JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 7 NUMBER 5 1984

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CODEN: JLCHD8 7(5) i-viii, 861-1060 (1984)
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Manuscript Preparation and Submission. See the last page of this issue.

Subscription Information. *Journal of Liquid Chromatography* is published in fourteen numbers and two supplements in January, February, March (2 numbers), April, May (2 numbers), June, July (2 numbers), August, September, October (2 numbers), November, and December by Marcel Dekker, Inc., 270 Madison Avenue, New York, New York 10016. The subscription rate for Volume 7 (1984), containing fourteen numbers and two supplements, is $350.00 per volume (prepaid). The special discounted rate for individual professionals and students is $175.00* per volume. To secure this special rate, your order must be prepaid by personal check or may be charged to MasterCard or VISA. Add $40.00 for surface postage outside the United States. For airmail to Europe, add $72.32; to Asia, add $91.52.

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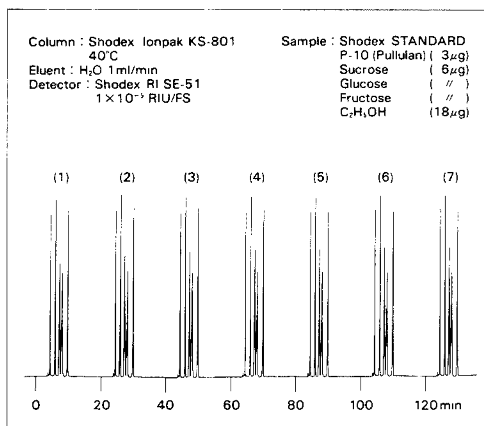

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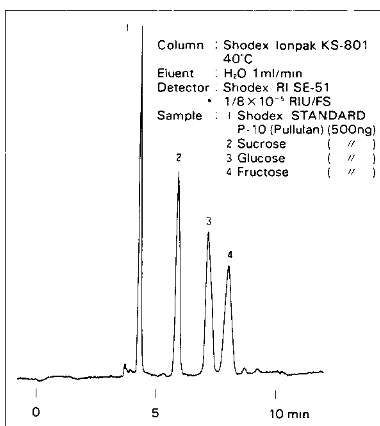

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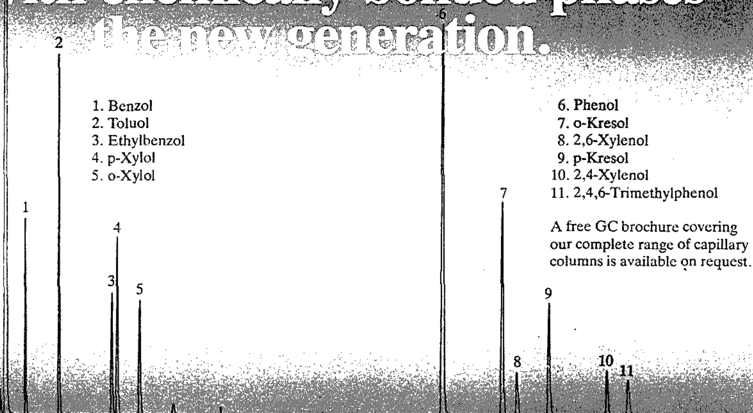

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TRACE ANALYSIS AND SPECIATION FOR ARSENIC ANIONS BY HPLC-HYDRIDE GENERATION-INDUCTIVELY COUPLED PLASMA EMISSION SPECTROSCOPY

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ABSTRACT (26)

High performance liquid chromatography (HPLC) has already been successfully interfaced with inductively coupled plasma (ICP) emission spectroscopy for arsenic analysis and speciation. However, in many instances the overall minimum detection limits (MDLs) are inadequate for many environmental type samples. Arsine generation in a continuous, on-line fashion has been shown to provide for significantly improved MDLs by direct-ICP approaches. This hydride generation-ICP (HY-ICP) derivatization approach has now been successfully interfaced with paired-ion, reversed phase HPLC. This provides a doubly hyphenated technique, namely HPLC-HY-ICP in order to perform true metal/nonmetal speciation. Such methods of arsenic speciation have now been perfected with regard to minimum detection limits, linearity responses over several orders of magnitude, separation of various arsenic species from possible sample interferences, and related analytical matters. The final approaches have been applied both to spiked water samples, as well as to actual environmental drinking water supplies from the New England region. These results demonstrate an ability to qualitatively and quantitatively speciate for arsenate and arsenite at levels ranging from 50 ppb and above in each species. The ability to speciate drinking water supplies (wells) is also demonstrated by these overall application results.

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0148-3919/84/0705-0861$3.50/0

INTRODUCTION (26)

Within recent years interest has grown concerning the development of newer analytical methods for the successful and accurate speciation of metal or nonmetal derivatives or species (1-5). This has occurred because of the growing realization that different forms or species of a metal can exert different toxicological and biological properties in animal and human systems (6-9). Thus, the belief that toxicological observations correlate with total metal content has now fallen into some disrepute. Many published toxicological studies that attempt to relate total metal analysis or content to observed toxicological properties must therefore be reconsidered in light of the more recent suggestions that individual metal or nonmetal species can have very different biological effects in man. Total metal content in place of metal or nonmetal speciation type analyses tells us very little about what toxic properties are really due to individual metal or nonmetal species present. In order to provide valid toxicological results and interpretations, one must therefore have valid analytical speciation methods for just those metal or nonmetal species present in any given sample. This is not always an easy or straight-forward analytical task. In addition, many toxicologists do not have the background to undertake speciation analyses along with their desired toxicological studies. There has thus evolved an extensive metal and non-metal toxicology literature which involves little real inorganic speciation data. This current state of affairs can only be corrected when the analytical chemist develops suitable trace methods of analysis <u>and</u> speciation for many of those metal or nonmetal species of interest to environmentalists and toxicologists or biologists.

Arsenic has long been known to have severe toxic properties in mammalian systems, but much less is known with regard to the toxic properties of various oxyanion derivatives. The trivalent forms of arsenic are more toxic than the pentavalent forms. At a level of 10 ug/g (ppm), sodium arsenite has produced embryotoxicity and teratogenic effects in mice, while at the same level, sodium arsenate had no obvious effect (7). Epidemiologic studies have suggested that arsenic in drinking water may be related to an increased incidence of skin cancer. Arsenate is the valence form most prevalent in nature, and in this form, it tends to be rapidly excreted and probably does not accumulate. Arsenite is the trivalent species ($NaAsO_2$), while arsenate is the pentavalent form (Na_2HAsO_4). All of the arsenic species of interest exist mainly as oxyanions, with valences usually of -1 or -2. Within recent years, a problem has appeared within various New England states, such as New Hampshire, wherein high levels of total arsenic have been found in certain drinking water supplies. Apparently lead arsenate has been used as an agricultural

chemical and pesticide in various fruit orchards in New England. Residues of this arsenic derivative have gradually leached from the orchards into the soil, and from there into various wells used for drinking water. Total levels of arsenic have been found as high as 200 ppb to 250 ppb (parts-per-billion), but they are more commonly 100 ppb or below. Methods for conveniently and reliably performing arsenic speciation of such drinking water supplies have not, in general, been applied to well water samples from New England or elsewhere.

We, as well as others, have demonstrated the capabilities of paired-ion, reversed phase HPLC (RP-HPLC) for performing metal anion analyses, with an emphasis on speciating various arsenic oxyanions (1, 3, 10-17). Conventional ion exchange HPLC or ion chromatography are also fully capable of resolving the various arsenic oxyanions, and such approaches have already been interfaced with inductively coupled plasma (ICP) emission spectroscopy, graphite furance atomic absorption (GFAA) spectroscopy, flame atomic absorption (FAA) spectroscopy, direct current plasma (DCP) emission spectroscopy, electrochemical detection (EC), conductivity detection (CD), and similar specific or nonspecific HPLC detection methods (1). Earlier, we had described an HPLC-ICP approach for speciating at least three different arsenic derivatives (3). These methods of speciation, at least in our hands, did not provide useful and practical minimum detection limits (MDLs). This precluded their direct application to environmental samples, such as well water from New Hampshire.

It occurred to us that there were at least two approaches for immediately improving (lowering) the MDLs with HPLC-ICP, especially for arsenic speciation purposes. One of these might have involved an initial sample preconcentration step using electrothermal carbon cup vaporization of the HPLC eluents just prior to ICP sample introduction (23). Indeed, this particular approach has recently been described at some length by Caruso _et al_. (24). We have always felt that this approach, though general for virtually all metals or nonmetals and capable of providing, in principle, usable MDLs, might at the same time have certain difficult-to-achieve interfacing problems for continuous HPLC-ICP operation. The recent report by Caruso _et al_. that has used electrothermal carbon cup vaporization of the individual HPLC analytes for ICP detection operated in an entirely off-line manner. We have believed that a successful tandem analytical approach should be able to operate on a continuous, on-line basis, with as little operator intervention as possible. Indeed, it should also be able to analyze a large number of environmental samples in an automated fashion, similar to HPLC-ultraviolet (UV) detection or liquid chromatography-electrochemical detection (LCEC).

Another possible solution to the problem of suitable MDLs in HPLC-ICP applications appeared to reside in the use of continuous hydride formation or

generation post-column, with efficient introduction of such metal or nonmetal hydrides into the ICP. Though this particular approach had never been used with HPLC-ICP interfacing, the formation of arsine from various arsenic derivatives has long been known. This approach has been used to improve MDLs for FAA and ICP (18, 19, 25). It therefore occurred to us, as it has to others, that hydride generation (HY) after the HPLC separation step, just before the ICP detection step, might vastly improve overall MDLs for the final speciation of arsenicals (20, 21). This same approach should just as readily be applicable to all other metal or nonmetal species capable of forming hydrides by suitable reaction with sodium borohydride or other reagents. We have, as yet, only applied HPLC-HY-ICP for certain arsenic species, with excellent overall analytical results. The approach provides MDLs of about 50 ppb for environmental type samples or artificially spiked water samples, with a high degree of accuracy and precision. The methods have been applied to a number of artificially spiked water samples for both arsenate and arsenite. In addition, they have been used for a number of New Hampshire well water samples, using both HPLC-HY-ICP for speciation purposes and direct HY-ICP for total arsenic determinations.

EXPERIMENTAL

Reagents

Sodium arsenite, sodium arsenate and sodium hydroxide were Baker Analyzed, reagent grade chemicals from VWR Scientific, Inc. (Boston, Mass.). Sodium dimethylarsenate (SDMA) was obtained from Pfaltz and Bauer, Inc. (Stamford, Conn.). External arsenic standards for Direct-HY-ICP were Baker Analyzed. Mobile phase water for HPLC was taken from a custom made still by Barnstead Co., Division of Sybron, Inc. (Boston, Mass.). Sodium borohydride, 98% $NaBH_4$, was obtained from Alfa Products, Thiokol Corporation, Ventron Division (Danvers, Mass.). Mobile phase and sodium borohydride solutions were filtered through 0.45 um filters (Gelman Sciences, Inc., Ann Arbor, Mich.). Hydrochloric acid (HCl) was of Baker high purity grade Ultrex acids.

Apparatus

This work was performed using an Instrumentation Laboratory (IL) Model Plasma-200 (Instrumentation Laboratory, Inc., Andover, Mass.). A standard cross flow nebulizer spray chamber with a modified drain trap was used to introduce the HPLC eluent to the plasma region. The drain trap was positioned directly beneath the spray chamber. Waste flowed directly into the covered drain trap reservoir by means of a conical plastic attachment. The HPLC system consisted of a Laboratory Data Control (LDC) (Riviër Beach, Fla.) Constametric I pump,

a Rheodyne Model 7125 syringe injection valve (Rheodyne Corp., Cotati, Calif.) fitted with a 200 ul loop and a Honeywell Corp. (Minn., Minn.) dual pen strip chart recorder. Data reduction of the ICP emission intensity data was performed with a Radio Shack TRS-80 Model II microcomputer (Tandy Corp., Fort Worth, Texas). The computer was used to calculate peak areas from raw ICP emission data.

HPLC separations were performed on a number of commercial C_{18} columns as follows: 1) Excalibar Spherisorb ODS (5 um, pre-packed column, 4.6-mm x 15-cm)(Applied Science, State College, Penna.); 2) an in-house slurry packed (4.6-mm x 25-cm) column using 10 um Lichrosorb C_{18} (MCB Chemicals, Inc., Cinc., Ohio); 3) an in-house slurry packed column (4.6-mm x 15-cm) using 5 um Ultrasphere ODS (Altex/Beckman, Berkeley, Calif.); or 4) a pre-packed 10 um High Performance (HP) guard column (10-cm x 3.2-mm)(Alltech Assocs., Deerfield, Ill.).

The hydride generator was constructed from two glass tees (Technicon Corp., Tarrytown, N.Y.), part no. 116-0200-045, connected with 1/16" Teflon tubing running from the end of the HPLC column to the ICP nebulizer. Reagent solutions were introduced with a dual channel peristaltic pump, Figure 1, which was home-made. Direct HY-ICP was done using the IL Plasma Hydride Device.

Methods

The HPLC mobile phase consisted of 5 mM PIC A (tetrabutylammonium phosphate reagent (Waters Associates, Milford, Mass.) in distilled water, prepared according to the manufacturer's directions with a final pH of 7.15. Columns were operated at room temperature at a flow rate of normally 1 ml/min. Each column was washed at the end of each working day with 50:50 MeOH:HOH, to ensure complete removal of all ion-pairing reagent and salts.

Solutions of $NaBH_4$ were prepared in 0.25% sodium hydroxide (NaOH). Three to four percent solutions were made for HPLC-HY-ICP and filtered under vacuum. The plasma would become unstable at more concentrated solutions due to the increase in hydrogen gas formed. At lower $NaBH_4$ concentrations the overall arsine conversion efficiency would decrease. Concentrated HCl was used as received, and introduced separate from the other reagents, all at the same flow rate of 0.25 ml/min. Arsenic standards for HPLC were prepared fresh each day by dissolving the inorganic salts in distilled water, as used for the mobile phase. Injection volumes of 200 ul were used for all blanks, standards, spiked water and environmental well water samples.

Direct-Hydride Generation-ICP was done using the IL Plasma Hydride Device (PHD) with a 2% solution of $NaBH_4$. Samples were adjusted to 3M HCl just prior to analysis. External standards for Direct-HY-ICP were prepared by

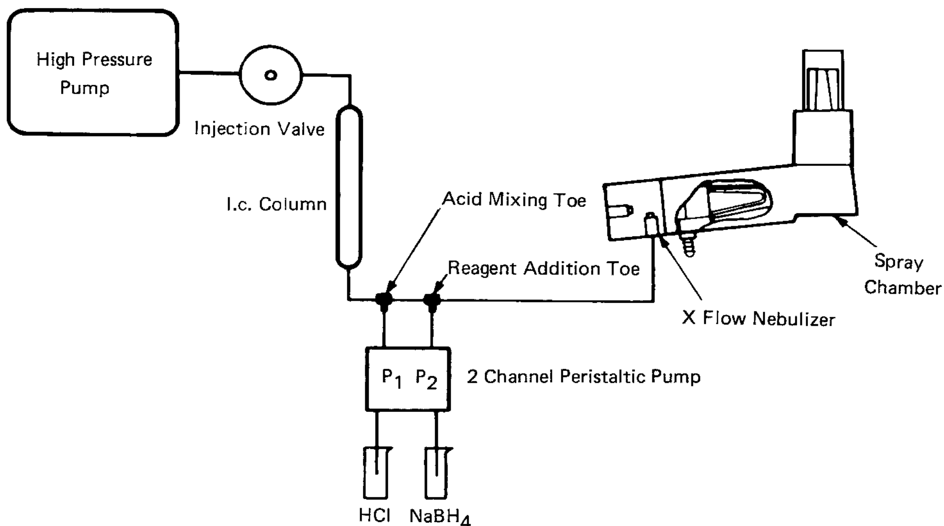

Figure 1. Schematic diagram of the total HPLC-HY-ICP instrumentation.

appropriate dilution of 1000 ppm solutions of Baker Analyzed arsenic standard in 3M HCl.

Well water samples were collected from New Hampshire in glass jars with no acid or preservatives added and were refrigerated on receipt. No sample preparation was involved prior to HPLC-HY-ICP analysis. A minimum of three (3) injections were made of each sample and blank injections were made both before and after all sample injections.

The ICP operating parameters used throughout these studies with both Direct-HY-ICP and HPLC-HY-ICP were as follows: forward power (watts) = 1,000; bandpass (nm) = 0.02; observation height (mm) = 14; gas flows: coolant = 15 l/min; auxillary = 5 l/min; sample = 1 l/min. The 228.81 nm emission line for arsenic was used throughout all analyses.

Arsine conversion efficiences were determined for each arsenic species by measuring peak heights or emission intensity readings as a function of systematically varying the hydride generation conditions, including reagent concentrations, flow rates of reagents, mixing tees, contact times, and similar reaction parameters. It is believed that this experimental approach led to maximization of arsine formation and introduction into the ICP plasma, but thus far this is still an assumption. We have not yet demonstrated a 100% efficiency for the formation of arsine from any of these arsenic species, but

in principle there is at least one approach to demonstrate this experimentally. Studies are now underway in order to fully demonstrate the actual efficiencies of hydride generation as a function of the individual arsenic species. Despite the current assumption of maximum hydride formation, rather than absolute demonstration, our final MDLs for Direct-HY-ICP and HPLC-HY-ICP are adequate for practical environmental sample applications, as below.

RESULTS AND DISCUSSION

Minimum Detection Limits (MDLs) and Linearity of Calibration Plots

Figure 1 illustrates the overall HPLC-HY-ICP experimental apparatus, wherein the hydrides are formed in the Plasma Hydride Device after the HPLC separation. The hydride (arsine) together with the HPLC mobile phase and excess hydride generation reagents are all introduced into the conventional ICP cross-flow nebulizer. This acts as a gas-liquid separator, wherein most or all of the arsine is transferred to the ICP plume, but most (95-99%) of the aqueous solution eventually ends up not entering the same plume region. The cross flow nebulizer acts in HPLC-HY-ICP just as it does in conventional Direct-ICP work, or in Direct-HY-ICP applications.

Figure 2 indicates a typical HPLC-HY-ICP separation for a mixture of three arsenic oxyanion standards, all baseline resolved within 9 mins. The specific HPLC conditions used for this analysis are indicated in the Figure. Arsenite and arsenate are completely resolved from one another, and these have been the only two species commonly found in well water samples thus far (22). It is possible that other well water samples from different regions could contain organoarsenical species, in addition to or instead of these two.

The initial intent of utilizing continuous, on-line hydride generation with HPLC-ICP was to substantially lower the MDLs from those initially realized by HPLC-ICP alone (3). The current studies with these particular arsenic species have indicated a MDL improvement of at least one to two orders of magnitude by HPLC-HY-ICP, Table 1. The MDLs are defined here as the minimum concentration of analyte that produces a signal-to-noise ratio three times the standard deviation of the background noise level. This is a somewhat different definition of MDLs than that normally used by chromatographers, but it is actually the approach to MDLs generally employed by spectroscopists. We have discussed the potential advantages of using this approach to determining MDLs previously (3). It is clear from Table 2 that the ICP response for each arsenic species has been enhanced (improved) by at least a factor of about 1,000 over the similar response in the absence of any hydride generation. Theory would have predicted an MDL improvement, at best, of two orders of magnitude (100%), in going from HPLC-ICP to HPLC-HY-ICP. This is because in

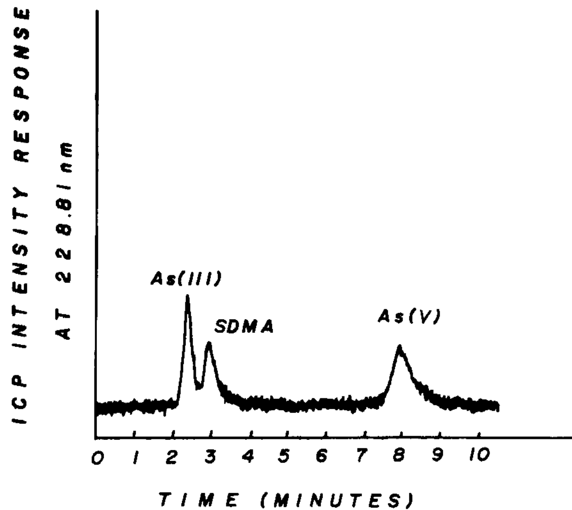

Figure 2. HPLC-HY-ICP chromatogram of arsenite (III), sodium dimethyl arsenate (SDMA), and arsenate (V) at the 200 ppb levels in each, using PIC A in the mobile phase at 1 ml/min flow rate with a 10 um, 25-cm C-18 reversed phase column and 200 ul injections.

Table 1. HPLC-HY-ICP and HPLC-ICP Minimum Detection Limits (MDLs) (ppm)

COMPOUND NAME	HPLC-HY-ICP[a]	HPLC-ICP[b]
Arsenite	0.050	48.0
Arsenate	0.050	24.0
Sodium Dimethyl Arsenate (SDMA)	0.105	199.0

a. HPLC-HY-ICP conditions used an Excalibar RP-18 column, 4% $NaBH_4$ solution and concentrated HCL. Injection volume = 200 ul.
b. HPLC-ICP conditions used an Excalibar RP-18 column and injection volumes of 20 ul.

Table 2. Analysis of Spiked Distilled Water Samples for Arsenate and Arsenite (ppb)

SAMPLE NO.	HPLC-HY-ICP[a]			HY-ICP	ACTUAL LEVELS			
	ARSENITE	ARSENATE	TOTAL	TOTAL	ARSENITE	ARSENATE	TOTAL	% ERROR[b]
1	52 ± 14[c]	39 ± 13[c]	91	93 ± 1	52	56	118	-23
2	74 ± 8	63 ± 6	137	119 ± 1	62	65	127	+8
3	54 ± 3	----	54	68 ± 0	84	--	84	-36
4	112 ± 50	173 ± 51	285	260 ± 2	87	141	228	+25
5	101 ± 33	69 ± 9	169	198 ± 4	104	97	201	-15
6	104 ± 45	88 ± 27	192	209 ± 4	104	97	201	-4
7	166 ± 5	---	166	124 ± 1	140	--	140	+19
8	271 ± 15	203 ± 13	474	407 ± 2	208	195	403	+17
9	322 ± 32	282 ± 32	604	509 ± 11	261	243	504	+19
10	301 ± 34	---	301	280 ± 1	279	--	279	+9
11	404 ± 122	428 ± 49	832	977 ± 9	397	527	924	-11

a. HPLC-HY-ICP conditions used an Excalibar RP-18 column, hydride conditions of 4% $NaBH_4$ solution and concentrated HCl.

b. % Error refers to difference between total arsenic content as determined by HPLC-HY-ICP and actual total arsenic levels spiked.

c. Numbers represent the average ± standard deviation for at least three separate runs made on the same day. Low standard deviations for HPLC-HY-ICP may be due to the small number of analyses done at each sample level.

the absence of hydride generation, only about 1% of the total HPLC eluent ever enters the ICP plume region, due to the current use of the cross flow nebulizer. With a 100% hydride formation occurring, presumably all of this should enter the plasma/plume region once it enters the cross flow nebulizer. This is due almost entirely to the greater volatility and gaseous nature of the arsine as opposed to arsenate or arsenite anions in solution. Our observation of a 1,000 fold improvement (lowering) of MDLs, Table 1, is due to the fact that we are here comparing a 20 ul injection by HPLC-ICP versus a 200 ul injection(s) by HPLC-HY-ICP. This therefore adds another order of magnitude to lowering of the final HPLC-HY-ICP MDL for almost all three species. Thus, MDLs with a mass sensitive detector such as the ICP as an HPLC-ICP system, could be routinely lowered just by going to larger injection volumes than the normally employed 20 ul or 25 ul.

The precision of the HPLC-HY-ICP method at the MDL was studied by injecting a standard mixture of arsenite and arsenate at the 50 ppb levels in each species. This solution was injected at least ten times (n=10), and each sample injection was preceded by a blank injection prepared as for the sample. The percent relative standard deviation (%RSD) was 16% for arsenite and 23% for arsenate. This is expected for a level determined near the MDL, where the theoretical precision would be 33% (signal-to-noise = 3:1).

Our observation in Table 1 that we are realizing at least a two fold lowering of the MDLs in going to HPLC-HY-ICP strongly suggests that we may have near 100% arsine conversion/formation efficiencies from arsenate and arsenite. However, we have not conclusively demonstrated this as yet. Complete conversion of the parent arsenic anions to their desired hydride form is not absolutely necessary, as long as the reaction extent occurs reproducibly.

Calibration plots for each of the three arsenic anions were linear over four orders of magnitude, ranging from the low part-per-billion (ppb) to mid-part-per-million (ppm) region. This includes the region of most interest for environmental samples. The correlation coefficients for these calibration plots ranged from 0.988 to 0.999.

Spiked Distilled Water Analysis

A series of eleven (11) spiked distilled water samples, at known concentration levels of both arsenite and arsenate, have been studied now by HPLC-HY-ICP, Table 2. These results indicate a general agreement between the levels of arsenic species spiked and the values determined. A linear regression analysis of these results and the actual spiked values gave correlation coefficients of 0.979 and 0.967 for arsenite and arsenate respectively. The total arsenic level in each of these samples were also determined by

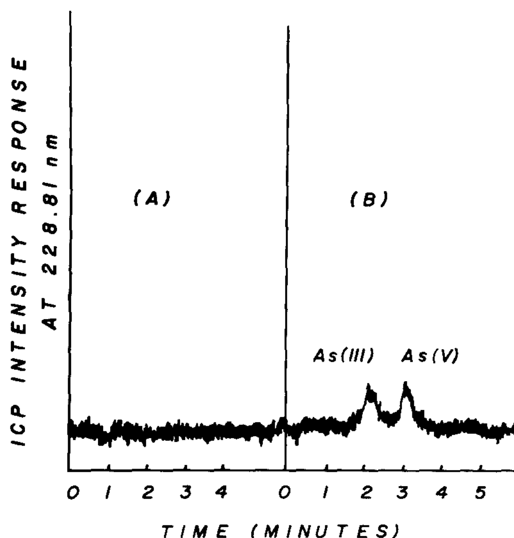

Figure 3. HPLC-HY-ICP chromatograms for (A) a 200 ul blank injection of distilled water; and (B) a 200 ul injection of a spiked distilled water sample prepared at the 40 ppb to 60 ppb levels in both arsenate and arsenite.

Direct-HY-ICP, Table 2. In general, there is good agreement between these values and the actual total arsenic content. As one might expect, the % error tends to get worse at lower and lower concentration levels, especially as these approach the known, already demonstrated MDLs. Also, the accuracy of the individual arsenic determinations falls off as the spiked levels approach MDLs.

Figure 3 illustrates a typical HPLC-HY-ICP chromatogram of a spiked distilled water sample, with the concentration levels as indicated. Even at this level of 40 ppb to 60 ppb in each of the two arsenic anions, each HPLC peak is clearly discernible and substantially above the background noise levels. In going from a 25-cm long column to a 15-cm one, Figures 2 and 3, the total analysis time has now been cut about 50%.

Application to Environmental Well Water Sample Analysis

This HPLC-HY-ICP technique has now been applied to actual well water samples from the state of New Hampshire, wherein initial analyses for total arsenic content had demonstrated surprisingly high levels, at times 200 ppb to 250 ppb or thereabouts. Our methods of sample preservation have been indicated above (Experimental), but no chemical method was worked out by us

in order to effectively preserve arsenic species content. Other workers have demonstrated that ascorbic acid added to water samples can prevent the interconversion of arsenite to arsenate on standing (22). Our own interests have been in determining how much of species interconversion does occur in these particular well water samples on standing in a refrigerator, without the initial addition of any chemical preservatives. Table 3 indeed summarizes these results, wherein we have analyzed two such well samples as a function of time after their removal from the well itself. There is a relatively small decrease in the arsenite content and a corresponding increase in the arsenate levels when we compare Day 1 with Days 2 and 4. Such results suggest that analysis of these particular samples anywhere from Days 1 to 4 would probably provide accurate results for the levels of arsenic species originally present in the well itself. It is indeed possible that sample integrity, based on the relative changes in arsenite/arsenate from Days 1 to 4, could be retained for even one week after the samples were taken. However, by Day 19, which was the next point of analysis in this study, all of the initial arsenite had been converted to arsenate. Clearly, in order for this analysis to be absolutely representative of the arsenic species originally present in the well itself, samples should be analyzed immediately after collection in order to preserve sample integrity. Future utilization of HPLC-HY-ICP or alternative approaches for environmental water speciation should/must take sample preservation and sample integrity into consideration (22).

In order to determine species differences from one well to another, we have applied these techniques to another six well water samples, Table 4. As indicated in the last column on the right of Table 4, these samples were analyzed from six days to four months after they were collected, and stored as already indicated. Thus, for the first three samples, numbers 3-5, in all probability there has been complete conversion of any originally present arsenite to arsenate. This would clearly explain why these three samples only show the presence, at the time of their analysis, of arsenate. These results therefore do not necessarily represent what was originally present in the well water at the time of collection or in the well itself. However, the final values indicated for these particular samples are indicative of what was present at the time of analysis. On the other hand, for samples 6-8, these were analyzed within one week after the time of collection, and on the basis of our somewhat limited stability studies, Table 3, the speciation indicated here is probably indicative of what was originally present in these wells at the time of sample collection. The results by HPLC-HY-ICP and Direct-HY-ICP are consistent with regard to total arsenic present for a given sample on that day of analysis. Sample number 6 contains both arsenate and arsenite,

Table 3. Determination of Arsenite and Arsenate Stability in Well Water Samples

SAMPLE NO.	As SPECIES[a]	DAY 1[b]	DAY 2[b]	Day 4[b]	Day 19[b]
1	ARSENITE	188 ± 18	126 ± 10	143 ± 5	0 ppb
	ARSENATE	56 ± 4	89 ± 23	76 ± 0	232 ± 40 ppb
	TOTAL	244 ± 22	216 ± 33	219 ± 5	232 ± 40 ppb
2	ARSENITE	183 ± 14	140 ± 13	156 ± 0	0 ppb
	ARSENATE	53 ± 14	75 ± 20	69 ± 4	216 ± 16 ppb
	TOTAL	236 ± 28	215 ± 33	225 ± 4	216 ± 16 ppb

a. HPLC-HY-ICP conditions used an Alltech HP Guard Column, 3% $NaBH_4$ and concentrated HCl for hydride generation.
b. All numbers represent the average ± standard deviation for at least three (3) separate analyses performed on the same working day.

Table 4. HPLC-HY-ICP and Direct-HY-ICP Analyses of Well Water Samples (ppb)

SAMPLE NO.	HPLC-HY-ICP[a, b]		TOTAL As	DIRECT-HY-ICP	DAYS OF
	ARSENATE	ARSENITE		TOTAL As	STORAGE
3	182 ± 4	ND[c]	182	212 ± 4	99
4	196 ± 12	ND	196	226 ± 1	86
5	235 ± 24	ND	235	243 ± 1	86
6	78 ± 14	99 ± 7	177	193 ± 1	7
7	ND	126 ± 3	126	153 ± 0	6
8	ND	ND	ND	7 ± 3	6

a. HPLC-HY-ICP conditions used an Excalibar or 15-cm in-house slurry packed RP-18 column, other conditions as indicated in text.
b. Numbers represent the average ± standard deviations (ppb) for at least three separate analyses performed on the same working day.
c. ND indicates that no arsenite or arsenate could be detected in the sample at or above the MDL of 50 ppb.

Table 5. External Standard Versus Standard Additions Method of Analysis

SAMPLE COMPONENT	EXTERNAL STANDARD METHOD	STANDARD ADDN. METHOD
ARSENITE	99 ± 7	103
ARSENATE	78 ± 14	81

almost equally distributed, sample 7 contains only arsenite, clearly this has not converted yet to arsenate, and sample 8 contains no detectible arsenate or arsenite at these limits of detection.

The standard additions method was performed on two of the samples of Table 4, and Table 5 reports these results for sample number 6 above. Indicated here are the results of the direct analysis by the external standard method and the analogous results by the standard additions method. These overall results for the two different approaches on the same sample are identical within experimental error. A similar study with another well water sample of Table 4 provided results which also were identical within exptl. error. Of interest in Table 4 is the fact that samples 7 and 8 were drawn from different, but nearby wells, and these contain extremely different levels of arsenic species and total arsenic. This speciation approach could therefore have utility for tracing the path or source of underground arsenic contamination of drinking water supplies.

CONCLUSIONS

We have developed and optimized new HPLC-HY-ICP approaches which can now provide a rapid and direct method of speciating well water supplies for total arsenic levels and individual arsenic species levels. Such approaches therefore provide an approach to determine variations of arsenic species between wells or other water supplies. The methods are totally usable down to the 50 ppb level for arsenate and arsenite, which is apparently at or below the demonstrated level of these arsenic species in those wells already studied here. It is hoped that these newer approaches will now find widespread acceptance and utilization by others interested in determining arsenic species levels in a variety of environmental, biological, industrial, and toxicological samples.

ACKNOWLEDGEMENTS

We wish to acknowledge the assistance of S. Colgan and R. Shansky at NU in their preparation of various spiked water samples. C. Baxter and C. Nelson of the N.H. Water Supply Division were helpful in providing us with various well water samples. This work was supported by a research and development contract from the Analytical Instrument Division of Instrumentation Laboratory, Inc. to NU, which has allowed us to undertake and complete the work described. Additional support was provided, in part, by a grant from the NIH Biomedical Sciences Research Support Grant No. RR07143, Department of Health and Human Services, to NU. We are very grateful for these sources of financial assistance. This is contribution number 153 from The Barnett Institute of Chemical Analysis and Materials Science at Northeastern Univ.

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26. Abbreviations used: HPLC = high performance liquid chromatography; HY-ICP = hydride generation-inductively coupled plasma emission detection; HY = hydride generation; ICP = inductively coupled plasma emission detection; PHD = plasma hydride device; IL = Instrumentation Laboratory, Inc.,; ppb = parts-per-billion (ng/g, ng/ml); ppm = parts-per-million (ug/g, ug/ml); SDMA = sodium dimethylarsenate; cm = centimeter; mm = millimeter; MDLs = minimum detection limits; RP = reversed phase; GFAA = graphite furnace atomic absorption; DCP = direct current plasma; FAA = flame atomic absorption; UV = ultraviolet; LCEC = liquid chromatography-electrochemical detection; $NaBH_4$ = sodium borohydride; HCl = hydrochloric acid; NaOH = sodium hydroxide; HP = high performance; %RSD = percent relative standard deviation;

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 877-882 (1984)

ENTHALPY-ENTROPY COMPENSATION OF OCTYLSILICA STATIONARY PHASE IN REVERSED-PHASE HPLC

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ABSTRACT

Enthalpy-entropy compensation has been investigated in reversed-phase high performance liquid chromatography with octylsilica stationary phase. The compensation temperatures were determined for this system, and the results show that their change with the composition of the mobile phase was almost similar to that with octadecylsilica stationary phase. It is concluded that the retention mechanism of the separation of alkylbenzenes in both systems with the mobile phase exceed 20 % water content is the same.

INTRODUCTION

The selection of satisfactory separation conditions still remains a major problem in high performance liquid chromatography (HPLC). In recent years, the role of column temeprature in HPLC has been recognized (1-11) and we have reported improved separation with a relatively low temperature column in micro-HPLC and suggested the importance of low

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0148-3919/84/0705-0877$3.50/0

temperature influence for the separation of thermally labile compounds (8-12).

In these works, we took an enthalpy-entropy compensation approach (4, 11-14). Linear enthalpy-entropy compensation in liquid chromatography has been investigated and some results suggest that an enthalpy-entropy compensation can demonstrate the identity of the retention mechanism underlying reversed-phase separation systems. Since compensation behavior is generally attributed to the effect of the solvent on the interacting species, comparison of the compensation temperature obtained with appropriate data can serve as a diagnostic tool for variations in the retention mechanism on changing the conditions such as the mobile phase composition and column temperature. In the previous work (12) we have demonstrated that enthalpy-entropy compensation occurs in the low temperature reversed-phase micro-HPLC with octadecylsilica stationary phase. This short contribution describes the application of the enthalpy-entropy compensation approach to investigate the reversed-phase system with octylsilica stationary phase and to evaluate the similarity of the retention mechanism in both systems.

EXPERIMENTAL

A model MF-2 microfeeder (Azuma Electric, Co.Ltd., Tokyo, Japan) was used as the pump. The detector was a Jasco Uvidec 100-III UV spectrophotometer set at 207 nm. The column used was prepared by packing a 0.5 mm i.d. x 12.6 cm long PTFE tubing with octylsilica bonded material (FineSIL, C-8, 10 um, Jasco) by the slurry technique.

The temperature of the column was controled by a Komatsu DW-620 thermostat (Tokyo,Japan). The temperature range examined was from - 1°C to 50°C.

