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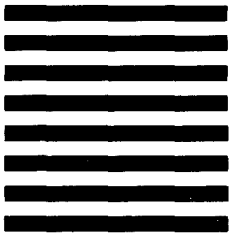

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MUTUAL SEPARATION OF HOLMIUM, ERBIUM, AND YTTRIUM BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

KENICHI AKIBA[1], HIROYUKI HASHIMOTO[1], SHIGETO NAKAMURA[1], AND YOSHIHIRO SAITO[2]

[1]*Institute for Advanced Materials Processing*
Tohoku University
Katahira-2, Aoba-ku
Sendai 980-77, Japan
[2]*Shimadzu Corporation*
1, Nishinokyo-Kuwabaracho, Nakagyo-ku
Kyoto 604, Japan

ABSTRACT

The separation of yttrium and chemically-similar heavy lanthanoids has been studied by countercurrent chromatography (CCC) equipped with a multilayer coiled column containing a toluene solution of 2-ethylhexylphosphonic acid mono-2-ethylhexyl ester as a stationary phase. The retention of the stationary phase in a coiled tubing was enhanced with increasing revolutional speed and with decreasing flow rate of the mobile phase. This high retention of the stationary phase improved the peak resolution. The mutual separation of rare earths with low separation factors was satisfactorily accomplished with good resolution through a single step of CCC.

INTRODUCTION

Lanthanoid series elements remarkably resemble each other in physical and chemical properties. Furthermore, yttrium ion has an ionic radius close to the value for holmium ion owing to lanthanoid contraction. The lanthanoid elements and yttrium are usually classed as rare earths because of their general occurrence

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in mineral resources and closely similar properties, and multistage processes are required for the mutual separation of individual elements.

Different types of liquid–liquid partition chromatography with support–free stationary phase have been proposed (1–6). This chromatographic method is attractive because it offers the wide choice of two immiscible solvent phases on the basis of liquid–liquid extraction properties. Centrifugal partition chromatography (CPC) has been previously introduced to the separation of a series of lanthanoids, and adjacent elements were isolated with reasonable resolution (7–10). The separation of yttrium and lanthanoids is still so difficult, because the extractability of some heavy lanthanoids is very close to that of yttrium. In a preceding study, the separation of yttrium was performed by two steps of CPC processes (11). Yttrium and accompanying heavy lanthanoids with low separation factors were first fractionated by CPC with a stationary phase containing an acidic organophosphorus compound, and further isolation of yttrium from heavy lanthanoids was accomplished by subsequent CPC treatment through a versatic acid stationary phase.

An alternative high–speed countercurrent chromatography (CCC) using a multilayer coiled column has also afforded great possibility for the separation of chemically–similar materials (1,2). The separation of lanthanoid ions was successfully achieved by passing a mobile phase through a coiled column, yielding excellent peak resolution (12).

An acidic extractant, di(2–ethylhexyl) phosphoric acid (DEHPA) has been most commonly used for the separation of rare earths owing to its high extractability and relatively high separation factor between these elements (13,14). Other acidic extractant, 2–ethylhexylphosphonic acid mono–2–ethylhexyl ester (EHPA) has also excellent properties for the extraction of rare earths (8). Its low extractability compared with DEHPA is rather desirable for a stationary phase component because a mild acidic solution might be available for the mobile phase.

In the present paper, a liquid–liquid extraction process was applied to multistage separation by means of CCC provided with the stationary phase of an organic solution of EHPA having better selectivity for heavy lanthanoids. Chromatographic procedures were performed for the mutual separation of the most difficult separable mixture of yttrium, holmium and erbium.

EXPERIMENTAL

Materials

The extractant, 2-ethylhexylphosphonic acid mono-2-ethylhexyl ester (EHPA; PC-88A, Daihachi Chemical Ind. Co. Ltd.) was washed with a sodium hydroxide solution to remove acidic impurities and diluted with toluene (8). Arsenazo III (Dojindo Lab.) was used as a post column reagent.

Apparatus

A prototype high-speed countercurrent chromatograph apparatus (HCC-1A; Shimadzu Corporation) holds two identical tubing columns symmetrically on the rotary frame. Each column holder undergoes a synchronous planetary motion around own axis and revolution around the central axis of the centrifuge with a 10 cm orbital radius in the same direction. A multilayer coiled column was prepared from a polytetrafluoroethylene (PTFE) tubing with a 1.5 mm inner diameter and ca 150 m length by winding onto two cylindrical column holders with 15 cm diameter and 14 cm length, firming three coiled layers with total about 300 helical turns. The total capacity of the two connected multilayer coil was measured as 265 cm^3. This apparatus was housed in an air-thermostated box regulated at 35°C.

For continuous analysis of elution stream, the detection system consists of a post column-reactor, a photometric detector with a flow cell and a pen recorder (8).

Procedure

In liquid-liquid extraction, a toluene solution (10 cm^3) of 0.02 M (M = mol dm^{-3}) $(EHPA)_2$ was shaken for 1 h at 35°C with an equal volume of an aqueous phase containing 2×10^{-5} M of Dy, Ho, Y and Er. The aqueous pH was adjusted to an appropriate value with 0.1 M $(H,Na)Cl_2CHCOO$. The concentrations of rare earths were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES).

In CCC experiments, the column was first filled with a toluene solution containing 0.02 M $(EHPA)_2$. The aqueous mobile phase was pre-equilibrated by

contacting with the corresponding organic solution, degassed with a on-line degasser and pumped "head to tail" into the column under operating conditions, i.e., a revolutional speed of 800 rpm, a flow rate of 5 cm^3 min^{-1} at 35°C, unless otherwise noted in the text. After an equilibrium had been reached between two phases, a sample solution containing 5×10^{-4} - 1×10^{-3} M of Dy, Ho, Er and Y was loaded through a sample loop (1 cm^3). Metal ions were eluted with the mobile phase of an appropriate pH buffered with 0.1 M (H,Na)Cl_2CHCOO. The eluted rare earths were monitored by measuring the absorbance at 650 nm for rare earth complexes by a post-column reaction with 0.14%(w/v) Arsenazo III in 50%(v/v) ethanol.

The volume of mobile phase (V_m) was obtained as the elution volume for an unretained component, and that of stationary phase was evaluated by subtracting V_m from the total column volume (V_c). These values were confirmed to be fair agreement with the volumes of column contents expelled by introduction of nitrogen gas after a series of CCC experiments.

RESULTS AND DISCUSSION

Liquid-Liquid Extraction of Rare Earths

Extraction equilibrium of yttrium and lanthanoids having very close extractability with 2-ethylhexylphosphonic acid mono-2-ethylhexyl ester (EHPA) was examined at 35°C in order to select chemical conditions for CCC procedures of the mutual separation of rare earths. As Fig. 1 shows plots of log D against pH gave a series of parallel lines with slopes close to 3 for yttrium and some heavy lanthanoids. Further, the third power dependency of the distribution ratio on the reagent concentration was confirmed. Overall extraction equilibrium for the rare earth ions into the toluene solution of EHPA can be expressed by

$$M^{3+}_{aq} + 3(HA)_{2,org} \rightleftarrows M(HA_2)_{3,org} + 3H^+_{aq} \quad (1)$$

$$K_{ex} = \frac{[M(HA_2)_3]_{org}\,[H^+]^3_{aq}}{[M^{3+}]_{aq}\,[(HA)_2]^3_{org}} \quad (2)$$

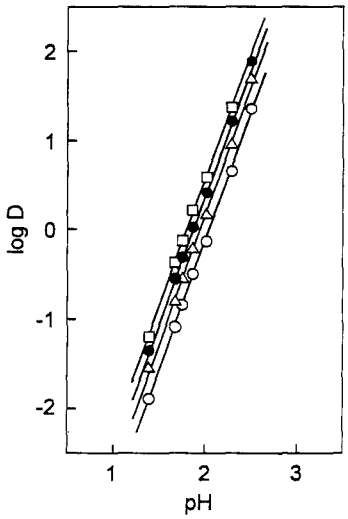

FIGURE 1 Extraction of rare earth elements by EHPA. Organic phase: 0.02 M $(EHPA)_2$ in toluene; aqueous phase: 0.1 M $(H,Na)Cl_2CHCOO$; 35°C. (●) Y, (○) Dy, (△) Ho, (□) Er.

where M denotes the rare earth and $(HA)_2$, the dimer of EHPA. The extraction constant (K_{ex}) and the separation factor (α_D) given by the ratio of their K_{ex} values are summarized in Table 1. The K_{ex} values were in the order Dy < Ho < Y < Er. The separation factors between these ions were small as 1.91 for Ho/Dy, 1.86 for Y/Ho and 1.51 for Er/Y, suggesting particular difficulties in their mutual separation.

