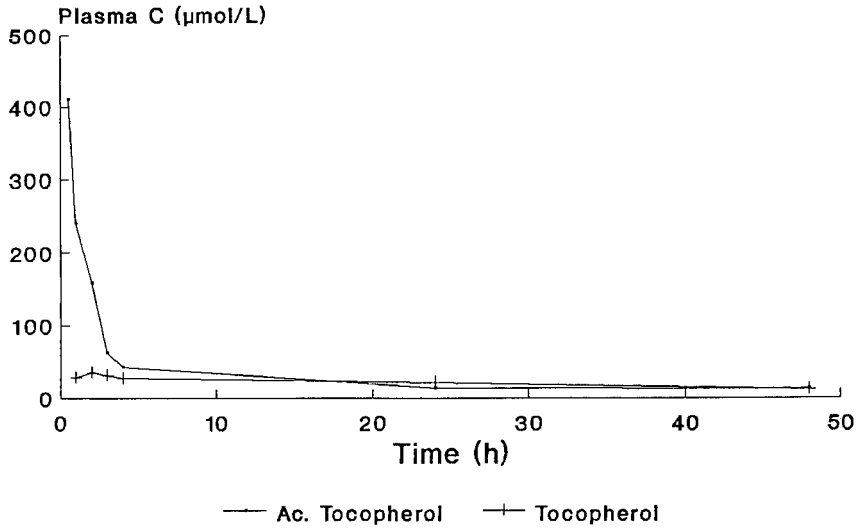

FIGURE 3. Representative plasma concentration-time profile of Tocopherol acetate and Tocopherol after a single 8 mg/Kg i.v. of Tocopherol acetate solution.

respectively, similar to the figure 2 and their plasma concentrations for **1** and **2** were inside of the representation concentration versus time profile (figure 3). Examination of **1** and **2** stability in mobile phase following extraction indicated to be stable upon storage at 4 C for up to 48 h, with RSD values of 1.6% for **2** and 2.8% for **1**. Therefore, to ensure sample integrity; HPLC analysis should be finalized before 48h.

Conclusions

Compared to other chromatographic procedure, this HPLC method has the advantage to be a very simple and

rapid procedure. Complete pharmacokinetic characterization of these drugs requires a sensitive and selective assay for both drug in plasma samples. We report here such an assay for the analysis of **1** and **2** from dog plasma. The major advantage of this method is the direct analysis without the need for a previous treatment of the different plasma samples.

Under the experimental conditions described the linearity, detection limit and recovery was best in tocopherol acetate that tocopherol free analyses. Inter and intra-day precision, expressed as the relative standard deviation (RSD), was always lower than 6.32%.

For the selectivity of the procedure and his rapidity, this HPLC assay is quite suitable for routine analysis in pharmacokinetic study.

ACKNOWLEDGEMENT

This work was supported by a project of FIS nº 90/0257 and nº 92/1227.

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LIQUID CHROMATOGRAPHY CALENDAR

1995

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MAY 21: Techniques for Polymer Analysis and Characterization, a short course, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

MAY 22 - 24: 8th International Symposium on Polymer Analysis and Characterization, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 23: Miniaturization in Liquid Chromatography versus Capillary Electrophoresis, Pharmaceutical Institute, University of Ghent, Ghent, Belgium. Contact: Dr. W. R. G. Baeyens, Univ of Ghent, Pharmaceutical Inst, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

MAY 31 - JUNE 2: 27th Central Regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 11 - 14: 1995 International Symposium and Exhibit on Preparative Chromatography, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 16: Capillary Electrophoresis, Routine Method for the Quality Control of Drugs: Practical Approach (in English); L'Electrophorese Capillaire, Methode de Routine pour le Controle de Qualite des Medicaments: Approche Pratique (in French), Monpellier, France. Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Inst. Europeen des Sciences Pharmaceutiques Industrielles de Montpellier, Ave. Charles Flahault, 34060 Montpellier Cedex 1, France.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

JULY 7 - 8: FFF Workshop, University of Utah, Salt Lake City, UT. Contact: Ms. Julie Westwood, FFF Research Center, Dept. of Chem., University of Utah, Salt Lake City, UT 84112, USA.

JULY 10 - 12: FFF'95, Fifth International Symposium on Field-Flow Fractionation, Park City, Utah. Contact: Ms. Julie Westwood, FFF Researcvh Center, Dept. of Chem. Univ. of Utah, Salt Lake City, UT 84112, USA.

JULY 23 - 28: 35th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency, Denver, Colorado. Contact: Patricia Sulik, Rocky Mt. Instrum. Labs, 456 S. Link Lane, Ft. Collins, CO 80524, USA.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 4 - 7: 13th International Symposium on Biomedical Applications of Chromatography and Electrophoresis and International Symposium on the Applications of HPLC in Enzyme Chemistry, Prague, Czech Republic. Contact: Prof. Z. Deyl, Institute of Physiology, Videnska 1083, CZ-14220 Prague 4, Czech Republic.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography and Extraction, Indianapolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco.. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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