The eluents used were as follows; acetonitrile/water: water content 0.1 %, 10 %, 20 %, 30 %. A HPLC grade acetonitrile was purchased from Kanto Chemicals (Tokyo, Japan). Water was purified and distilled. The test substances (alkylbenzenes) were obtained from Tokyo Kasei (Tokyo, Japan).

Prior to measurements the column was washed with the mobile phase until a constant value for the retention volume of the test substances was obtained. The test substances were injected into the column as 1000 ppm solution in each mobile phase. For t_0 measurements, sodium nitrite of 100 ppm (15, 16) water solution was used. The capacity factor, k', was calculated by the normal way such as $k'=(t_R-t_0)/t_0$ in which t_R is the retention time of a solute. All measurements were made in triplicate. The average reproducibility was better than 0.3 %.

RESULTS AND DISCUSSION

In the enthalpy-entropy compensation as described by Melander et. al.(4), a plot of ln k' against 1/T (van't Hoff plot) yields a straight line if the mechanism of the process is the same over the temperature range investigated and the enthalpy is constant.

$$\ln k' = -\Delta H^{o}/RT + \Delta S^{o}/R + \ln\phi \qquad (1)$$

where ϕ is the phase ratio of the column.

The plots obtained for alkylbenzenes are indeed linear and the slopes are positive; hence, for these substances in the system with octylsilica the enthalpy is constant and negative over a temperature range investigated. The regression coefficients are listed in Table-1.

To calculate the compensation temperature β , following equation was used;

$$\ln k'_T = -\Delta H^{o}(1/T - 1/\beta)/R - \Delta G^{o}/R + \ln\phi \qquad (2)$$

Table-1 Data of the van't Hoff plots for alkylbenzenes.

water content in mobile phase	ΔH^{o}, kcal/mole ethyl-benzene	n-butyl-benzene	n-hexyl-benzene	n-octyl benzene
0.1 %	1.504 (0.927)	1.417 (0.971)	1.596 (0.956)	1.638 (0.961)
10 %	0.656 (0.960)	0.836 (0.966)	0.893 (0.991)	1.394 (0.981)
20 %	1.021 (0.983)	0.983 (0.942)	1.286 (0.999)	1.502 (0.974)
30 %	0.676 (0.961)	0.845 (0.983)	1.253 (0.989)	1.411 (0.977)

The number in a parenthesis shows the regression coefficient for each instance.

where k'_T is the capacity factor at temperature T. The temperature T should be near the harmonic mean of the experimental temperatures used for the evaluation of the enthalpies and 293 K is used in this investigation.

After the data have been replotted according to the equation-(2), it is confirmed that the enthalpy-entropy compensation occurs in all the instances investigated. The calculated compensation temperatures are summarized and compared with those with octadecylsilica stationary (12) phase in Table-2.

Identity or close similarity of the compensation temperatures would strongly suggest that the retention mechanism is the same under the conditions examined.

The results shown in Table-2 are almost similar to those with octadecylsilica stationary phase system, and provide the following information items;

1) the compensation temperature is dependent on the water contents of the mobile phase.

Table-2 Compensation temperatures.

water content in mobile phase	compensation temperature, β , K	
	octylsilica	octadecylsilica
0.1 %	360(293)	381(273)
10 %	464(293)	517(278)
20 %	586(292)	639(278)
24 %	-	682(293)
30 %	721(293)	-
ref.4	639(-)*	
ref.13	794(-)**	

The number in a parenthesis is k'_T for each instance.

* methanol/water system with Pharmaphase ODS for substituted benzene derivatives.

** methanol/water system with octylsilica and octadecylsilica for heptylbenzoates homologues.

2) the compensation temperature increases with increasing water contents of the mobile phase.

3) the compensation temperatures are almost similar to those in normal phase system (385 K in n-hexane-silica system (4) and 480 K in FC-78-silica system (11)) at the water content lower than 10 % in mobile phase.

4) the compensation temperature of 586 K and 721 K for water content of 20 % and 30 % in the aqueous mobile phases, respectively, are consistent with the values of 639 K and 794 K described for other reversed-phase systems.

From above four information items, it is concluded that the retention mechanism with octylsilica-aqueous mobile phase system is almost the same to that with octadecylsilica-aqueous mobile phase system. This has been confirmed by the different experimental ways in elsewhere (17).

The reversed-phase mechanism (e.g., solvophobic theory or sorption theory) contributes to the retention of alkylbenzenes in both systems of octyl- and octadecylsilicas stationary phases where the water content of the aqueous mobile phase exceed 20 %, otherwise very similar to the mechanism in the normal phase systems at lower water content in the mobile phase, although the chromatographic data shown here does not prove whether the interactions are directly with the reversed-phase (solvophobic theory) or with solvent molecules absorbed on the surface of the reversed-phase (sorption theory).

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 883-891 (1984)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATIONS USING COLUMNS PACKED WITH SPHERICAL ODS PARTICLES - III. EFFECT OF COLUMN DIMENSIONS ON THE RESOLUTION OF A COMPLEX MIXTURE*

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ABSTRACT

Separations on short columns, (3 and 5 cm, packed with 3 μ ODS spherical materials) and somewhat larger ones (10 cm and 20 cm columns having 2.1 mm and 4.6 mm diameters packed with 5 μ ODS spherical materials) were compared using Aroclor 1254. With simple mixtures, the results showed that short columns can give separations comparable with those on longer columns when the percentage of the organic modifier in the mobile phase is adjusted. This was not so with more complex mixture. The results also showed that columns which have a comparable volume do not produce comparable separation. The longer column, 200 mm x 2.1 mm gave better resolution than the shorter 50 mm x 4 mm column. Also a shorter column, (100 mm x 4.6 mm), which had double the volume of a longer column. (200 mm x 2.1 mm), gave better resolution of the Aroclor 1254 test solution.

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*Presented in part at the Eastern Analytical Symposium, New York, NY, November, 1983.

INTRODUCTION

In a previous study (1) the separation of a mixture was compared on 5 cm and 10 cm columns packed with 3 μ and 5 μ spherisorb ODS spherical particles using standard high performance liquid chromatography (HPLC) equipment without modification. The results indicated that separations on the 10 cm column were not significantly better than those on 5 cm columns packed with supports of the same size and physical properties.

In a later study (2) the instrument was modified to accommodate the requirements of 3 cm, 5 cm, and 10 cm columns packed with 3 μ ODS spherical particles. The results showed that the 10 cm column gave much better resolution than the 3 cm and 5 cm columns under the same experimental conditions, i.e., mobile phase compositions and flow rate. The results also show that the 3 cm and 5 cm columns can give resolutions of simple mixtures, comparable with those on the 10 cm column if the composition of the mobile phase is adjusted to meet the requirement of longer solute residence time in the shorter column.

In this study, the separation of a complex mixture, Aroclor 1254, was compared on short 30 mm x 4 mm and 50 mm x 4 mm packed with 3 μ ODS particles, and 200 mm x 2.1 mm, 200 mm x 4.6 mm, 100 mm x 2.1 mm, and 100 mm x 4.6 mm packed with 5 μ spherical ODS material. The best resolution of the mixture was obtained when the 200 mm x 4.6 mm column was used. The effect of column length, diameter and volume is also discussed.

EXPERIMENTAL

Materials: Aroclor 1254 was received from Dr. Anderson, NCI-Frederick Cancer Research Facility. A solution of 4.9μg/μl Aroclor 1254 in acetonitrile was used. Acetonitrile (ACN) was glass distilled (Burdick and Jackson). Distilled deionized water was used.

Apparatus: A liquid chromatograph model 1090 equipped with a variable wave length detector, an oven, variable volume automatic injector and personal computer model HP-85 was used: results were printed on a HP-3390A reporting integrator. All these instruments were manufacctured by Hewlett-Packard. Both columns used (30 mm x 4 mm and 50 mm x 4 mm) were packed with 3 μ Spherisorb ODS packings obtained from Phase Separations, Inc. (see reference 1 for packing physical properties, and column packing procedure).

The other four columns (100 mm x 2.1 mm, 100 mm x 4.6 mm, 200 mm x 2.1 mm and 200 mm x 4.6 mm) were prepacked with 5 μ spherical C_{18} particles (Hewlett-Packard).

The experiments were run at 40°C, unless otherwise specified, using a mobile phase of ACN/H_2O.

RESULTS AND DISCUSSION

High speed liquid chromatography (HSLC) is becoming increasingly popular. Generally in HSLC short columns (30-60 mm) with a diameter of 4-7 mm are used. In a previous study, (2) we have shown that when a simple mixture is used the 30 mm x 4 mm gives results similar to those obtained on 100 mm x 4 mm packed with 3 μ ODS material, if the organic modifier in the mobile phase is adjusted, lowering the percentage of the organic modifier for the shorter column to increase the solute residence time. The present study shows that, although this will hold for simple mixtures, it may not be the case when complex mixtures are being separated on shorter columns. Note that the longer columns are packed with different material than the short columns (3 cm and 5 cm), and this may affect the results. The shorter columns are packed with 3 μ ODS materials which is, theoretically, more efficient.

Figure 1 shows the separation of Aroclor 1254, using the 3 cm and 5 cm columns. The 5 cm column gave better separation than the 3 cm

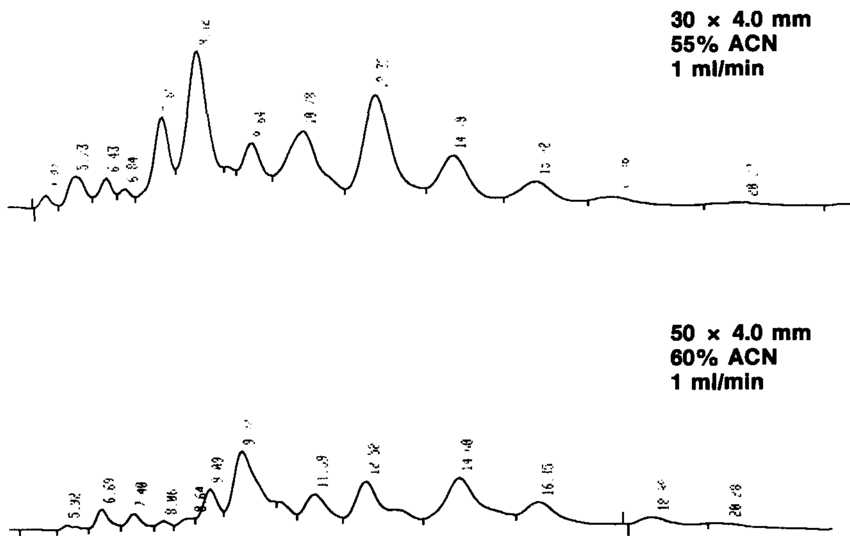

Figure 1. Separation of Aroclor 1254 on 30 mm x 4.0 mm and 50 mm x 4.0 mm columns packed with 3 μ spherisorb ODS material using a mobile phase of 55% and 60% ACN/H_2O respectively at a flow rate of 1 ml/min. Detection was carried out at 230 nm. Sample solution injected was 0.2 ul.

column although the mobile phase was adjusted to produce comparable residence time in the shorter column.

Figure 2 shows that the separation of Aroclor 1254 was improved when the 100 mm x 4.6 mm column was used rather tnan the 100 mm x 2.1 mm column. Note that the narrower column gave better sensitivity.

Figure 3 compares the separation of Aroclor 1254 using a 200 mm x 2.1 mm and 200 mm x 4.6 mm columns using 70% ACN/H_2O. The results show, as in figure 2, that, although both columns are of the same length, the wider column gave better separation. A comparison of the separation of Aroclor 1254 on the six columns tested clearly indicats the superiority of the

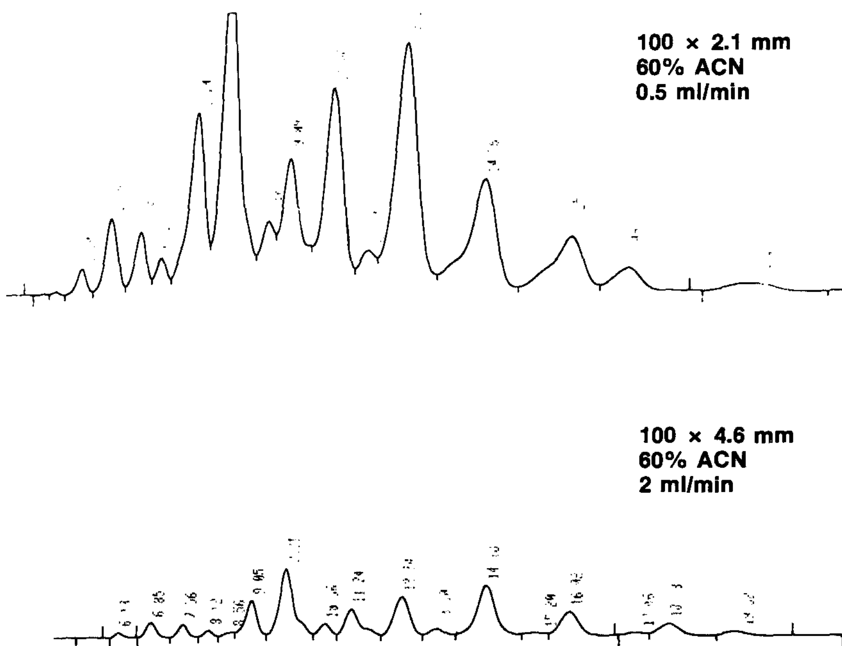

Figure 2. Separation of Aroclor 1254 on 100 mm x 2.1 mm and 100 mm x 4.6 mm columns packed with 5 μ spherical ODS material using a mobile phase of 60% ACN/H_2O at a flow rate of 0.5 ml/min and 2 ml/min respectively. Other conditions as in figure 1.

200 mm x 4.6 mm column over the others under the same conditions. However, when the organic modifier was adjusted (Figure 4), both 200 mm columns gave comparable results.

To find out the effect of column volume on separation, the 200 mm x 2.1 mm and 100 mm x 4.6 mm, which has double the volume, were packed with the same material and compared under the same mobile phase composition and flow rate (Figure 5a and 5b). The results show that the elution times were longer using the 100 mm x 4.6 mm column. As a result, the resolution

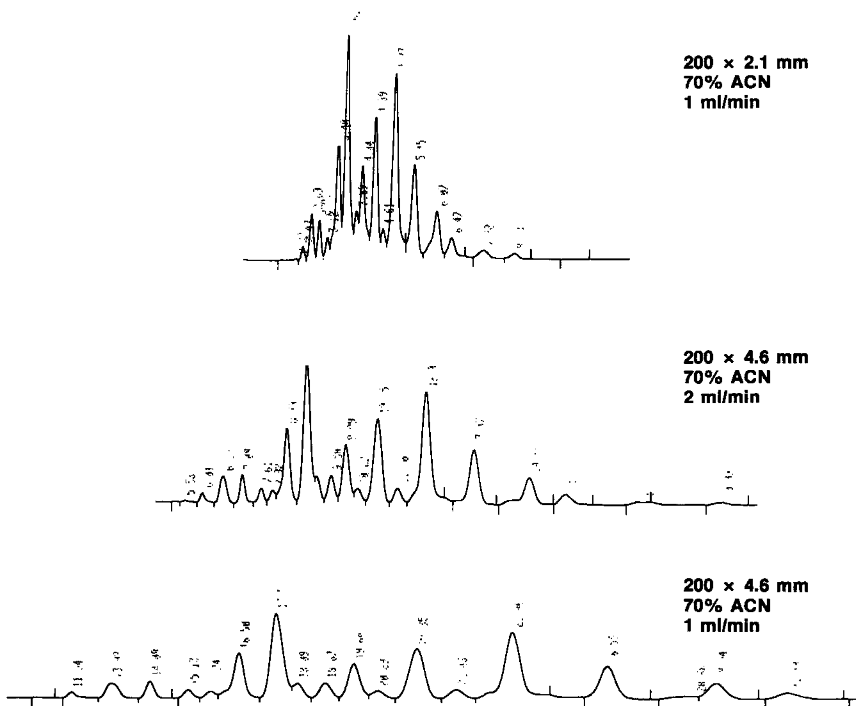

Figure 3. Same as in figure 2 except 200 mm x 2.1 mm and 200 mm x 4.6 mm columns using 70% ACN/H_2O.

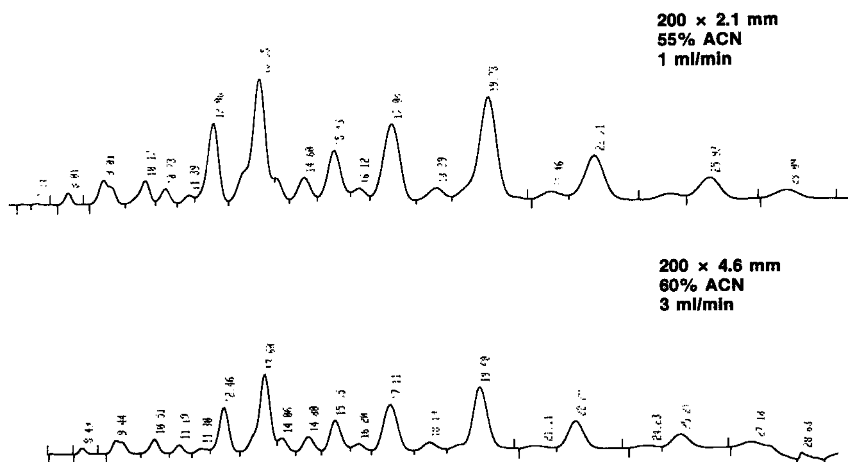

Figure 4. Same as in figure 3, but different percentage of organic modifier and flow rate.

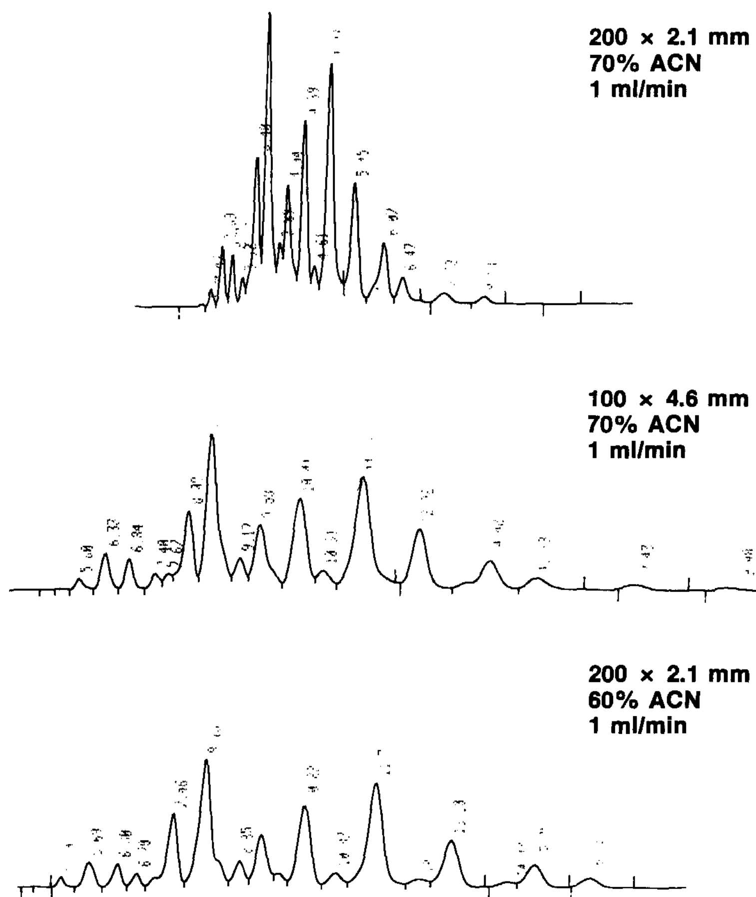

Figure 5. Comparison of the separation of Aroclor 1254 on 50 mm x 4.0 mm column packed with 3 μ spherisorb ODS material, 100 mm x 4.6 mm and 200 mm x 2.1 mm packed with spherical ODS material; (a) top, (b) middle, (c) bottom.

improved but the sensitivity did not. When the mobile phase was changed from 70% to 60% ACN for the 200 mm x 2.1 mm column (Figure 5c) the results were comparable with those obtained with the 100 mm x 4.6 mm and 70% ACN. Again, when the results obtained using the 50 mm x 4 mm column (Figure 1) are compared with those using the 200 mm x 2.1 mm column (Figure 5) both having comparable volume, the longer column gave better resolution of Aroclor 1254. It is clear, therefore, that not only is the column volume important, but also the length and the internal diameter. A detailed study of the relationship and effect of column volume, length and diameter on resolution is being conducted and the results will be published later.

CONCLUSION

The choice of column is governed by the type and size of sample (sensitivity) and its complexity. For small size samples HSLC will suffice, while for complex samples an efficient column will be needed, such as 200 x 4 mm, if the sample size and detection method permit.

Although short columns can be made to give comparable separations with those obtained on longer columns for simple mixtures, this was not the case when Aroclor 1254 was tested. Although the concentration of the modifier in the mobile phase was adjusted, there are limits to the level of the organic modifier in the mobile phase can be lowered in the attempt to achieve comparable results between short and long, wide and narrow columns packed with the same material. In the final analysis this is governed by (a) the solubility of solutes in the mobile phase; (b) instrument back pressure; and (c) sensitivity.

ACKNOWLEDGEMENT

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The author would like to thank Mark Shuman of Hewlett-Packard and John Klose of Chemical Synthesis and Analysis Laboratory, for technical assistance.

This work was supported by Contract NO. N01-CO-23910, with the National Cancer Institute, NIH, Bethesda, MD 20205.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 893-905 (1984)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMPHIBIAN PEPTIDES. SELECTIVITY CHANGES INDUCED BY pH.

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ABSTRACT

The effect of pH on the retention behavior under reversed- phase liquid chromatography conditions of a series of peptides was examined. Isocratic conditions were used with either methanol or acetonitrile as organic modifiers. The intrinsic hydrophobicity of the peptides was altered by changes in the pH of the eluent mixture. Increased retention at pH 7 relative to pH 4 was correlated with the presence of a histidine residue in a hydrophobic environment. An experimental parameter, α_{pH}, was defined as the positive quotient of capacity factors at pH 4 and pH 7 for a given eluent. These α_{pH} values are interpreted as reflecting changes in peptide hydrophobicity introduced by variations in solvent and pH. Identical α_{pH} values were obtained for homologous peptides, particularly histidine containing peptides. This approach to selectivity effects yielded diagnostic conditions for the analysis of bombesin, a peptide touted as a potential marker for human small-cell lung carcinoma.

INTRODUCTION

The combined use of reversed-phase HPLC and radioimmunoassay (RIA) has evolved as a powerful tool for the identification and quantitation

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of peptides in tissue extracts [1-3]. Problems inherent to radioimmunoassays such as low specificity or cross-reactivity with other structurally related compounds present in samples [4], are minimized by the prior separation of the peptides by reversed-phase HPLC [3]. The resolving power of this chromatographic method allows discrimination among peptide analogs based on minimal structural differences [4]. However, despite the impressive results obtained by the joint application of these techniques to the analysis of peptides from tissue samples the peptides are commonly defined as having "immuno-like reactivity" only because structural homology is defined by antigenic sites. Therefore, we investigated ways of enhancing and exploiting specific chemical characteristics of peptides and their interaction with the bonded phase during HPLC.

Our approach was based on the notion that a predominant factor in the separation of peptides on reversed-phase HPLC is the extent and magnitude of hydrophobic interactions between the bonded-phase material and the peptide molecule [5]. Quantitative expressions have been developed to establish this correlation [6-11]. Based on these assumptions, if one could modify the intrinsic hydrophobicity of a peptide in a predictable fashion, this altered hydrophobicity might be anticipated on theoretical and experimental bases to be reflected in the reversed-phase HPLC behavior of a peptide. This modified behavior could then be compared with the "immuno-like reactivity" found in tissue samples, providing an experimental parameter directly correlated to the parent peptide. The variables chosen for this study were pH, with emphasis on ionic changes induced in the imidazole ring of

histidine residues, and the organic solvent components of the mobile phase. The peptides selected for the initial studies belong to the bombesin family and selected tachykinins (physalaemin-related peptides) [12]. This choice was based on the increased attention given to the possible application of bombesin and physalaemin as a potential markers for human lung small-cell carcinoma [13-15], and the presence of immunoreactivities to these peptides in mammalian tissues [16].

MATERIALS AND METHODS

The peptides physalaemin, physalaemin, kassinin, eledoisin, litorin, and ranatensin were obtained from Penisula Laboratories, San Carlos, CA; bombesin was purchased from Bachem Inc., Torrance, CA. Peptide solutions for HPLC were prepared in 30% methanol/water at a concentration of 1 mg/ml. From these stock solutions, the peptide was diluted in the isocratic solvent and injections of 6-10 μg of peptide material were made. The peptide solutions were made fresh at weekly intervals.

The buffer solutions used for HPLC elution were: a) 15 mM ammonium acetate (ca. 1g/liter) brought to pH 4 with glacial acetic acid; and b) 10 mM tris-(hydroxymethyl)aminomethane (Tris-base) buffered to pH 7 with concentrated phosphoric acid. Methanol (50% v/v) and acetonitrile (30% v/v) were used as organic modifiers. The HPLC eluent consisted of a premixed solution of buffer and organic solvent; i.e., a single pump isocratic elution.

The instrumentation used consisted of a M6000A pump, 440 UV absorbance detector (280 nm), U6K injector, 720 system controller, and a

730 data module, all from Waters Associates. The column used was a Whatman Partisil-5 ODS (4.6 mm ID x 25 cm) equipped with a Brownlee 5 micron Spherisorb RP-18 precolumn. The flow rate was 1 ml/min. The eluted peaks of bombesin and physalaemin were further identified by RIA analyses described elsewhere [14,17] to ensure that the UV absorbance trace indeed correlated with the peptide in question.

RESULTS AND DISCUSSION

The rationale behind this study was the premise that hydrophobic binding interactions play a major role in the separation of peptides by reversed-phase HPLC [5]. Supporting evidence for this argument is found in work coming from different laboratories [6-11]. This hydrophobic effect has been quantitatively expressed and determined to be an additive property reflecting the cumulative hydrophobicity of the amino acid residues present in the peptide. A correlation was found between the sum of hydrophobic constant values and the elution order of peptides which was qualified as having a predictive value for peptides ranging from 5-20 residues [6-11].

Another important aspect, not as fully explored, is the accesibility and extent of the peptide surface available for hydrophobic binding [20]. It was this particular feature, namely the modification of the accessible or effective surface on the peptide, that we felt could be specifically exploited in the case of bombesin. By operating at acid (pH 4) and neutral (pH 7) conditions the ionic character of carboxyl and imidazole groups would be effected: lysine and arginine residues would remain unchanged at both pH valves; the carboxyl group

would be ionized at pH 7 and the negatively charged residues would show little affinity for the HPLC column, whereas the imidazole group (histidine) would be neutralized at pH 7 (pKa 5.5-7) with an anticipated higher retention (Fig. 1).

In the case of bombesin, the histidine residue at position 12 may be manipulated to produce a disrupted peptide surface by protonation (pH 4) or an extended hydrophobic area by neutralization (pH 7). In order to establish that changes in elution were correlated to the presence of histidine residues, a series of histidine (H) and non-histidine (NH) containing peptides was examined (Table 1). The solvent strength was maintained constant so as to isolate the effect of pH. Isocratic conditions also prevented fluctuations in pH due to varying amounts of organic solvent in the mobile phase [21].

The first set of experiments using methanol as organic modifier is illustrated in Fig. 2. Litorin and bombesin (H-peptides) and physalaemin (NH), were analyzed isocratically (50% methanol) at pH 4 and pH 7. It is evident from Fig. 2 that, in accordance with the expectations, bombesin experienced a dramatic shift in retention. Results for all peptides examined are shown in Table 2. The $\Delta k'$ is the difference in k' values at pH 7 and pH 4. For the NH-peptides, physalaemin and eledosin showed no k' variation while for kassinin there is a small, but significant decrease in k' at pH 7. For the H-peptides a more consistent pattern developed: litorin and ranatensin experienced substantial increases in k' at pH 7, and for bombesin this effect was magnified with a dramatic shift in k' ($\Delta k' = +30.7$).

In order to establish the role of organic solvent on this pH induced selectivity effect, we also examined acetonitrile as a

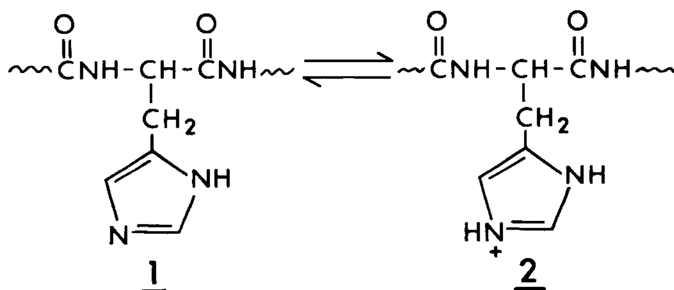

Figure 1. Ionic equilibrium for a histidine residue. The unionized imidazole (1) is anticipated to bind more effectively to a RP-HPLC column.

TABLE 1. Peptides Used In This Study

Peptide	Sequence
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Kassinin	Asp-Val-Pro-Lys-Ser-Asp-Glu-Phe-Val-Gly-Leu-Met-NH_2
Litorin	pGlu-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
Ranatensin	pGlu-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
Bombesin	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

modifier. The results obtained with acetonitrile (30% v/v) are illustrated in Fig. 3. From the peptides examined only bombesin showed a significant increase in k' as shown in Table 3. The NH-peptides showed a modest decline in k' at pH 7. The H-peptides, litorin and ranatensin were unaffected by pH whereas bombesin showed a substantial increase in k' at pH 7.

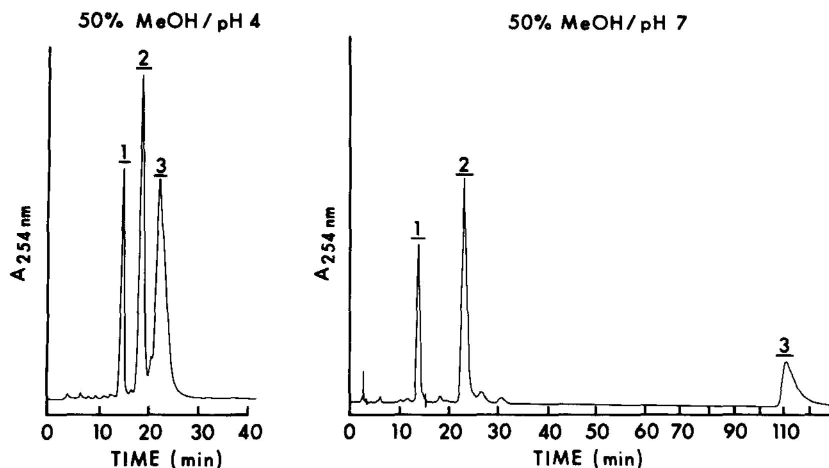

Figure 2. RP-HPLC of Physalaemin (1), Litorin (2) and Bombesin (3) using 50% methanol in 0.015 M ammonium acetate, pH 4 (left panel) and in 0.01 M Tris-phosphate, pH 7 (right panel); 2 and 3 are histidine-containing peptides.

Table 2. Reversed-phase HPLC capacity factors (k') for bombesin and related peptides. Isocratic elution with 50% (v/v) methanol.

Peptides	pH 4	pH 7	Δk' at pH 7 relative to pH 4
Non-histidine (NH)			
Physalaemin	3.6 (0.22)[a]	3.4 (0.23)	-0.2
Eledoisin	0.2 (0.21)	2.4 (0.22)	+0.5
Kassinin	3.6 (0.21)	2.4 (0.22)	-1.2
Histidine (H)			
Litorin	4.6 (0.23)	6.6 (0.22)	+2.0
Ranatensin	15.1 (0.40)	21.1 (0.34)	+6.0
Bombesin	5.1 (0.15)	35.8 (0.26)	+30.7

[a]Mean ± S.D. in parenthesis; n=6 except for bombesin where n=3.

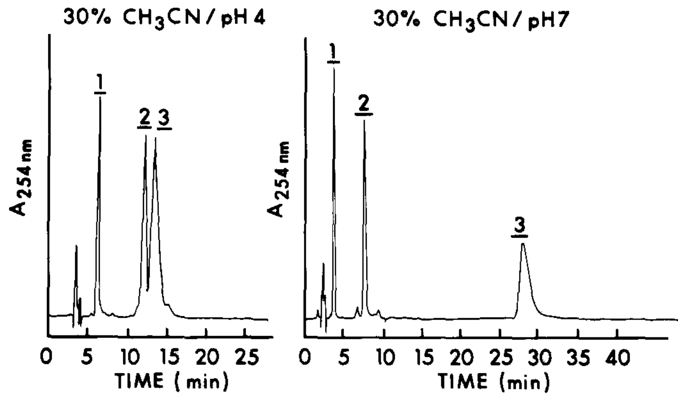

Figure 3. RP-HPLC of Physalaemin (1), Litorin (2) and Bombesin (3) using 30% acetonitrile in 0.015 M ammonium acetate, pH 4 (left panel) and in 0.01 M Tris-phosphate, pH 7 (right panel); 2 and 3 are histidine-containing peptides.

Table 3. Reversed-phase HPLC capacity factors (k') for bombesin and related peptides. Isocratic elution with 30% (v/v) acetonitrile.

Peptides	pH 4	pH 7	Δk' at pH 7 relative to pH 4
Non-histidine (NH)			
Physalaemin	0.9 (0.11)[a]	0.7 (0.06)	-0.2
Eledoisin	1.8 (0.04)	1.1 (0.06)	-0.7
Kassinin	0.6 (0.00)	0.4 (0.04)	-0.2
Histidine (H)			
Litorin	2.4 (0.08)	2.4 (0.06)	0
Ranatensin	5.1 (0.06)	4.9 (0.06)	-0.2
Bombesin	3.1 (0.04)	11.0 (0.00)	+7.9

[a]Mean ± S.D. in parenthesis; n=3.

The results obtained are in agreement with the basic postulate that protonation of histidine residues results in disruption of the hydrophobic surface of the peptide with a concomitant effect on k' values. Litorin, ranatensin, and bombesin illustrate this effect (Tables 2 and 3). A complementary solvent effect was uncovered with litorin and ranatensin in which no k' variation occurred in the presence of acetonitrile (Table 3). The magnitude of the shift observed for bombesin is larger than that anticipated from only the hydrophobic residues involved. The amino acid residues adjacent to the histidine group in litorin and bombesin are illustrated in Table 4. The extended surface generated at pH 7 would have a Phe in litorin versus a Leu in bombesin. Although Phe is more hydrophobic than Leu, this is apparently not reflected in a proportional increase in k' values. This observation, plus the lower magnitude of the effect of acetonitrile on bombesin relative to methanol, is suggestive that other factors are operating in bombesin. A conformational effect is an attractive possibility, hydrogen bonding between Arg^3 and His^{12} may occur at pH 7 and the resulting folded conformer exhibits retention characteristics different from those predicted by the hydrophobic theory. An example of stable conformers of a cyclic peptide separable by HPLC has been reported [5].

The effect of pH on the retention of these peptides, particularly H-peptides, may be more easily visualized by defining a new experimental parameter, α_{pH}, as the positive quotient of k' values at pH 7 and pH 4 for a given mobile phase. These values are tabulated in Table 5. The α_{pH} parameter is interpreted as reflecting changes in hydrophobi-

Table 4. Relative hydrophobicity of selected amino acid residues in the vicinity of histidine residue in litorin and bombesin.

Peptide	Carboxyl terminal sequence
Litorin	Gly-His-Phe-Met NH_2
Bombesin	Gly-His-Leu-Met NH_2
Relative hydrophobicity[a]:	Trp > Phe > Ile ≃ Leu > Tyr

[a]References [6-11]

Table 5. Selectivity expressed as a function of pH (α_{pH}).

	α_{pH} [a]	
Peptides	50% CH_3OH	30% CH_3CN
Non-histidine (NH)		
Physalaemin	1.06	1.29
Eledoisin	1.05	1.64
Kassinin	1.50	1.50
Histidine (H)		
Litorin	1.43	1.00
Ranatensin	1.40	1.04
Bombesin	7.02	3.55

[a]Ratio of k' values at the pH 4 and pH 7.

city introduced by variations in solvent and pH. For physalaemin and eledoisin, α_{pH} values with methanol indicated low sensitivity toward changes and these small selectivity differences were of equal magnitude in both cases; this parallelism was lost with acetonitrile where

eledoisin showed a more pronounced response, i.e., a larger α_{pH}, than physalaemin. Kassinin showed that selectivity was affected by pH (Table 2), but not by organic solvent as evidenced by the identical α_{pH} values. Litorin and ranatensin showed identical behavior under both solvent conditions; i.e. selectivity effects were greater with methanol (α_{pH} = 1.40), and negligible with acetonitrile (α_{pH} = 1.00). It should be emphasized that the magnitude of the shift is the same in both cases, a situation resembling that of the NH-peptides physalaemin and eledoisin (Table 5). Bombesin is particularly sensitive to pH variations, (large α_{pH} values) and also experiences a solvent effect evidenced by a lower α_{pH} with acetonitrile (Table 5).