Retention of Stationary Phase

In the present CCC process, it is significantly important to provide the satisfactorily stable retention of a suitable organic solution as the stationary phase in the tubing coil. The volume of the stationary phase retained in the column has been found to widely vary depending on operating conditions such as the revolutional speed and the flow rate (15).

TABLE 1
Extraction Constants and Separation Factors

Rare earth	log D at pH 2.0	log K_{ex}	α_D
Er	0.55	−0.35	
			1.51
Y	0.37	−0.53	
			1.86
Ho	0.10	−0.80	
			1.91
Dy	−0.18	−1.08	

Organic phase: 0.02 M $(EHPA)_2$ in toluene.
Aqueous phase: 0.1 M $(H,Na)Cl_2CHCOO$.
Temperature: 35°C.

Figure 2 shows the effect of the revolutional speed on the retention of the toluene solution of EHPA. The retention ratio (V_s/V_c) defined as the ratio of the stationary phase volume (V_s) to the total column volume (V_c) increased along with the revolutional speed ranging from 400 to 800 rpm tested, as indicated by the line (a) in Fig. 2, and no retention was obtained at lower speeds below 400 rpm. After the stationary phase had been retained at a low revolutional speed of 600 rpm, its volume remained almost unchanged even though the revolutional speed further increased up to 800 rpm, as indicated by the line (b) in Fig. 2.

Figure 3 shows the effect of the flow rate of the mobile phase on the retention ratio. As indicated by the line (a) in Fig. 3, the retention ratio was found to decrease with an increase in the flow rate. When the stationary phase had been retained at a high flow rate of 7 $cm^3\ min^{-1}$, the retention ratio remained almost constant irrespective of the decreasing flow rate from 7 to 2 $cm^3\ min^{-1}$, as indicated by the line (b) in Fig. 3. The retention ratio of the stationary phase thus can be adjusted to an appropriate value by controlling operating conditions; the favorable retention of the stationary phase was obtained with a revolutional speed above 600 rpm and a flow rate below 10 $cm^3\ min^{-1}$.

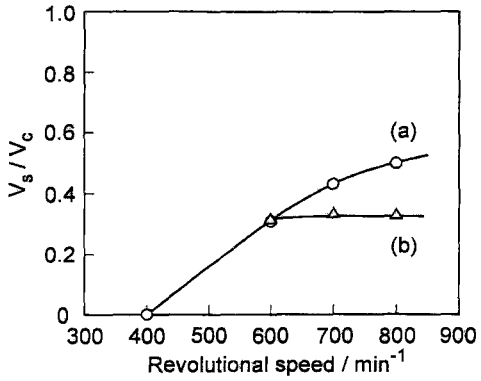

FIGURE 2 Effect of the revolutional speed on the retention of the stationary phase. V_c: column volume; V_s: stationary phase volume; flow rate: 5 cm^3 min^{-1}; 35°C. (a) The stationary phase was retained at different revolutional speeds; (b) the revolutional speed increased after the retention of the stationary phase at 600 rpm.

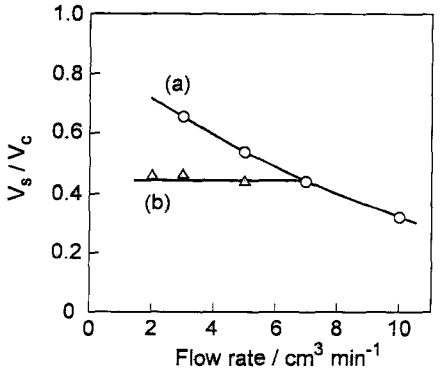

FIGURE 3 Effect of the flow rate of the mobile phase on the retention of the stationary phase. Revolutional speed: 800 rpm; (a) the stationary phase was retained at different flow rate; (b) the flow rate decreased after the retention of the stationary phase at 7 cm^3 min^{-1}.

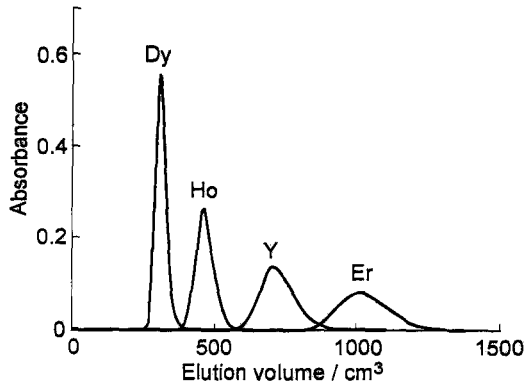

FIGURE 4 Elution peaks of individual rare earth elements. Revolutional speed: 800 rpm; flow rate: 5 cm^3 min^{-1}; V_s/V_c = 0.51; sample: 10^{-3} M RE, 1 cm^3; mobile phase: pH 2.05.

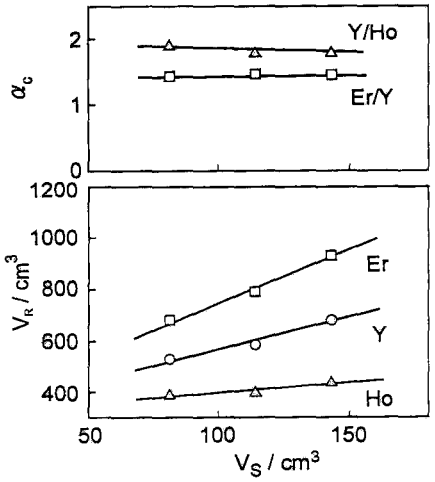

FIGURE 5 Effect of the stationary phase volume on the retention volume and the separation factor. Flow rate: 5 cm^3 min^{-1}; the stationary phase volume was varied by controlling the revolutional speed.

Effect of Stationary Phase Volume on Retention of Rare Earths

The volume of the stationary phase retained in the column would be an important factor governing the retention behavior of desired species and optimizing separating conditions. The elution of each rare earth ion was tested by means of CCC having the stationary phase of EHPA in toluene, and the individual eluted peaks of the rare earth ions are illustrated in Fig. 4. These ions were eluted in the order Dy < Ho < Y < Er in agreement with the order in D values; this implies the possibility of adequate separation of the closely resemble rare earths without any serious overlap of adjacent peaks.

In chromatography, the retention volume (V_R) of a desired component is given by the volumes of the mobile and stationary phases and the distribution ratio (D)

$$V_R = V_m + D V_s \quad (3)$$

where V_m is the mobile phase volume.

Figure 5 shows the V_R value evaluated from individual peaks at different stationary phase volumes produced by varying the revolutional speed. The V_R value increased linearly with the V_s value yielding individual slopes for each rare earth.

The separation factor (α_c) in chromatography is evaluated from the retention volumes of two adjacent peaks ($V_{R,2} > V_{R,1}$)

$$\alpha_c = \frac{V_{R,2} - V_0}{V_{R,1} - V_0} \quad (4)$$

where V_0 is the elution volume for an unretained component, that is, corresponds to V_m.

The separation factor was almost unaltered independent of variation in the V_s value as shown in Fig. 5, because the V_R value for related rare earths increased linearly with the V_s value.

Retention Volume and Distribution Ratio of Rare Earths

The distribution ratio of the related species is of importance in determining retention volume of the peaks. Figure 6 shows a plot of the V_R value obtained from chromatograms at a constant volume of stationary phase, i.e, V_s = 135 cm^3 at

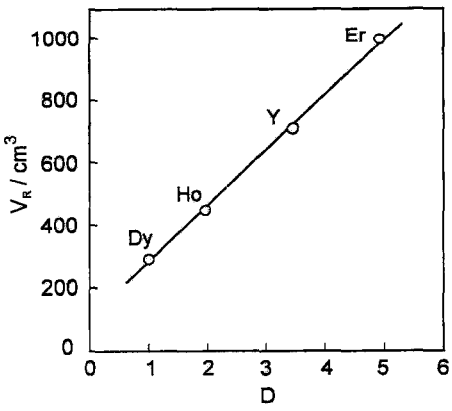

FIGURE 6 Correlation between the retention volume and the distribution ratio. V_s : 135 cm^3; V_m : 130 cm^3; pH 2.05.