The two major factors contributing to this selectivity effect are: a) pH, which changes the ionic nature of the peptide; and b) organic solvent, which modifies both the column bonded-phase and the eluate. Considering these factors, a possible scheme emerges which may help explain the differences observed: for physalaemin and eledoisin the ionizable groups are identical (Asp, Lys) but whose position in the peptide chain differ relative to each other (Table 1), whereas litorin and ranatensin only contain a single histidine residue at the same position in the carboxyl terminal region. In each of these pairs, the ionic changes introduced are identical: when the residue undergoing this change is in a homologous sequence (litorin and ranatensin) an identical α_{pH} value is observed. On the other hand, if these ionizable groups differ in their relative sequence (physalaemin and eledosin), this lack of structural continuity is detected by a solvent effect.

For the peptides examined, increased retention in reversed-phase HPLC at pH 7 relative to pH 4 correlates well with the presence of a

histidine residue in a hydrophobic environment. By using a solvent effect, this predictive shift becomes diagnostic for bombesin which satisfies the original objective of this study. As to why other H-peptides do not exhibit this quality when acetonitrile is used might be answered by examining the comformational behavior of bombesin as a function of pH.

In summary, the experimental parameter α_{pH} represents a potentially valuable observation for the identification and structural correlation of peptides by HPLC. Peptides may be distinguished by reversed-phase HPLC on the basis of their intrinsic chemical properties due to hydrophobic and ionizable residues (k' values), their hydrophobicity altered by changing pH (acid vs neutral) and affecting retention with organic modifiers (methanol and acetonitrile) at both pH limits to give α_{pH} values. By following this protocol substantial information regarding the degree of structural homology among a group of peptides may be obtained in a non-destructive manner using only minute amounts of sample as required for RIA. Further work along these lines is in progress.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 907-916 (1984)

SEPARATION OF HMG PROTEINS BY REVERSE-PHASE HPLC

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ABSTRACT

A high-pressure liquid chromatographic method is described for separating the high mobility group (HMG) proteins on a 5-μm Nucleosil C18 column with the use of the ion pairing agent trifluoroacetic acid (TFA) in the mobile phase. With a multistep acetonitrile-TFA gradient, the calf thymus HMG proteins elute from this column as separate peaks in the order HMG 17, 14, 2, and 1. Protein elution is monitored by measuring the absorbance at 214 nm in a 3-mm flow cell, and the identity and purity of the peaks are confirmed by polyacrylamide gel electrophoresis and amino acid analysis. Both HMG 1 and 2 elute as multiple peaks, suggesting the presence of major variants of these two proteins. Other peaks in the chromatogram include degradation products of HMG 1 and histone H1.

INTRODUCTION

One subset of nonhistone proteins that has been isolated and studied in considerable detail during the past several years is the high mobility group, or HMG, proteins. Unlike the histones, HMG proteins are deficient in basic amino acids

and rich in acidic amino acids and proline. They are extracted from chromatin in 0.35 M sodium chloride and remain soluble in 2% trichloroacetic acid (TCA).

Four proteins, HMG 1, 2, 14, and 17, have been identified by gel electrophoresis and column chromatography as the primary HMG components associated with chromatin in a variety of somatic tissues. Numerous recent reports (1-4) have demonstrated that two of these proteins, HMG 14 and 17, may be preferentially associated with active chromatin, and that they may somehow modulate changes in chromatin structure required for gene activation. Various other proteins and peptides that coextract with the HMGs, e.g., ubiquitin and proteolytic fragments of HMG 1 and H1 histone, have also been identified (5,6).

Existing methods for separating these proteins preparatively require a combination of ion exchange chromatography and selective TCA precipitation (6). While this approach is reliable, reproducible, and permits the isolation of large quantities of at least two of the HMG proteins (HMG 1 and 2), sample preparation and chromatography times are extensive. To separate all four HMGs, additional fractionation steps (such as selective TCA precipitation) must be performed on fractions that coelute with HMG or histone degradation products.

To circumvent these difficulties, we have devised a rapid (80-min) preparative method for separating the HMG proteins by reverse-phase high-pressure liquid chromatography (HPLC) on Nucleosil 100, a spherical octadecyl-silicic acid support, using the ion pairing agent trifluoroacetic acid (TFA) in the mobile phase. With this approach, each of the major HMG proteins and specific proteolytic fragments of H1 histone and HMG 1 are resolved into separate, well defined peaks. In addition, both HMG 1 and 2 resolve into at least two distinct

peaks. Amino acid analyses suggest that the HMG 1 species may represent sequence variants that differ in the number of their glutamic acid and alanine residues, while the two HMG 2 proteins are probably not sequence variants.

MATERIALS AND METHODS

HPLC-grade acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ). Lots were chosen that had a relative absorbance (vs water) at 200 nm (in a 1-cm cell) of 0.017 or lower. Trifluoroacetic acid was obtained from Eastman Kodak Co. (Rochester, NY).

Total HMG protein was extracted from calf thymus using the method of Bhullar and Candido (7). Approximately 10 mg was dissolved in Buffer A (aqueous 0.1% TFA), and centrifuged prior to injection to remove insoluble material. The proteins were separated on a 5-μm Nucleosil 100 column using a multistep acetonitrile gradient. The mobile phase was initiated at 20% Buffer B (40% acetonitrile, 0.1% TFA), and the Buffer B concentration was increased at a rate of 1.33%/min for 30 min to a final 60% Buffer B. After 30 min, the rate of Buffer B addition was then changed to 15%/min for 1 min, 0.43%/min for 35 min, and finally 10%/min for 1 min. Eluent emerging from the column was routed to the 214-nm detector, fractions collected, and the pooled samples lyophilized.

The separations were performed on an Altex Model 332 liquid chromatograph (Altex Scientific, Berkeley, CA), using two Model 110A pumps, a microprocessor-controlled solvent programmer (Model 410), and a Pharmacia UV-1/214 detector fitted with a 3-mm flow cell (Piscataway, NJ). Ten grams of Nucleosil 100 (5 μm) in methanol was slurry-packed into a 7.8 x 300 mm column fitted with 2-μm frits, by using a pneumatic pump (Jones Chromatography Co., Stoe, OH) at 6000 psi for 30 min. A

precolumn (4.5 x 250 mm, dry-packed with 37-53 μm precolumn silica, Whatman Chemical Co., Clifton, NJ) was installed before the injector, and a guard column (4.5 x 70 mm, dry-packed with 30 μm C18-derivatized silica particles, Serva Biochemical Co., Long Island, NY) was placed before the Nucleosil column.

After separation, the isolated HMG fractions were identified by electrophoresis (3 h, 130 V, 20 mA) in 10-cm acid-urea gels (8) containing 2.5 M urea. Amino acid analyses of each fraction were also obtained on a Beckman 120C amino acid analyzer following hydrolysis of the lyophilized proteins in 4 M methanesulfonic acid at 110 °C for 40 h.

RESULTS AND DISCUSSION

Eight major and several minor peaks were obtained following chromatography of total HMG protein on Nucleosil 100, using the multistep acetonitrile gradient shown in Fig. 1. The proteins in each peak were identified by collecting fractions across the middle of the peak (to limit contamination from adjacent peaks) and subjecting the isolated (lyophilized) proteins to electrophoresis in acid-urea gels and amino acid analysis.

HMG 17, identified by its electrophoretic mobility in gels and by its characteristic amino acid composition (Table 1), eluted as the first major peak (peak A) at approximately 55% Buffer B. HMG 14, a minor component in the HMG preparation used in these experiments, eluted as a small peak (peak B) 5 min later. The amino acid compositions of these two HMG peaks were essentially identical to those reported previously for HMG 14 and 17 (9).

Peak C, which eluted at 40 min, did not contain an identifiable HMG protein but contained numerous peptides that migrated throughout the length of the gel. This peak was substantially larger in older (6-month) HMG samples that showed

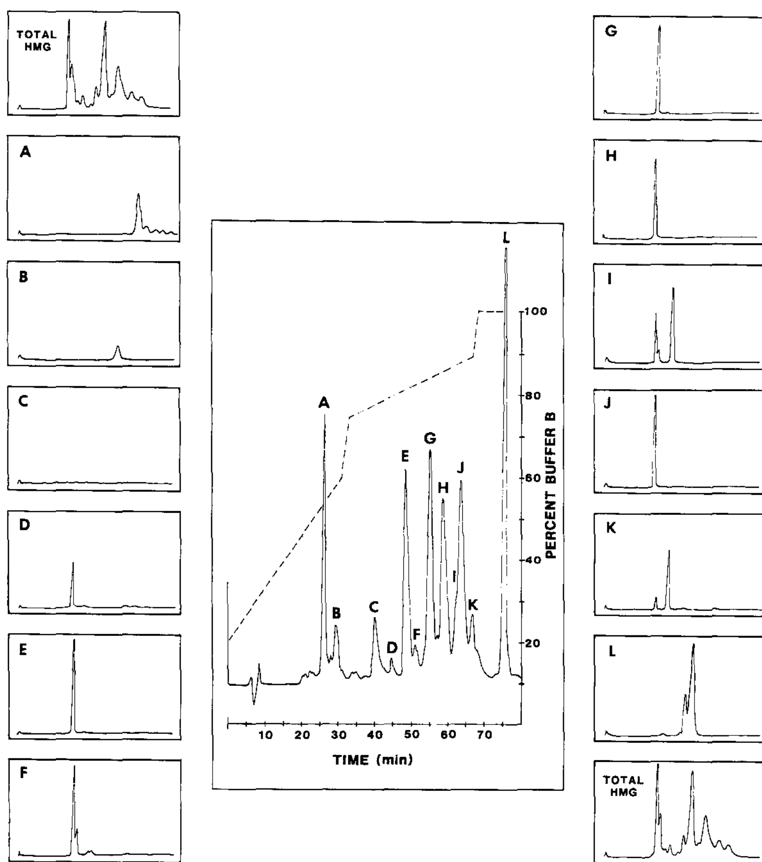

Figure 1 Separation of HMG proteins by HPLC and analysis of the isolated fractions by disc gel electrophoresis. CENTER PANEL: Absorption profile (214 nm) of total HMG protein separation on a Nucleosil 100 column as described in Materials and Methods. Dashed line: Buffer B gradient 100% Buffer B is 40% acetonitrile; 0.1% TFA. PERIPHERAL PANELS: Microdensitometer scans of Naphthol blue black stained HMG fractions following electrophoresis in acid-urea gels at 120 V for 3 hr. The labeled fractions in these panels correspond to the isolated HPLC peaks in the center panel. Electrophoresis is from the left (cathode) to right (anode). Peak A: HMG 17; Peak B: HMG 14; Peak C: numerous peptides of various sizes; Peak D: HMG 1; Peaks E and G: HMG 2; Peaks F and I: HMGs 1, 2, and 3; Peaks H and J: HMG 1; Peak K: HMG 3, the N-terminal end of HMG 1; and Peak L: fragments of H1 histone.

TABLE 1

Amino Acid Compositions† of Peaks Obtained Following HPLC of Total HMG Protein on Nucleosil 100

Peak*:	A	Expected	B	Expected	E	G	Expected	H	J	Expected	K	L
HMG:	17	17	14	14	2	2	2	1	1	1	3	8
Lysine	24.5	24.3	21.9	21.1	19.9	19.5	19.4	20.9	21.1	21.3	18.4	20.4
Histidine	0	0	0	0.2	1.7	1.8	2.0	1.3	1.2	1.7	1.4	0.1
Arginine	4.4	4.1	5.3	5.4	4.2	4.2	4.7	3.6	3.5	3.9	3.7	3.1
Aspartic acid	10.9	12.0	8.0	8.3	9.9	10.7	9.3	10.9	10.3	10.7	9.3	3.6
Threonine	1.7	1.2	4.1	4.1	2.1	2.3	2.7	2.3	2.6	2.5	4.0	5.2
Serine	2.9	2.3	7.3	8.0	7.6	7.9	7.4	5.0	5.0	5.0	5.2	8.3
Glutamic acid	11.6	10.5	18.1	17.5	17.6	17.3	17.5	18.4	17.0	18.1	11.6	5.8
Proline	12.7	12.9	9.0	8.1	7.9	7.8	8.9	6.7	6.8	7.0	7.5	7.2
Glycine	9.7	11.2	6.5	6.4	6.6	6.5	6.5	5.4	5.4	5.3	6.7	8.5
Alanine	18.5	18.4	14.3	14.8	7.6	7.5	8.1	9.0	10.8	9.0	10.6	21.2
Half-cystine	0	0	0	0	0.3	0	trace	0.6	0	trace	0	0
Valine	2.0	2.0	3.9	4.0	1.7	1.7	2.3	2.0	2.1	1.9	6.6	6.1
Methionine	0	0	0	0.1	1.8	1.8	0.4	1.9	1.9	1.5	1.8	0
Isoleucine	0	0	0	0.3	1.2	1.2	1.3	1.7	1.7	1.8	2.4	1.4
Leucine	1.0	1.0	1.8	2.0	2.1	2.4	2.0	2.1	2.3	2.2	4.2	7.4
Tyrosine	0	0	0	0.2	2.9	2.9	2.0	3.2	3.1	2.9	2.8	0.9
Phenylalanine	0	0	0	0.3	4.1	3.7	3.0	4.2	4.1	3.6	3.7	0.8
Tryptophan	0	0	0	nd	0.7	0.8	nd	0.8	0.9	nd	0	0

*Letter description as shown in Figure 1.

† Amino acid analyses of each fraction were obtained on a Beckman 120C amino acid analyzer following hydrolysis of the lyophilized proteins in 4 M methanesulfonic acid at 110°C for 40 h. Expected compositions are those published by Johns (9).

signs of degradation by gel electrophoresis, suggesting that these peptides are fragments of histone or HMG proteins.

Two well separated peaks eluting between 80 and 85% Buffer B at 48 min (peak E), and 55 min (peak G), respectively, were found to contain a protein with an electrophoretic mobility and amino acid composition characteristic of HMG 2. Except for minor differences in their aspartic acid and half-cystine contents, the amino acid compositions of the proteins in these two fractions were virtually identical. Thus these proteins may not be amino acid sequence variants of HMG 2. They may, however, differ in their extent of amidination (number of glutamine or asparagine residues) or degree of modification by acetylation, phosphorylation, or methylation. Multiple species of calf thymus HMG 2 were also observed by Goodwin et al. (6,10,11) following chromatography of whole HMG 2 on carboxymethyl-sephadex. Significant differences in the amino acid compositions of the two major and two minor HMG 2 species were not detected in their analyses either.

Similarly, three peaks were found to contain proteins that coelectrophoresed with HMG 1. The amino acid compositions (Table 1) of the proteins in the two larger peaks, H and J, were also characteristic of HMG 1 (sufficient sample was not available for analyzing peak D). The proteins in these two peaks differed only in their glutamic acid, alanine, and half-cystine contents. Although these differences were small, they suggest the possibility that the two major HMG 1 species observed here may be amino acid sequence variants. The presence of several HMG 1 species was suggested earlier by Goodwin et al. (11), and was based on their observation that the isoelectric focusing pattern of total HMG 1 contained multiple peaks. It was thought that the complexity might arise as a result of aggregation. Multiple species were never obtained by chromatography.

Minor peaks F and I were found to contain a mixture of HMG 1, 2, and 3. HMG 3, the N-terminal end of HMG 1, eluted as the major component of peak K at 67 min. Peak I, which contained considerable HMG 3 (probably because of incomplete separation of adjacent peaks upon collection), also contained a small amount of HMG 2. This protein (HMG 2) could not be accounted for by overlap from adjacent peaks (since peaks H and J are both HMG 1), and its presence suggests that another HMG 2 variant may exist and elute as a shoulder (labeled I in Fig. 1) on peak J. Similar results were observed for peak F. The major component of this peak was HMG 1, which suggests that this peak is a minor HMG 1 variant that elutes beween two HMG 2 variants.

The last peak, L, eluted from the column at 75 min and 100% Buffer B. The proteins (at least two) in this fraction displayed an electrophoretic mobility midway between HMG 2 and HMG 17, similar to that observed for fragments of H1 histone. The acetonitrile concentration at which this peak elutes (40%) is very close to that observed for intact H1 histone (12). Amino acid analysis of this peak (Table I) also supports the idea that these proteins are fragments of H1; the protein is rich in lysine and alanine and low in glutamic and aspartic acids.

This approach offers several advantages over currently used methods for separating and analyzing the HMG proteins. The method is rapid, requiring only 80 min per run. Each HMG protein, several potential HMG variants, and various contaminating histone H1 and HMG degradation products all separate as distinct peaks in one chromatographic run. Using the preparative column, several milligrams of each HMG protein may be obtained without sacrificing the quality of the resolution. Separations may be performed in the analytical mode by using an analytical or microbore column and a 1-cm flow

cell, or by postcolumn derivatization of the proteins with a fluorescent tag. Because intact histones begin eluting at an acetonitrile concentration greater than that required for elution of the final protein in the HMG fraction (peak L, H1 peptides), this approach may be modified to permit the separation of both histones (12) and HMG proteins in a single run. By using entirely volatile buffers, extensive and time-consuming dialysis steps are eliminated, and the isolated fractions may be lyophilized directly.

ACKNOWLEDGMENTS

Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-ENG-48.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 917-924 (1984)

DANSYLATION OF AMINO ACIDS AND BYPRODUCT FORMATION

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ABSTRACT

An ion pair reversed-phase HPLC method was developed for the determination of flourescent DNS-AA and by-products DNS-OH, DNS-NH_2, and DNS-$N(CH_3)_2$ which form during the dansylation reaction. The method permits the separation of the four fluorescent compounds and ultraviolet absorbing DNS-Cl in 25 min. Dansylation of amino acids was carried out under different reaction conditions and evaluated with regard to major products formed and not only the DNS-AA. Optimum reaction conditions can readily be obtained for the dansylation of amino acids using this method.

INTRODUCTION

Dansyl (5-dimethylaminonapthalene-1-sulfonyl) chloride reacts with amino acids to form fluorescent derivatives which can be detected at extremely low concentrations (1). The reason why this reagent is not widely used, however, is the formation of multiple derivatives of several amino acids and other fluorescent by-products (2). Some of the by-products known to form are DNS-OH[1] from the hydrolysis of DNS-Cl (3), DNS-NH_2[1] from the decomposition of DNS-amino acids (4), and DNS-$(CH_3)_2$[1] probably from the

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decomposition of DNS-CL[1] (5). Considerable effort has been spent on developing optimal reaction conditions for the formation of DNS-amino acids (2,3,6), but few reports have appeared in the literature on the effect of reaction parameters on the formation of the fluorescent by-products. Therefore, a method that permits the rapid determination of optimal reaction conditions for the dansylation of amino acids will be helpful.

In this paper we describe a reversed-phase ion pair HPLC[1] method for monitoring the formation of DNS-OH, DNS-NH_2, DNS-$N(CH_3)_2$ by-products, and DNS-AA directly during the dansylation reaction under different reaction conditions. Ultraviolet absorbing dansyl chloride is also monitored.

MATERIALS AND METHODS

Apparatus

Chromatographic separations were carried out on a Waters Associate (Milford, MA) HPLC system which included the following components: Model 6000A[2] solvent delivery system, Model U6K injector, 30 cm X 4 mM I.D. μ Bondapak C_{18} column, Model 420 fluorescent detector. For isocratic conditions the mobile phase consisted of 5 mM t-butylammonium hydroxide in 10 mM K_2HPO_4 adjusted to pH = 7.00 and 40% v/v acetonitrile. The flow rate was 1 ml/min. The filters used were 370 ± 110 nm excitation and 500 nm long pass emission.

Reagents

Amino acids, gold label (Calbiochem-Behring Corp., La Jolla, CA), dansyl chloride, dansylamide, dansylamino acids (Pierce Chemical Co., Rockford, IL), t-butylammonium hydroxide, 40% w/v (Aldrich Chemical Co., Inc., Milwaukee, WI) were used without further purification. Dansyl acid was prepared by hydrolyzing dansyl chloride with 20% KOH v/v. Completeness of hydrolysis was determined chromatographically by the absence of DNS-Cl absorbance at 250 nM. Dansyl dimethylamide was synthesized and purified according to a published procedure (7). Dansyl dimethylamide purity was determined chromatographically. Water used in all determinations was deionized and twice distilled, obtained from a MegaPure System (Corning, Corning, NY).

Reaction Conditions

The dansylation of leucine is given as a typical reaction. The ratio of DNS-Cl:leucine was 10:1 at pH = 9.

DNS-Cl, 0.5 ml (4.3 mg/ml acetonitrile, 0.016 M) was added to 0.5 ml leucine (0.21 mg/ml, 0.0016 M), dissolved in buffer (0.1 M Na_2CO_3), adjusted to pH 9 with 6 N HCl, then stored in the dark. Periodically, 0.1 ml samples were withdrawn and diluted to 2 ml with mobile phase and 20 μl were injected on the column.

Calculations

The amount of DNS-AA and DNS-NH_2 found in the reaction mixture was determined by their fluorescent response and expressed as the percentage of the initial concentration of amino acid. The amount of DNS-$N(CH_3)_2$ and DNS-OH found was also determined by their fluorescent response and expressed as the percentage of the initial concentration of DNS-Cl. Chromatographically DNS-Cl was 98% pure based on the presence of DNS-OH as an impurity.

DISCUSSION

To monitor the formation of DNS-OH, DNS-NH_2, DNS-$N(CH_3)_2$, and DNS-AA during the dansylation of amino acids, a reversed-phase ion pair HPLC method was developed. Separation of these fluorescent derivatives required the addition of tetrabutylammonium phosphate to the mobile phase to prevent DNS-OH from eluting with the Vo. A typical separation of fluorescent products for the dansylation of leucine was accomplished in 10 min under isocratic conditions. Dansyldimethylamide **4**, which results from the decomposition of DNS-Cl, can elute with the other fluorescent components within 25 min by gradient elution (Fig. 1). Dansyl chloride **5** does not fluoresce but its ultraviolet absorbance can be monitored at 250 nm and included in the chromatograph (Fig. 1). Dansyl dimethylamide results from the decomposition of DNS-Cl since it was present in the blank at the same concentration that it was found in the DNS-AA reaction mixture but not as an impurity in DNS-Cl. The formation of DNS-$N(CH_3)_2$ is a function of pH but not temperature. After 30 min at pH = 8, 0.07% DNS-$N(CH_3)_2$ was found compared to 1.4% at pH = 10.

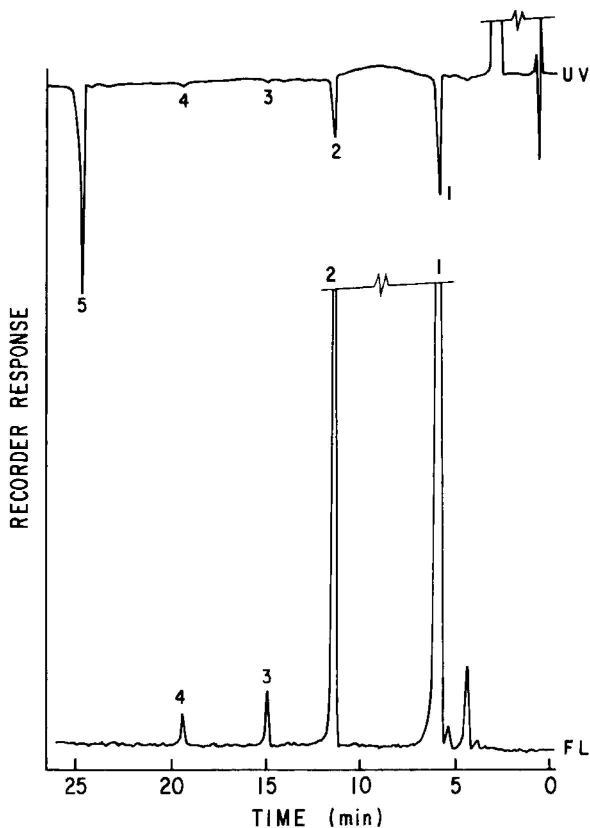

Fig. 1. Gradient elution of dansyl derivatives solvent: A = 5mM tetrabutylammonium phosphate; B = CH_3CN; gradient 40%-70% B, curve 6 (linear), 25 min. Peaks 1, DNS-OH; 2, DNS-leu; 3, $DNS\text{-}NH_2$; 4, $DNS\text{-}N(CH_3)_2$; 5, DNS-Cl.

Rate of formation of DNS-AA was a function of excess DNS-Cl. At equimolar concentrations of DNS-Cl and leucine, a maximum yield of 81% DNS-leu was obtained after 2 h (Fig. 2). During the same reaction period 18% of DNS-OH was formed from the hydrolysis of DNS-Cl and less than 2% $DNS\text{-}NH_2$ resulted from the decomposition of the DNS-leu product. Quantitative yields of DNS-leu were obtained after 20 min and 5 min when the ratio of DNS-Cl:leucine

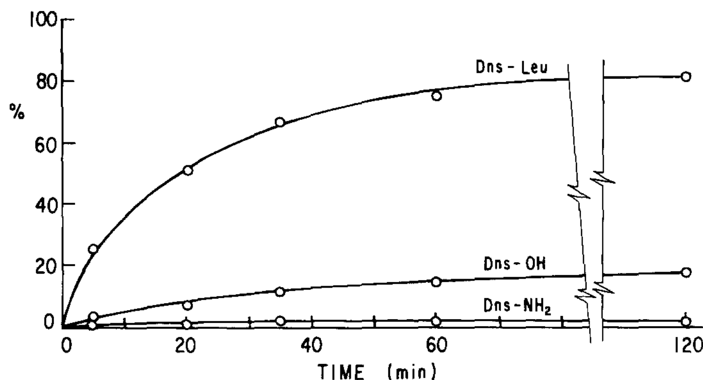

Fig. 2. Dansylation of leucine. Conditions, DNS-Cl:leu, 1:1, pH = 8, 25°C.

was increased to 10:1 and 20:1, respectively. Increasing the DNS-Cl:leucine ratio, however, resulted in a significant increase in the amount of DNS-NH_2. A 20-fold increase in DNS-Cl increased the DNS-NH_2 concentration 40 times after 2 h (Fig. 3).

Dansylation of amino acids is usually carried out under alkaline conditions. We have found that dansylation of leucine can proceed at pH = 7; a maximum yield of 95% was reached after 50 min (Fig. 4). At more alkaline conditions, pH 9 and 10, quantitative yields of DNS-leu were obtained in 5 min. The amount of DNS-OH formed during the reaction was essentially constant for pH 7, 8, and 9, however, at pH = 10, 51% DNS-OH was found after 60 min (Fig. 4). The amount of DNS-NH_2 formed was constant over the pH range 7-10.

Elevated reaction temperatures had an adverse effect on the formation of dansylated amino acids. A maximum yield of 66% DNS-leu was obtained after 5 min at 35°C and 60°C. Reported attempts to accelerate the dansylation of amino acids with elevated temperatures also resulted in lower yields of dansylated amino acids (6). By quenching the reaction with $EtNH_2$ poor yields were presumed to be due to rapid hydrolysis of DNS-Cl at the higher temperature, as evidenced by the very low chromatographic peak observed for DNS-NHEt (6). We found, by following the formation of dansyl product as well as dansyl by-products directly during the reaction, that the poor

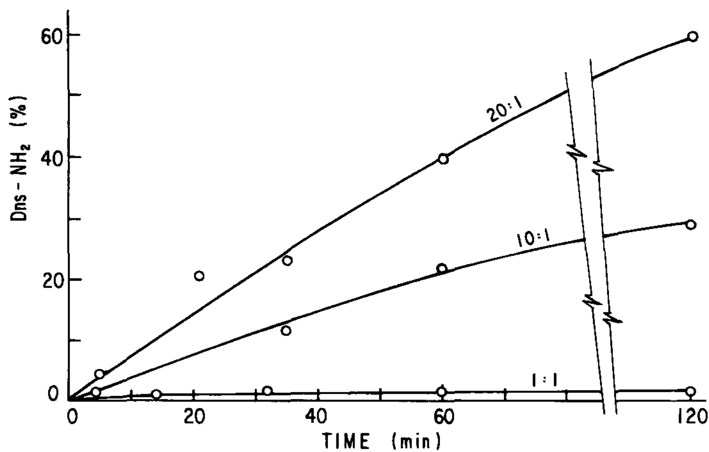

Fig. 3. Effect of DNS-Cl concentrations on $DNS-NH_2$ formation. Conditions, DNS-Cl:leu, 1:1, 10:1, 20:1, pH = 8, 25°C.

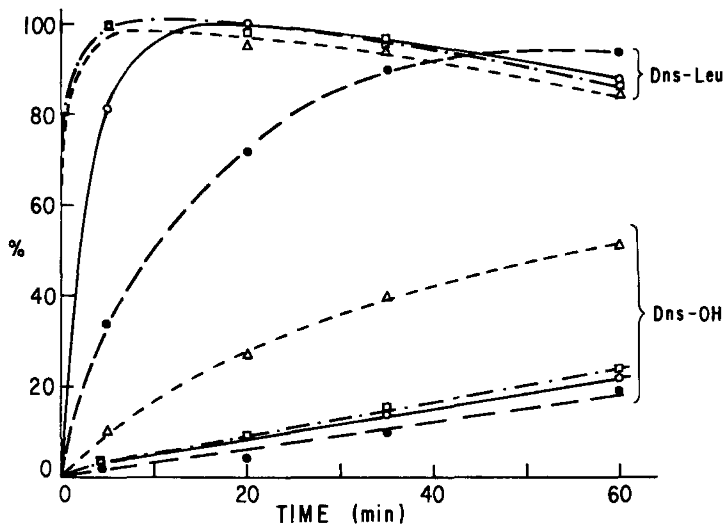

Fig. 4. Effect of pH on dansylation of leucine. Conditions, pH = 7(•); 8(O); 9(□); 10(Δ).

yields resulted from a combination of rapid hydrolysis of DNS-Cl and decomposition of DNS-leu to DNS-NH_2. At 60°C, 68% DNS-OH and 38% DNS-NH_2 were found after 20 min.

The amount of DNS-NH_2 formed during the dansylation of amino acids depends on the amino acid as well as the excess of DNS-Cl. Between 12%-16% DNS-NH_2 was formed for leucine, cysteine, and phenylalanine after 1 h with a 10-fold excess of DNS-Cl at pH = 8 and 25°C. However, for proline and asparagine, less than 0.5% DNS-NH_2 was found under the same reaction conditions and time period. Neadle and Pollitt (4) found 11%-15% DNS-NH_2 formed for the dansylation of α-alanine, valine, leucine, and norleucine using 10% excess of DNS-Cl. A method has been reported for the dansylation of amino acids which provides high yields independent of the ratio of DNS-Cl to amino acids over a 1,000-fold range (6). Using this procedure for the dansylation of leucine (40 mM Li_2CO_3, pH = 9.5, 25°C, and 10-fold excess DNS-Cl), we found that near quantitative yield was reached after 10 min but DNS-leu decomposed and 25% DNS-NH_2 formed after 1 h.

In conclusion, we have developed a reversed-phase HPLC method for following the formation of DNS-AA product and by-products directly. Using this method we improved the understanding of the dansylation reaction and were better able to arrive at optimum conditions for the dansylation of amino acids. Dansylation of amino acids was carried out under different reaction conditions and evaluated with regard to major products formed and not only the DNS-AA. Similar advantages should be obtained for the dansylation of N-terminal amino groups of small peptides hydrolysates using this method.

ACKNOWLEDGMENTS

We thank W. I. Kimoto and R. Greenberg for their helpful suggestions.

FOOTNOTES

[1] Abbreviations used: HPLC, high performance liquid chromatography; DNS-Cl, -OH, -NH_2, -$N(CH_3)_2$, -AA; dansyl-chloride, -acid, -amide, -dimethylamide, -amino acid; Vo, void volume.

[2] Reference to brand or firm name does not constitute endorsement by the

U.S. Department of Agriculture over others of a similar nature not mentioned.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 925-934 (1984)

STUDIES ON STEROIDS CLXXXIV. SEPARATION OF CATECHOL ESTROGEN MONOGLUCURONIDES AND MONOSULFATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION[1]

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ABSTRACT

Separation of catechol estrogen monoglucuronides and monosulfates by high-performance liquid chromatography with electrochemical detection on a reversed-phase column has been carried out. The effects of composition and pH of mobile phases on the capacity factor were investigated with a TSKgel ODS-120T column. Each group of isomeric monoglucuronides and monosulfates of 2- and 4-hydroxyestrogens was efficiently resolved on this column when the 0.5% ammonium dihydrogen phosphate-tetrahydrofuran-acetonitrile system was used as a mobile phase.

INTRODUCTION

Since the reports on the occurrence of 4-hydroxyestrogens as well as the well-known 2-hydroxyestrogens in pregnancy urine by three groups (2-4), considerable attentions have been drawn to the metabolic fate of catechol estrogens in connection with their potent physiological activities. The catechol estrogen conjugates were determined by gas chromatography-mass spectrometry (5), radioimmunoassay (6), and high-performance liquid chromato-

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graphy (HPLC) (4) involving prior hydrolysis and/or solvolysis of the conjugates. These procedures, however, have inevitable disadvantages: the lack of reliability in the results and the loss of informations on the conjugated forms. It appears to be attractive to develop a method for the direct determination of catechol estrogen conjugates without deconjugation. The present paper deals with the separation of isomeric monoglucuronides and monosulfates of 2- and 4-hydroxyestrogens by HPLC with electrochemical detection (ECD).

EXPERIMENTAL

Instruments

The apparatus used for this work was a Yanagimoto L-4000W high-performance liquid chromatograph equipped with a Yanagimoto VMD 101A electrochemical detector (Yanagimoto Co., Kyoto, Japan). The potential of the detector was set at +0.9V vs a Ag/AgCl reference electrode. A TSKgel ODS-120T (5 μm) column (25 cm x 0.4 cm i.d.) (Toyo Soda Co., Tokyo, Japan) was used under ambient conditions. The pH of the mobile phase was adjusted with phosphoric acid or ammonium hydroxide. All solvents were degassed by sonication. The mobile phase was used at a flow rate of 1 ml/min.

Chemicals and Reagents

The catechol estrogen conjugates were synthesized in these laboratories by the methods previously reported (7,8). All the reagents used were of analytical reagent grade. Solvents were purified by distillation prior to use.

RESULTS AND DISCUSSION

In reversed-phase HPLC, the methanol-, acetonitrile-, and tetrahydrofuran (THF)-buffer systems are usually employed as

mobile phases. Initially, our effort was directed to the separation of the isomeric monoglucuronides and monosulfates of 2- and 4-hydroxyestrogens with these solvent systems. Among several commercially available columns tested a TSKgel ODS-120T column provided the most promising result. Therefore, suitable conditions for the separation were examined in detail with this column.

First, acetonitrile, methanol, or THF combined with 0.5% ammonium dihydrogen phosphate (pH 3.0) at various ratios was employed as a mobile phase. The capacity factors (k') of all the substrates increased with a decreasing ratio of organic solvent. Satisfactory separation, however, was not attained by any binary solvent systems. For instance, when acetonitrile-buffer (5:16) was used, 2-OHE$_1$ 3-S and 2-OHE$_2$ 2-S were not resolved, and 2-OHE$_1$ 2-S was eluted with the retention of more than 1 hr. The similar chromatographic behaviors were also observed for other groups of catechol estrogen conjugates.

These results prompted us to use a ternary solvent system containing an additional organic modifier, THF, which has proved to be effective for the separation of the ethereal compounds (9) and amino acid derivatives (10,11). As for the 0.5% ammonium dihydrogen phosphate-THF-acetonitrile system, the effect of the ratio of THF to acetonitrile on the retention value was investigated. The k' values of catechol estrogen ring A conjugates relative to the 17-conjugate in each group were plotted against the ratio of THF to acetonitrile in the mobile phase. The results obtained for 4-hydroxyestrogen sulfates and glucuronides are shown in Fig. 1a and 1b, respectively. The relative k' value of each substrate was reduced remarkably with an increasing ratio of THF to acetonitrile. This phenomenon indicated that THF would be more effective than acetonitrile for the early elution of ring A conjugates relative to ring D conjugates. The relative k' values of 2-hydroxyestrogen conjugates at various ratios of THF

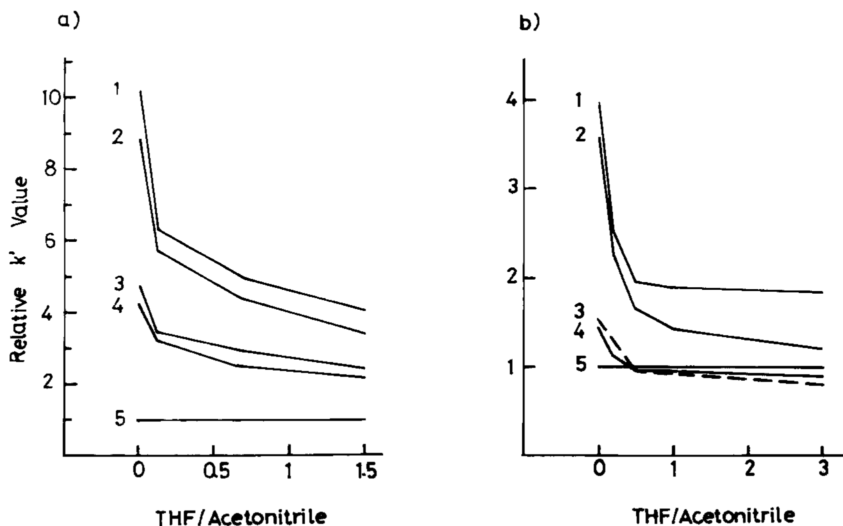

FIGURE 1. Effect of the Ratio of THF to Acetonitrile in the Mobile Phase on Relative k' Values of 4-Hydroxy-estrogen Sulfates (a) and Glucuronides (b).
a) Organic solvent-buffer (5:16). 1, 4-OHE_1 4-S; 2, 4-OHE_1 3-S; 3, 4-OHE_2 3-S; 4, 4-OHE_2 4-S; 5, 4-OHE_2 17-S. b) Organic solvent-buffer (3:10). 1, 4-OHE_1 4-G; 2, 4-OHE_1 3-G; 3, 4-OHE_2 3-G; 4, 4-OHE_2 4-G; 5, 4-OHE_2 17-G.

to acetonitrile were also estimated. The results obtained were similar to those of 4-hydroxyestrogen conjugates (Table 1). The use of a suitable concentration of THF in the mobile phase improved the separation and brought about an earlier elution, providing a sharp peak of theoretical shape on the chromatogram. Based upon these data we arrived at a conclusion that THF would be an efficient modifier in the mobile phase for the separation of these conjugates. The suitable concentrations of THF in the mobile phases were found to be 14.3% for 2-hydroxyestrogen sulfates, 9.5% for 4-hydroxyestrogen sulfates, 11.5% for 2-hydroxy-estrogen glucuronides, and 17.3% for 4-hydroxyestrogen glucuronides.

TABLE 1
Effect of the Ratio of THF to Acetonitrile in the Mobile Phase on Relative k' Values of 2-Hydroxyestrogen Conjugates

Compound	THF/acetonitrile				
	0	0.25	0.5	1	1.5
2-OHE_1 2-S	4.8*	3.0	2.6	2.3	2.0
2-OHE_2 2-S	3.4	2.9	2.7	2.4	2.2
2-OHE_1 3-S	3.4	2.3	2.0	1.7	1.6
2-OHE_2 3-S	1.8	1.5	1.4	1.3	1.2
2-OHE_2 17-S	1.0	1.0	1.0	1.0	1.0
2-OHE_1 2-G	1.0**	1.0	1.0	1.1	1.1
2-OHE_2 2-G	2.0	1.6	1.4	1.3	1.3
2-OHE_1 3-G	1.2	0.8	0.7	0.7	0.6
2-OHE_2 3-G	0.4	0.4	0.4	0.4	0.4
2-OHE_2 17-G	1.0	1.0	1.0	1.0	1.0

* Organic solvent-buffer (5:16), ** organic solvent-buffer (3:10)

The effect of pH on the k' value was then examined with 0.5% ammonium dihydrogen phosphate-THF-acetonitrile. The k' values of these substrates were plotted against pH of the buffer in the mobile phase. It is of particular interest that the effects of pH on the k' values were quite different among the four groups. The k' values of 4- and 2-hydroxyestrogen glucuronides decreased remarkably with an increasing pH value in the range of 3.0 to 5.0 (Fig. 2a,b). The compounds having a glucuronic acid moiety (pK_a 3.2) exhibited greater k' values at pH 3.0 where undissociated species are predominant. With an increasing pH value of the mobile phase the dissociation of these conjugates increases and hence, their k' values decreased. The result was compatible with

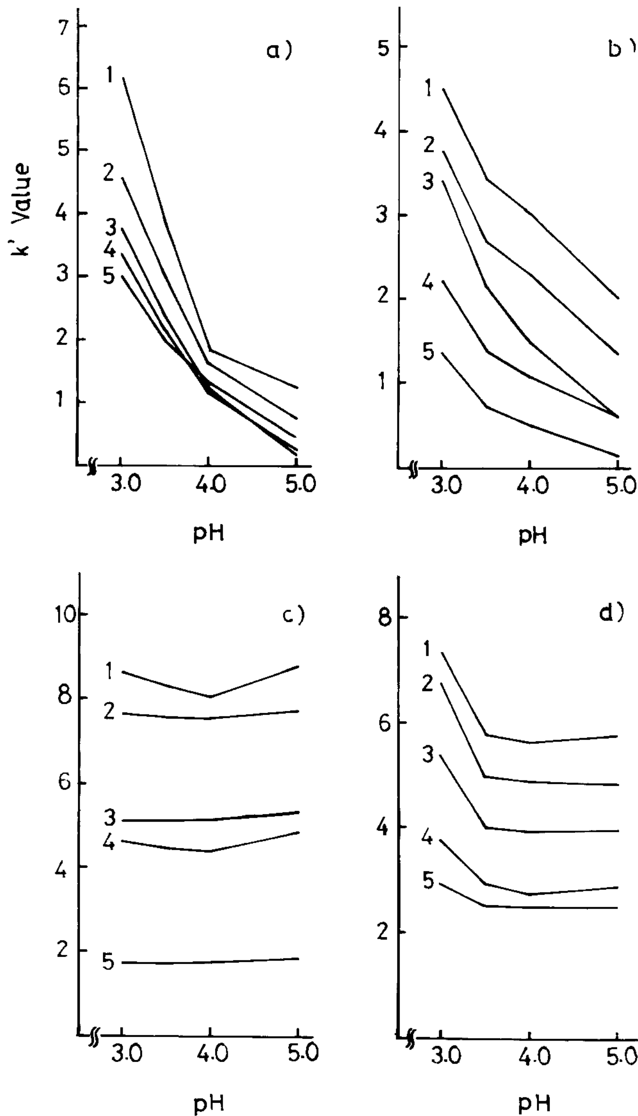

FIGURE 2. Effect of pH of Mobile Phase on k' Values of 4-Hydroxyestrogen Glucuronides (a), 2-Hydroxyestrogen Glucuronides (b), 4-Hydroxyestrogen Sulfates (c), and 2-Hydroxyestrogen Sulfates (d).
a) 1, 4-OHE$_1$ 4-G; 2, 4-OHE$_1$ 3-G; 3, 4-OHE$_2$ 17-G; 4, 4-OHE$_2$ 4-G; 5, 4-OHE$_2$ 3-G. b) 1, 2-OHE$_2$ 2-G; 2, 2-OHE$_1$ 2-G; 3, 2-OHE$_2$ 17-G; 4, 2-OHE$_1$ 3-G; 5, 2-OHE$_2$ 3-G. c) 1, 4-OHE$_1$ 4-S; 2, 4-OHE$_1$ 3-S; 3, 4-OHE$_2$ 3-S; 4, 4-OHE$_2$ 4-S; 5, 4-OHE$_2$ 17-S. d) 1, 2-OHE$_2$ 2-S; 2, 2-OHE$_1$ 2-S; 3, 2-OHE$_1$ 3-S; 4, 2-OHE$_2$ 3-S; 5, 2-OHE$_2$ 17-S.

TABLE 2
k' Values of Catechol Estrogen Conjugates

Compound	k'*	Compound	k'**
2-OHE_2 17-S	2.9	2-OHE_2 3-G	0.7
2-OHE_2 3-S	3.7	2-OHE_1 3-G	1.4
2-OHE_1 3-S	5.4	2-OHE_2 17-G	2.2
2-OHE_1 2-S	6.8	2-OHE_1 2-G	2.7
2-OHE_2 2-S	7.3	2-OHE_2 2-G	3.4
4-OHE_2 17-S	1.7	4-OHE_2 3-G	3.0
4-OHE_2 4-S	4.6	4-OHE_2 4-G	3.4
4-OHE_2 3-S	5.1	4-OHE_2 17-G	3.8
4-OHE_1 3-S	7.6	4-OHE_1 3-G	4.6
4-OHE_1 4-S	8.7	4-OHE_1 4-G	6.2

* t_0: 4.4 min, ** t_0: 4.8 min.

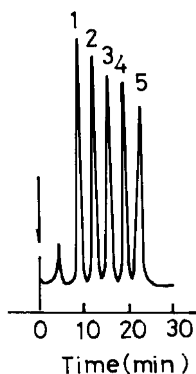

FIGURE 3. HPLC Separation of a Synthetic Mixture of 2-Hydroxyestrogen Glucuronides.
1, 2-OHE_2 3-G; 2, 2-OHE_1 3-G; 3, 2-OHE_2 17-G; 4, 2-OHE_1 2-G; 5, 2-OHE_2 2-G.
Conditions: column, TSKgel ODS-120T; mobile phase, 0.5% ammonium dihydrogen phosphate (pH 3.5)/THF/acetonitrile (20:3:3), 1 ml/min; detection, ECD at +0.9V.

the previous finding on the resolution of estriol 16-glucuronide and 17-glucuronide (12). The most satisfactory separation was obtained for 4-hydroxyestrogen glucuronides at pH 3.0 (Fig. 2a) while for 2-hydroxyestrogen glucuronides at pH 3.5 (Fig. 2b). As for 4-hydroxyestrogen sulfates no significant difference in the k' value was found in the range of pH 3.0 to 5.0 (Fig. 2c). The k' values of 2-hydroxyestrogen sulfates decreased remarkably from pH 3.0 to 3.5 and then were constant in the range of pH 3.5 to 5.0 (Fig. 2d). Although no plausible explanation is at present available, these phenomena may be ascribable to the difference in ionization of the solutes in the mobile phase (13).

On the basis of these data 0.5% ammonium dihydrogen phosphate (pH 3.0)-THF-acetonitrile (16:3:2, 16:2:3, and 40:9:3) were chosen as mobile phases suitable for 2-hydroxyestrogen sulfates, 4-hydroxyestrogen sulfates, and 4-hydroxyestrogen glucuronides, respectively. For 2-hydroxyestrogen glucuronides 0.5% ammonium dihydrogen phosphate (pH 3.5)-THF-acetonitrile (20:3:3) was found to be the most suitable mobile phase. Each group of isomeric monosulfates and monoglucuronides of 2- and 4-hydroxyestrogens was efficiently resolved by using the above solvent systems. The k' values of these catechol estrogen conjugates are listed in Table 2. A typical chromatogram of 2-hydroxyestrogen glucuronides is illustrated in Fig. 3. The detection limits of ring A sulfates and glucuronides of catechol estrogens were estimated to be 1 ng and 5 ng, respectively, while that of catechol estrogen 17-conjugates was 500 pg (S/N=2 at 2 nA full scale).

The application of the present method to the determination of catechol estrogen conjugates in biological fluids will be reported elsewhere (14).

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan. The authors are indebted to Miss Yoshiko Yumura for her technical assistance.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 935-942 (1984)

DETECTION OF PHOMENONE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN TOMATO PLANTS INFECTED BY *PHOMA DESTRUCTIVA* PLOWR.[+]

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ABSTRACT

Identification and quantitative determination of phomenone in ethyl acetate extracts from Tomato plants infected by *Phoma destructiva* Plowr., was carried out by high-performance liquid chromatography with UV detection, and ethanol-water (30/70, v/v) as mobile-phase on Perkin-Elmer RP-18/10 stainless column, at 20°C and 0.9 ml min^{-1} flow rate. Detection limit was 5.5 ng, with standard deviation of ± 3%, and retention time of 6.22 min. Analysis of extracts from spiked tomato fruits shows the same parameters. The method appears to be adequate for detection and quantitation of phomenone in contaminated tomato leaves and fruits using a pre-column packed with Lichroprep RP-18/25-40 µm.

[+]This research has been supported by the Italian National Research Council. Special "ad hoc" program "Chimica Fine e Secondaria" - Subprojiect Ca.

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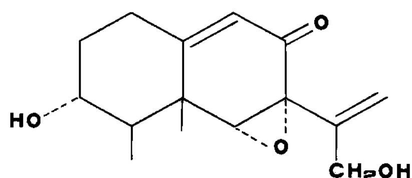

Fig. 1 - Chemical structure of phomenone.

INTRODUCTION

Phomenone (Fig. 1) is an eremophilanic type sesquiterpene produced by some phytopathogenic Phoma species (1,2,3). It was recently identified as the phytotoxin of Phoma destructiva Plowr., the causal agent of a wilt disease of Tomato, and found in wilted leaves of infected Tomato plants (4-6).

Because of the toxicity of phomenone to plants and animals (7,8) and of its potential occurrence in agricultural commodities infected by Phoma species and their fruit-rotting isolates (9-13) the development of a simple and rapid analytical method is of importance. This paper reports on the identification and quantification of phomenone by high-performance liquid chromatography (HPLC) in infected tomato leaves and fruits. A method based on three steps, i.e.: extraction of toxin by ethyl acetate; purification by a Lichroprep RP-18 column; and chromatographic separation and quantification by HPLC was developed.

EXPERIMENTAL

Reagent. All reagents were analytical-grade chemicals. HPLC grade water and ethanol for eluting solvents were purchased from Fluka

AG. A pure sample of phomenone was purified from culture filtrates of *P. destructiva* as previously described (5). A solution of 4.3 mg of toxin in 100 ml methanol was used as standard.

Extraction and preliminary purification of phomenone from Tomato plants.

The infected material was obtained from artificially infected Tomato plants as reported elsewhere (6). The lyophilized plant material was extracted 4 times, with ethyl acetate (32 ml/g), at room temperature, in a Warring blendor, for 5 min. The combined ethyl acetate extracts were filtered through a sintered glass funnel and then evaporated under reduced pressure to give a green powder. This residue was dissolved in a small volume of methylene chloride, and the solution mixed with Lichroprep RP-18 (Merck, particle size 25-40 μm). Then the mixture was evaporated and the dry residue was loaded on a Lichroprep RP-18 small column (cm 10 x 0,8), which was eluted under vacuum water-pump with a single volume of water-ethanol (70:30, v/v). The colourless extract was concentrated and analyzed by HPLC.

Extraction and preliminary purification of phomenone from tomato fruits.

A pure sample of phomenone (0.1 mg) and ethyl acetate (192 ml) were added to lyophilized tomato juice (6g). This mixture was treated as described above for the plant material.

Thin-layer chromatography.

Analytical TLC was performed on silica gel plates (Merck, Kieselgel 60, F_{254}, 0.25 mm, eluted with ethyl acetate-n-hexane, (90:10); or with chloroform-methanol (85:15), and on K/C_{18} F plates (Whatman, 0.25 mm, eluted with water-ethanol (70:30), or with acetonitrile-water (90:30). After elution, the chromatograms were air dried and observed under 254 nm UV light. Phomenone appeared as quenched fluorescence spot and was visualized as blu-green spot on chromatograms sprayed with 5% sulforic acid and 3% phosphomolybdic acid in methanol, and heated for 10 min at 110°C.

High-performance liquid chromatography.

A Perkin - Elmer series 3B microcomputer controlled pump module, equipped with a variable wavelenght Perkin-Elmer LC-75 Spectrophotometric Detector set at 240 nm was used in connection with a Perkin-Elmer Sigma 10B chromatografic data station. Liquid chromatografic separations were performed on a prepacked Perkin--Elmer RP-18 (particle size 10 µm) column (cm 25x0.46, stainless steel). Analyses were performed at 20°C, employng a mixture of water-ethanol (70:30, v/v) as mobile-phase at 0.9 ml min^{-1} flow rate.

RESULTS AND DISCUSSION

Before performing the analysis on the previously decolorized tomato extracts, the optimum conditions of phomenone analysis were determined.

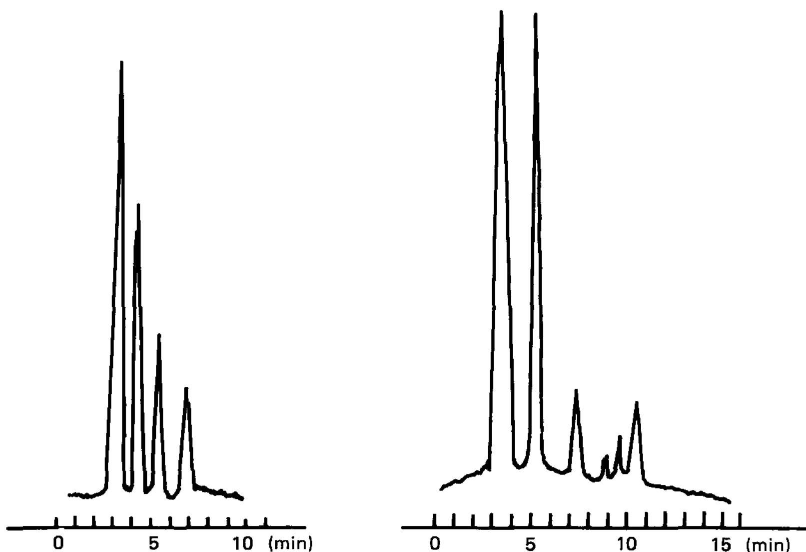

Fig. 2 - a) Chromatogram of extract from healty tomato fruits.
b) Chromatogram of extract from healty Tomato plant.
Column: Perkin-Elmer RP-18 (cm25x0.46, 10 μm) at 20°C. Mobile phase: water-ethanol (70:30, v/v) at 0.9 ml min^{-1} flow rate. Detector: UV absorption at 240 nm.

The minimum detectable amount of the phytotoxin was 5.5 ng, and the retention time was 6.22 $\pm$ 0.05 min. The standard deviation calculated from eight repeated injection with four different volumes, was in the range of $\pm$ 3%. Linear calibration curve from 0-50 ng.

The analysis performed on healthy tissue extracts no peak with retention time as phomenone (Fig. 2).

Furthermore, the analysis of healthy plant and fruit material spiked with phomenone led to a recovery of 85%. Finally, the

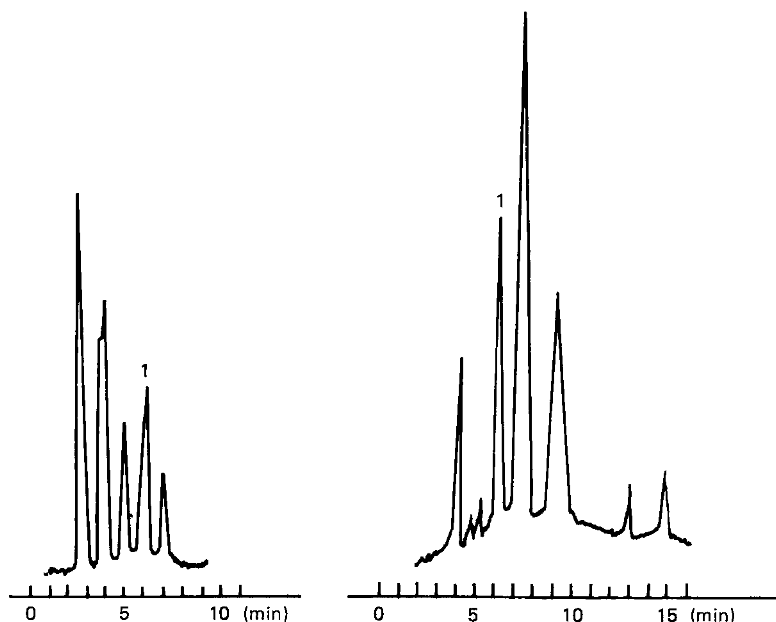

Fig. 3 - a) Chromatogram of extract from tomato fruits added with phomenone.

b) Chromatogram of extract from Tomato plant infected by Phoma destructiva.

1, phomenone (6,22 min). Column: Perkin-Elmer RP-18 (cm 25x0.46, 10 μm) at 20°C. Mobile phase: water-ethanol (70:30, v/v), at 0.9 ml min^{-1} flow rate. Detector: UV absorption at 240 nm.

analysis performed on the extracts from Tomato plants infected by P. destructiva and on the extracts from tomato fruits spiked with phomenone led to identification of phomenone, by the appearance of an individual peak at 6.22 min (Fig. 3).

The nature of the compound responsible of this signal was confirmed by co-injection of the positive extracts with a reference sample of

phomenone. The spectroscopic, physicochemical and chromatographic properties of a purified fraction of infected plant extracts collected from the liquid chromatograph at the phomenone retention time were identical to those of a reference sample of toxin. Moreover, the contaminated extracts of the Tomato plants, treated with a mixture of acetic anhydride and pyridine, showed, by HPLC analysis, a compound with chromatographic properties identical to a pure sample of acetylphomenone.

In Tomato plants infected by P. destructiva, phomenone was found in amount up to 0.096 mg of toxin per Kg of plant, fresh weight.

Conclusions

High-performance liquid chromatography represents a feasible method for separation and determination of phomenone in infected tomato leaves and fruits.

This technique can be regarded as applicable to agricultural commodities infected by toxigenic Phoma species.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 943-952 (1984)

COMPARISON OF TWO NOVEL TECHNIQUES FOR ELIMINATING LIQUID CHROMATOGRAPHY GRADIENT GHOST PEAKS

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INTRODUCTION

Distilled water to organic gradients in reversed phase liquid chromatography are often plagued with UV-detectable "ghost" peaks that can obscure sample peaks and complicate interpretation of results.[3,4] These contaminants usually come from the distilled water[3] although it is possible for them to originate in the organic eluent.[4] The ghost-peak problem can be eliminated by using specially prepared "LC-grade" water, although this is expensive. In addition water can be cleaned with the Milli-Q ion-exchange system, containing a carbon absorption column[5] but not where these systems contain buffers or organic amine eluting agents such as triethylamine phosphate. Gurkin and Ripphahn have shown that a C-8 silica column can be used off-line in a low-pressure mode to clean water.[6]

A very effective on-line clean-up system that can be used with buffered or amine containing eluents was recently described.[4]

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This involves inserting a large particle "porous polymer" styrene-divinylbenzene column in the high pressure aqueous line before the gradient mixer. This "eluent conditioner column" removes ghost peaks contributed not only by the water but also from such buffer additives as triethylamine, phosphoric acid, sodium hydroxide, boric acid and ammonium hydroxide.

Several publications have shown that UV irradiation of batches of distilled water also eliminates UV detectable ghost-peaks.[7,8] Two batch UV irradiators are now commercially available (Barnstead, Boston, MA, U.S.A. and Photronix, Medway, MA, U.S.A.).

This work shows that a flow-through, on-line UV irradiator may also be used to eliminate water-derived ghost peaks with the advantage that no water transfers are needed and the usual 1/2 to 3 hour wait[4] is not required. This on-line UV irradiator is shown here to be as effective for eliminating water derived ghost-peaks as the aqueous eluent conditioner column if water only is used as an eluent.

The flow through irradiator described herein is made by using Teflon tubing wrapped around the UV lamp in a batch irradiator for the water flow line. Scholten _et. al_.[9] showed earlier that Teflon is transparent to UV light.

Low wavelength 189 nm UV irradiation of water is believed to eliminate ghost peaks by photo-oxidizing trace level contaminating organic compounds. The principle of photo-oxidation is well established in organic chemistry[10] and in areas such as the LC halide photo-conductivity detector[11] and in the organic carbon analyzer[12] for environmental analysis.

EXPERIMENTAL SECTION

Materials and Methods

Figure 1 shows the modified Photronix (Medway, MA, USA) Model 816 HPLC batch UV irradiator. The flow-through section was constructed by wrapping Teflon tubing (0.15 cm I.D. x 0.30 cm O.D. part 200-32, Rainin Instrument Co., Woburn, MA, USA) in 33 coils (205 cm total length) around the 35 watt UV lamp used in the Photronix reservoir. The ends of the Teflon tubing were tied in place with wire. The hold-up volume is ca. 3.6 mL. The lamp with the coiled tubing was then inserted back into the protective quartz test tube sealed to the top of the Photronix reservoir. Further the final chamber can be used to batch clean water for buffer preparation for one LC and simultaneously operate as a flow-through irradiator for a second LC. Additionally, water in the batch irradiator acts as a coolant for the flowing water system.

A Hewlett-Packard (Avondale, PA) 1084D liquid chromatograph was used and at least four replicate runs were obtained for each gas sparging or lamp on-off condition. Cylinder gas bubbled through a frit, was used for each condition of oxygen saturation, air saturation, or helium sparging. Distilled water (Belmont Springs, Belmont, MA, USA) was from 5 gallon polyethylene bags in cardboard boxes. Acetonitrile was HPLC grade (Burdick and Jackson, Muskegon, MI, USA).

For work using the porous polymer, this column was installed in the aqueous eluent line just after the pump and before the gradient mixer. This 25 x 0.46 cm column was dry tap-packed with 150-200 um Chromosorb 101 styrenedivinylbenzene porous polymer (Supelco, Bellefonte, PA). Each morning this column was cleaned

FIGURE 1. Photronix HPLC reservoir with the lid raised to show the Teflon tubing for the flow-through UV irradiator coiled around the lamp and running to an auxilliary flask of distilled water.

by turning a valve arrangement so that ca. 15-25 mL of acetonitrile flushed the aqueous line. A dozen flush cycles between aqueous and acetonitrile may be required for initial cleaning.

RESULTS AND DISCUSSION

Figures 2 and 3 show that a flow-through irradiator having a water residence time of only 0.9 min works very well. Figure 2 shows that the 254 nm detectable ghost peaks (lower row) are removed when the flow-through irradiator is turned on (upper row). This is true for flows as high as 4 ml/min for water saturated with oxygen (chromatogram b), water saturated with air (chromatogram d) and for water "degassed" of nitrogen and oxygen by sparging with helium (chromatogram f). For helium sparged water passed through the on-line UV irradiator, the disappearance of peaks found with 254 nm UV detection was also shown with detection at 210 nm (Figure 3, chromatogram a, lamp off. vs. chromatogram b, lamp on). Since levels of dissolved oxygen should be low in this case, the mechanism of "photo-oxidation" may be more complicated than the simple reaction of major levels of oxygen with the organic impurities under the influence of UV light. Also with 210 nm detection, the ghost peaks were shown to disappear using the flow-through UV irradiator with air saturated water (chromatogram c, lamp off vs. chromatogram d, lamp on) but in this case the baseline shift between 4% and 96% acetonitrile was less than with helium degassing. Part of this better match between the initial 4% acetonitrile baseline and the final 96% acetonitrile baseline

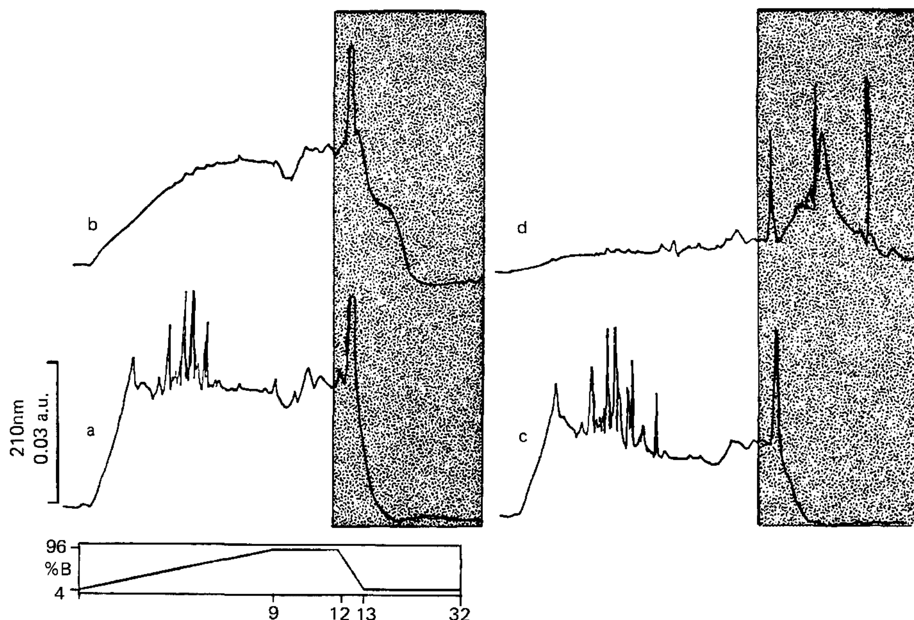

FIGURE 2. Gradient liquid chromatograms showing that 254 nm detectable ghost peaks from commercial distilled water (lower row) are eliminated with the Teflon flow-through irradiator (upper row) for water saturated with oxygen (left), water saturated with air (middle), and water degassed with helium (right). Flows of 4 ml/min are used with a reversed phase Waters C-18 Radial Comparession column (10 x 0.5 cm), 10 micron particles. The gradients are from 0 to 96% acetonitrile using the gradient shape shown at the bottom with initial isocratic water for 19 min (76 ml). The hatched portion is on the return of the gradient.

comes from the fact that turning on the irradiator causes the 4% acetonitrile baseline to shift up by 0.009 absorbance when air saturated vs. a shift up to 0.005 absorbance when helium degassed. The cause of these shifts was not investigated.

In runs similar to those shown in Figure 2 (using 4 mL/min), but using 2 mL/min and 1 mL/min (irradiated residence times of 1.8 and 3.6 min, respectively), the baseline showed nearly complete

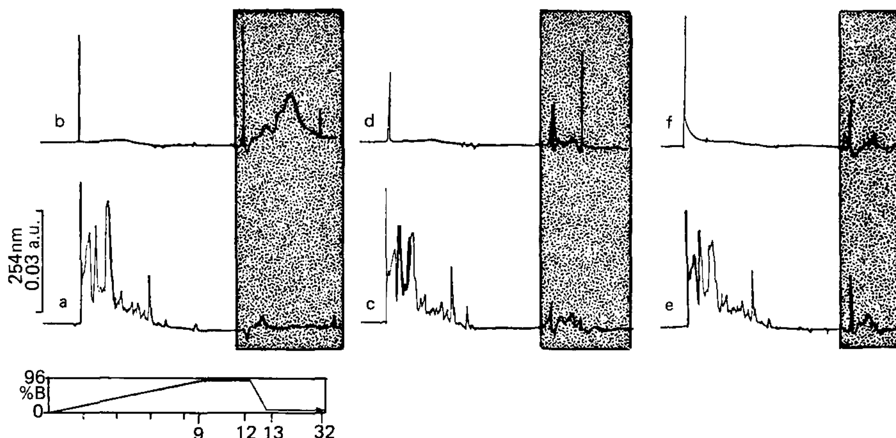

FIGURE 3. Gradient liquid chromatograms showing that 210 nm detectable ghost peaks from commercial distilled water (lower row) are eliminated with a Teflon flow-through irradiator (upper row) for water degassed with helium (left) and water saturated with air (right). Conditions are as in Figure 2 except that the gradient is from 4 to 96% acetonitrile and detection is at 210 nm.

absence of ghost peaks for all three conditions of gas purging (oxygen, air, and helium). Thus it appears that major levels of oxygen are not necessary to "photo-oxidize" the contaminants in water.

One complication was found with air-saturated water when using slow 1 mL/min flows with the on-line UV irradiator. Gas that bubbled out of solution as the water warmed in the irradiator caused occasional flow irregularities. Bubble formation was not observed with the helium-degassed water. Thus helium-degassed water allows slow flows through the irradiator with no flow irregularities.

It should also be stressed that UV irradiation is not useful with water containing phosphates or organic additives. Potassium

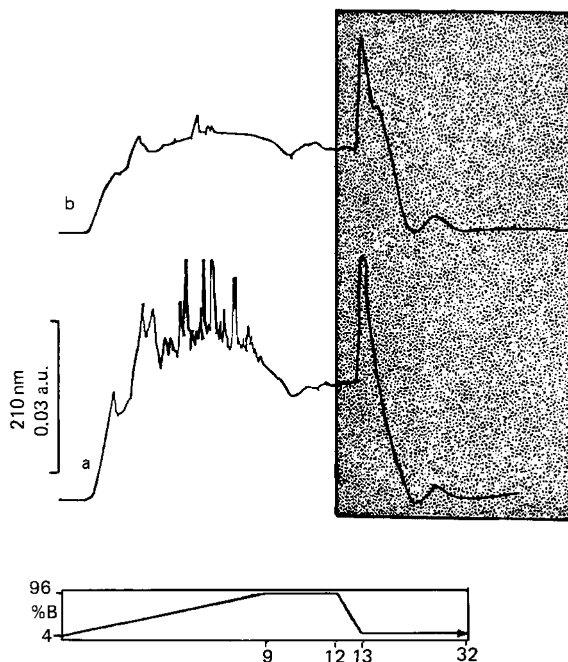

FIGURE 4. Gradient liquid chromatograms (4 to 96% acetonitrile) showing that 210 nm detectable ghost peaks from commercial distilled water (a) are mostly eliminated with a porous polymer (Chromosorb 101) "eluent conditioner column" in the aqueous line (b). Conditions are as in Figure 2 except that a 3 cm Brownlee C 18 guard column and a 15 cm Zorbax C 18 column are used at 3 ml/min and no UV irradiator is used.

phosphate buffer decomposes on UV irradiation forming highly UV-absorbing species that "break-through" the C-18 column after many gradient cycles.

When a porous polymer eluent conditioner column was placed in the aqueous eluent line after the pump (Figure 4, chromatogram b), the 210 nm ghost-peak level was found to be similar to that found with helium degassed water (Figure 3, chromatogram b). The disad-

vantage of this porous polymer eluent conditioner column is that it requires re-conditioning about every 2 days with a 5 min acetonitrile wash. However, it does remove most ghost peaks contributed by buffer or amine additives put in the water.

This work shows that for liquid chromatography using water only as one reversed-phase gradient component, ghost peaks can be eliminated by simply continuously degassing the water with helium and running the water through the flow-through on-line UV irradiator described here. Showing similar effectiveness is the porous polymer eluent conditioner column which in addition removes most ghost peaks contributed by buffer or amine additives.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. Leon E. Rubin of Polaroid for his kind support of this work.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 953-960 (1984)

ANALYSIS OF PENTACHLOROPHENOL IN DRINKING WATER AND HUMAN URINE BY HPLC WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

High performance liquid chromatography with electrochemical detection (HPLC-EC) was applied to the analysis of pentachlorophenol (PCP) in drinking water and human urine. Lower detection limits for PCP in drinking water was approximately 1-5 ppb while the chlorinated phenol could be detected in human urine at about 10 ppb concentration. Analyses of PCP in drinking water can be made with no sample pretreatments, while analyses involving human urine required acid hydrolysis, absorption on an anion exchange resin, and desorption with methanol. In each case dramatic savings in analysis times and increases in sample through-put are realized using analysis based on HPLC-EC rather than the more traditional procedures relying on gas chromatography with electron capture detection. Method sensitivities were approximately equivalent to methods using gas chromatography.

INTRODUCTION

Pentachlorophenol is a widely used pesticide and preservative. Its use is especially prevalent in the southern portions of this country, particularly in the hot, high humidity regions of the Gulf and Atlantic coasts. It is employed throughout these areas for treating wood products to prevent attack by microorganisms. Because of its wide use PCP has become a ubiquitous environmental pollutant, and has been found to be widely present in human urine in this country at low to mid ppb (ng/ml) concentrations (1). Accumulation of PCP in the human population of this country presumably results from contact with wood products such as paper and cardboard, ingestion of contaminated food-stuffs or water supplies, and contact with materials used in construction of dwellings for human habitation. Like many halogenated organic compounds PCP is toxic and readily accumulates in high lipid-content animal tissues.

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Traditional protocol (1-3) for analysis of PCP and related chlorophenols in human urine is based on gas chromatography with electron capture detection (GC-EC), a technique not notably compatible with the aqueous media of drinking water and human urine. The initial step in the protocol for urine consists of acid hydrolysis to free PCP from its glucose or sulfate conjugates. Free phenol is extracted with benzene (a suspected carcinogen), methylated with diazomethane (a toxic explosive suspected carcinogen) and the resulting fraction analyzed by gas chromatography or purified by column chromatography and then subjected to analysis. These wet chemical manipulations may require several hours to effect. Analysis of municipal drinking water could be accomplished using the above procedure with elimination of the acid hydrolysis step.

Analysis of aqueous media for PCP was approached in the present study by substituting HPLC-EC for GC-EC. Since HPLC-EC typically employs reversed phase columns with mobile phases of aqueous buffers mixed with varying amounts of water-miscible organic solvents to control retention the technique is compatible with urine or other aqueous samples. In addition, electrochemical detection is highly sensitive to phenols. Thus, it seemed reasonable that an analytical method based on HPLC-EC might greatly reduce sample preparation time and markedly increase sample through-put.