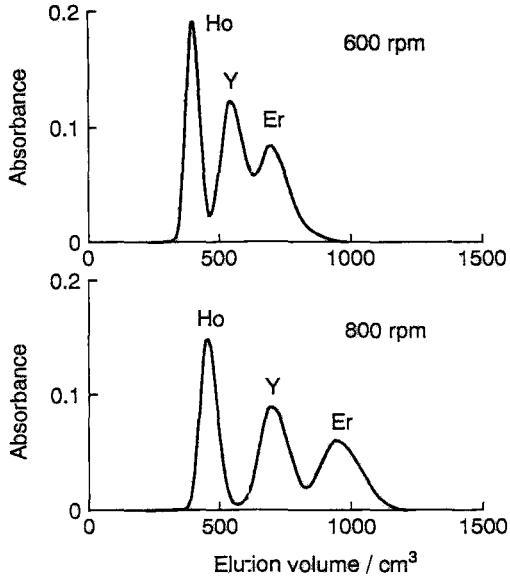

FIGURE 7 Chromatograms at different revolutional speeds. Flow rate: 5 cm^3 min^{-1}; sample: 5x10^{-4} M each RE, 1 cm^3.

800 rpm, against the equilibrium distribution ratio. The retention volumes for individual rare earths were in proportion to their distribution ratios according to Eq.(3). Thus the position of the eluted peak can be predicted from the D value provided the values of V_m and V_s are known.

Separation Parameters

The separation of desired species can be characterized by some chromatographic parameters (16). The number of theoretical plate (N) is related to the retention volume and peakwidth (W):

$$N = 16 (V_R / W)^2 \quad (5)$$

The resolution (R_s) of adjacent peaks is defined as

$$R_s = 2 \frac{V_{R,2} - V_{R,1}}{W_1 + W_2} \quad (6)$$

Figure 7 illustrates typical chromatograms obtained for a mixture of Y, Ho and Er, at different revolutional speeds. The elution profiles at a high speed of 800 rpm show good separation of individual peaks. Figure 8 shows the effect of the revolutional speed on the separation parameters. The variation in N was rather minor, though some scatters were observed, and the resolution was appreciably improved along with revolutional speed. This high resolution would be attributable to an increase in the volume of the stationary phase retained from V_s = 81 cm^3 at 600 rpm to 143 cm^3 at 800 rpm.

In order to examine the effect of the revolutional speed at a fixed V_s, the subsequent CPC procedure was performed by increasing the revolutional speed after the stationary phase had been previously retained at a low revolutional speed of 600 rpm. Separation parameters evaluated at the constant volume of the stationary phase, V_s = about 85 cm^3, are shown in Fig. 9, as a function of revolutional speed. In this case, the V_R values of given rare earths remained almost unaltered owing a given V_s. The values of N and R_s were also little affected by the revolutional speed provided the stationary phase volume was kept constant. The high retention of the stationary phase resulted from the high speed of revolution would thus contribute to the enhancement of the separation parameters.

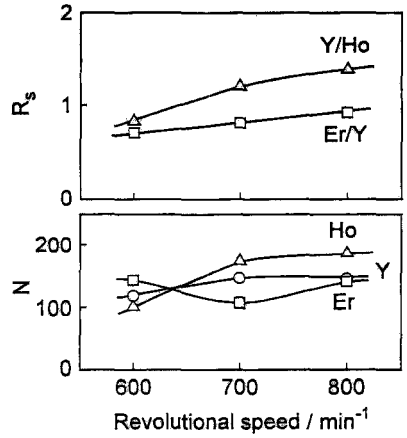

FIGURE 8 Effect of the revolutional speed on the chromatographic parameters. Flow rate: 5cm^3 min^{-1}; pH 2.06.

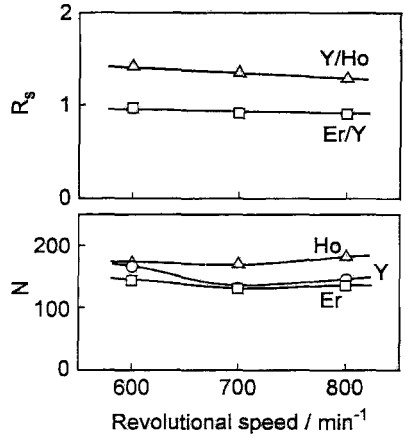

FIGURE 9 Effect of the revolutional speed on the chromatographic parameters at a fixed volume of the stationary phase. The stationary phase was initially retained at 600 rpm. V_s = 85 cm^3; pH 2.17.

Effect of Flow Rate

Further improvement of peak resolution was attempted by varying the flow rate of the mobile phase in the range from 3 to 10 cm^3 min^{-1} at a constant revolutional speed of 800 rpm. As Fig. 10 shows, the values of N and R_s were found to decrease with an increase in the flow rate. However, these findings also include an effect of a decrease in the stationary phase volume, that is, from V_s = 176 at a flow rate of 3 cm^3 min^{-1} to V_s = 87 cm^3 at 10 cm^3 min^{-1}.

Variations in separation parameters were examined at a fixed volume of the stationary phase, i.e., V_s = 122 cm^3, by initially retaining the stationary phase at a high flow rate of 7 cm^3 min^{-1}. As Fig. 11 shows, the values of N and R_s were found to be almost independent of the flow rate as long as the V_s value was kept constant.

Thus, the high resolution at the low flow rate noted in Fig. 10 was mainly caused by the high retention ratio of the stationary phase, e.g., V_s/V_c = 0.66 at a flow rate of 3 cm^3 min^{-1}.

Effect of pH of Mobile Phase

Chromatographic separation of Y, Ho and Er was performed by passing the aqueous mobile phases of different pH. The flow rate of mobile phase was adjusted to 3 cm^3 min^{-1}, after two phase equilibration at 5 cm^3 min^{-1}. Figure 12 illustrates resulted chromatograms at different pH. At a lower pH of 1.95, the peaks were not well separated each other. The V_R values increased with increasing pH and the degree of separation of metal ions was much improved. The separation parameters evaluated are summarized in Table 2. The V_R value for each rare earth increased along with pH governing the D value, and the separation factor was almost unchanged as 1.75 for Y/Ho and 1.45 for Er/Y. The higher pH value appreciably led an excellent peak resolution and then the R_s value attained near 1 at pH 2.06 even for the pair of Er/Y with the low separation factor of 1.47.

It is important to control the pH of the mobile phase in order to optimize the distribution ratio of each component for adequate separation. When the D value is low, the resolution will be poor due to low retention on the stationary phase, while at high D value a long time for elution will be required.

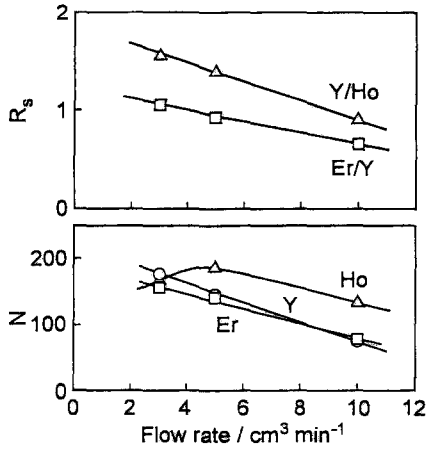

FIGURE 10 Effect of the flow rate on the chromatographic parameters. Revolutional speed: 800 rpm; pH 2.06.

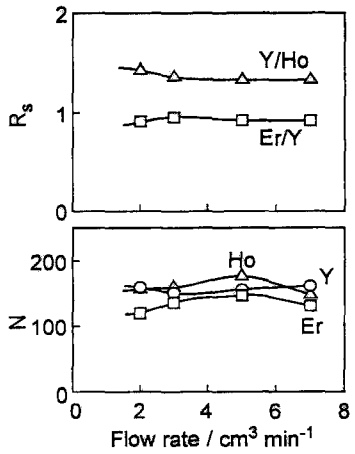

FIGURE 11 Effect of the flow rate on the chromatographic parameters at a fixed volume of the stationary phase. Revolutional speed: 800 rpm; the stationary phase was initially retained at 7 cm^3 min^{-1}; V_s = 122 cm^3, pH 2.09.