Other researchers have appreciated the promise held by HPLC for analysis of biological materials for chlorinated phenols. Lores, Edgerton, and Moseman used HPLC-EC to confirm the presence of a large number of chlorinated phenols in human urine after primary analysis by gas chromatography(4). Earlier these researchers had reported recoveries of chlorinated phenols (10 bbp) from fortified urine of from 79 to 88% after adsorption and desorption from XAD-4 resin into 10% 2-propanol in hexane (5). During the latter study GC-EC was employed as the analytical method.

In this study we surveyed recoveries and background interferences arising during isolation by resin adsorption and desorption of pentachlorophenol in fortified drinking water and urine and during isolation of PCP from these matrices by acid-base solvent extractions.

MATERIALS AND METHODS

High Performance Liquid Chromatography

HPLC experiments were carried out using several modular liquid chromatographs assembled in this laboratory. Mobile phase delivery systems used single piston minipumps (Laboratory Data Control, Riveria Beach, FL) with pulse dampners (Handy and Harman Tube Co., Norristown, PA) and pressure gauges (Alltech Associates, Deerfield, IL). The electrochemical detectors consisted of glassy carbon electrodes maintained at 1.0 V vs. Ag/AgCl reference electrodes by a commercial potentiostat (Bioanalytical Systems, West Lafayette, IN), a model 174 Polarographic Analyzer (Princeton Applied Research, Princeton, NJ), or a potentiostat constructed in the laboratory with a circuit similar to one described earlier (6). Chromatography columns were 4.6 mm i.d. by 250 mm stainless steel obtained commercially or packed in this laboratory. Spherisorb ODS, 5 μm, (Alltech Associates), laboratory prepared and packed trimethylsilyl reversed phase on 8 μm sperical silica gel, and cyanopropylsilyl bonded phase on 10 μm silica (Alltech Associates) gave approximately equivalent retention times for PCP when mobile phases of 0.1 M $NH_4H_2PO_4$ with 70, 50, and 30% acetonitrile, respectively, were pumped through the columns at 1 ml/min.

Sample introduction was by valve-loop injection using a 7000 psi valve (Valco Instruments, Houston, TX).

Acid Hydrolysis Of Urine

Urine samples were hydrolyzed according to a published procedure (4) or by the use of sulfuric acid at 100°C (3 ml conc. H_2SO_4, 5 ml urine, 30 min). Hydrolyses were conducted in culture tubes with teflon-faced screw caps.

Solvent Extraction And Resin Adsorption Of PCP From Hydrolyzed Urine

Solvent extractions of PCP from urine (and from distilled water) was performed according to the method outline in Fig. 1. Extractions were carried out in screw-capped culture tubes with as few sample transfers as possible.

Anion exchange resins (AG1-X2, and AG1-X8) and BioBeads S-X8 were purchased from BioRad Laboratories, Richmond, CA and Amberlite XAD-2 and Tenax from Alltech Associates. The resins were washed with methanol (3 X 5 ml) and with distilled water (3 X 5 ml) after being packed into disposable Pasteur pipets.

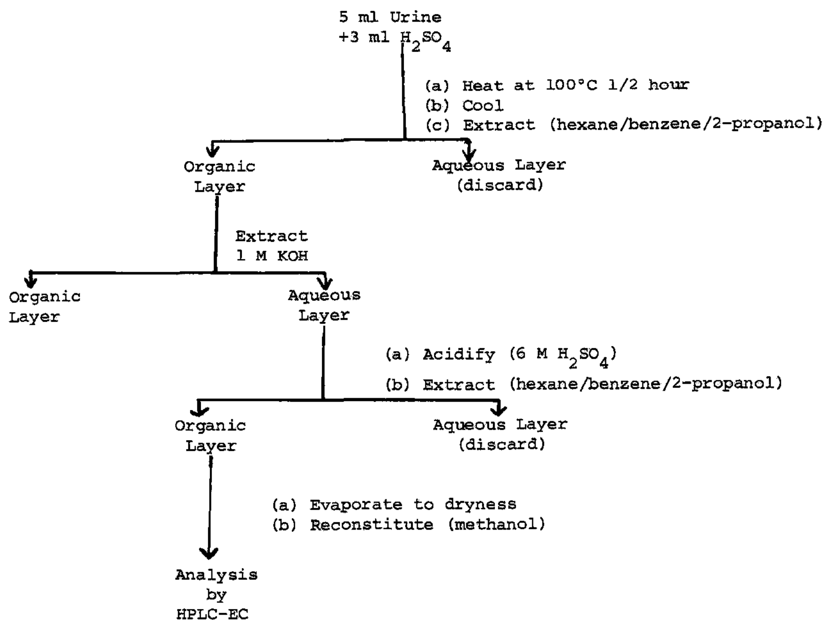

Figure 1. Flow chart for Hydrolysis and Extraction of Urine Samples.

The resin columns (ca. 3 cm bed height) were used to adsorb 5 ml samples of urine hydrosylate and washed with deionized water (3 X 5 ml). Adsorbed material was desorbed with 5 ml of methanol and the elute was analyzed directly by HPLC-EC. Recoveries were estimated by comparison with PCP standards of appropriate concentration.

RESULTS

To test the utility of HPLC-EC for analysis of PCP in aqueous solutions we first ran a series of drinking water samples to which varying amounts of the chlorinated phenol had been added. The water samples were injected directly with no sample clean-up. Chromatograms from analysis of 20 µl of drinking water spiked with 20 ppb PCP (A) and drinking water without added phenol (B) are shown in Fig. 2. These results indicate that analysis of about 2 ppb PCP in drinking water, is possible with the method as described without any pre-analytical sample manipulation. Furthermore, limit of detection could be easily increased by

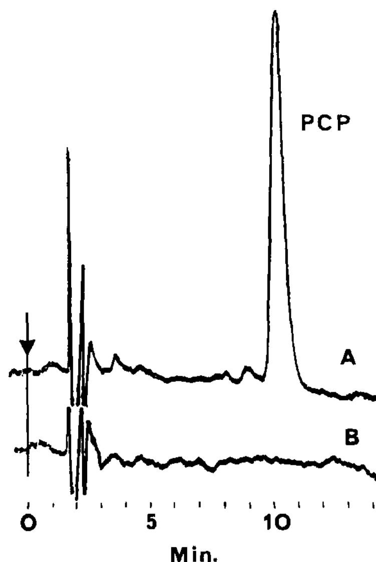

Figure 2. HPLC-EC analysis of (A) 20 ppb PCP in municipal drinking water and (b) water without added PCP. Conditions: 50 μl injections; sensitivity 10 nAFS; cyanopropyl reversed phase column; 25% CH_3CN in 0.1 M $NH_4H_2PO_4$, pH 4.0; 1.2 ml/min.

using shorter chromatography columns, chromatography columns with smaller internal diameters, larger sample sizes, or a combination of these or other modifications. Mid-ppt measurements should be attainable.

We next turned to human urine as a sample matrix. While urine can be injected directly onto the chromatography columns, the many protein constituents of the materials will cause rapid degradation of expensive columns. Furthermore the many electroactive constituents will lead to high backgrounds and decreased sensitivity, and since a substantial portion of PCP is present in urine as conjugates, much of the material will not be detected.

We first used an acid hydrolysis step followed by an acid/base/solvent extraction procedure as outlined in Fig. 1. The resulting chromatograms from HPLC-EC analysis of human urine (A) and human urine which had been spiked with 100 ppb PCP (B) are shown in Fig. 3. While PCP is easily identified and measured at this level, there are considerable electroactive substances isolated during

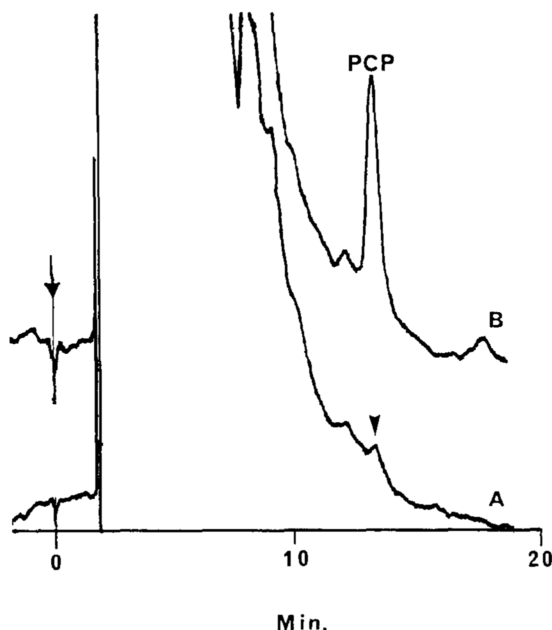

Figure 3. HPLC-EC analysis of (A) hydrolyzed human extract and (B) extract of hydrolyzed human urine fortified with 100 ppb PCP. Conditions: 20 µl injections; sensitivity, 50 nAFS; trimethylsilyl reversed phase column; 50% CH_3CN in 0.1 M $NH_4H_2PO_4$, pH 4.0; 1.0 ml/min.

work-up. These substances prevent substantial increases in sensitivity and appear to cause fouling of the working electrode. Sample work-up requires considerable time and effort.

The utility of resin adsorption of acid hydrolyzed fortified urine was then tested. Urine samples fortified with varying amounts of PCP were hydrolyzed using the procedure outlined in Fig. 1, or as previously described (4). The urine was passed through short resin columns as described in the methods section, the columns were washed with water and the PCP was then desorbed with methanol. The methanolic solutions were analyzed directly by HPLC-EC. A representative chromatogram from the analysis of human urine fortified with 100 ppb is given in Fig. 4. The resin employed in obtaining this chromatogram was AG1-X2 anion exchange resin which gave the best combination of high recovery and

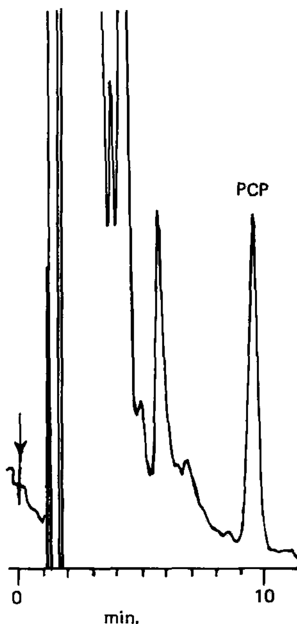

Figure 4. HPLC-EC analysis of PCP (100 ppb) isolated from fortified human urine by adsorption on AG1-X2 resin as described in the text. Conditions: 50 µl injection; sensitivity 50 nAFS; Spherisorb ODS column; 70% CH_3CN in 0.1 M $NH_4H_2PO_4$, pH 4.0; 1.0 ml/min.

relative freedom from interfering materials. Recoveries from fortified urine (200 ppb level) were 108 ± 9% while recoveries from distilled water (200 ppb level) processed according to this method were 93 ± 3% (mean ± sample standard deviation of ten or more samples).

DISCUSSION

The combination of analyte isolation by anion exchange resin adsorption and HPLC-EC analysis allows monitoring of human urine for PCP at low ppb levels. The pre-analytical sample manipulations are simple, they involve few transfers from one container to another, and employ a minimum of reagents. There are no steps involving evaporation of organic solvents, which is both expensive and time consuming, and derivatization is not required. Because of the simplicity and speed of the method and its high sensitivity it is a logical candidate for monitoring PCP contamination in large populations.

ACKNOWLEDGMENT

This research was supported by cooperative agreement No. 808055010 between the United States Environmental Protection agency and the University of Southern Mississippi.

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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) ASSAY METHOD FOR CEFTIZOXIME

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ABSTRACT

We improved a high-performance liquid chromatographic method for the quantitative determination of ceftizoxime in human serum and urine using cefotaxime as internal standard. It employs a μ Bondapak Alkyl Phenyl column, elution with acetonitrile-phosphate buffer and measurement of UV absorption at 254 nm. Results obtained using the HPLC assay were compared to those obtained using a microbiological assay. The correlation coefficient was 0.987 (n:25). The method is rapid, accurate and reproducible with a sensitivity of 2.5 μg/ml of ceftizoxime. Cefotaxime and its major metabolite, the desacetylcefotaxime, can also be quantitated by this procedure.

INTRODUCTION

Ceftizoxime (FK 749, SK & F 88373-Z){sodium (6R, 8R)-7-{(Z)-2-(2-imino-4-thiazolin-4-yl)-2-methoxy-iminoacetamido}-8-oxo-5-thial-azabicyclo {4.2.0} oct-2-ene-2-carboxylate} is a new parenteral third-generation cephalosporin. The structural formula is shown in Figure 1. Suzuki et al. (1) used high-performance liquid chromatography (HPLC) for the analysis of ceftizoxime in rat serum, bile and urine. This method did not use an internal standard and need to be modified to quantitate ceftizoxime in human samples. We report here an improved method for determination of ceftizoxime in human serum and urine, using cefotaxime as an internal standard. The total time needed to complete the analysis of ceftizoxime in individual serum samples is about 20 minutes and the amount of serum required for the assay is 0.5 ml.

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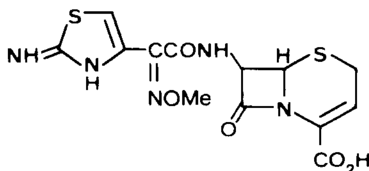

Fig. 1. Chemical structure of ceftizoxime

MATERIALS AND METHODS

Sodium ceftizoxime was obtained from Smith Kline and French Laboratories, Philadelphia, PA (lot number, 81225). Cefotaxime (lot number RP-3717) and desaceytlcefotaxime (lot number RO28628A) were kindly provided by Hoechst-Roussel Pharmaceuticals Inc, Sommerville, NJ. Acetonitrile, potassium phosphate monobasic and phosphoric acid 85%, all HPLC grade, were purchased from Fisher Scientific Co, Pittsburg, PA.

The chromatographic system consisted of a model 6000 A solvent delivery system, a U-6K injector and a variable - wavelength model 450 detector (Waters Associates, Milford, MA.) set at 254 nm and 0.1 A.U.F.S. Separation was accomplished on a μ Bondapak Alkyl Phenyl column (30 cm of length X 4 mm I.D.; Waters Associates, Milford, MA.). A mixture of 13% acetonitrile in 0.02 M (pH 2.6) phosphate buffer was used as the chromatographic eluant, at a flow-rate of 1.5 ml/min (pressures of 1,500 to 2,000 psi). The buffer solution was filtered through an HA 0.45-μ filter (Millipore Corp. Bedford, MA.) and the acetonitrile through a FH 0.45-μ filter (Millipore Corp. Bedford, MA.). The mobile phase was deaerated with an ultrasonic water bath for 20 minutes. The elution profile was recorded on a Perkin-Elmer recorder, (model 023 Perkin-Elmer, Norwalk, CT) using a chart speed of 30 cm/h and was set at 1.0 mV.

A standard solution of sodium ceftizoxime was prepared in HPLC-grade water at a concentration of 1,000 μg/ml. This solution was further diluted ad 10 ml with pooled lyophilized frozen human serum (freshly reconstituted with ten ml of HPLC-grade water) to give concentrations of 160, 120, 80, 60, 30, 20 and 10 μg/ml. These concentrations represent serum concentrations to be expected from the administration of 1 g dose of ceftizoxime (2). The standard solution was freshly prepared each day and kept under refrigeration when not in use. The standard solution of sodium cefotaxime, the internal standard, was prepared similarly.

After adding 75 µl of the internal standard solution (sodium cefotaxime, 1,000 µg/ml) to a 0.5 ml aliquot of serum sample containing ceftizoxime, an equal volume (0.5 ml) of acetonitrile was added. The sample was vortexed for 20 sec at maximum speed, to ensure complete protein precipitation. The mixture was centrifuged at 3,000 rpm for ten minutes. A supernatant aliquot of 10 or 20 µl was injected into the HPLC.

Fresh urine from a healthy volunteer (Clinitest R, negative) to which graded concentrations of ceftizoxime were added (150, 100, 75, 50, 25, 12.5 and 6.25 µg/ml) was used to construct the urine calibration curve. These urine concentrations are expected from the administration of 1 g dose of ceftizoxime (3).

Unknown urine samples were diluted with HPLC-grade water to a concentration in the range of the standard curve. To 0.5 ml of diluted urine, 75 µl of sodium cefotaxime solution (1,000 µg/ml), as internal reference standard, and 0.1 ml of phosphate buffer-acetonitrile 13% mixture were added. The volume injected varied between 5 to 20 µl.

To compare the HPLC assay and the microbiological assay, we used 25 serum samples from human volunteers who had received a single 1 g dose of ceftizoxime (4), and pooled fresh serum to which known amounts of ceftizoxime were added. The microbiological assay was performed using a disc agar diffusion method with _Bacillus subtilis_ ATCC 6633 spores that were seeded into penicillin assay seed agar prepared in 1% phosphate buffer, pH 6. Incubation was at 30°. Standards for assay of serum were prepared in pooled human serum devoid of background antimicrobial activity. Assays were performed according to the method of Fare et al (5). The microbiological assay was linear between 0.75 and 5.0 µg/ml.

RESULTS AND DISCUSSION

Figure 2 shows a typical chromatogram of ceftizoxime and cefotaxime, these peaks were not disturbed by other serum components. Typical chromatogram of urine sample is shown in Figure 3; no interfering peaks can be observed although the sample was injected without prior clean up. The retention time was 6.0 min for ceftizoxime and 8.5 min for cefotaxime. As shown in Figure 4a and 4b, a linear relationship exists for known concentrations of ceftizoxime in serum and diluted urine respectively over a 2 to 200 µg/ml range as plotted against the corresponding peak height ratios. Linear regression analysis of the standard calibration

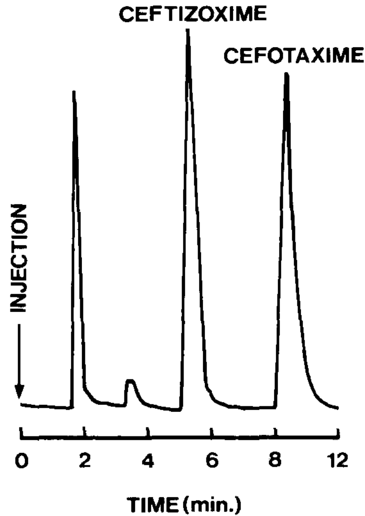

Fig. 2. Typical chromatogram for a serum sample containing 108.5 μg of ceftizoxime/ml

lines yielded the respective equations: y = 0.010 x - 0.021 (r, 0.998) for serum, and y = 0.023 x - 0.031 (r, 0.998) for urine. These correlations indicated an excellent linearity. The precision of the HPLC method was determined by assaying five replicate serum samples containing 60 and 120 μg/ml of ceftizoxime. Each sample was assayed in triplicate. The low coefficients of variation obtained (2.04 and 1.93%) show the precision of this HPLC method. Reproducibility data were obtained from five frozen serum samples containing 60 and 120 μg/ml of ceftizoxime and thawed on five different days; the coefficients of variation from day to day were 3.70 and 2.75% for both ceftizoxime concentrations. Three different ceftizoxime concentrations in pooled human serum and in phosphate buffer (pH, 7.4) were used to determine the precipitation recovery ratio. The protein precipitation recovery ratio was 1.04 ± .05 (mean ± S.D.).

Some antibiotics commonly used and probenecid were tested for interference with ceftizoxime and cefotaxime peaks; serum samples containing gentamicin (10 μg/ml), tobramycin (10 μg/ml) and probenecid (1,0 g given P.O. to healthy volunteer) (4) did not interfere with the analysis. To verify the application of this HPLC method to the serum

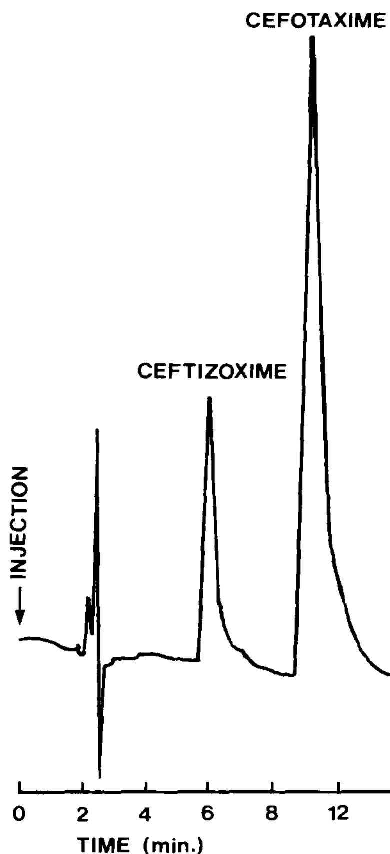

Fig. 3. Typical chromatogram for a urine sample containing 16.3 μg of ceftizoxime/ml.

determination of cefotaxime using ceftizoxime as an internal standard, expected concentrations of desacetylcefotaxime (the active metabolite of cefotaxime) were added to serum samples. The desacetyl metabolite separated very well, with a retention time of 3.5 min. However, ampicillin (20 μg/ml) would interfere with the assay of cefotaxime, with a retention time close to the desacetyl cefotaxime. The sensitivity limit of assay for serum samples was 2.5 μg/ml when a signal-to-noise ratio of 2 or greater was used as a criterion for a significant response. This is in contrast with the determination limit reported by Suzuki and

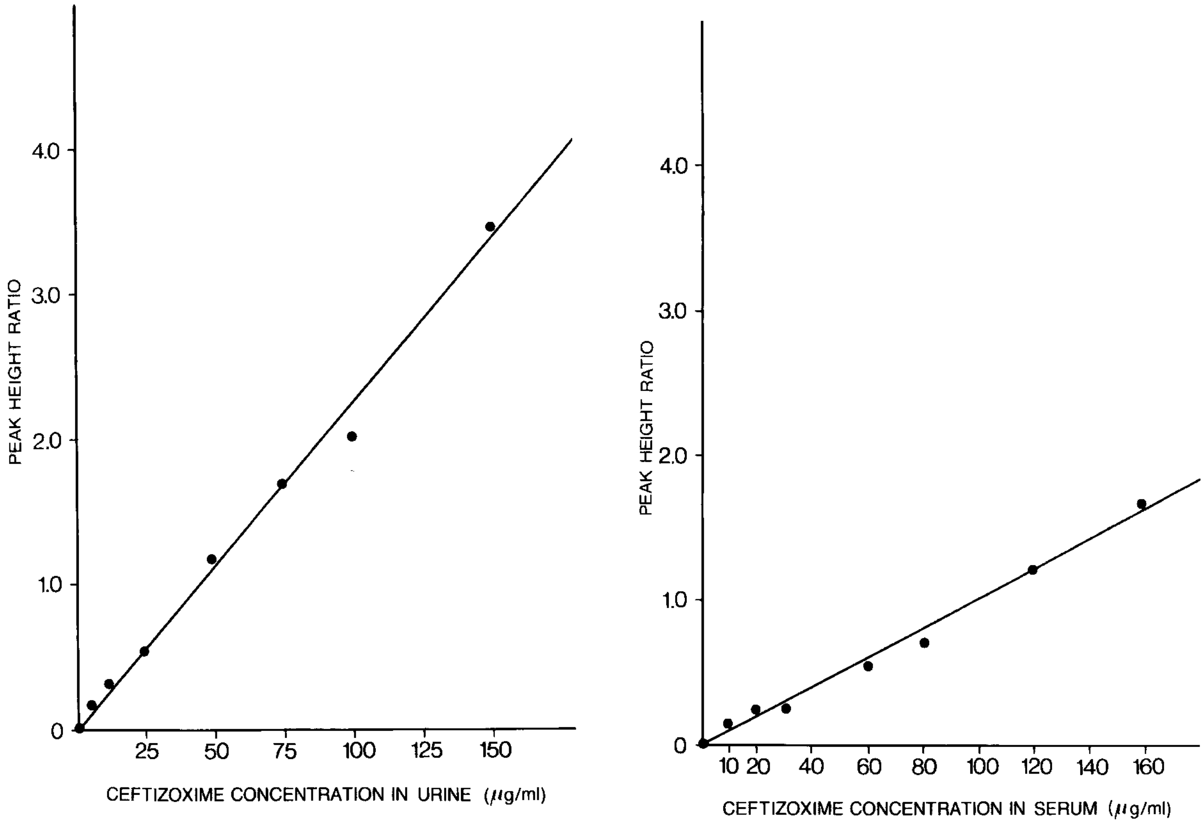

Fig. 4. Standard calibration curve of ceftizoxime in serum (a) and in diluted urine (b).

TABLE 1

Comparison Between HPLC Determination of Ceftizoxime and Microbiological Assay (25 Serum Samples)

		HPLC (μg/ml)	Microbiological assay (μg/ml)
pt 13, day 2	(0.25 h)	28.3	30.3
	(0.5 h)	43.4	38.8
	(0.75 h)	39.0	34.3
	(1.0 h)	38.6	39.2
	(1.5 h)	46.4	39.0
	(2.0 h)	33.9	31.1
	(3.0 h)	26.1	22.2
	(4.0 h)	17.7	14.9
	(6.0 h)	9.9	8.4
	(8.0 h)	6.6	6.1
	(10.0 h)	6.4	2.2
pt 3, day 1	(0.06 h)	177.1	205.1
	(0.25 h)	74.1	99.3
	(0.5 h)	57.4	56.3
pt 3, day 1	(0.75 h)	41.0	40.4
pt 2, day 1	(3.0 h)	14.6	11.3
	(10.0 h)	2.9	1.0
pt 10, day 1	(1.0 h)	36.2	27.0
	(1.5 h)	32.7	25.4
blank		0	0
blank		0	0
spiked plasma	(250 μg/ml)	248.2	226.6
" "	"	260.2	294.7
" "	(60 μg/ml)	60.5	59.6
" "	"	60.3	64.6

coworkers (0.2 μg/ml for serum at 0.01 A.U.F.S.) (1), but this may be explained by a larger dilution effect in the sample preparation (1.0 ml vs 0.3 ml) and a wider criterion for the evaluation of the limit of sensitivity.

The Table 1 shows the actual data obtained by microbiological assays. The comparison of these two methods yielded a correlation coefficient of 0.987. In the lower range of ceftizoxime serum concentrations, the microbiological assay showed slightly lower values than did the HPLC assay. The last ceftizoxime concentration used in constructing HPLC standard serum curve was 10 μg/ml; this may explain the lack of precision of the HPLC method at low ceftizoxime concentrations.

Although microbiological methods are more widely used they have their share of disadvantages. The HPLC assay generated results within 10-15 minutes after starting a limited number of samples; microbiological assays are more time consuming. When an antibiotic has active metabolite(s) or forms active decomposition products or when two antibiotics are administered, the highly specific HPLC methods are preferable(6-8).

The HPLC method described here may allow simultaneous determination of ceftizoxime and cefotaxime, which has distinct advantage over conventional methods for studies of comparative tissue penetration.

ACKNOWLEDGEMENTS

We would like to thank the late Tracy F Woodman 3 rd and Dr George P. Lewis for their guidance and support.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 969-977 (1984)

A SIMPLE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROCEDURE FOR THE DETERMINATION OF N^1-METHYLNICOTINAMIDE IN URINE

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ABSTRACT

A simple high performance liquid chromatography procedure for the determination of N^1-methylnicotinamide in urine samples is described. The procedure eliminates the need for extraction or ion exchange clean-up of urine samples prior to their analysis. Human and rat urine samples can be analyzed for N^1-methylnicotinamide directly following a simple pH adjustment. The metabolite was separated and quantitated on a 5μ Ultrasphere ODS (C18) reverse-phase column. The mobile phase contained 10 mM K_2HPO_4 and 10 mM sodium 1-octanesulfonate in 8% acetonitrile at pH 7.0. The system has been used to conduct over 1000 determinations during a period of three months without reduction in performance or efficiency.

INTRODUCTION

Recently high performance liquid chromatography (HPLC) has been used to measure niacin and two metabolites important in assessing niacin status in humans, N^1-methylnicotinamide (N^1MN)

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and N^1-methyl-2-pyridone-5-carboxamide (2-PYR) (1,2). In 1981, Sandhu and Fraser (3) described an HPLC method for the measurement of niacin metabolites in rat urine. Samples were purified by a modified extraction procedure of Hengen et al. (4), and analyzed by reverse-phase HPLC using a methanolic potassium citrate buffer as the mobile phase. In 1982, Carter (5) described an HPLC method for the quantification of niacin metabolites in human urine. Interfering substances were removed from the samples by anion exchange column chromatography prior to HPLC analysis. N^1MN was analyzed on a reverse-phase column with a mobile phase containing 10 mM potassium phosphate, 5 mM sodium 1-octanesulfonate as an ion pairing agent, and 10% acetonitrile by volume at pH 7.0. The same column was used for 2-PYR analysis with a mobile phase containing 10 mM potassium phosphate, 2% acetonitrile, and no sodium 1-octanesulfonate. Because the ion exchange clean-up step resulted in a 3- to 25-fold dilution of the samples, Carter (5) suggested that for very dilute urines or samples from deficient subjects, the purified samples may require lyophilization prior to HPLC analysis.

This paper describes a modification of Carter's HPLC procedure which eliminates the need for extraction or ion exchange clean-up of samples prior to N^1MN analysis by HPLC. Human and rat urine samples can be analyzed directly, with minimal dilution and without lyophilization.

MATERIALS AND METHODS

Samples

Random urine samples or 24-hour collections from normal subjects admitted to the Human Nutrition Unit of this Center for metabolic studies, and 24-hour urine collections from adult male Wistar rats fed either a corn-based control diet or a corn-based niacin-deficient diet were used.

Sample Preparation

One-tenth mL of a concentrated buffer solution [0.5 M K_2HPO_4 and 0.25 M sodium 1-octanesulfonate (Eastman Kodak Company, Rochester, NY), pH 7.0] was added to 0.25 - 0.75 mL urine and adjusted to pH 7.0 with dilute H_3PO_4 or KOH as necessary. Distilled water was added as required to provide a final volume of 1.0 mL. The sample was then vortexed and filtered through a 0.2 micron nylon filter (Rainin Instrument Company, Woburn, MA) into a vial for HPLC analysis.

Standard Solutions

The solutions used to construct the standard curve were prepared by mixing graded amounts of N^1MN stock solution (0.1 mg/ml; Sigma, St. Louis, MO) with 0.1 mL of the concentrated buffer solution (as above) and diluting each standard solution to 5 mL with water. The prepared standard solutions ranged from 4 to

24 µg/mL. The standard solutions were mixed and filtered through 0.2 micron filters into HPLC vials. The volume of each standard solution injected on the column was 150 µL.

High Performance Liquid Chromatography Analysis

A Hewlett-Packard 1084B liquid chromatograph with a variable wavelength ultraviolet detector (Hewlett-Packard, Santa Clara, CA) was fitted with a 25 cm x 4.6 mm Altex Ultrasphere ODS (C18) reverse-phase analytical column (Rainin Instrument Company, Woburn, MA) and a 3 cm Brownlee Spheri-5 RP-18 guard column (Rainin Instrument Company, Woburn, MA). The particle size of both columns was five microns. The mobile phase contained 10 mM K_2HPO_4 and 10 mM sodium 1-octanesulfonate in 8% acetonitrile (Burdick and Jackson, Muskegon, MI) at a final pH of 7.0. The buffer was filtered through a 0.2 micron nylon filter and degassed before it was introduced into the HPLC system. Conditions were isocratic with a 1.25 mL/minute flow rate, a 30°C column and mobile phase temperature, and a wavelength setting of 264 nm in the sample cell. The reference cell wavelength was 430 nm. Injection volumes varied from 25 to 150 µL. Urinary N^1MN was quantitated by comparing its peak area with that of N^1MN standards analyzed under the same conditions.

RESULTS

Identification

N^1MN in urine samples was identified by comparison of its retention time with that of an N^1MN standard. Both were about

12.1 minutes, with a small day-to-day variation due to slight differences in mobile phase composition. The retention time for 20 samples run in an 8-hour interval was 12.09 minutes (C.V.=0.2%). For 25 samples run 5 per day on 5 separate days over a 3-week interval, it was 12.16 minutes (C.V.=5.5%). N^1MN standard added to urine samples co-eluted with the urinary N^1MN. Scans of the ultraviolet spectrum between 190 and 350 nm showed the same wavelength of maximum absorbance (264 nm) for N^1MN both in standards and urine samples. Scanning both slopes of the urinary N^1MN peak produced the same wavelength of maximum absorbance (264 nm), providing additional evidence that no other substances were co-eluting with N^1MN. In addition, N^1MN peaks from samples of pooled normal rat urine were collected and reanalyzed using a mobile phase containing no counter-ion and only 2% acetonitrile. In each case the chromatogram corresponded to that of N^1MN standards run under the same conditions.

Standard Curve

A standard curve was constructed by plotting absorbance, expressed in units of area, versus μg N^1MN injected onto the column for five standard points ranging in concentration from 4 to 24 μg/mL. The volume injected was 150 μL. The plot was linear over this range and fit the equation $y=84981x - 8995$, where y is integration units and x is μg N^1MN injected ($r=0.9998$). When multiple 150 μL injections of standard were made, the coefficient of variation at each level was $\leq$ 1.00% (n=5

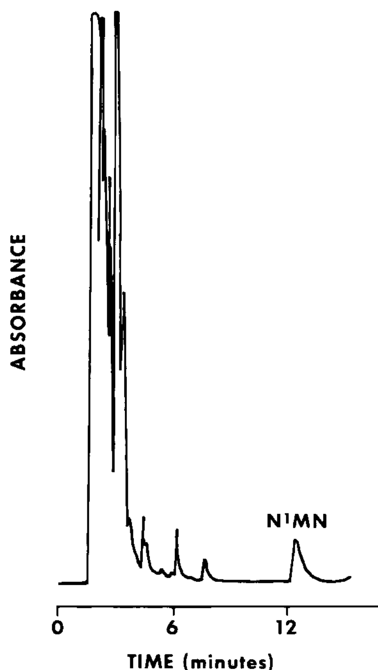

FIGURE 1. Chromatogram of a random sample of normal human urine. A 0.5 mL aliquot of urine was prepared as described in the text. Injection volume of the prepared sample was 25 µL. Attenuation setting of the ultraviolet detector = 2^8.

at each level). For multiple 25 µL injections of a urine sample containing 99 µg/mL, the coefficient of variation was 1.10% (n=10).

Recovery Studies

Recovery studies were performed by determining N^1MN concentrations in urine samples with and without the addition of an equal volume of 0.1 mg/mL N^1MN stock standard to 0.5 mL of each urine sample during the sample preparation step. For three

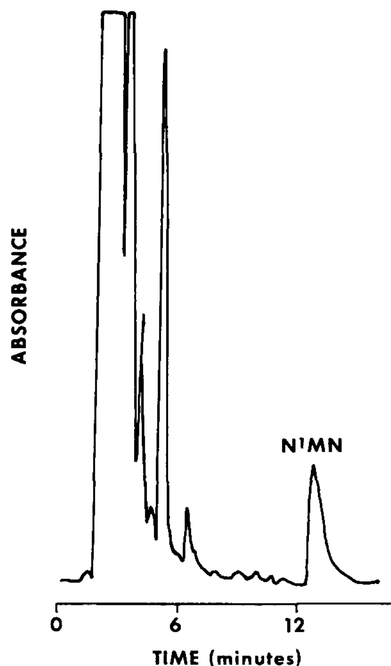

FIGURE 2. Chromatogram of a 24-hour collection of normal rat urine. A 0.5 mL aliquot of urine was prepared as described in the text. Injection volume of the prepared sample was 50 μL. Attenuation setting of the ultraviolet detector = 2^8.

normal rat urine samples, the recoveries of added N^1MN were 105.2%, 100.3%, and 99.7%; for a normal human urine sample, the recovery was 105.2%. Chromatograms of human and rat urine samples without added N^1MN standard are shown in Figures 1 and 2, respectively.

DISCUSSION

The application of HPLC techniques to the determination of urinary niacin metabolites represents an advance in nutrition

status assessement. HPLC methods are simpler, faster, and more sensitive than conventional colorimetric and fluorometric methods (1,5). However, HPLC methods have required a preliminary sample clean-up step by either extraction or ion exchange column chromatography. Direct assay of the urine sample saves time, eliminates the possibility of losses due to poor recovery, and avoids excessive sample dilution. The latter is especially important when working with small urine volumes and/or deficient states. By modifying the mobile phase composition of Carter's analysis procedure, the N^1MN retention time can be increased enough to isolate it from the substances present in urine which would otherwise interfere with HPLC analysis. This is accomplished by doubling the counter-ion concentration from 5mM to 10 mM and reducing the acetonitrile content from 10% to 8%. Using these modifications, no interference is observed in human or rat urine chromatograms, while reproducibility and recovery are maintained. The method has been used successfully to analyze N^1MN in the urine of niacin-deficient rats and in dilute 24-hour collections of human urine. The system described in this paper incorporates a guard column and has been used to conduct over 1000 determinations during a three-month period without significant reduction in performance or efficiency. Attempts to eliminate the ion exchange column clean-up step from Carter's 2-PYR analysis procedure by mobile phase modification were not successful. Changing the acetonitrile concentration did not sufficiently isolate 2-PYR

from the neighboring UV-absorbing substances present in both normal and deficient human or rat urine to allow its quantitation in unpurified samples. An entirely different mobile phase or HPLC column may be required for the analysis of 2-PYR in these samples.

Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 979-987 (1984)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CEFTAZIDIME IN SERUM, URINE, CSF AND PERITONEAL DIALYSIS FLUID

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ABSTRACT

A rapid, sensitive and specific high performance liquid chromatographic method is described for the determination of ceftazidime in serum, urine, CSF and peritoneal dialysis fluid. The procedure employs reversed-phase chromatography, using hydrochlorothiazide as an internal standard. The assay only requires 100 µl of sample with direct injection of diluted urine, CSF, peritoneal dialysis fluid or protein precipitated serum. Stability studies indicate good drug recovery if urine and serum are stored under proper conditions. The method is specific for ceftazidime in the presence of amikacin, gentamicin, kanamycin, tobramycin, methicillin, penicillin G, ampicillin, chloramphenicol and caffeine. The method has been successfully employed in the assay of over 700 samples obtained during human clinical trials.

INTRODUCTION

Ceftazidime (Fortaz™), developed by Glaxo Group Research, is a beta-lactamase resistant cephalosporin antibiotic for parenteral administration. Ceftazidime exhibits good *in vitro* activity

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against a wide range of gram negative pathogens and most gram positive organisms. Particularly noteworthy is its excellent activity against Pseudomonas aeruginosa (1).

The present study concerns the development of a HPLC method for the determination of ceftazidime, using hydrochlorothiazide as an internal standard. The method employs the direct injection of diluted urine, CSF, peritoneal dialysis fluid or protein precipitated serum into the HPLC, and is based on an earlier method developed for cefoperazone (2).

EXPERIMENTAL

Reagents and Materials

HPLC grade acetonitrile and methanol and ACS grade sodium hydroxide and glacial acetic acid were from Fisher Scientific Company (Fair Lawn, NJ). The peritoneal dialysis fluid was Dianeal 137 with 1.5% dextrose from Travenol Laboratories, Inc. (Deerfield, IL). Glaxo Inc. (Research Triangle Park, N. C.) supplied the ceftazidime pentahydrate. Hydrochlorothiazide was supplied by Merck Sharp and Dohme (West Point, PA). Methicillin, ampicillin, penicillin G, kanamycin, tobramycin and gentamicin were obtained from Pfizer Quality Control (Brooklyn, NY). Chloramphenicol was supplied by Parke Davis (Detroit, MI) and amikacin was supplied by Bristol Labs (Syracuse, NY). Caffeine was purchased from Sigma (St. Louis, MO).

Chromatography Equipment and Conditions

The HPLC system (Waters Assoc., Milford, MA) consisted of a model 6000A solvent delivery system; a U6K loop injector; a model

440 UV absorbance detector with a 254 nm filter; a guard column containing Bondapak phenyl/corasil; and a prepacked 30 cm x 3.9 mm i.d. stainless steel column containing 10 μm C_{18} μ-Bondapak. The chromatograms were recorded by a 10 mv strip chart recorder (Fisher Recordall, Series 500, Fairlawn, NJ) at a chart speed of 0.25 cm/minute. The chromatographic system was operated at ambient temperature with a flow rate of 2.0 ml/minute and a column pressure of approximately 2000 psi. The detector was employed at 0.02 or 0.05 a.u.f.s., and a 10-15 μl sample injection volume was used.

The mobile phase was prepared by combining 20 ml of glacial acetic acid, 200 ml deionized water, and 120 ml of acetonitrile. The resultant solution was thoroughly mixed before bringing the final volume to 2000 ml with deionized water. The pH of the solution was adjusted to pH 4.0 with a 6N sodium hydroxide solution. The mobile phase was filtered through a Millipore filter, type HA, pore size 0.45 μm (Millipore, Bedford, MA) prior to use.

Standard Solutions

Four different concentration ranges of ceftazidime in methanol were prepared for the analysis of serum, urine, CSF and peritoneal dialysis fluid samples. Standard curves for serum employed methanol solutions containing 25, 50, 100 and 200 μg/ml of ceftazidime. For urine assays 100, 200, 400 and 800 μg/ml methanol standards were employed. For CSF assays 1.25, 2.5, 5 and 10 μg/ml methanol standards were used, and 50, 100, 200 and 400 μg/ml methanol standards were used for the peritoneal dialysis assays. These standards were prepared fresh each day. The internal standard, hydrochloro-

thiazide was also prepared in methanol at concentrations of 100, 400 and 5 μg/ml for the assay of plasma, urine and peritoneal dialysis fluid, and CSF, respectively. Methanol solutions of hydrochlorothiazide were stable for at least 6 months, stored at $4^{o}C$.

Standard Curves and Patient Samples

A 100 μl aliquot of each sample was transferred to a 15ml conical centrifuge tube with a micropipet, along with 100 μl of ceftazidime methanol standard and 100 μl of internal standard. The mixture was vortexed (Vortex-Genie, Ace Scientific Supply, Linden, NJ) for 30 seconds, then centrifuged at 1400 xg and $0^{o}C$ in a refrigerated centrifuge (Beckman, Model J6, Palo Alto, CA) for 15 minutes. Serum and urine standard curves employed pooled, drug free human serum and urine. Standard curves for CSF were prepared using deionized water. Dianeal 137 with 1.5% dextrose was employed to prepare standard curves for peritoneal dialysis fluid. Blank samples for the standard curves were prepared using 200 μl of methanol instead of the drug and internal standard solutions, and patient samples were assayed by substituting 100 μl of methanol for the drug standard solution. Quantitation of ceftazidime concentrations employed least squares regression of plots of peak height ratio (ceftazidime/internal standard) versus ceftazidime concentration.

Assay Specificity

Samples containing 1000 μg/ml of gentamicin, kanamycin, tobramycin, penicillin G, methicillin, amikacin, ampicillin, chloramphenicol and caffeine were assayed to determine the possibility of interference should these compounds be present in patient samples.

Stability Studies

Pooled human serum, human urine of pH 5 and pH 8, and Dianeal 137 with 1.5% dextrose (pH 5.1) were fortified with ceftazidime in concentrations of 100, 400, 400 and 100 μg/ml, respectively. The samples were then assayed after storage for 0, 0.5, 1, 2, 4, 8, 24 and 48 hours at ambient temperature.

Individual 1 ml aliquots of serum and pH 5.5 urine, containing 100 μg/ml and 400 μg/ml of ceftazidime, respectively, were stored at -15°C and -70°C for 30 days. These samples were assayed after 1, 3, 14 and 30 days of storage, and the results were compared to those from freshly prepared samples.

RESULTS AND DISCUSSION

Figure 1 illustrates typical chromatograms for the assay of patient serum, urine and CSF samples. The retention times for the internal standard and the ceftazidime were about 6 min and 11 min, respectively. Blank samples containing no drug or internal standard were free from any interfering peaks in the vicinity of the drug and internal standard. Peaks observed with serum samples fortified with 1000 μg/ml of other antibiotics and caffeine were well resolved from both the drug and internal standard. The retention times were 5, 10, 18 and 37 minutes for amikacin, ampicillin, caffeine and chloramphenicol, respectively. No peaks were found in samples containing kanamycin, gentamicin, tobramycin, penicillin G or methicillin, and these drugs presumably eluted with the solvent peak or were retained on the column.

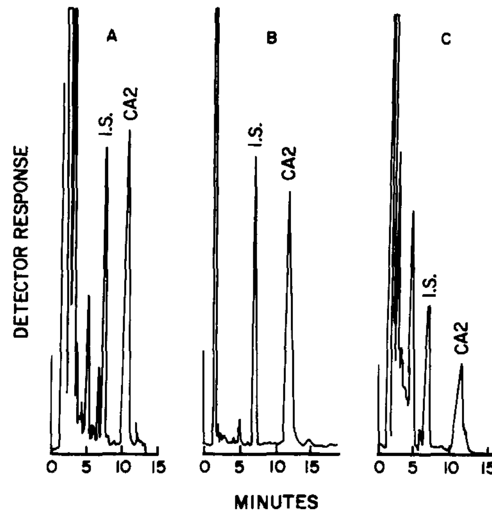

FIGURE 1 - Chromatograms from the assay of samples obtained from patients receiving intravenous ceftazidime (CA2). (A) urine containing 417 µg/ml. (B) serum containing 97 µg/ml. (C) CSF containing 4.7 µg/ml. I.S. is internal standard.

Calibration curves for peak height ratio versus ceftazidime serum, urine, CSF and dialysis fluid all exhibited excellent linearity, with negligible intercepts and correlation coefficients which were consistently in the range of 0.998-0.999. The lower limit of detection was 0.5 µg/ml, and the recovery of drug was consistently at least 95 percent. Table I summarizes the results of the precision studies for four serum, urine and CSF standards assayed on a single day, or individually on four separate days.

The ambient temperature stability studies exhibited ceftazidime recoveries after 24 and 48 hr of 100 and 99 percent for dialysis fluid, 101 and 96 percent for pH 5 urine, 86 and 80 percent

TABLE I

WITHIN-DAY AND BETWEEN-DAY PRECISION OF CEFTAZIDIME ASSAY*

Within-Day Precision

Serum Standards		Urine Standards		CSF Standards	
Added (μg/ml)	Found (μg/ml)	Added (μg/ml)	Found (μg/ml)	Added (μg/ml)	Found (μg/ml)
21.5	20.8±0.6	86.2	85.1±3.4	1.08	0.97±0.04
43.1	42.5±0.9	172.4	172.3±2.7	2.16	2.00±0.03
86.2	86.1±1.2	344.8	342.4±3.3	4.31	4.26±0.29
172.4	168.7±2.3	689.6	686.0±8.4	8.62	8.42±0.35

Between-Day Precision

Serum Standards		Urine Standards		CSF Standards	
Added (μg/ml)	Found (μg/ml)	Added (μg/ml)	Found (μg/ml)	Added (μg/ml)	Found (μg/ml)
21.5	20.4±1.1	86.2	84.1±2.5	1.08	0.95±0.05
43.1	42.9±1.3	172.4	168.4±5.5	2.16	2.03±0.04
86.2	85.4±1.9	344.8	338.9±8.6	4.31	4.10±0.10
172.4	170.4±4.5	689.6	683.8±9.7	8.62	8.86±0.55

*N = 4 replicates for serum, urine and CSF (±S.D.)

for pH 8 urine, and 29 and 4 percent for serum, respectively. The marked deterioration of ceftazidime in serum samples at ambient temperature precludes the storage of serum samples containing ceftazidime for any appreciable length of time at ambient temperature. The stability of ceftazidime in peritoneal dialysis fluid over 48 hr at ambient temperature makes possible the advance preparation of ceftazidime dialysis fluids for intraperitoneal admin-

istration. Ceftazidime recovery after storage at -15°C for 3, 14 and 30 days was 105, 94 and 88 percent for pH 5.5 urine, and 99, 108 and 86 percent for serum, respectively. The stability of the samples increased when stored at -70°C, with ceftazidime recoveries of 99 and 101 percent for the urine, and 97 and 91 percent for the serum, respectively, after 3 and 30 days of storage.

Assay of Patient Samples

This assay method has been employed for over one year in the analysis of human serum, urine, CSF and peritoneal dialysis fluid from patients participating in clinical trials. Serum ceftazidime concentrations have usually been in the range of 10-200 µg/ml, in agreement with earlier work (3). Urine drug concentrations were usually between 100-800 µg/ml. Urine concentrations above this range were easily diluted prior to assay. The CSF and peritoneal dialysis fluid drug concentrations have usually been in the range of 1-10 µg/ml and 10-100 µg/ml, respectively.

It is concluded that the method is rapid, sensitive and does not exhibit interferences from a number of other drugs which may be present in samples obtained from patients. The primary advantage of this method is the use of hydrochlorothiazide, which is quite stable, as the internal standard. An earlier assay (4) employed cephalexin as the internal standard, and this drug is considerably less stable (5,6) than is hydrochlorothiazide.

ACKNOWLEDGEMENTS

This study was supported by Glaxo, Inc., Research Triangle Park, N.C.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 989-1002 (1984)

THE USE OF HIGH PERFORMANCE MOLECULAR SIEVING COLUMNS FOR THE STUDY OF LYMPHOCYTE PRODUCTS

I. MACROPHAGE TRANSGLUTAMINASE INTERACTION WITH PRODUCTS OF CON A-STIMULATED MOUSE SPLEEN CELLS

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ABSTRACT

Partially purified transglutaminase from mouse peritoneal macrophages has been prepared and shown to utilize mouse

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lymphokines as substrates. Biosynthetically labeled mitogen-stimulated spleen cell products were fractionated by high performance liquid chromatography on a molecular sieving column. A fraction known to contain macrophage migration inhibition factor (MIF) of molecular weight 15,000 kd was reacted with the transglutaminase and rechromatographed. Higher molecular weight labeled components which did not dissociate in the presence of 6M guanidine HCl were observed. These data suggest that the molecular heterogeneity often reported for lymphokines may be the result of transglutaminase modification of their native structures. The relationship of these findings to possible regulatory functions in the immune response is suggested.

INTRODUCTION

Recent data obtained during purification studies on mouse (1) and human (2) macrophage migration inhibition factor (MIF) shows that these activities exists in multiple molecular weight fractions and indicates that polymerization of some type is involved. Preliminary studies using dissociating solvents such as guanidine HCl suggest that this association involves formation of covalent bonds. Since both Concanavalin A (Con A) -stimulated lymphocytes (3) and various macrophages (4) are known to produce a transglutaminase that can form epsilon-(gamma-glutamyl)lysine linkages between susceptible substrates, we felt the presence of

these cell types in biological systems employed normally for generation of MIF might explain this observation. Using HPLC molecular sieving columns and dissociating solvents, we prepared enzyme from murine macrophages and studied its ability to polymerize biosynthetically labeled, mitogen-stimulated spleen cell products, tentatively identified as lymphokines.

MATERIALS AND METHODS

Assay of Transglutaminase Activity. Transglutaminase activity was assayed as described by Schroff, et al (4), a measurement of ^{14}C-putrescine incorporation into casein. The reaction mixture (85 ul) contained final concentrations of 100 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 10 mM $CaCl_2$, 3 mM dithiothreitol, 4 mg/ml alpha-casein, and 0.65 mM ^{14}C-putrescine (5-10 uCi/mMole). The reaction was initiated by addition of 15 ul of the enzyme fraction and the mixtures were incubated for 2 hr. Control samples were prepared by first heating enzyme preparations to 100C for 10 min. Duplicate aliquots were removed from each vial and placed onto glass fiber filter paper strips that had been pretreated with 0.1% unlabeled putrescine. The filters were dipped immediately into 10% trichloroacetic acid (TCA) and were washed three times by floatation on 5% TCA solutions. The filters were washed in ethanol/acetone 1:1 (v:v) and then in

acetone only and dried. Radioactivity was counted in an LKB liquid scintillation counter. Control values were subtracted and specific activity calculated based on umoles putrescine incorporated/mg/min.

Preparation of Transglutaminase Fractions. Transglutaminase was prepared from thioglycollate induced mouse peritoneal exudates. Peritoneal exudate cells (6.0×10^{8}) were harvested from 20 mice and allowed to adhere to teflon bags for 2 days in Dulbecco's complete medium supplemented with 5% heat inactivated fetal calf serum. The adherent cells were washed twice in Dulbecco's complete medium without serum. The remaining cells (1.6×10^{8}) were recovered in 2 ml of lysis buffer consisting of 100 mM Tris-HCl, 50 mM NaCl, 1mM EDTA, and 3 mM dithiothreitol. The cell suspension was lysed by sonic disruption at 4C and clarified by centrifugation at 37,000 x g for 30 min. The transglutaminase-containing supernatant was partially purified by high performance liquid chromatography (HPLC) on a molecular sieving column (Toyosoda TSK 2000SW). The column was equilibrated in 0.05M phosphate buffered saline, pH 7.2, and had been calibrated previously with standard proteins of known molecular weights. The supernatant (200 ul containing 2.8 mg protein) was loaded onto the column and chromatographed at 0.5 ml/min. Fractions were collected every 30 seconds and assayed for

transglutaminase activity as described above. The fractions containing transglutaminase activity were pooled.

Preparation of Biosynthetically Labeled Lymphokines. Biosynthetically labeled lymphokines were prepared from mouse spleen cells as described previously (5). Spleens were perfused with complete Dulbecco's Minimal Essential Medium containing 5% heat inactivated fetal calf serum. After homogenization and filtration through cheesecloth, cells were harvested by centrifugation at 400 x g for 10 minutes and erythrocytes were lysed by osmotic shock. The cell pellet was resuspended in 10 ml of Dulbecco's without FCS and viability and cell count were determined by trypan blue exclusion. Cells were added to 24 well plates at 1×10^{7} cells/well in 1.0 ml aliquots. Cells were stimulated by the addition of 10 ugrams of Con A/well and allowed to incubate for 2 hr at 37C in 7% CO_2. Duplicate cultures were prepared without Con A stimulation. After 2 hr, the medium and nonadherent cells were removed and leucine-free medium was added to each well. ^{3}H-leucine (50 uCi) was added to each well and the cells were allowed to incubate for 24 hr. After incubation, the supernatants were harvested and desalted over Sephadex G-25 and lyophilized. The labeled molecules were partially purified by HPLC as described above on a molecular sieving column. Fractions were counted in a liquid scintillation counter and that portion

of biosynthetically labeled material eluting at a molecular weight of approximately 15 kd was used in the remaining studies. Our previous studies have shown the labeled material to co-purify with MIF activity.

Reaction of Lymphokines with Transglutaminase. The 15 kd tritium labeled MIF-containing fraction obtained from HPLC was incubated with the transglutaminase active fraction (approx 80 kd) for 2 hours at 37C and was then rechromatographed on the same HPLC column to determine if the labeled MIF would appear at higher molecular weight ranges because of transglutaminase activity. In order to insure that any changes were due to enzyme activity, controls were prepared in which an excess of putrescine, a substrate for transglutaminase, was added and chromatography was carried out similarly. The covalent nature of the interaction was differentiated from non-covalent association by repeating the chromatographic separation in the presence of 6M guanidine HCl.

RESULTS

Partial Purification of Transglutaminase. The method for partial purification of transglutaminase from thioglycollate induced macrophages was sufficient for these applications. The technique described allowed preparation of sufficient amounts of enzyme with an increase of 38-fold in specific activity. More

importantly, the technique allowed quick separation of transglutaminase activity from other contaminating proteins which enabled us to determine shifts in lymphokine elution in other molecular weight ranges. Figure 1 shows the elution profile of the macrophage cell lysate when chromatographed on the TSK2000SW HPLC column. The major portion of transglutaminase activity elutes at a molecular weight of about 80 kd which is consistent with the reported molecular weight for many types of these enzymes (6).The fraction indicated was used for the remaining studies.

Preparation of Low Molecular Weight Lymphokines. We have previously reported the utility of ^{3}H-amino acid incorporation into mitogen stimulated lymphocyte products and their subsequent identification as lymphokines (5). This technique was used to prepare labeled proteins and a fraction rich in MIF was selected for further study. Figure 2 shows the elution profile of labeled lymphokine on the same molecular sieving column as above. The low molecular weight fraction was collected and incubated with transglutaminase (Fig. 3). After incubation with the enzyme, the lymphokine fraction was observed to elute at higher molecular weights corresponding to those where biological activity for MIF has been reported previously (1). This increase in apparent molecular weight did not occur in an excess of an alternate transglutaminase substrate. To determine whether this change in elution was due to covalent linkage of the small labeled species,

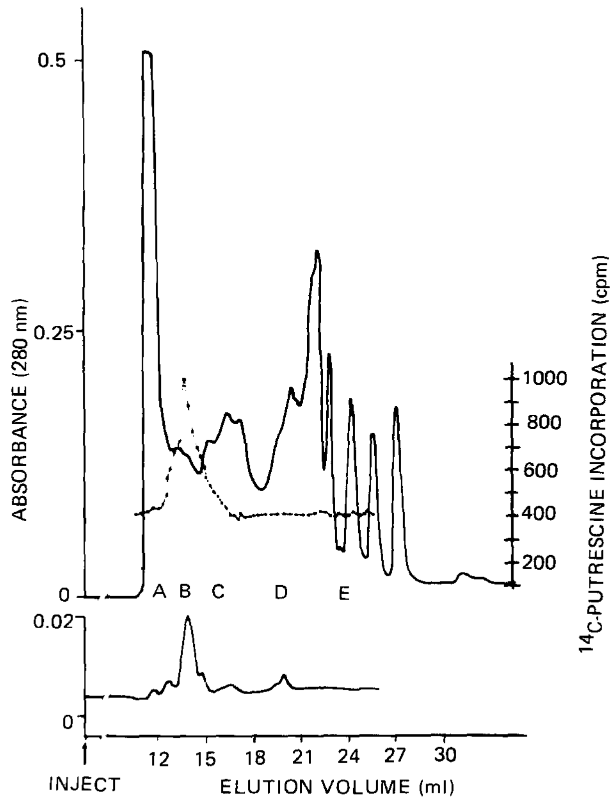

Figure 1. Molecular sieving HPLC of macrophage lysate. Cell lysate (200 ul containing 2.8 mg protein) was loaded onto a 0.75 x 60 cm Toyosoda TSK2000SW column which was pumped with PBS, pH 7.2 at a flow rate of 0.5 ml/min. Optical density was monitored at 280 nm. Fractions (0.25 ml) were collected and assayed for transglutaminase activity as described in the text. The solid line is 280 nm absorbance, the broken line is enzyme activity expressed as ^{14}C cpm. The calibration proteins marked are: A- Thyroglobulin, 670 kd; B- IgG, 158 kd; C- Ovalbumin, 44 kd; D- Myoglobin, 17 kd; and E- Vitamin B-12, 1.35 kd. Transglutaminase activity is shown to elute at approximately 80 kd. The lower portion of the figure shows a sample of the fraction selected for further studies rechromatographed as above.

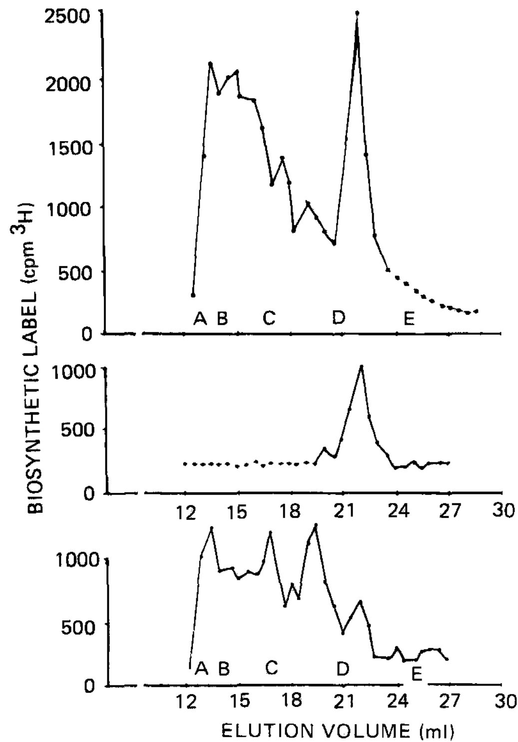

Figure 2. Molecular sieving of biosynthetically labeled lymphokine fractions from mouse spleen cells. ^{3}H-labeled lymphokines were prepared as described in the text and HPLC was performed as in Fig 1. Fractions were collected and counted by liquid scintillation. Fig 2 shows a profile of labeled mitogen treated mouse spleen cell lymphokine supernatants (upper). The fraction at approximately 15 kd was collected and rechromatographed (middle). After incubation of this 15 kd lymphokine with the transglutaminase fraction from Fig 1, the HPLC was repeated (lower). Note the shift toward higher molecular weight as determined by elution position.

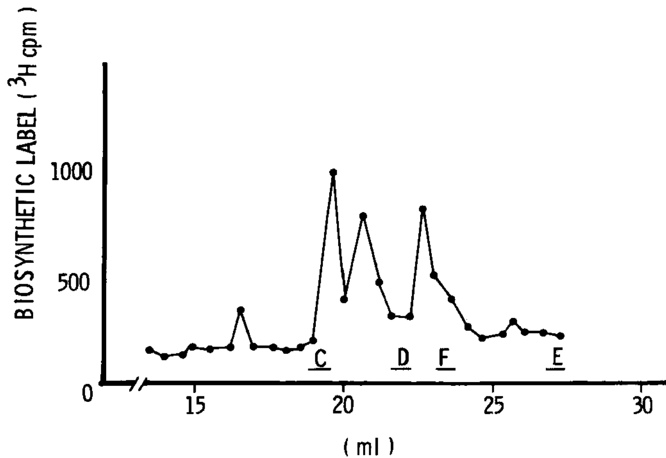

Figure 3. HPLC in the presence of 6M guanidine HCl on a TSK4000SW column. The column was a 0.75 x 60 cm Toyosoda TSK4000SW pumped at 0.5 ml/min with 6 M guanidine HCl. Calibration standards C, D, and E as in Fig 1. F=cytochrome C, 12.5 Kd. Fractions were collected and counted as above. Peaks are at multiples of 15 Kd. Guanidine HCl did not dissociate these higher molecular weight species, suggesting covalent bonds have been formed.

the chromatography was repeated in the presence of 6M guanidine HCl. The higher molecular weight species remained intact, suggesting that their increased size was, indeed, the result of covalent linkage by transglutaminase.

DISCUSSION

The molecular heterogeneity of lymphokines from various systems has been reported often (1,2,9-12). The relationship of these

activities has remained undefined largely because of the lack of sufficient material for exacting chemical analysis. In some instances, higher molecular weight active species are merely complexed with contaminating serum proteins such as albumin and can be readily dissociated by altering solvent composition (8). In the case of murine MIF, these higher molecular weight forms are not dissociated easily and appear to involve covalent bond formation. Sorg has shown that different species of MIF predominate depending upon culture conditions, e.g., as incubation time increases, molecular weight increases (1) as determined by bioassay of Sephadex fractions. Schroff et al have demonstrated that macrophages contain transglutaminase activity and have shown by staining techniques that the enzyme can be used as a marker for macrophage activation (4). The heterogeneity of mouse MIF may be due to its ability to serve as a substrate for the transglutaminase which is present from contaminating macrophages (12).

The present data confirm that covalent bond formation via transglutaminase does increase the molecular weight of biosynthetically labeled proteins present in the supernatant of mitogen-stimulated spleen cells. Because of limitations in bioassay sensitivity, we have not shown that these larger forms are active, yet they do correspond with the elution of bioactive molecules obtained from preparative procedures. It is difficult to determine whether these higher molecular weight forms of MIF

are artifacts of *in vitro* processing, or if they are representative of *in vivo* states that may be regulated by a system in which transglutaminase participates. One could speculate concerning the potential regulatory interaction of lymphokines, polyamines, transglutaminase, alpha-2-macroglobulin, lymphocytes and macrophages. Since all of these elements affect various immune functions (13-18), the ability of both alpha-2-macroglobulin and transglutaminases to bind amines might well be of some regulatory significance.

The present bioassay techniques for MIF and the lack of completely chemically characterized MIF do not allow quantitative comparison of the heterogeneous species; therefore, any further speculation about the physiological significance of these findings is unwarranted. Additionally, appearance of multiple active fractions, although resulting from transglutaminase action, may be an *in vitro* artifact with no *in vivo* basis. The importance of these findings in terms of *in vivo* correlation can be evaluated only when measurement of MIF by direct chemical or immunoassay procedures becomes available.

ACKNOWLEDGMENTS

This work was supported by grant No. CA29145 from the National Cancer Institute, National Institutes of Health, DHHS, and funds from the DFG. Portions of the work was conducted in laboratories

furnished and equipped by the generous contributions of the Missouri Order of Eastern Star and the Fraternal Order of Eagles Art Ehrmann Cancer Fund. The authors are grateful to Mary Lou Kaltenbach for editorial assistance in the preparation of this manuscript.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 1003-1012 (1984)

ASSAY OF YOHIMBINE IN HUMAN PLASMA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

Yohimbine is a selective α_2 adrenoreceptor antagonist used in the study of α_2 adrenoreceptors in man. In order to better improve administration regimens for the study of yohimbine in man, we have developed an assay for the determination of yohimbine in plasma utilizing reverse phase high performance liquid chromatography with electrochemical detection. Using a C_{18} column and a methanol:acetate (60:40) mobile phase, we detected yohimbine in plasma following a simple chloroform extraction. Reserpiline was used as an internal standard. The assay was linear over a concentration range of 50-250 ng/ml in spiked plasma and had a lower limit of sensitivity of 10 ng/ml. It was used to detect yohimbine in plasma sampled from 4 volunteers during an infusion of the alkaloid.

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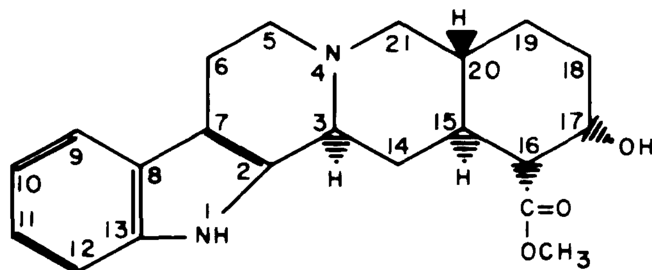
INTRODUCTION

Yohimbine (Figure 1) is an α_2 adrenoreceptor antagonist which has been a useful probe for the study of the physiology and pharmacology of α_2 adrenoreceptors (1). α_2 receptors are important regulators of central sympathetic outflow and may also be involved in such disparate functions as regulation of catecholamine release at the sympathetic noradrenergic terminal, mediation of vascular smooth muscle contraction and platelet aggregation, and in the regulation of intermediary metabolism. In studying yohimbine in man we have noted that the alkaloid raises blood pressure at doses of 16 to 125 μg/kg (2). Based on the time course of these effects and the kinetics of reserpine, a chemically similar alkaloid (3), we designed an infusion regimen which was estimated to produce steady-state levels of about 50 ng/ml plasma (about 10^{-7}M) and used this regimen to study the influence of yohimbine on plasma catecholamines (2) and vasoconstrictor responses to epinephrine and phenylephrine (4). Although the chosen regimen (125 μg/kg bolus, 1 μg/kg/min infusion) elicited the anticipated pharmacologic effects(2,4), it was felt that future studies of the influence of yohimbine on other aspects of sympathetic function, required more rigorous design of regimens to achieve steady-state levels and verification of these levels. Accordingly, we have developed an analytical method for the determination of yohimbine in human plasma using high performance liquid chromatography with electrochemical detection and



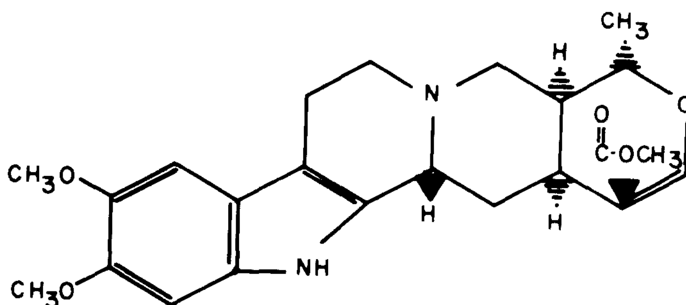


Figure 1. Chemical structures of yohimbine and the internal standard for yohimbine, reserpiline.

reserpiline as an internal standard (Figure 1). We have applied this method to the analysis of samples during infusions at the empirically chosen rate.

MATERIALS

Reagents

Methanol and chloroform were purchased from Burdick and Jackson Laboratories, Inc. Muskegon, Michigan. Sodium

acetate was purchased from Sigma Chemical Co. St. Louis, Mo. Glacial acetic acid and ammonium hydroxide were from Fisher Scientific Co. Yohimbine HCl was purchased from Sigma Chemical Co. (St. Louis, Mo.). The internal standard, reserpiline, was purchased from K and K Rare and Fine Chemicals, Plainview, N.Y.

Standards

A stock solution of yohimbine HCl (100 μg/ml) was prepared daily and diluted in methanol. A stock solution of reserpiline (30 μg/ml) was prepared in methanol and stored in the refrigerator.

Instrumentation

Chromatography was performed with a model PM-30A solvent delivery system and a Rheodyne manual injector (both purchased from Bioanalytical Systems, Inc., West Lafayette, IN). A Waters Associates C_{18} μBondapak column (300 mm x 3.9 mm I.D.) was used for chromatographic separation of yohimbine and reserpiline from plasma constituents. A model LC4B amperometric detector (Bioanalytical Systems) with a glassy (vitreous) carbon electrode was used to detect yohimbine and reserpiline in extracts of plasma. A strip chart recorder (10 mv input) was used.

METHODS

Sample Extraction

Blood samples were collected in EDTA and centrifuged to separate the plasma which was stored frozen at -20° C

for periods of 1 to 3 months. To 3 ml of plasma 30 μg of the internal standard, reserpiline, was added. In dropwise fashion, 4 N NH_4OH was added to adjust the pH to 9 (usually about 100 μl). Three ml of chloroform were added to the plasma in 20 ml polyethylene tubes which were gently inverted 20 times. The samples were centrifuged for 5 minutes at 1100 G. The organic layer was removed using a pipette and saved and the plasma was extracted two more times. The 9 mls of chloroform containing yohimbine were evaporated to dryness with a stream of air at room temperature. The residue was redissolved in 1 ml of chloroform and 0.5 ml of 0.1 N acetic acid was added and vigorously vortexed. This mixture was centrifuged and the acid layer removed for injection onto the column in volumes of 50-100 μl. Using [^{3}H] yohimbine, this extraction resulted in a recovery of about 75%. In preliminary studies it was noted that extracted samples could be stored overnite prior to injection onto the chromatographic column without alteration of the peak-height ratio.

Chromatographic Conditions

The chromatographic system was operated at room temperature. The mobile phase consisted of a 60% methanol and 40% acetate buffer (0.4 M, pH 6.0) and was carefully degassed prior to use. The flow rate of the mobile phase was 1.5 ml/min. An applied potential of 950 mV across the glassy carbon electrode was used. The mobile phase was not recycled.

Quantitation

Peak height ratios of yohimbine to reserpiline were plotted against concentration. A least-squares regression analysis of this line was used to calculate yohimbine concentration in samples.

RESULTS AND DISCUSSION

Figure 2 shows a typical chromatogram of blank plasma and plasma containing 105 ng/ml yohimbine. Yohimbine elutes approximately 4 minutes after the injection while the internal standard appears at 8 minutes. No peaks are found in blank plasma which interfere with yohimbine or the internal standard. Figure 3 shows a typical standard curve. Current techniques limit sensitivity to 10 ng/ml. Three clinical samples were assayed in triplicate and produced levels of 57 ± 8 (S.D.), 80 ± 10 and 185 ± 23 ng/ml.

Four normal volunteers were given yohimbine at the previously cited regimen (125 μg/kg bolus, 1 μg/kg/min infusion) and plasma was sampled 10,20,30,60,90 and 120 minutes after administration of the bolus. Results of this study are shown in Figure 4. Levels peaked rapidly after the infusion was begun and administration of the initial bolus (175 ± 38 ng/ml at the 10 minute sample point) and reached an approximate steady state by the 30 minute sample point. At this time, levels were 66 ± 14 ng/ml. After 2 hours of infusion, levels had fallen to 46 ± 14 ng/ml.