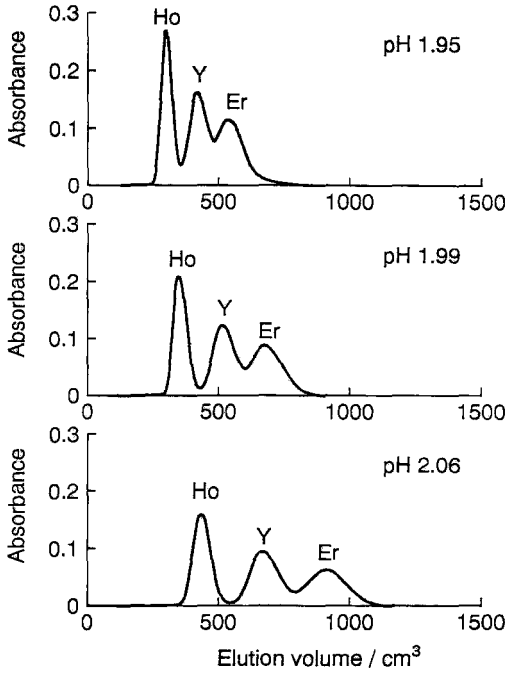

FIGURE 12 Chromatograms at different pH of the mobile phase. Revolutional speed: 800 rpm; flow rate: 3 cm^3 min^{-1}; the stationary phase retained at 5 cm^3 min^{-1}, V_s = 136 cm^3.

Peak Resolution

The resolution of elution peaks usually depends on several chromatographic parameters (17). The resolution between adjacent peaks can be presented with the aid of Eqs. (4)–(6) as

$$R_s = \frac{k'_2 (\alpha_c - 1)}{2\,\alpha_c (N_1^{-1/2} + N_2^{-1/2}) + 2\,k'_2 (N_1^{-1/2} + \alpha_c N_2^{-1/2})} \quad (7)$$

where k' denotes the capacity factor and is related to V_R or α_c as follows:

$$V_R = V_0(1 + k') \quad (8)$$

$$\alpha_c = k'_2 / k'_1 \quad (9)$$

TABLE 2
Chromatographic Separation of Y,Ho and Er

Rare earth	V_R/cm^3	α_c	N	R_s
pH 1.95				
Ho	290	1.73	182	1.01
Y	407	1.45	124	0.653
Er	532		81	
pH 1.99				
Ho	337	1.78	159	1.18
Y	499	1.45	141	0.815
Er	666		121	
pH 2.06				
Ho	430	1.78	155	1.36
Y	665	1.47	160	0.954
Er	915		133	

Stationary phase: 0.02 M $(EHPA)_2$ in toluene initially retained at 5 cm^3 min^{-1}.
Mobile phase: 0.1 M $(H,Na)Cl_2CHCOO$, 3 cm^3 min^{-1}.
CCC : 800 rpm, 35°C.

Figure 13 shows plots of the R_s values for Y/Ho and Er/Y against the capacity factor for Y. The solid lines (a) and (b) indicate the R_s values calculated from Eq.(7) for Y/Ho and for Er/Y, respectively. Here, the values evaluated at pH 2.06 (Table 2) were used for calculation; $N_{Ho} = 155$, $N_Y = 160$, $N_{Er} = 133$, $\alpha_c(Y/Ho) = 1.78$, and $\alpha_c(Er/Y) = 1.47$. The calculated value of R_s increased with increasing k' and was asymptotic to a plateau. The observed value of R_s varied along the calculated trend. The deviations from the calculated lines are probably due to the variation in the N values which tend to increase with increasing V_s and pH. From Eqs.(3) and (8), k' can be related to D as

$$k' = D V_s / V_m \quad (10)$$

Increases in the stationary phase volume and the distribution ratio bringing about the higher capacity factor are thus desirable for improving mutual separation.

The separation parameters are mainly governed by the stationary phase volume, and hence a high retention volume of the stationary phase would results in the

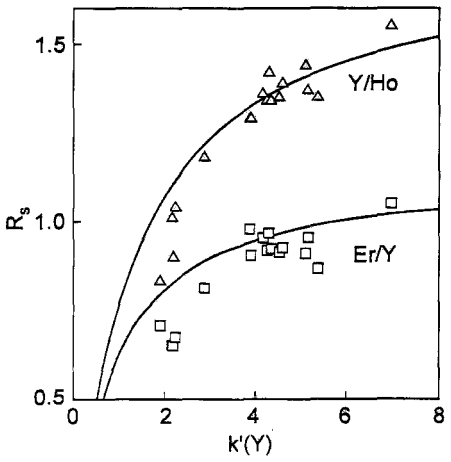

FIGURE 13 Correlation between the resolution and the capacity factor.

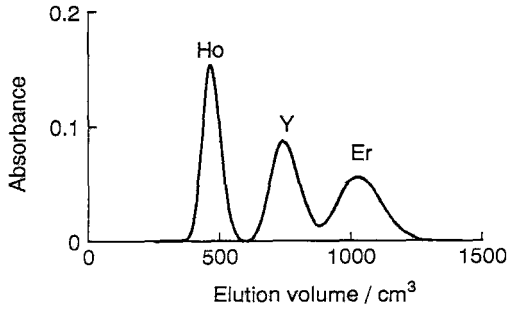

FIGURE 14 Mutual separation of Ho, Y and Er. Revolutional speed: 800 rpm; flow rate: 3 cm^3 min^{-1}; pH 2.05; V_s = 174 cm^3.

good separation of related species. The mutual separation of three rare earths was performed under the optimum conditions of a high revolutional speed of 800 rpm and a low flow rate of 3 cm^3 min^{-1} at pH 2.05. The resulted peaks were sufficiently separated as illustrated in Fig. 14. The R_s value representing the actual separation of elution peaks was found to be above 1 corresponding to a complete peak separation for each pair of Y/Ho (α_c=1.77, R_s=1.55) and Er/Y (α_c=1.45, R_s=1.05).

CONCLUSIONS

The results of the present studies with CCC are summarized as follows:

1. The extractability of yttrium and the heavy lanthanoids, Ho and Er, with EHPA was very close each other giving low separation factors less than 2.0.
2. The retention of the stationary phase in a coiled column increased with increasing revolutional speed and with decreasing flow rate of the mobile phase.
3. The retention volume for each rare earth was found to vary linearly with D and V_s; $V_R = V_m + D\,V_s$.
4. The high retention ratio of the stationary phase improved the mutual separation of the rare earths.
5. The separation parameters, N and R_s, were found to be practically independent of the revolutional speed and flow rate provided the retention ratio of the stationary phase remained constant.
6. The mutual separation of rare earths of low separation factors as α_D=1.86(Y/Ho) and 1.51 (Er/Y) was almost completely separated through a single step of CCC with good resolution, R_s=1.55 for Y/Ho and 1.05 for Er/Y.

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EVALUATION OF 9-METHYLAMINO-METHYLANTHRACENE AS A CHEMICAL LABEL FOR TOTAL REACTIVE ISOCYANATE GROUP: APPLICATION TO ISOCYANATE OLIGOMERS, POLYURETHANE PRECURSORS, AND PHOSGENE

ROY J. RANDO, HALET G. POOVEY, AND RACHELE A. GIBSON
Section of Environmental Medicine
Tulane University Medical Center
New Orleans, Louisiana 70112

ABSTRACT

9-Methylamino-methylanthracene (MAMA) is a secondary amine compound which can be used for derivatization and quantitation of isocyanate compounds by HPLC with detection by fluorescence and ultra-violet light absorbance The compound has potential as a chemical label for determination of airborne total reactive isocyanate group (TRIG) compounds arising from partially polymerized polyurethanes and thermal decomposition products of fully cured polyurethanes Six isocyanate oligomers and polyurethane precursors based on methylene-bis-(phenylisocyanate) (MDI), 1,6-hexamethylene-diisocyanate (HDI), and 2,4- and 2,6-toluenediisocyanate (TDI) were assayed for isocyanate content using MAMA reagent After reaction of isocyanate with MAMA, the urea derivatives were analyzed by reversed phase HPLC using three detection / quantitation modes: fluorescence with excitation at 245 nm and emission at 414 nm, UV absorbance at 245 nm and UV absorbance at 370 nm The ratio of absorbance at 245 nm and at 370 nm identified multiple peaks originating from isocyanate-containing compounds in each sample The total amount of TRIG in each sample was then quantitated by absorbance of these peaks at both of the UV wavelengths For the test samples, observed recoveries of TRIG by MAMA-HPLC assay ranged from

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96 to 105%, in comparison to a reference titration assay. The technique was also evaluated for chemical interference from phosgene gas. A single compound was found after reaction of MAMA with phosgene which responded as two equivalents of TRIG.