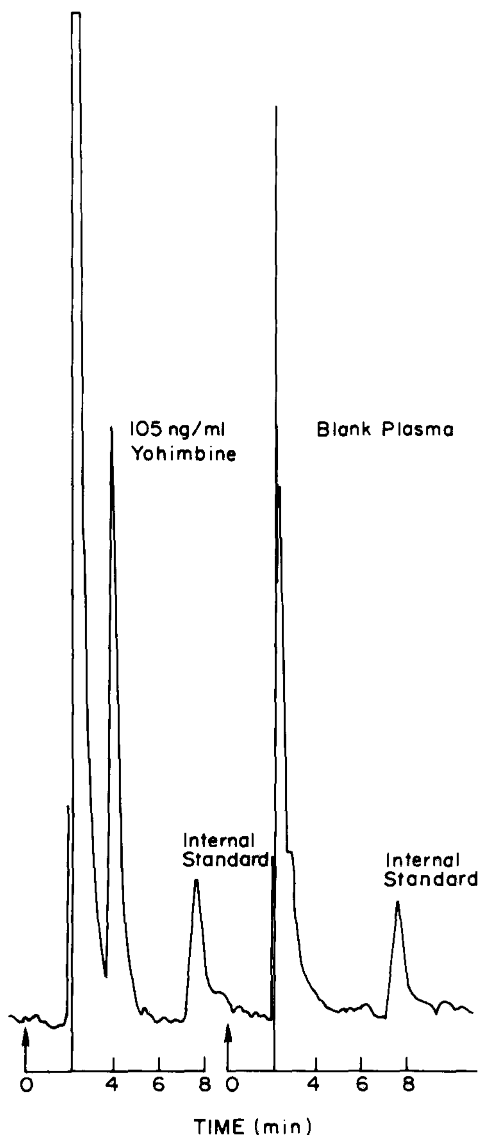


Figure 2. Chromatograms of samples of the same plasma before (right) and after administration of yohimbine intravenously. The sample was injected on the column at each arrow. 30 μg internal standard was added to the plasma

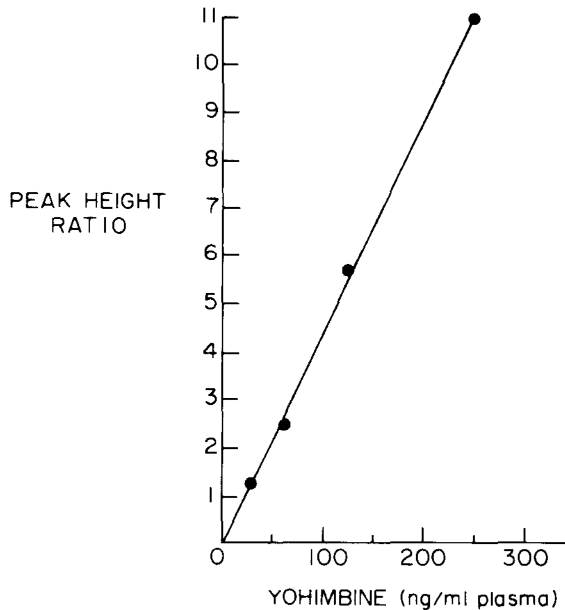


Figure 3. Standard curve determined in plasma relating known concentration of yohimbine to the ratio between the heights of the yohimbine and internal standard peaks (peak height ratio). The curve was linear from 25 to 250 ng/ml yohimbine.

These data show that the electrochemical detector can be used to measure yohimbine in human plasma. The methods employed appear generally applicable to the study of yohimbine in man and will prove useful in evaluating the effects of steady-state levels of yohimbine on autonomic function and in monitoring oral therapy with yohimbine in conditions which may include impotence (5) and autonomic dysfunction (6).

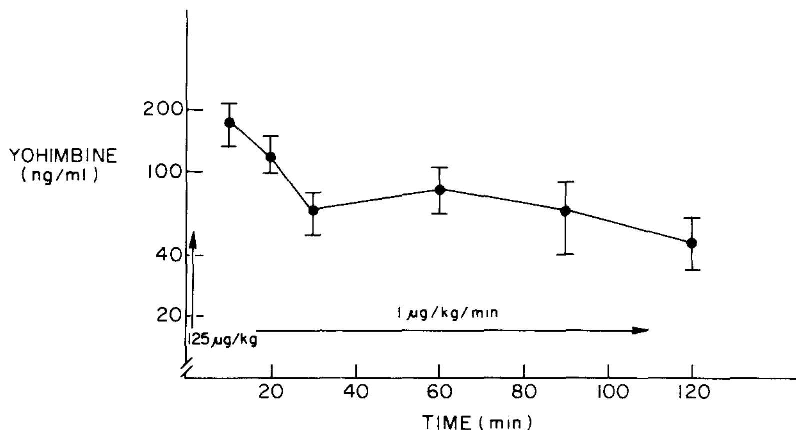


Figure 4. Plasma levels of yohimbine (mean ± SE) in 4 volunteers given a bolus of yohimbine HCl (125 μg/kg) followed by an infusion at a rate of 1 μg/kg/min.

ACKNOWLEDGEMENTS

This work was supported by grants 5-M01RR00095, GM31304, and HL14192 from the National Institutes of Health and by a grant from the Hrafn Sveinbjarnarsson Foundation. Dr. Goldberg was Clinical Associate Physician, Elliot V. Newman Clinical Research Center during the period of these investigations. His current address is Lilly Research Laboratories, 307 E. McCarty Street, Indianapolis, Indiana 46285. Dr. Robertson is recipient of a Research Career Development Award from the NIH GM00494.

Ms. Betty Campbell and Mrs. Carolyn Rork helped with preparation of the manuscript.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 1013-1021 (1984)

THE APPLICATION OF UV-RADIOACTIVITY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TO THE STUDY OF HYPOXANTHINE TRANSPORT IN HUMAN ERYTHROCYTES

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ABSTRACT

A procedure is presented for the simultaneous measurement of concentrations of labeled and non labeled hypoxanthine by HPLC in order to study hypoxanthine transport in erythrocytes. A radioactivity detector connected on-line to the high performance liquid chromatograph in series with a UV detector provides on-line quantitative monitoring of hypoxanthine in erythrocytes or incubation medium. The procedure provides a rapid, sensitive and convenient means for the study of hypoxanthine transport.

INTRODUCTION

A rapid and selective reversed phase high performance liquid chromatographic method for the simultaneous determination of two important purine bases hypoxanthine and xanthine, in biological fluids has recently been developed in our laboratories (1). During analysis of hypoxanthine and xanthine in plasma and erythrocyte samples by this method, we observed an evolution of the hypoxanthine level in plasma samples during the time elapsed between sampling and centrifugation (2). This evolution would seem to be due to the increase of hypoxanthine in erythrocytes with time and at the release of

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hypoxanthine into the plasma. For these reasons, we set up a chromatographic system to study the transport of hypoxanthine in erythrocytes. This system consisted of a radioactivity detector connected on-line to the high performance liquid chromatograph in series with a UV detector.

The transport of hypoxanthine in erythrocytes has already been examined (3 - 8) however, the transport mechanism has not yet been clearly understood generally for technological reasons.

In the procedure presented here, the coupling of radioactivity and UV detectors makes possible the simultaneous measurement by direct means of the labeled and unlabeled hypoxanthine concentrations in the erythrocytes and the incubation medium. Furthermore given the sensitivity of the method, the studies can be carried out at physiological concentrations.

MATERIALS AND METHODS

Reagents

Potassium dihydrogenophosphate, sodium chloride, trichloracetic acid 20% were obtained from Merck (Darmstadt, GFR). Potassium chloride and glucose were obtained from Prolabo (Paris, France). Hypoxanthine was purchased from Sigma (St Louis, Mo, USA). ($8 - {}^{14}C$) hypoxanthine (52 mCi/mmol) was obtained from CEA (F 91191, Gif sur Yvette, France). Liquid scintillator, Lumaflow II was obtained from Roche Kontron (Montigny le Bretonneux, France).

Saline medium : the composition of the saline medium was the following: 0.01 M sodium phosphate buffer, pH 7.40, KCl 5.6 mM, NaCl 153 mM and glucose 5mM.

Apparatus and Chromatographic Conditions

Chromatographic analyses were done with an integrated unit, a S P 8000 high performance liquid chromatograph (Spectra Physics, Orsay les Ullis, France). The column (15 cm x 4.6 mm I.D.) and a precolumn (5 cm x 4.6 mm I.D.) were packed with Hypersil ODS 3 µm (Shandon, Cheshire, Great Britain) by the slurry packing technique described by Coq et al (9). The mobile phase consisted of 0.02 M KH_2PO_4, the pH of which was adjusted to 3.65 with phosphoric acid. The flow rate was 1.5 ml/min. Detection was carried at 254 nm.

Radioactivity was measured using a Flo-One radioactive flow detector (Roche Kontron, Montigny le Bretonneux, France) connected on-line to the high performance liquid chromatograph in series with the UV detector, so that the column effluent first passed through the UV detector, then through the radioactivity detector where it was mixed with scintillator fluid before passing into the cell and out to waste.

The radioactivity detector is equipped with a 0.5 ml volume cell. The liquid scintillator used was Lumaflow II, the flow rate was 1.5 ml/min.

This apparatus was coupled to a 10 mV data recorder (Servotrace, Sefram, Paris, France). The Flow-One detector was used for the measurement of low energy, Beta-emitting radionuclides such as Carbon - 14. This instrument is equipped with a microprocessor which regulates the pump, operating programs and system controls. In the integrate mode, the peak elution times and total counts accumulated under the peak were printed out.

Blood Samples

Blood samples (5 to 10 ml) from different donors were collected in a heparinized tube and immediately centrifuged at 3000 r pm. Plasma, leucocytes and the upper layer of erythrocytes were removed.

The Time Course of Hypoxanthine Uptake by Erythrocytes

The time course of hypoxanthine uptake was measured at three different concentrations of $(8 - {}^{14}C)$ hypoxanthine : 10, 40, 80 $\mu mol.l^{-1}$. The assays were carried out with a Beckman microfuge using 0.4 ml polyethylene tubes. An aliquot of erythrocytes was suspended in an equal volume of saline medium preloaded with $(8 - {}^{14}C)$ hypoxanthine. The incubations were done at 25°C. At fixed times, the incubations were terminated by centrifuging the erythrocyte suspension. The $(8 - {}^{14}C)$ hypoxanthine taken up by the erythrocytes was determined by HPLC analysis after deproteinisation with trichloracetic acid 12%.

Hypoxanthine Distribution between Erythrocytes and Saline Medium at the Steady State

The saline medium was preloaded with $(8 - {}^{14}C)$ hypoxanthine at different concentrations : 0, 10, 20, 40, 80, 160 $\mu mol.l^{-1}$. The erythrocytes

from six different donors were incubated with the medium (1 vol/1vol) for five minutes at 25°C with gentle shaking, and the cells were separated from the saline medium by centrifugation. Both the erythrocytes and the saline medium were analysed by HPLC.

RESULTS AND DISCUSSION

Figure 1 shows the chromatograms from UV - radioactivity analysis of human erythrocytes (a) and saline medium (b) before incubation. The chomatograms of the analysis of the same sample after incubation are shown in figure 2. In this example the saline medium is preloaded with 10 $\mu mol.l^{-1}$ (8 - ^{14}C) hypoxanthine. Figure 2 shows that hypoxanthine crosses the cells membrane of erythrocytes. The radioactive peak follows the UV peak by a time factor which depends on the flow rate and volume between detector cells.

Linearity studies show that in radioactivity, the relation between the total counts measured under the peak and the concentration of (8 - ^{14}C) hypoxanthine is perfectly linear up to 40 $\mu mol.l^{-1}$. In UV, the linearity is excellent up to 50 $\mu mol.l^{-1}$.

The detection limit in UV for hypoxanthine is about 2.5 pmol. In radioactivity, the detection limit is four to five times lower, however at high sensitivities, the baseline is noisier in radioactivity than in UV detection.

The time course of the uptake of hypoxanthine by erythrocytes was measured at the three following concentrations : 10, 40 and 80 $\mu mol.l^{-1}$ (8 - ^{14}C) hypoxanthine. The incubations were carried out at 25°C and not at 37°C so as to allow the necessary time to carry out the study, the rate of uptake being less at lower temperatures (3).

Figure 3 shows that the equilibrium was reached within one to two minutes at 25°C which indicates that the uptake process is rapid at physiological concentrations of hypoxanthine.

During the radioactivity analysis we checked that no Inosine monophosphate peak was in fact present on the chromatograms. A hypoxanthine peak was the only radioactive compound found which proved that no metabolic conversion took place during the experiments.

The coupling of the radioactivity detector on-line with the UV detector made possible the simultaneous determination of the labeled and unlabeled

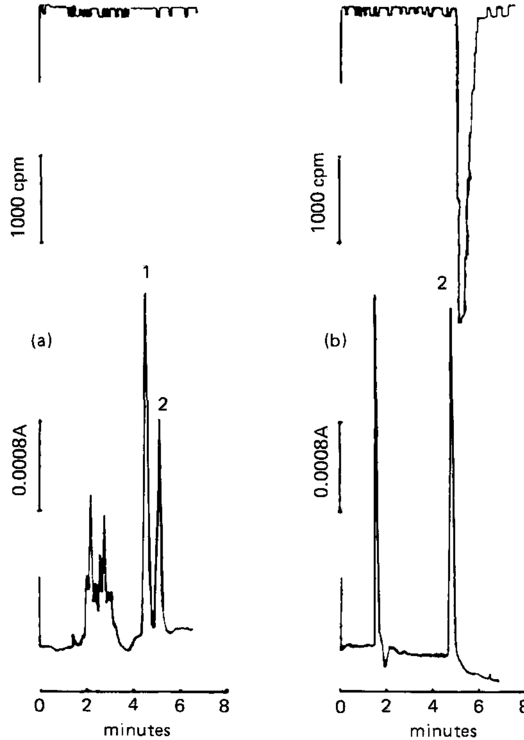

Figure 1 :

Chromatograms from the UV - radioactivity analysis of human erythrocytes (a) and of a saline medium (b) before incubation.

Analysis conditions : injection volume : 10 µl. Column : Hypersil ODS 3µm. Mobile phase : 0.02 M KH_2 PO_4, pH 3.65 ; flow rate : 1.5 ml/min. UV detection : 254 nm. Liquid scintillator : Lumaflow II ; flow rate : 1.5 ml/min.

Peaks - 1 : uric acid, 2 : hypoxanthine (Hyp)

Radioactivity analysis : (a) Hyp labeled : 0 µmol.l^{-1}
(b) Hyp labeled : 10 µmol.l^{-1}

UV analysis (a) Hyp unlabeled : 4.5 µmol.l^{-1}
(b) Hyp labeled : 10 µmol.l^{-1}

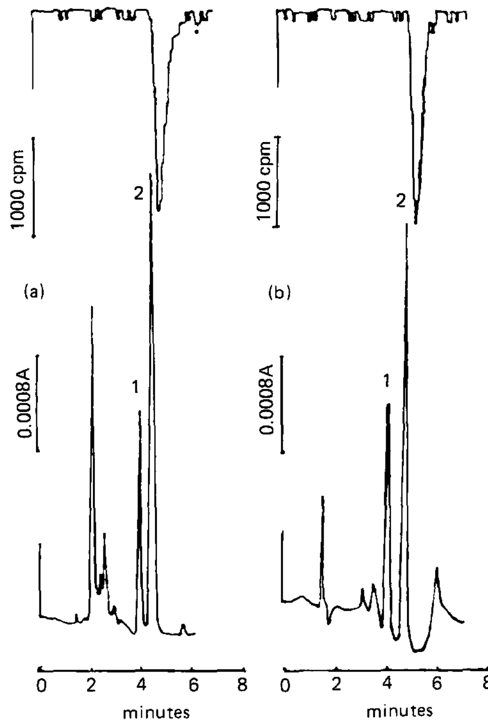

Figure 2 :

Chromatograms from the UV - radioactivity analysis of the same samples as in figure 1, erythrocytes (a), saline medium (b), but after incubation.

Peaks - 1 : uric acid, 2 : hypoxanthine

Radioactivity analysis : (a) Hyp labeled : 4.8 $\mu mol.l^{-1}$
(b) Hyp labeled : 5.0 $\mu mol.l^{-1}$

UV analysis (a) Hyp (labeled + unlabeled) : 7.2 $\mu mol.l^{-1}$
(b) Hyp (labeled + unlabeled) : 7.1 $\mu mol.l^{-1}$

hypoxanthine concentrations in both erythrocytes and saline medium after incubation. Using this procedure, we considered the distribution of labeled and unlabeled hypoxanthine between erythrocytes and saline medium at the steady state. An example of this hypoxanthine distribution between erythrocytes of a healthy subject and saline medium loaded with (8 - ^{14}C) hypoxan-

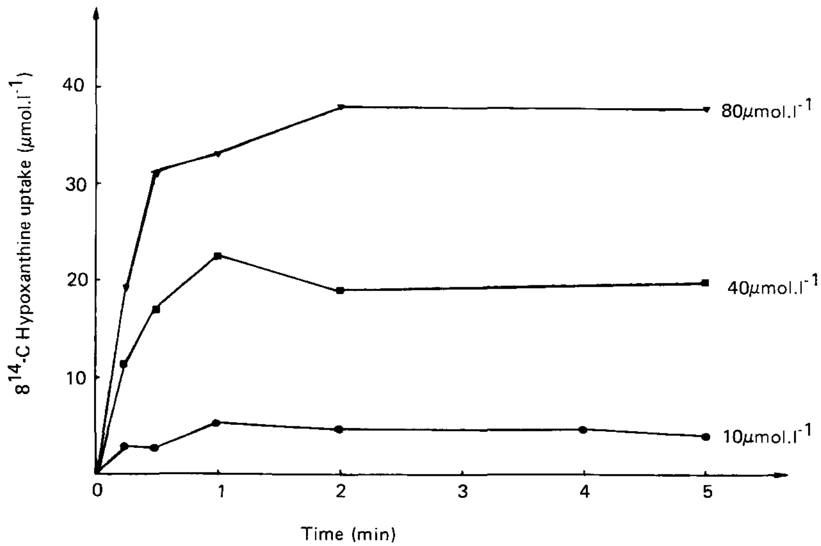

Figure 3 :

The time course of hypoxanthine uptake by human erythrocytes from 10, 40, 80μmol.l^{-1} (8 - ^{14}C) hypoxanthine in the incubation medium.

TABLE 1

Distribution of Labeled and Unlabeled Hypoxanthine between the Erythrocytes of a Healthy Subject and a Saline Medium after Incubation

Erythrocytes		Saline medium	
Hyp	8 - ^{14}C	8 - ^{14}C	Hyp
9	0	0	8
8.5	4.8	5.0	9
9	10	9.0	8
9	20	19.5	7.5
9	39	39.0	8
7.5	72.5	90.0	9.0

thine at different concentrations ranging from 0 to 160 $\mu mol.l^{-1}$ is presented in table 1. This table indicates that after incubation at 25°C for five minutes both labeled and unlabeled hypoxanthine reach approximately equal concentrations in the cells and in the medium. The results obtained from the experiments on erythrocytes of five healthy subjects are similar.

Conditions of the experiment :

- Initial Hyp concentration in erythrocytes : 17 $\mu mol.l^{-1}$
- Initial labeled Hyp concentrations in saline medium : 0, 10, 20, 40, 80, 160 $\mu mol.l^{-1}$.
- Incubation : 5 minutes at 25°C.

The data show that hypoxanthine crosses the cell membrane of erythrocytes in both directions : the hypoxanthine in the medium is partially taken up by the erythrocytes, and the hypoxanthine initially present in the erythrocytes is partially released into the medium. These observations lead us to conclude that the increase of hypoxanthine concentration in plasma samples left in contact with erythrocytes may in fact be due to a hypoxanthine release from the erythrocytes into the plasma.

As a more general conclusion, we feel that the system described in this paper, may indeed be one of the most suitable for the monitoring of labeled and unlabeled compounds in transport studies.

ACKNOWLEDGMENTS

The authors acknowledge their debt to Mister Bard and Mister Granjon of Roche Kontron who kindly loaned the Flo-One detector. We also wish to thank Professor J. Cotte, in whose laboratory this work was conducted and Professor M. Porthault for his interest in this work.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 1023-1032 (1984)

ASSAY OF ERYTHROMYCIN FROM HUMAN SERUM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A sensitive and selective method has been developed for the determination of serum concentrations of erythromycin A by high performance liquid chromatography with electrochemical detection. Erythromycin was extracted from alkalinized serum samples with methyl t-butyl ether. After evaporation of the ether, the samples were reconstituted in acetonitrile/ammonium acetate and washed with hexane. Aliquots were injected onto a Sepralyte diphenyl column. The mobile phase consists of acetonitrile/sodium perchlorate/ammonium acetate/methanol under isocratic conditions. Eluted peaks were detected by dual coulometric electrodes operated in the oxidative screen mode. The recovery of erythromycin from serum was 84%. Assay limit of quantification was 0.05 µg/ml serum, and dynamic linear range was 0.05-1.5 µg/ml. This method was used to quantitate both erythromycin and its gastric degradation products from human serum. Additionally, other macrolide antibiotics could be quantified by electrochemical detection. Analytical results for erythromycin compared favorably with those obtained with a standard microbiological assay.

INTRODUCTION

The 14-membered ring macrolide antibiotic, erythromycin A, is routinely measured by microbiological assays (1-4). While having an appropriate limit of detection, bioassays lack specificity; active metabolites and prodrug esters of erythromycin, as well as other antibiotics, are likely to interfere. A number of high performance liquid chromatography methods employing spectrophotometric detection have been reported for the separation and quantification of macrolides (5-9). They were judged unsuitable for determination of serum concentrations of erythromycin expected from therapeutic doses of this antibiotic. Improved detection

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limits have been achieved by an HPLC-fluorescence assay (10-11) and used for the clinical serum determination of erythromycin, but the assay requires complex post-column derivatization.

Electrochemical detection has been successfully applied to trace analysis of electroactive compounds in clinical and environmental samples (12-14). This paper describes a practical and specific method for the quantification of erythromycin in serum based on HPLC with electrochemical detection. The method provides a sufficient lower limit of detection for clinical samples and is applicable to the quantification of other macrolides.

MATERIALS

Stock solutions of erythromycin A (Pfizer Taito Lot No. 906-636005) and internal standard at 1 mg/ml, prepared in acetonitrile, were stable for several months at 4°C. Dilutions were made with acetonitrile/20 mM ammonium acetate (1/1). Hexane, acetonitrile, methanol, methyl t-butyl ether and water were all HPLC grade (Burdick and Jackson, Muskegon, MI, USA). All other chemicals were reagent grade.

METHODS

Instrumentation

A Spectra-Physics SP 8770 (San Jose, CA) liquid chromatograph was equipped with a 21 mm x 3.0 mm I.D. 40 µm glass bead guard column and a 25 mm x 4.6 mm I.D. column packed with 5 µm Sepralyte diphenyl (Analytichem International, Harbor City, CA). Samples were automatically injected using an HPLC autosampler (Micromeritics Model 725, Norcross, GA) equipped with a 100 µl sample loop. An ESA 5100A electrochemical detector (Environmental Sciences Associates, Bedford, MA) was interfaced with a Spectra-Physics 4100 computing integrator and peak height recorded.

The electrochemical detector was equipped with an ESA Model 5020 guard cell placed in line before the injector in order to electrolyze components of the mobile phase (1.0 volt potential) which could contribute to the background current. The

ESA Model 5010 dual electrode cell was operated in the oxidative screen mode with electrochemical cell I voltage set at 0.7 V and cell II at 0.8 V. To prevent pressure build-up on the graphite electrodes, in-line filters were used. A 0.5 µm stainless steel filter (Rainin, Woburn, MA) was placed before the guard cell and a similar 2 µm filter before the injection loop. A 0.5 µm carbon filter (ESA No. 5100-A-50) was used before the dual electrodes. These filters were replaced periodically to maintain total system pressure below 2,500 psi. A mobile phase of acetonitrile/20 mM sodium perchlorate/20 mM ammonium acetate/methanol (50/32/8/10), pH apparent of 7.0, was filtered through a 0.2 µm Nylon 66 filter (Rainin). Mobile phase was pumped at 1.0 ml/minute, recycled into a 1 L reservoir and replaced weekly. When the response of the electrode decreased by 50%, the analytical cell was flushed with 6N HNO_3 for 30 minutes and washed overnight with acetonitrile/20 mM sodium perchlorate (1/1).

Cyclic voltammetry was conducted on a BAS Model CV-1B (West Lafayette, Indiana) equipped with a glassy carbon working electrode and Ag/AgCl reference electrode. Compounds were dissolved in mobile phase to give a final concentration of 0.5 mg/ml. Current was recorded over a voltage range of + 1.3 to -1.0 volts.

Microbiological Assay

An automated microbiological agar diffusion assay using *Micrococcus luteus* (ATCC #9341) was used as a bioassay for erythromycin A in serum (3). Neomycin assay agar and potassium phosphate buffer solution were used.

Sample Preparation

Frozen human serum samples from males dosed with erythromycin were thawed at room temperature, and 0.25 ml aliquots were added to disposable 16 mm x 100 mm culture tubes. If samples were found to contain concentrations of erythromycin greater than 1.5 µg/ml by this assay, the samples were diluted with control serum, processed and analyzed again. After addition of saturated K_2CO_3 (50 µl), 1 ml of HPLC-grade water, fortified with internal standard, was added to the samples. (The internal standard was an analog of erythromycin.) The sample was immediately extracted with 5 ml of methyl t-butyl ether on a Vortex mixer for 30 seconds. The organic layer was transferred to another tube and evaporated to

dryness at 40°C using a Buchler Vortex evaporator. The residue was reconstituted in 1.0 ml acetonitrile/20 mM ammonium acetate (1/1) and the reconstituted sample washed with 1 ml hexane. The phases were separated by centrifugation and the aqueous layer removed and analyzed for erythromycin A by HPLC with electrochemical detection. Samples at this stage were stable for at least 48 hours. Five-point calibration curves were established using drug extracted from fortified serum samples.

RESULTS AND DISCUSSION

Chromatography

Of the several stationary phases tested (μBondapak C_{18}, μBondapak C_8, μBondapak CN, μBondapak phenyl, Sepralyte diphenyl, Absorbisphere C_{18}, Spherisorb C_{18}), the Sepralyte diphenyl column yielded minimal tailing of macrolide peaks. Buffers tested in the mobile phase with regard to their effects on background current and electrode response to erythromycin A were potassium phosphate, ammonium acetate, monochloro-acetic acid and sodium perchlorate. Only the sodium perchlorate did not lead to a loss in electrode sensitivity after several days of electrode use. However, a mobile phase supplemented with sodium perchlorate, with a pH below 6.5, caused the degradation of erythromycin. Consequently, a mixture of acetonitrile/20 mM sodium perchlorate/20 mM ammonium acetate/methanol (pH 7) (50/32/8/10) was used to prevent this degradation but still maintain electrode sensitivity to erythromycin for at least ten days. The low ionic strength of the buffer used in the mobile phase minimized background current from the detectors. As noted by previous investigators (8), retention time for erythromycin was greatly affected by the pH of the mobile phase; the lower the pH, the shorter the retention time. However, at higher pH the electrochemical oxidation of erythromycin was facilitated. Thus, the pH of the mobile phase was maintained between 7 and 7.5.

Detection

Macrolides exhibit a relatively high oxidation potential on glassy carbon electrodes ($\geq$1.20 volts). This made electrochemical detection of these compounds

more difficult because of oxidation of mobile phase and extraneous sample constituents. Amperometric response given by a glassy carbon electrode could detect $\geq$100 nanogram quantities of erythromycin A, but the detector rapidly lost sensitivity. Alternatively, coulometric response given by a porous graphite electrode could detect nanogram quantities of compound, but the detector generated a large background current, particularly critical in this application of high oxidation potential. Thus, it was necessary to use an electrochemical detection system with dual coulometric electrodes in the oxidative screen mode. In this manner, many components in the extracted serum samples were irreversibly oxidized at the upstream electrode (0.7 volt potential) and did not contribute to the detected current at the downstream electrode (0.8 volt potential). Operation of the electrochemical detector in the oxidative screen mode not only minimized sample background current but enhanced the selectivity as well. Slow loss of electrode response still occurred upon repeated injections (>1,000 injections) of serum extracts but could be regenerated by acid flushing of the electrodes.

Limit of detection for a standard solution of erythromycin was 0.5 ng injected. For analysis of human serum samples, a limit of quantification of 50 ng/ml was adequate (about 1 ng injected). Thus, the procedure reported here provided a similar limit of quantification as the HPLC-post column derivatization-fluorescence method (11) with potential for lower detection limits by the use of greater detector gain settings and extraction of larger sample volumes.

Specificity

In spite of the use of the oxidative screen mode of the electrochemical detector, occasionally extraneous serum and reagent chromatographic peaks were detected. However, by judicious choice of mobile phase pH, the peaks did not interfere with the analytes of interest (Figure 1a). A drug-related peak was observed in the serum of subjects orally dosed with erythromycin suspension (Figure 1c) but was absent in the serum of subjects orally dosed with enteric coated erythromycin (Figure 1b). The retention time of this peak was identical with that of the internal spiroketal of erythromycin A (Table 1), the degradation product of erythromycin A that formed under the acid conditions of the stomach.

The assay method proved to be applicable to the quantification of several classes of macrolide antibiotics. The analyzed macrolide antibiotics which contained

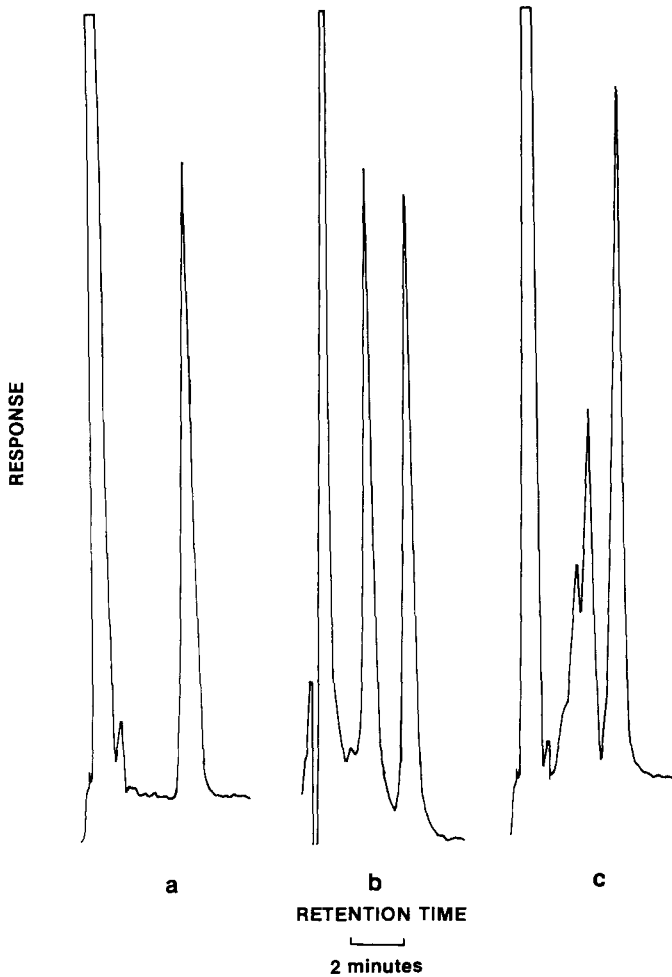

FIGURE 1. Chromatographic determination of erythromycin extracted from human serum. LC-EC conditions are noted in Methods.

a) Extract of human serum prior to erythromycin dose (sample fortified with internal standard).

b) Extract of human serum after oral dose enteric coated erythromycin (erythromycin and internal standard).

c) Extract of human serum after oral dose of erythromycin suspension (erythromycin, spiroketal and internal standard).

Table 1
Relative Retention Time and Relative Peak Area of
Various Macrolide Antibiotics and Erythromycin Derivatives

Macrolide Antibiotics	Relative Retention Time	Relative Peak Area	Erythromycin A Derivatives	Relative Retention Time	Relative Peak Area
Erythromycin A	1.00	1.00	Dihydro	0.96	1.02
Erythromycin B	1.19	0.93	Anhydro	1.26	1.06
Erythromycin D	1.02	1.04	Enol Ether	2.70	1.16
Tylosin	0.51	0.69	Spiroketal	1.25	1.66
Josamycin	0.66	0.94	Descladinose	0.59	1.70
Oleandomycin	0.88	0.79	N-demethyl	0.88	ND*
			N-didemethyl	0.80	ND*
			2-Propionate	1.01	0.75
			2-Ethylsuccinate	1.00	0.92

*N.D. = not detected by electrochemical detector. Retention time was determined by U.V. detection at 214 nm of a 10 µg injection.

a basic sugar were detectable by electrochemical oxidation (Table 1). Additionally, other erythromycins, erythromycin esters and erythromycin degradation products could be separated and quantified by this procedure. Only the two N-desmethyl erythromycins were undetectable (Table 1). Cyclic voltammetry on glassy carbon electrode confirmed the absence of an oxidation potential below 1.3 volts for these latter two compounds, which suggested the importance of the tertiary amine of the desosaminyl sugar in determining the oxidation potential of the molecule.

Linearity

Peak heights for drug and internal standard (I.S.) were recorded in electronic integrator units and expressed as a ratio of drug to I.S. Three point standard curves were analyzed with authentic samples. A five point standard curve validated assay linearity between 0.05-1.5 µg/ml, with correlation coefficients of 0.9920-0.999.

Recovery

Preparation of serum sample for injection onto the HPLC-electrochemical detection system required only a simple extraction procedure. The recovery of

Table 2
Erythromycin Standard Curve from Fortified Human Serum

Erythromycin A Concentration (ng/ml)	Intraassay Precision* (n = 4)		Interassay Precision** (n = 5)	
	ng/ml	RSD	ng/ml	RSD
50	60	8.7	60	30
150	160	5.1	160	22
500	460	3.3	470	5.4
1,000	1,000	4.7	1,000	5.1
1,500	1,510	2.0	1,500	3.0

* Samples were prepared, processed and analyzed on the same day.
** Samples were processed and analyzed on consecutive days.

erythromycin (determined by comparing the response of known drug amounts with the extracted fortified samples) averaged 84% over the dynamic range of the assay (n = 20).

Precision

Instrumental precision was 3.5% (n = 5), determined by repeated injections of 50 ng erythromycin. Intraassay relative standard deviation ranged from 3% to 9%. The maximum interassay relative standard deviations varied from 3% to 30% (Table 2). In order to correlate the HPLC assay data with those of the microbiological assay, serum samples of subjects dosed with erythromycin were assayed by both procedures. Comparable values were obtained by these assays, giving a correlation coefficient of >0.99 and a slope of 0.87.

Acknowledgements

The author would like to express appreciation to Ms. D. Farrell and Mr. S. Bartolucci for their technical assistance, Dr. J. Stankewich and Dr. A. English for conducting the microbiological assays and Dr. K. Bratin and Dr. F. Falkner for their valuable discussions.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 1033-1050 (1984)

SENSITIVE DETECTION AT LOW-WAVELENGTH FOR METHANOL GRADIENT ELUTION IN REVERSED-PHASE CHROMATOGRAPHY

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ABSTRACT

The use of a water-methanol gradient in conjunction with UV detection at low-wavelength in reversed-phase liquid chromatography was studied. In order to correct the baseline drift, formamide was added to the initial eluent. By using this initial eluent, gradient elution with water-methanol system could be performed at four times greater sensitivity. Sodium nitrate appeared to function similarly to formamide in enhancing the sensitivity. A wide selection of double wavelength detection was made possible by the simultaneous addition of formamide. This method seems to be convenient and practical since it allows the addition of acid and inorganic salt to the adjusted mobile phase. The simultaneous determination of 7 medical materials in cosmetics was carried out with this method, and satisfactory results were obtained both in the recovery and the variation coefficient.

INTRODUCTION

Gradient elution has been used in the field of modern high performance liquid chromatography (HPLC).

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This technique has the following advantages in overcoming general elution problems in HPLC (1). 1) Total analysis time can be significantly reduced. 2) Effective sensitivity becomes very high because of a negligible variation in the peak shape. 3) Elution and separation of multiple components having a wide difference in polarity can be accomplished simultaneously.

"General detection" of most of the organic compounds is carried out by UV absorption at low-wavelength near 210 nm. However, such a gradient technique at 210 nm can cause drastic baseline drifts. The elution technique of a methanol gradient combined with low-wavelength detection has been rarely reported because the absorption of methanol begins in that region. With methanol, therefore, a narrow gradient and low sensitive detection must usually be employed. V. Berry (2) has presented a sensitive gradient technique at low-wavelength using acetonitrile. In this system, detection at 210 nm with a sensitivity of 0.1 a.u.f.s. becomes feasible, baseline drift is less than 5%, and no ghost peaks appear. With his method, three problems related to methanol as eluent, namely, a mid-gradient hump, UV mismatch, and ghost peaks have been solved.

Otherwise, methanol is widely used because of its low price and toxity. The UV absorbance mismatch between initial and final eluents in the water-methanol system is greater than that in the water-acetonitrile system, because methanol

has an absorption at 183 nm. This paper shows how the addition of formamide to the initial eluent of the methanol gradient makes possible detection at 214 and 205 nm. This method can be performed conveniently and practically since it allows the addition of acid and inorganic salt to the adjusted eluents. All chromatographic profiles were obtained at high sensitivity with this method, and we show its application to the simultaneous determination of multiple medical materials in cosmetics.

EXPERIMENTAL

Reagents. Nine reagents in a series of phenones from acetophenone to tetradecanophenone were purchased from Tokyo Kasei (Tokyo, Japan). Methyl p-hydroxybenzoate, salicylic acid, butylated hydroxytoluene were purchased from Wako Pure Chemical (Osaka, Japan). Monoammonium glycyrrhizinate, tocopheryl acetate and diphenhydramine hydrochloride were purchased from Tokyo Kasei. Pantothenyl ethyl ether was purchased from Daiichi-Seiyaku (Tokyo, Japan). All these compounds were guaranteed reagent grade and were used without further purification. Methanol for HPLC (absorbance at 210 nm was 0.70 a.u. maximum and at 220 nm was 0.30 a.u. maximum) was purchased from Wako Pure Chemical and water was obtained from a Milli-R/Q-Reagent-Grade water system (Millipore, Bedford, MA, U.S.A.). Formamide and phosphoric acid were purchased from Wako Pure

Chemical and sodium perchlorate was purchased from Kanto Chemical (Tokyo, Japan).