INTRODUCTION

The isocyanates are an important group of commercial chemicals used in the production of polyurethanes and of certain carbamate pesticides. These compounds are of particular interest in industrial hygiene due to their documented ability to act as respiratory irritants and skin and respiratory sensitizers.[1] The collection and measurement of specific airborne isocyanate monomers has become fairly routine using various secondary amine derivatizing agents with high performance liquid chromatographic determination. These compounds react with isocyanates to form substituted urea derivatives which can be detected by ultraviolet absorbance, fluorescence, or amperometry. Examples of some of these amine reagents include N-(n-propyl)-N-(4-nitrobenzyl)-amine,[2,3] methoxyphenyl-piperazine,[4] 1-(2-pyridyl)-piperazine,[5] 3-(2-aminoethyl)indole [tryptamine],[6] and 9-methylamino-methylanthracene [MAMA].[7]

Measurement of total reactive isocyanate group (TRIG) in air is of more recent interest. TRIG is comprised of any compound containing free isocyanate groups, this includes the monomeric diisocyanates commonly used in industry, as well as their oligomers which are becoming increasingly more popular due to their lower vapor pressures and consequently lower degree of hazard. In addition, TRIG includes any partially polymerized material containing free isocyanate such as would be found during the course of reaction between an isocyanate and a polyol to form a polyurethane.

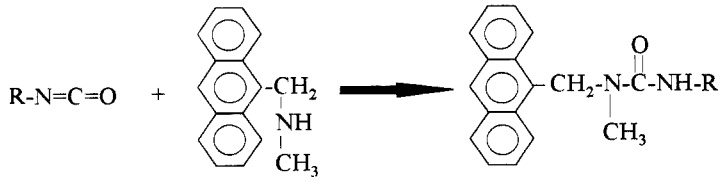
Essentially the only current working method for TRIG in air is that developed by the United Kingdom's Health and Safety Executive (HSE). HSE's

Method MDHS 25[8] uses a solution of methoxy-phenylpiperazine (MOPIP) in an impinger for collection of airborne TRIG. TRIG compounds react with the MOPIP to form urea derivatives which absorb in the ultraviolet region, are electrochemically active, and can be separated by HPLC. For a given diisocyanate monomer and its oligomers, a constant ratio between UV absorbance and electrochemical detector response is reportedly obtained.[4] Thus, a peak in the HPLC chromatogram is identified as an isocyanate compound, and the amount of TRIG is quantitated using the specific monomer as a standard. Total TRIG content of a sample is taken as the sum of the amounts of isocyanate equivalent for each of the identified TRIG peaks. The amount of isocyanate equivalent is determined from the electrochemical response of a given TRIG compound in comparison to standards made from the parent monomer compound.

The technique has had problems with specificity and the inability to deal with TRIG derived from more than one parent monomer.[9,10] In addition, TRIG compounds which are not simple oligomers of a given monomeric diisocyanate, such as might be found in polyurethane pyrolysis fumes, cannot be accurately quantified since they may have no direct relationship to the analytical response of the monomer standard. Additionally, recent work has shown that TRIG contained in partially polymerized urethanes made from toluene-diisocyanate (TDI) and ethylene glycol exhibited inconsistent ratios of response by electrochemical and UV absorbance detection as the number of polymer units increased.[11]

In the past, some work has been done on the determination of specific forms of TRIG using 9-(N-methylaminomethyl)-anthracene (MAMA) as the derivatizing agent. For example, the method was applied with some success to measure isocyanate monomer and oligomer in paint overspray.[12] Also, MAMA was used to determine TRIG in the emissions from thermal degradation of polyurethane binders.[13] More recently we have evaluated the potential of using MAMA as a chemical label for TRIG forms whose exact chemical structures are not known.[14] The basis of this application is the reaction of MAMA with

isocyanate groups to yield substituted urea derivatives which contain the anthracenyl moiety



The anthracenyl group in MAMA is a highly specific chromogen and fluorogen which exhibits strong absorption at a primary wavelength of about 245 nm and shows a series of weaker absorption maxima between 350 and 400 nm.[7,14] The compound is also highly fluorescent, with an emission maximum at about 414 nm, and with major excitation maxima corresponding to the UV absorbance maxima.

In our previous work, the consistency of response by UV and fluorescence detection was evaluated in a series of urea derivatives prepared from MAMA and 11 isocyanate compounds.[14] Aliphatic, aromatic, mono- and di-isocyanates were included in order to evaluate possible structure / response relationships. The ratios of response factors (response factor = integrated area ÷ isocyanate concentration) for absorbance at 245 and 370 nm were relatively constant across isocyanates, with a mean of 10.46 and standard deviation of 0.87 (RSD = 8.3%). All eleven derivatives were also highly fluorescent, but they exhibited wide variability in response factors (RSD = 55%). The results suggested that the ratio of UV absorbance at 245 nm to that at 370 nm may be used for reliable identification of MAMA-derivatized TRIG, and that quantitation of MAMA-derivatized TRIG may be achieved with satisfactory accuracy using absorbance detection at either 245 or 370 nm.

The purpose of this work was to extend the evaluation of MAMA as a chemical label for TRIG by applying it to the assay of several oligomers and polyurethane precursors derived from three diisocyanate monomers: 1,6-hexamethylene diisocyanate (HDI), 2,4- and 2,6-toluenediisocyanate (2,4-TDI, 2,6-TDI), and 4,4'-methylene-bis-(phenylisocyanate) (MDI). These materials were first assayed for isocyanate content using a reference titrimetric procedure. They were subsequently derivatized with MAMA and analyzed by HPLC. The reliability of the method was further assessed by investigating potential interference from phosgene gas.

MATERIALS AND METHODS

9-Methylamino-methylanthracene was obtained from Aldrich Chemical Co. (Milwaukee, WI) in a purity of 99%. MDI (Rubinate 44®) was obtained from Rubicon Chemical Co. (Geismar, LA). HDI (98%) was obtained from Aldrich.

The following oligomeric isocyanate containing materials were evaluated in this study:

- **CMDI**, obtained from BASF Corp., Geismar, LA. This material nominally contains 58 - 68 % MDI monomer, with the remainder being oligomers of MDI.
- **Lupranate M20S**, obtained from BASF Corp., Geismar, LA. This material nominally contains 40% MDI monomer, and 60% MDI oligomers.
- **PMPPI [poly(methylene(polyphenylisocyanate))]**, obtained from Aldrich Chemical Co. This material is an oligomeric form of MDI.
- **Desmodur N100**, obtained from Mobay Corp., Baytown, TX. This is a homopolymer of HDI, with HDI-biuret (a trimer of HDI) being predominant. A maximum of 1.6% HDI monomer is expected after 3 to 6 months storage. The material was approximately 18 months in storage when used in this work.

- **Imron paint activator 192S**, manufactured by DuPont Automotive Products, Wilmington, DE. This material is a solution of about 33% HDI oligomers in butyl acetate, ethyl acetate, and trimethylbenzene.
- **Rexthane**, manufactured by the Sherwin-Williams Co., Cleveland, OH. This is a moisture-curing, heavy duty, polyurethane varnish containing 2,4- and 2,6-TDI monomers, polyurethane prepolymers (polyhydric alcohols modified with TDI), and xylene.

Reference Assay of Isocyanate Content

The isocyanate content of the study materials was determined using a standard titration procedure based on reaction of isocyanate with di-n-butylamine [15] An aliquot of the test material (0.5 to 1 g) was weighed out and subsequently dissolved in 6 mL toluene previously dried over molecular sieve 5A. To the sample was added 2 0 N di-n-butylamine in toluene at a ratio of 7.5 mL to 1 gram of isocyanate-containing material The sample was loosely capped and allowed to react for 15 minutes Thirty-four mL of isopropanol were then added to the mixture, along with 75 μL bromocresol green indicator solution (0.1% in 0.02 N NaOH). The mixture was placed on a magnetic stirrer and the excess di-n-butylamine was titrated with standardized HCl solution, (~1.0 N). The isocyanate content of the test materials was calculated based on the number of equivalents of di-n-butylamine consumed during reaction in comparison to a reagent blank:

$$wt\%NCO = \frac{4.2N(B-S)}{W},$$

where N = normality of the HCl titrant,

B= volume of HCl required for titration of reagent blank,

S = volume of HCl required for titration of sample,

and W = mass of sample assayed.