Apparatus. The spectrophotometer used in this work was UVIDEC-610 (Japan Spectroscopic, Tokyo, Japan). The HPLC equipment consisted of two Waters 6000A pumps (Waters Assoc., Milford, MA, U.S.A.), a Waters 720 System Controller, a Waters U6K septumless loop injector, a Waters 441 UV detector and a Japan Spectroscopic UVIDEC100-2 variable wavelength UV detector. A column (6 mm id x 200 mm) packed with TSK-LS-410 (5µ) (Toyo Soda, Tokyo, Japan) was used and this column was preceded by a Brownlee Labs guard column (Rheodyne, Berkley, CA, U.S.A.).

Procedures. Methanol was allowed to stand in contact with air at room temperature for at least one day after cutting the seal. With gradients from 25 to 100% methanol, the initial eluent, a mixture of water and methanol (75/25) was first degassed by vacuum under ultrasonic waves. The flow rate was set at 1.5 ml/min and column temperature was maintained at 40°C with circulating warm water. It took 5.1 minutes to detect the eluent passed through the column after mixing two eluents. Therefore, the final eluent was held for 10 minutes after which the system controller indicated the end of gradient, by considering the equilibrium of the final eluent in the detector cell. The concentration of methanol added to each phenone was 100 µg/ml, and 15 µl of the mixture was injected. Table 1

TABLE 1. The symbols of medical materials in cosmetics

Ⓐ	Pantothenyl ethyl ether	■
Ⓑ	Methyl p-hydroxybenzoate	△
Ⓒ	Salicylic acid	○
Ⓓ	Diphenhydramine hydrochloride	□
Ⓔ	Butylated hydroxyanisol	◎
Ⓕ	Monoammonium glycyrrhizinate	▣
Ⓖ	Butylated hydroxytoluene	●
Ⓗ	Tocopheryl acetate	▲

shows the medical materials in cosmetics used in this work. The monoammonium glycyrrhizinate was dissolved in a mixture of ethanol and water (50/50) and the other 7 materials were dissolved in 95% ethanol at various concentrations. The injection volume was 20 μl.

RESULTS AND DISCUSSION

Methanol gradient at low-wavelength detection.

The UV mismatch on the detection at 214 nm was about 0.35 a.u. through the gradient from water to methanol. The baseline was concave upward against the linear gradient. The UV mismatch was about 0.02 a.u. through the gradient from water to 25% methanol, therefore, the analysis could be performed sufficiently at 214 nm with a sensitivity of 0.1 a.u.f.s.. It is clear that the UV mismatch takes place more than 25% methanol in the gradient. Therefore, at higher concentrations of methanol than 25% the UV mismatch must be corrected.

The correction method is discussed in the following section.

Selection of the additive to correct the baseline.

In general, the baseline drift caused by UV mismatch is diminished by the addition of another compound to the initial eluent. Such a compound must absorb light at the wavelength of the detector. The following points seem to be required by such an additive. First, the additive must be soluble in water and it must not affect any characteristics of mobile phase. Preferably the maximum absorption of the additive appears in the vicinity of the detecting region and its molar extinction coefficient is large. Second, the additive should not be retained on the column, and it should not slow down recovery time from the final eluent to the initial eluent. Acetone is one of the representative additives having a detection region of 229 to 254 nm. However, 60 ml of acetone was required in 1 L of the initial eluent to match the absorbance of the initial eluent (water : methanol = 75 : 25) and the final eluent (methanol) at 214 nm detection, which is too much to avoid change in the characteristics of the mobile phase. Taking into account the various requirements described above, formamide was chosen from the typical LC solvents (3-4). Fig.1 shows the differential UV absorption between the final eluent and the initial

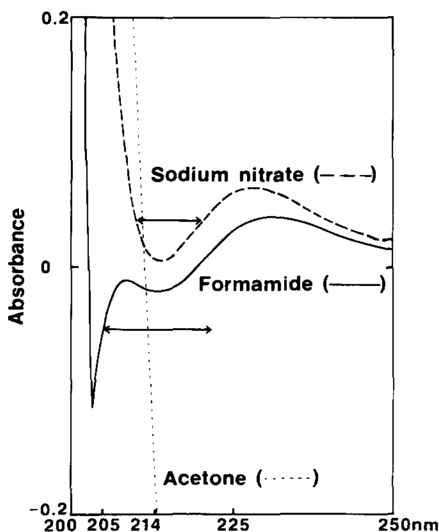

Fig.1 The differential UV absorption between the adjusted initial eluent and the final eluent at 214 nm. Sample side, the final eluent (methanol); reference side, the initial eluent (water:methanol=75:25) containing the additive;

eluent which was adjusted to near zero absorbance by addition of various additives at 214 nm. It is shown that the wavelength region is thereby expanded to a range of 205 to 222 nm for the permitted limit of ± 0.032 in UV absorption. The required volume of formamide per 1 L of the initial eluent was 125 µl. Sodium nitrate (5) which was used for the measurement of t_0 (void volume) was shown to be a good additive. It added at a rate of 340 µl of 1% aqueous solution per 1 L of the initial eluent. Fig.1 shows that the wavelength region is thereby expanded to a range of 211 to 221 nm for

the same permitted limit. The absorbance does not have to be matched precisely, because the absorbance of methanol is rising gradually in this range.

The effect of the gradient rate on the baseline.

Fig.2 shows the effect of the gradient rate on the baseline of a linear gradient of 25 to 100% methanol after matching the absorbance of the initial and final eluent. The absorption of formamide decreases linearly, whereas that of methanol increases concavely during the compositional change from the initial to final eluent, respectively. Therefore, the baselines at both wavelengths are concave. The curvature of the baseline increases remarkably with an increasing gradient rate. At a gradient rate of 5%/min or above was very the nonlinearity pronounced. The 2-component mobile phase does not seem to be at equilibrium in the detector cell at higher gradient rates. Additionary, a pronounced deviation of absorbance was observed at a methanol concentration of more than 75%. This is probably due to the interaction (6-7) of methanol molecules with residual oxygen molecules. From these results, the gradient rate has to be less than 2.5%/min to stabilize the baseline.

Reproducibility of peak heights for the quantitative analysis. It was shown that the use of formamide as an additive for baseline correction makes possible the use of a methanol gradient at low-wavelengths (214, 205 nm) and at high detector sensitivity (0.16 a.u.f.s.).

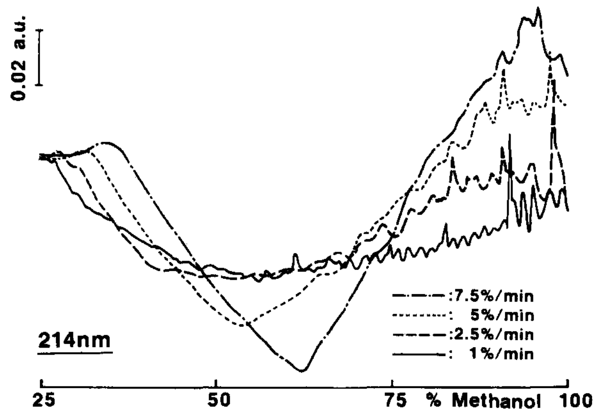

Fig.2 Effect of the gradient rate on the baseline.

Furthermore, elution profiles characteristic of multi component samples were obtained. To investigate the possibility of quantitative analysis, the reproducibility of peak height was further examined under these conditions. Following the suggestion of E.J.Kikta and A.E.Stange (8), phenones were employed as internal standard compounds with a wide range in polarity. Fig.3 shows the gradient elution profiles for 9 phenones at 214 and 205 nm detection. The variation coefficients of the peak heights of each component are summarized in Table 2. The variation coefficients of overscaled peaks should be at lower values, because ghost peaks appeared and baseline drift increased at methanol concentrations over 75%. The variation coefficients of peak heights were below 2%, except for the two highest phenones (n=10, 14). Therefore, quantitative analysis

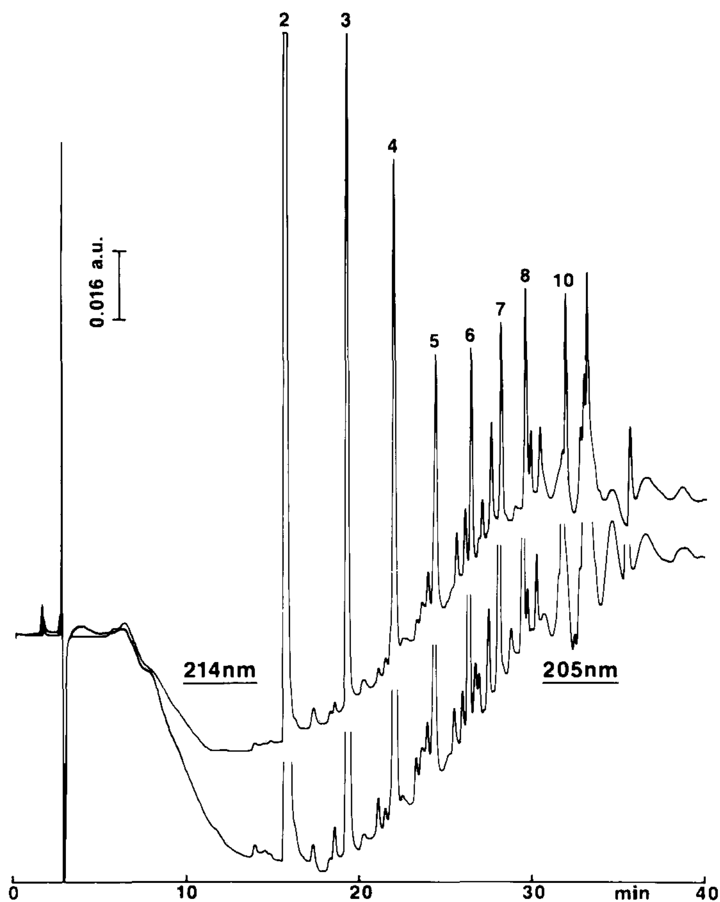

Fig.3 Gradient elution profile of 9 phenones. Mobile phase, 30 min linear gradient starting with a mixture of water:methanol=75:25, adjusted by formamide, and ending with methanol at a rate of 2.5%/min followed by 10 min of methanol. Sample size, 15 µl of each 100 µg/ml of 9 phenones; n = carbon side chain length for various phenones (for instance, n = 2 shows acetophenone).

TABLE 2. Variation coefficients of the peak heights of phenones

n	Detection wavelength	
	214 nm	205 nm
4	0.4	—
5	0.7	0.6
6	1.7	0.3
7	0.6	0.7
8	1.5	1.2
10	5.7	8.0
14	19.6	5.4 (%)

The reproducibility test was effected on repeating five times. n = carbon side chain length for various phenones.

seems possible within these limits. The reproducibility of peak height at 205 nm was more advantageous than that at 214 nm. This is probably because the sensitivity of phenone at 205 nm is higher than that at 214 nm. The solvents used for the mobile phase must be thoroughly purified for accurate analyses (9-10).

Application to the simultaneous determination of multiple medical materials in cosmetics. In general, various medical materials are contained in cosmetics and their amount is very small. Their solubilities differ from each other. Some materials are soluble in water and others are soluble in oil. It is convenient to apply the just described method to the simultaneous determination of multiple medical materials in cosmetics. Fig.4 shows the gradient elution profile for typical

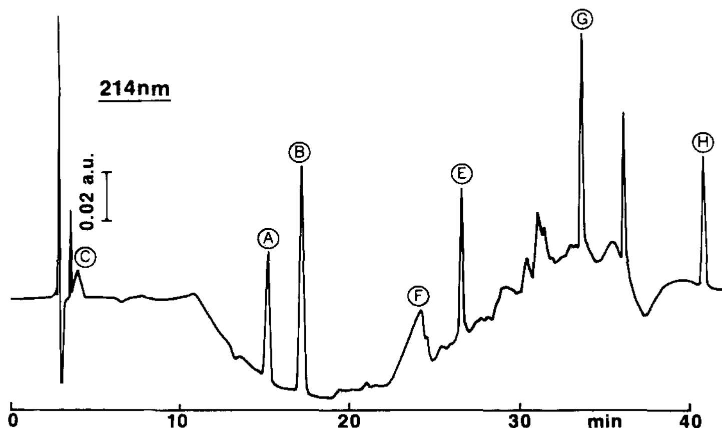

Fig.4 Gradient elution profile of typical medical materials.
Sample size, 20 µl of each concentration is Ⓐ : 300 µg/ml, Ⓑ : 20 µg/ml, Ⓒ : 10 µg/ml, Ⓓ : 20 µg/ml, Ⓔ : 20 µg/ml, Ⓕ : 750 µg/ml, Ⓖ : 20 µg/ml, Ⓗ : 25 µg/ml; the other conditions are the same as in Fig.3.

medical materials in cosmetics. The sample consists of 8 components. In this case, it is difficult to accomplish the elution and the separation by means of only the water-methanol eluent system. Actually, salicylic acid was hardly retained, monoammonium glycyrrhizinate did not show a sharp peak, and diphenhydramine hydrochloride was not eluted at all because of its adsorption on the column. Next, the addition of an acid and an inorganic salt into the mobile phase containing the corrective additive was attempted. Phosphoric acid was used since it has no absorption at low wavelength. This acid was added to the corrected initial eluent and the solution

was adjusted to pH 2.5. Taking into account the recovery time from the final eluent to the initial eluent, phosphoric acid was added to the final eluent at the rate of 1 ml per L. Sodium perchlorate was selected since it was very soluble in methanol. When a large amount of sodium perchlorate was contained in the eluent, an additional absorption appeared at low-wavelength. Therefore, the same amount of this salt was added into the initial and the final eluents. Fig.5 shows the separation of 8 components as mentioned above. Clearly, good elution and separation of the 8 components were obtained. With a mixture containing only phosphoric acid, the peak shape of monoammonium glycyrrhizinate was improved, salicylic acid and diphenhydramine hydrochloride were retained, but the two components were not separated. The addition of acetic acid allows to correct the baseline and to make an acidic mobile phase. In this case, the mobile phase has to be strictly pH 3.5 at 214 nm detection. Fig.5(b) shows the gradient profile for 8 components obtained with initial eluent without formamide. The difference between Fig.5(a) and (b) clearly shows the effect of a corrective additive. In Fig.5(b), it is difficult to determine and identify the trace peaks because of a sudden rise of the baseline. When 0.5 mol sodium perchlorate was added to the eluent, unknown peaks appeared at methanol concentrations over 75%. However, such peaks did not appear on the addition

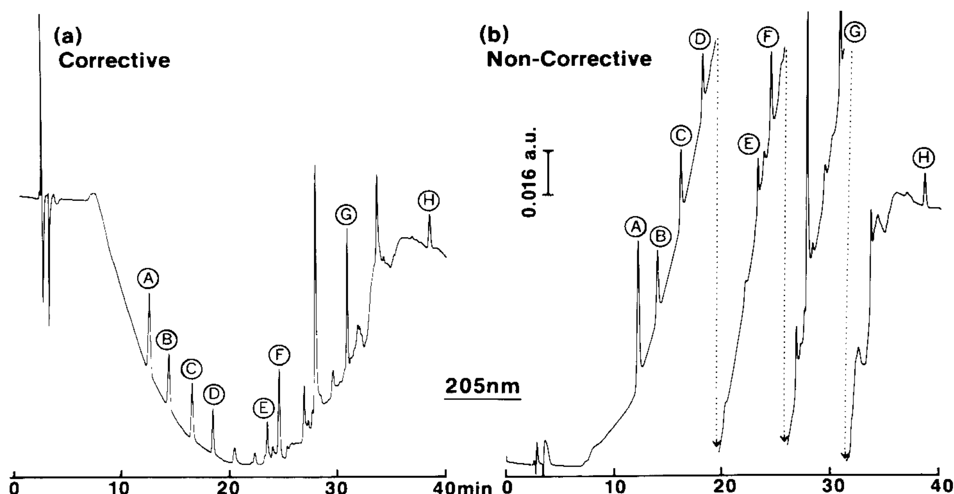

Fig.5 Separation of typical medical materials used in cosmetics.
Mobile phase, (a): 30 min linear gradient starting with 0.1 mol sodium perchlorate (water:methanol: phosphoric acid:formamide=750:250:5:0.125), and ending with 0.1 mol sodium perchlorate (methanol: phosphoric acid=1000:1) followed by 10 min of the final eluent, (b) excepts formamide from the initial eluent, and the other conditions are the same as in (a). Sample size, 20 µl of each concentration is Ⓐ : 100 µg/ml, Ⓑ : 5 µg/ml, Ⓒ : 2.5 µg/ml, Ⓓ : 5 µg/ml, Ⓔ : 5 µg/ml, Ⓕ : 250 µg/ml, Ⓖ : 5 µg/ml, Ⓗ : 5 µg/ml;

of 0.1 mol sodium perchlorate. Further, the baseline drift was reduced by the addition of inorganic salt. This eluent system was chosen for the final analysis. Fig.6 shows the calibration curves of 8 components under these conditions. Each calibration curve had good linearity and the correlation coefficients were greater than 0.998 at 214 nm and greater than 0.997 at 205 nm. The analysis required 55 minutes including

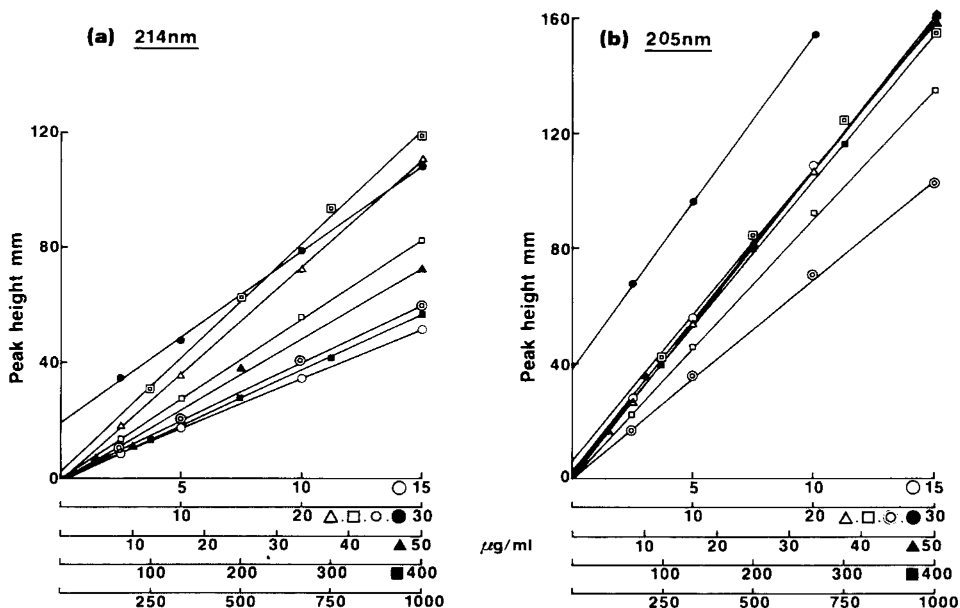

Fig.6 Calibration curves of 8 components for medical materials.
Sample size, 20 µl of mixed solution for various concentrations; the other conditions are the same as in Fig.5(a);

the recovery time of 15 minutes from the final eluent to the initial eluent. With manual injection, it seems to be difficult to complete all analyses in one day. The operation was repeated using new solvents under the same conditions. Good linearity was again observed on these data and the correlation coefficient was lower by 0.01 at both wavelengths. Only the calibration curve of butylated hydroxytoluene had a positive Y-intercept, and this was the case because the elution of butylated

TABLE 3. Recovery test from a known sample

Sample	Added (ug/5g)	No.1	No.2	No.3	No.4	No.5	Recovery(%)	c.v.(%)
Ⓐ	2500	2505	2518	2544	2531	2531	101.2	0.8
		2491	2594	2526	2526	2526	101.3	1.5
Ⓒ	75	80.7	83.5	79.7	81.6	82.6	108.8	1.8
		81.3	82.8	78.4	81.3	81.3	108.1	2.0
Ⓓ	150	158.1	162.5	158.1	157.0	161.4	106.3	1.5
		160.5	164.2	158.7	158.7	164.2	107.5	1.7
Ⓔ	150	150.2	153.1	150.2	151.7	148.8	100.5	1.1
		148.4	150.9	148.4	148.4	145.9	98.9	1.2
Ⓕ	5000	5085	5085	5052	5085	4987	101.2	0.8
		5152	5109	5067	5067	5109	102.0	0.7
Ⓖ	150	165.7	153.6	151.9	151.9	153.6	103.6	3.8
		168.8	152.0	150.3	148.6	148.6	102.4	5.6
Ⓗ	250	245.8	247.4	244.2	250.6	241.0	98.3	1.5
		——	260.5	253.8	263.8	250.4	102.9	2.4

c.v. : variation coefficient;
up line : 205 nm detection / down line : 214 nm detection;

hydroxytoluene took place on a ghost peak. The retention time and the peak height of this ghost peak were reproducible, and good linearity was obtained for the calibration curve consequently. The medical materials were added to a known lotion at a certain concentration and the recovery test was carried out with these calibration curves. Table 3 shows the result of the recovery test for 7 components. The result of methyl p-hydroxybenzoate was left out of the table since it was included in other cosmetic materials. Satisfactry results were obtained for both the recoveries and the coefficients of variation except for butylated hydroxytoluene. However, many practical problems will arise with the simultaneous deter-

mination of multiple components. For instance, in the case of extraction from oil rich cosmetics, effective extraction will be required for all components. So, simultaneous determination will be difficult for extreme differences of component ratios or response ratios. This problem may be overcome by improving the UV detector of the HPLC system.

CONCLUSION

The addition of a corrective additive to the initial eluent has proved to be very effective for stabilizing the baseline. This technique made possible the use of the methanol gradient technique with sensitive low-wavelength detection. Especially formamide was a good corrective additive. This method could be applied to the simultaneous determination of multiple micro components of cosmetics. The sensitivity of the analysis could be enhanced by more than four times by the addition of formamide into the initial eluent. It was possible to add acid and inorganic salt to the adjusted eluent. Double wavelength detection could be performed simultaneously on the basis of the exactly measured absorption of formamide. This analysis makes possible the detection of trace components like antiseptics contained in raw materials for cosmetics. This method should also be useful for profile analyses of natural medicines which consist of complex mixtures.

ACKNOWLEDGEMENTS

The authers thank Dr. Takeo Mitsui for allowing them to publish this paper and Dr. Yoshimitsu Kuroyanagi for helpful discussion.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5) 1051 (1984)

BOOK REVIEW

THE INTERNATIONAL BIOTECHNOLOGY DIRECTORY - 1984, J. Coombs, The Nature Press, New York, NY, 1983, 426 pages, $100.00 (US).

This comprehensive volume contains three parts. The first provides an overview of the current biotechnology industry. The second part presents activities of government agencies, societies and associations involved in biotechnology, with a listing by country. Finally, Part 3 serves as a "buyer's" guide for products, research and services.

The directory appears to cover all countries where biotechnology research and industry are active. Over 2000 organizations are included...commercial and non-commercial, companies, universities, and research centers...with a brief description of their activities. Almost half of the organizations covered are in the United States.

Although this is not the first directory of biotechnology, it is certainly one of the best ones this reviewer has seen. It should be on the desk of everyone who finds himself involved in this rapidly growing field.

Dr. Jack Cazes
Fairfield, CT, USA.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 1053-1054 (1984)

LC NEWS

CHROMATOGRAPHY CONTROLLER, designed to be used with the FPLC System, automates sample injection, controls up to eight motorized valves, integrates one monitor signal and uses another for control functions, and may be used to control multicolumn separation methods. A printer-plotter is included in the unit and serves to plot the chromatogram, report programmed information and peak integration values. Pharmacia Fine Chemicals, Inc., JLC/84/5, 800 Centennial Avenue, Piscataway, NJ, 08854, USA.

HPLC ABSORPTION MONITOR offers .001 AUFS sensitivity. It uses a low pressure mercury lAmp with a heated cathode for stability. It is microprocessor controlled with auto zero available over the entire sensitivity range. Sonntek, Inc., JLC/84/5, P. O. Box 8731, Woodcliff Lake, NJ, 07675, USA.

PHOTODIODE ARRAY DETECTOR FOR HPLC incorporates the latest microprocessor technology. It monitors at up to four different wavelengths simultaneously. The total UV spectrum is obtained within 0.1 second, t6thus eliminating the need for stop flow procedures. Absorbance ratio at two wavelengths can also be monitored continuously. LKB Instruments, Inc., JLC/84/5, 9319 Gaither Road, Gaithersburg, MD, 20877, USA.

CARBOHYDRATE COLUMN is packed with a proprietary 5 micron stationary phase. Analyses are performed at room temperature using a mobile phase of acetonitrile/water. Applications include analysis of sugars in beverages, food products, cough & cold remedies, and analgesics. IBM Instruments, Inc., JLC/84/5, P. O. Box 3020, Wallingford, CT, 06492, USA.

HPLC INJECTOR STATION for the Laboratory Automation System combines HPLC sample introduction with automated sample preparation procedures. Samples are automatically prepared by the system and then directly introduced to the HPLC via either syringe injection or aspiration from a sample tube. Zymark, Corp., JLC/84/5, Zymark Center, Hopkinton, MA, 01748, USA.

POLYACRYLAMIDE SOFT GEL FOR SEC/GPC may be operated at high pressures and undergoes minimum swelling and shrinkage when transferred between polar eluents. Uses include separations of

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polysaccharides, polyphenols, synthetic aqueous polymers, and biopolymers. Polymer Laboratories, JLC/84/5, Essex Road, Church Stretton, Stropshire, SY6 6AX, UK.

AUTOSAMPLER FOR HpLC offers repeatable and accurate sampling, high throughput, and total automation for up to 102 samples. Nine programs may be stored and positive sample identification is provided. Beckman Instruments, Inc., JLC/84/5, Altex Scientific Div., 1716 East 4th Street, Berkeley, CA, 94710, USA.

MICRO HPLC PUMP can be used at low flow rates. Two essentially pulse-free flows are combined in a micromixing system to produce linear gradients from 1,000 to 1 microliters/min. Syringe pumps are used with automatic refill after each run. Brownlee Laboratories, JLC/84/5, 2045 Martin Avenue, Suite 204, Santa Clara, CA, 95050, USA.

GPC+, FIRMWARE-BASED PROGRAM, calculates analytical results in gel permeation chromatography. It resides on a 8K chip enables the calculation of calibration curves via point-to-point, quadratic, or cubic fits of the data. Statistical data are presented to aid in selecting the best fit. Spectra-Physics Corp., JLC/84/5, 3333 North First Street, San Jose, CA, 95134, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(5), 1055-1059 (1984)

LC CALENDAR

1984

APRIL 29 - MAY 3: Analyical Applications of Supercritical Fluids - Supercritical Fluid Technol Symposium, at the meeting of the AOCS, Dallas, TX. Contact: Dr. J. W. King, CPC Internat'l, Moffett Tech Center, Argo, IL, 60501, USA.

MAY 17: Symposium: Therapeutic Drug Monitoring & Toxicology for the 80's: Clinical & Instrumental Perspectives, Farmington, CT, sponsored by the UConn Medical School & AAAC Connecticut Valley Chapter. Contact: Dr. Steven H. Wong, Dept. of Lab. Med., UConn Medical School, Farmington, CT, 06032, USA.

MAY 20 - 26: 8th Intl. Symposium on Column Liquid Chromatography, New York Statler Hotel, New York City. Contact: Prof. Cs. Horvath, Yale University, Dept. of Chem. Eng., P. O. Box 2159, Yale Stn., New Haven, CT, 06520, USA.

MAY 24 - 29: American Associatio for the Advancement of Science - Annual Meeting & Exhibit, New York City. Contact: AAAS Meetings Dept., 10th Floor, 1101 Vermont Avenue, NW, Washington, DC, 20005, USA.

JUNE 3-5: International Symposium on LCEC and Voltammetry, Indianapolis Hyatt Regency Hotel, Indianapolis, IN. Contact: The 1984 LCEC Symposium, P. O. Box 2206, West Lafayette, IN, 47906, USA.

JUnE 10 - 13: Annual Meeting of the Institute of Food Technologists, Anaheim, CA. Contact: IFT, 221 N. LaSalle Street, Suite 2120, Chicago, IL, 60601, USA.

JUNE 10-14: 14th Northeast Regional ACS Meeting, sponsored by the Western Connecticut and New Haven Sections, at Fairfield University, Fairfield, CT. Contact:D. L. Swanson, American Cyanamid Co., Stamford, CT, USA.

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JUNE 18-20: Second International Conference on Chromatography & Mass Spectrometry in Biomedical Sciences, sponsored by the Italian Group for Mass Spectrometry in Biochemistry & Medicine, Milan, Italy. Contact: Dr. A. Frigerio, via Eustachi 36, I-20129 Milan, Italy, or Dr. H. Milon, P. O. Box 88, CH-1814 La Tour-de-Peilz, Switzerland.

JUNE 18-21: Symposium on Liquid Chromatography in the Biological Sciences, Ronneby, Sweden, sponsored by The Swedish Academy of Pharmaceutical Sciences. Contact: Swedish Academy of Pharmaceutical Sciences, P. O. Box 1136, S-111 81 Stockholm, Sweden.

JUnE 19 - 23: SPI/SPE Plastics Show & Conf. East, Philadelphia, PA. Contact: SPE, 14 Fairfield Drive, Brookfield Center, CT, 06805, USA.

JUNE 21 - 22: Conference on Quantitative Characterization of Plastics & Rubber, McMaster University, Hamilton, Ont., Canada. Contact: John Vlachopoulos, Dept. of Chem. Eng., McMaster University, Hamilton, Ont., L8S 4L7, Canada.

JULY 1 - 7: 12th International Carbohydrate Symposium, Jaarbeurs Congress Centre, Utrecht, The Netherlands. Contact: J. F. G. Vliengenthart, Dept. of Bio-Organic Chem., State Univ. of Utrecht, P. O. Box 5055, NL-3502JB, Utrecht, The Netherlands.

JULY 9 - 13: 10th International Conference on Organic Coatings Science & Technol., Athens, Greece. Contact: V. Patsis, Materials Research Lab, CSB 209, State Univ. of NY, New Paltz, NY, 12561, USA.

JULY 15 - 20: International Conference on Ion Exchange, Cambridge College, UK. Contact: Conference Committee, IEX-84, Soc. Chem. Ind., 14 Belgrave Square, London, SWIX 8PS, UK.

AUGUST 21 - 24: 24th Int'l Conf on Analytical Chem. in Development, Sri Lanka. Contact: Secretary, Organizing Committee, Centre for Anal. Chem R & D, Dept. of Chem., University of Colombo, P. O. Box 1490, Colombo 3, Sri Lanka.

AUGUST 26-31: National ACS Meeting, Philadelphia, PA. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

SEPTEMBER 10-14: Advances in Liquid Chromatography, including the 4th Annual American-Eastern European Symposium on LC and the Int'l Symposium on TLC with Special Emphasis on Overpressured Layer Chromatography, sponsored by the Hungarian Academy of Sciences' Chromatography Committee & Biological Research Center and the Hungarian Chemical Society, in Szeged, Hungary. Contact: Dr. H.

Kalasz, Dept. of Pharmacology, Semmelweis University of Medicine, P.O.Box 370, H-1445 Budapest, Hungary, or Dr. E. Tyihak, Research Inst. for Plant Protection, P.O.Box 102, H-1525 Budapest, Hungary.

SEPTEMBER 16 - 21: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Marriott Hotel, Philadelphia, PA. Contact: D. B. Chase, DuPont Co., Experimental Station 328, Wilmington, DE, 19898, USA.

OCTOBER 1-5: 15th Int'l. Sympos. on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, D-6000 Frankfurt Main, West Germany.

DECEMBER 10-12: "TLC/HPTLC-84: Expanding Horizons in TLC," Sheraton-University City, Philadelphia, PA. Contact: J. C. Touchstone, University of Pennsylvania, Dept. OB-GYN, 3400 Spruce Street, Philadelphia, PA.

DECEMBER 16-21: International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, sponsored by the Chemical Inst. of Canada, Chemical Soc. of Japan, and the American Chem. Soc. Contact: PAC CHEM '84, International Activities Office, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

1985

FEBRUARY 11-14: Polymer 85, Int'l Symposium on Characterization and Analysis of Polymers, Monash University, Melbourne, Australia, sponsored by the Polymer Div., Royal Australian Chemical Inst. Contact: Polymer 85, RACI, 191 Royal Parade, Parkville Victoria 3052, Australia.

APRIL 28 - MAY 3: 189th National ACS Meeting, Miami Beach. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

JUNE 24 - 28: 59th Colloid & Surface Science Symposium, Clarkson College of Technology, Potsdam, NY. Contact: J. P. Kratohvil, Institute of Colloid & Surface Science, Clarkson College of Technology, Potsdam, NY, 13676, USA.

JULY 1-5: Ninth International Symposium on Column Liquid Chromatography, sponsored by the Chromatography Discussion Group and by the Royal Society of Chemistry's Chromatography & Electrophoresis Group, Edinburgh, Scotland. Contact: Prof. J. H. Knox, 9th ISCLC Secretariat, 26 Albany Street, Edinburgh, EH1 3QH, Great Britain.

JULY 4: 4th International Flavor Conference, Greece. Contact: C.

J. Mussinan, IFF R&D, 1515 Highway 36, Union Beach, NJ, 07735, USA.

SEPTEMBER 8-13: 190th National ACS Meeting, Chicago. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

SEPTEMBER 29 - OCTOBER 4: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Marriott Hotel, Philadelphia, PA. Contact: T. Rains, NBS, Center for Analytical Chemistry, Chemistry B-222, Washington, DC, 20234, USA.

1986

APRIL 6-11: 191st National Am. Chem. Soc. Mtng., Atlantic City, NJ. Contact: A. T. Winstead, ACS, 1155 16th Streeet, NW, Washington, DC, 20036, USA.

SEPTEMBER 7-12: 192nd National Am. Chem. Soc. Mtng., Anaheim, Calif. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

1987

APRIL 5-10: 193rd National Am. Chem. Soc. Mtng., Denver, Colo. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

AUGUST 30 - SEPTEMBER 4: 194th National Am. Chem. Soc. Mtng., New Orleans, LA. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

1988

JUNE 5 - 11: 3rd Chemical Congress of the North Americanmn Continent, Toronto, Ont., Canada. Contact: A. T. Winstead, ACS, 1155 Sixteenth St, NW, Washington, DC, 20036, USA.

SEPTEMBER 25 - 30: 196th ACS National Meeting, Los Angeles, CA. Contact: A. T. Winstead, ACS, 1155 Sixteenth Street, NW, Washington, DC, 20036, USA.

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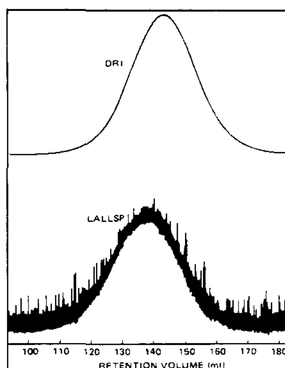

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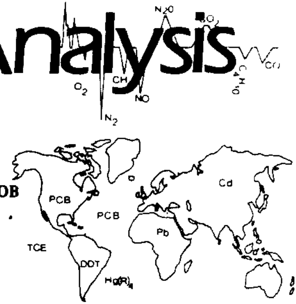

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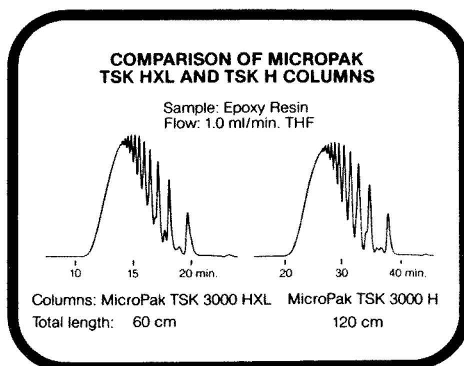

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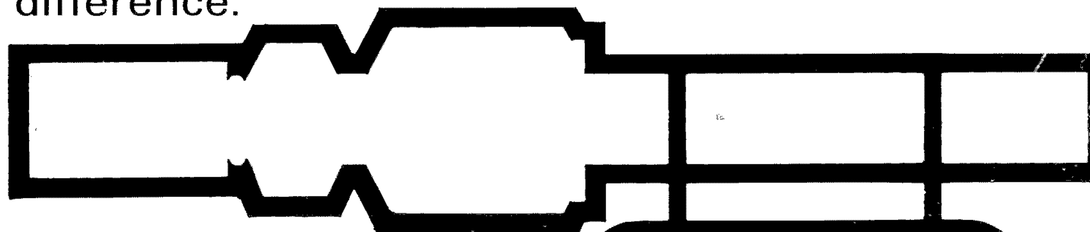

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