Assay of Isocyanate Content by MAMA / HPLC

The MAMA-urea derivatives of MDI, 2,4-TDI, 2,6-TDI, and HDI were synthesized in the laboratory for use as analytical standards. The general procedure for synthesis of an isocyanate-MAMA-urea was as follows: to a solution of MAMA in hexane or hexane/methylene chloride was added the isocyanate compound, either neat or as a solution in hexane; the reaction was allowed to proceed with constant stirring during which the urea derivative precipitated out of solution; the urea precipitate was recovered by filtration and dried; the crude derivative was then recrystallized from a suitable solvent. A two- to five-fold excess of MAMA was used in these procedures. Reaction rates for the aromatic isocyanates were very rapid, with most reactions going to completion within 30 minutes. The aliphatic isocyanate, HDI, reacted slower, with several hours allowed to ensure completion of reaction.

Reaction of the isocyanate containing materials with MAMA reagent was carried out in a similar fashion: a known mass of polymeric isocyanate (0.1- 0.3 grams) was weighed out and dissolved in an appropriate solvent (dioxane for the MDI-based materials, 50/50 dioxane/dimethylformamide for Imron 192S, and dimethylsulfoxide for Desmodur N100 and Rexthane). The expected number of moles of isocyanate present were calculated based on the reference assay. The samples were then reacted with a 3 to 5 fold excess of MAMA, based on a stoichiometric reaction of one mole of MAMA with one mole of isocyanate group. The MAMA reagent was dissolved in the same solvent as the isocyanate-containing material prior to its addition.

Assessment of Interference from Phosgene

Phosgene was evaluated as a possible interferant for the method. Phosgene gas was generated from a permeation device (VICI Metronics, Santa Clara, CA) housed in a standards generator (AID Model 360, Avondale, PA) held at a

constant 30°C with a flow of 1.0 L/min of house air dried over silica gel. The output of the standards generator was further diluted with dried house air to yield a final test concentration of 100 ppb phosgene. To evaluate the potential interference, samples from this test atmosphere were collected in midget impingers containing 10 mL of a solution of 0.1 mg MAMA / mL in acetonitrile at a flow rate of 1.0 L/min for 30 minutes. These samples were then analyzed by the same HPLC-UV-fluorescence technique that was applied to the TRIG compounds. For this procedure, HDI-MAMA-urea was used as the standard reference material.

High Performance Liquid Chromatography

Chromatographic analysis of the MAMA-derivatized samples and standards was performed with a Perkin Elmer Model 410 high pressure liquid chromatograph fitted with a Rheodyne injection valve. Peak detection was accomplished by fluorescence using a Shimadzu Model RF551 fluorometric detector with excitation at 245 nm and emission at 414 nm, and by ultraviolet absorption at two wavelengths using a Perkin Elmer Model LC90 UV detector set at a wavelength of 245 nm and a Waters Model 450 UV detector set at a wavelength of 370 nm.

Sample injection volumes were defined by a 20-μL sample loop. Samples were analyzed on a Supelcosil LC-8-DB octyl bonded phase column, 5 μm particle size (Supelco, Belafonte, PA). The column dimensions were 4.6 mm i.d. by 5 cm long. The mobile phase consisted of a mixture of acetonitrile and aqueous triethylammonium phosphate buffer (3% triethylamine in water, adjusted to pH 3.0 with phosphoric acid). The acetonitrile concentration was adjusted to ensure chromatographic separation of excess MAMA reagent and the individual isocyanate containing compounds in the samples, while minimizing chromatographic run times for the late eluting compounds. A concentration of 60% acetonitrile in the mobile phase at a flow rate of 1 mL/min was utilized for the

HDI-based samples; for analysis of the Rexthane sample, a mobile phase containing 54% acetonitrile was used at an initial flow rate of 2 mL/min which was increased to 4 mL/min after the elution of the TDI monomers. For the MDI-based materials, a mobile phase containing 60% acetonitrile was used at an initial flow rate of 1 mL/min for 22 minutes to allow elution of the MDI monomer and one oligomer, and then was increased to 2 mL/min. For those cases where the mobile phase flow rate was increased during a chromatography run, the measured areas of peaks of interest were corrected for the proportional change in integrated detector response due to the resulting reduction in residence times in the detectors.

All chromatograms were recorded and integrated using EZ CHROM software with data collection by a Strawberry Tree I/O board. Chromatograms were screened for presence of TRIG-derived peaks by comparison to the respective isocyanate parent monomer standard. The criteria for classification of a peak as being of TRIG origin was a detector response ratio for UV absorbance at 245 nm to that at 370 nm within ± 20% of that for the monomer standard. The 20%-criteria was derived as an approximation of the 95% confidence interval of the overall mean response observed in our previous work which applied the MAMA technique to eleven different isocyanate monomers.[14] Further confirmation of the identity of an unknown TRIG containing compound was provided by examination of its fluorescence response. In general, the flourescence of the identified TRIG compounds was of the same order of magnitude as their corresponding diisocyanate monomer standards, although there appeared to be a general trend of decreasing flourescence with increasing chromatographic retention time.

After identification of a TRIG compound, the amount of isocyanate present was determined in comparison to the response of the parent monomer standard. This was done for each of the UV absorbance detectors independently. Calibration of detector response for quantitation of TRIG in these compounds used conversion factors based on two moles TRIG / mole of diisocyanate monomer standard; i.e., for MDI, 0.121 μg TRIG / μg MDI-MAMA-urea; for HDI, 0.138 μg TRIG / μg

HDI-MAMA-urea; and for 2,4- and 2,6-TDI, 0.136 μg TRIG / μg TDI-MAMA-urea. In order to quantitate TRIG in the Rexthane sample, an average calibration curve was generated by combining the data for the 2,4- and 2,6-TDI standards and performing a linear regression of detector response vs. concentration with equal weighting for the responses of 2,4-TDI and 2,6-TDI.

RESULTS & DISCUSSION

Assay of TRIG Content of Test Samples

Figures 1 through 3 present representative chromatograms from each of the three classes of oligomeric isocyanates. In each case, excess MAMA reagent elutes at the beginning of the chromatogram, with MAMA-derivatized isocyanate-containing compounds, including that from the respective parent monomers, eluting later. In each case, baseline separation of excess MAMA from other constituents in the samples is obtained. The parent isocyanate monomers are the first eluting compounds of interest in each sample. Later eluting compounds are presumably either oligomers of the parent monomer isocyanate or polyurethane prepolymers formed by reaction of isocyanate with polyols or water. The elution order of these compounds is expected to be greatly influenced by molecular weight, with longer chain oligomers eluting later in the chromatographic run.

All three of the MDI-based materials contain significant amounts of MDI monomer, identified as peak number 1 in the chromatogram of the CMDI sample (Figure 1) and in Table I, which presents the results of the assays of each of the test materials. CMDI contains MDI monomer as well as two other peaks identified as isocyanate compounds. The third peak appears to actually be two unresolved compounds which are co-eluting. However, this does not interfere with the goal of assaying this sample for TRIG, and quantitating the contribution

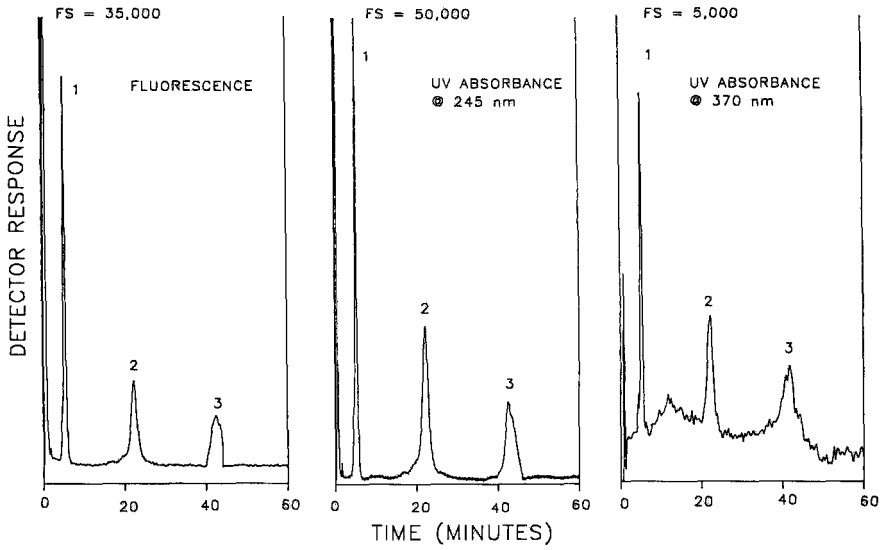


FIGURE 1 Chromatogram of a sample of CMDI containing 100 µmole TRIG per L. Chromatographic conditions: Supelcosil LC-8-DB column, 4.6 mm x 5 cm; 60% acetonitrile / 40% aqueous triethylammonium phosphate buffer; 1 mL/min for 22 minutes, then increased to 2 mL/min. Numbered peaks are TRIG containing compounds identified by ratio of response of UV detectors.

of these compounds to the total isocyanate content of the sample of CMDI is straightforward. In the other MDI-based samples, the monomer and only one other compound in the chromatogram are identified as containing isocyanate. PMPPI and M20S display very similar composition, suggesting these materials may actually be the same formulation distributed under different product names.

There is a high degree of consistency in the ratio of detector responses used to identify TRIG compounds in the three MDI-based materials. MDI monomer standard shows a detector response ratio of 9.9 for UV absorbance at 245 nm and 370 nm. The observed response ratios of the TRIG compounds identified in the MDI-based materials range from 9.6 to 10.5 (97% to 106% of the MDI standard).

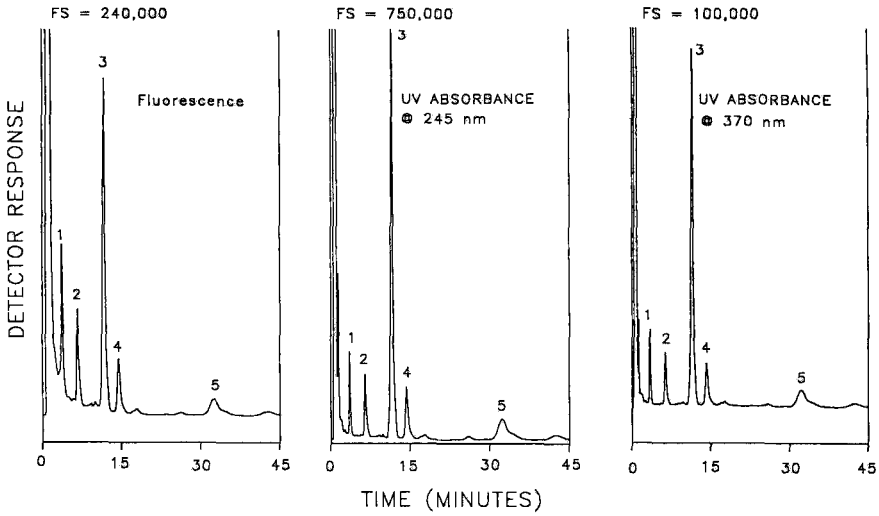


FIGURE 2 Chromatogram of a sample of Desmodur N100, containing 98.7 µmole TRIG per L. Chromatographic conditions: Supelcosil LC-8-DB column, 4.6 mm x 5 cm; 60% acetonitrile / 30% aqueous triethylammonium phosphate buffer, 1 mL/min. Numbered peaks are TRIG containing compounds identified by ratio of response of UV detectors.

Quantitation of total TRIG in the MDI-based samples using the MDI-MAMA-urea standard at either UV wavelength produces results that are not statistically different in comparison to the reference titration assay. For the M20S sample, a recovery of 105% is seen for quantitation at 245 nm in comparison to the reference assay for TRIG; for quantitation at 370 nm, 99% recovery is observed. For the CMDI sample - which includes the unresolved compounds identified as peak 3 - the respective recoveries are 105% and 104%, for the sample of PMPPI, recoveries of 95% and 96%, respectively, are obtained.

The data for the HDI-based materials is shown in Table II, while Figure 2 presents a chromatogram of the Desmodur N100 sample. In this chromatogram, four peaks are identified as having contained isocyanate. Peak 1 is HDI monomer

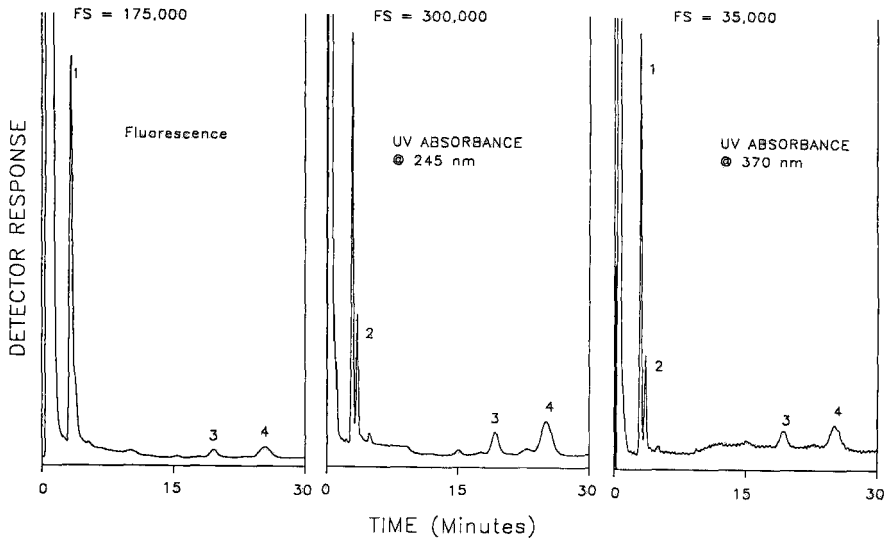


FIGURE 3 Chromatogram of a sample of Rexthane, containing 127 μmole TRIG per L. Chromatographic conditions: Supelcosil LC-8-DB column, 4.6 mm x 5 cm; 54% acetonitrile / 46% aqueous triethylammonium phosphate buffer, at 2 mL/min initially, increased to 4 mL/min. Numbered peaks are TRIG containing compounds identified by ratio of response of UV detectors.

TABLE I
MAMA Assay of TRIG Content of MDI-Based Samples

Material	Peak No.	Detector Response Ratio (245 nm: 370 nm)	weight % TRIG		
			MAMA (245 nm)	MAMA (370 nm)	Reference Assay*
MDI monomer standard	1	9.9 ± 3.0%	---	---	---
M20S	1	9.9 ± 10.0%	18.1 ± 8.9%	17.2 ± 6.4%	---
	2	10.5 ± 2.9%	4.9 ± 1.8%	4.5 ± 4.9%	---
	total	---	23.0 ± 6.7%	21.6 ± 4.7%	21.9 ± 10.5%
CMDI	1	9.6 ± 2.1%	7.2 ± 2.9%	7.2 ± 4.2%	---
	2	9.9 ± 2.0%	6.3 ± 4.4%	6.2 ± 3.7%	---
	3	9.7 ± 2.1%	10.6 ± 4.1%	10.6 ± 5.9%	---
	total	---	24.1 ± 3.0%	23.9 ± 3.9%	23.0 ± 1.7%
PMPPI	1	9.7 ± 3.1%	16.3 ± 5.0%	16.4 ± 7.3%	---
	2	9.6 ± 2.1%	5.7 ± 1.6%	5.8 ± 2.0%	---
	total	---	22.0 ± 3.6%	22.1 ± 5.3%	23.1 ± 5.6%

* reference assay by titration with di-n-butylamine / HCl
N.B.: all values are mean ± coefficient of variation

TABLE II
MAMA Assay of TRIG Content of HDI-Based Samples

Material	Peak No.	Detector Response Ratio (245 nm: 370 nm)	weight % TRIG		
			MAMA (245 nm)	MAMA (370 nm)	Reference Assay*
HDI monomer standard	1	8.3 ± 2.4%	---	---	---
Desmodur N100	1	8.7 ± 8.0%	0.7 ± 10.2%	0.7 ± 3.0%	---
	2	8.9 ± 3.4%	0.7 ± 1.0%	0.7 ± 2.5%	---
	3	8.4 ± 0%	11.5 ± 2.5%	11.3 ± 2.4%	---
	4	9.3 ± 3.2%	1.8 ± 2.4%	1.6 ± 2.7%	---
	5	15.5 ± 5.0%	0	0	---
	total	---	14.7 ± 2.5%	14.3 ± 4.4%	14.0 ± 10.0%
Imron 192S	1	none detected	---	---	---
	2	none detected			
	3	9.0 ± 5.6%	4.7 ± 1.9%	4.3 ± 6.3%	---
	4	8.9 ± 5.6%	0.6 ± 6.6%	0.6 ± 8.9%	---
	total	---	5.3 ± 1.7%	4.8 ± 5.8%	5.0 ± 2.6%

* reference assay by titration with di-n-butylamine / HCl
N.B.: all values are mean ± coefficient of variation

and accounts for less than 5% of the total TRIG in this sample. In contrast, no HDI monomer is detected in the sample of Imron 192S. Peak 3 is presumed to be HDI-biuret since it is clearly the largest component of both samples. Peaks 2 and 4 are TRIG-containing compounds of unknown structure which are presumably other oligomeric forms of HDI. Peak 5 in the chromatogram is not considered to have contained isocyanate since its detector response ratio is 15.5, which is outside of the selection criteria. The detector response ratios of the four TRIG compounds identified in these samples range from 8.4 to 9.3, equivalent to a range of 101 to 112% of that of the HDI-monomer standard which has an average detector response ratio of 8.3.

Quantitation of total TRIG in the HDI-based samples yields results that are not significantly different from that of the reference titration assay. For the Desmodur N100 sample, recoveries of 105% and 102% are obtained when quantitating TRIG by UV absorbance at 245 nm and 370 nm, respectively. For Imron 192S, these recoveries are 106% and 96%, respectively.

The Rexthane sample contains both 2,4-TDI and 2,6-TDI monomers as well as two other late-eluting compounds that contain isocyanate according to the selection criteria (Figure 3 and Table III). The detector response ratios of these two peaks are 11.8 and 12.7, respectively; whereas the monomer standards exhibit ratios of 9.8 and 10.9 (2,6-TDI and 2,4-TDI, respectively). The response ratios of the two unknown peaks are within the selection criteria of ± 20% when compared to 2,4-TDI. However, peak 4 is outside of the selection criteria when compared to 2,6-TDI (130% of the ratio for 2,6-TDI). It should be noted that the two TDI monomers - which are clearly identified in the Rexthane sample by retention time, UV absorbance, and fluorescence - also exhibit detector response ratios that are slightly higher than expected (10.5 and 12.6, respectively). This is felt to be a result of a shift in instrument performance between the times of analysis of the standards and the samples.

Quantitation of TRIG in the Rexthane sample yields recoveries of 107% and 100% for UV absorbance at 245 nm and 370 nm, respectively, in comparison to the titration assay. The contribution of TRIG in this sample from the two TDI monomers amounts to approximately 55% of the total. 2,6-TDI accounts for about three times as much TRIG as 2,4-TDI. This is in line with previously published data showing that in partially reacted polyurethane systems, free 2,6-TDI predominates over free 2,4-TDI.[3] This occurs in spite of the fact that typical commercial grades of TDI range from 80% to 65% 2,4-TDI. This phenomenon is a result of the much greater reactivity of 2,4-TDI in comparison to that of 2,6-TDI, with steric hindrance in the latter reducing its relative reactivity.

Overall, TRIG containing compounds in these six samples are readily identified by the screening criteria of UV absorbance detector response ratio.

TABLE III
MAMA Assay of TRIG Content of a TDI-Based Sample

Material	Peak No	Detector Response Ratio (245 nm: 370 nm)	weight % TRIG		
			MAMA (245 nm)	MAMA (370 nm)	Reference Assay*
2,6-TDI monomer standard	1	9.8 ± 6.1%	---	---	---
2,4-TDI monomer standard	2	10.9 ± 4.6%	---	---	---
Rexthane	1	10.5 ± 1.9%	0.7 ± 4.4%	0.7 ± 5.0%	---
	2	12.6 ± 9.5%	0.2 ± 5.2%	0.2 ± 7.7%	---
	3	11.8 ± 10.2%	0.3 ± 9.4%	0.3 ± 15.9%	---
	4	12.7 ± 6.3%	0.5 ± 7.8%	0.5 ± 16.1%	
	total	---	1.7 ± 7.5%	1.6 ± 5.9%	1.6 ± 1.9%

* reference assay by titration with di-n-butylamine / HCl
N.B all values are mean ± coefficient of variation

Additional confirmation is provided by fluorescence response. In line with our previous work on isocyanate monomers,[14] there appears to be a trend of enhanced UV absorbance response associated with the presence of aromatic structures and urea linkages in these compounds. This is especially reflected in the observed detector ratios since absorbance at 245 nm is likely to be significantly enhanced by these chemical structures, whereas absorbance at 370 nm would be minimally influenced. Quantitation of total TRIG by absorbance at 370 nm also appears to be slightly more accurate than that at 245 nm because of this effect. However the increased specificity at 370 nm comes at a significant loss in sensitivity - the response factors for absorbance at 245 nm being greater by approximately a factor of ten.

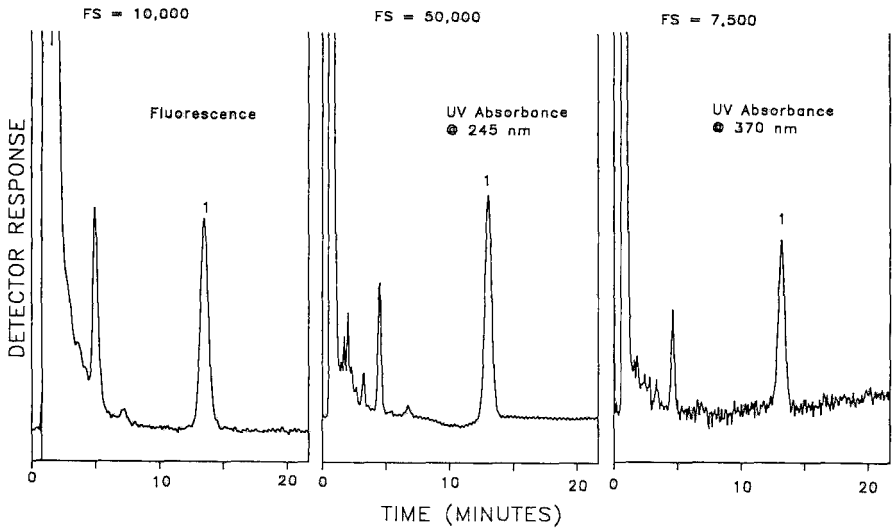
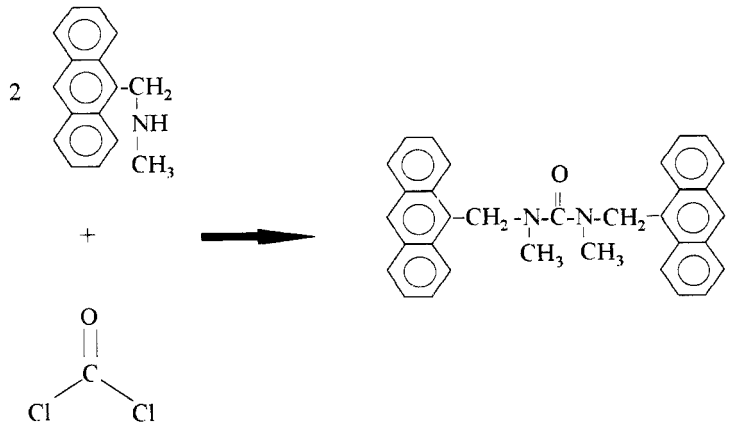


FIGURE 4 Chromatogram of a sample of MAMA reagent in acetonitrile reacted with phosgene gas. Chromatographic conditions: Supelcosil LC-8-DB column, 4.6 mm x 5 cm; 56% acetonitrile / 44% aqueous triethylammonium phosphate buffer, 1 mL/min. Peak 1 is a reaction product which responds as two equivalents of isocyanate group.

Interference from Phosgene

A chromatogram showing the result of reacting MAMA reagent with phosgene gas is shown in Figure 4. A peak with a retention time approximately twice that of HDI-MAMA-urea (13 minutes in comparison to 6.5 minutes for HDI-MAMA-urea) is apparent. In addition to being fluorescent, this compound exhibits a UV absorbance detector ratio of 10.5 ± 0.9 which is within 1% of that for the HDI standards used for comparison. Peak 1 in the chromatogram therefore falls within the criteria for classification as an apparent source of TRIG. The other unmarked peaks in the chromatogram are also present in the reagent blank and represent excess MAMA reagent and unknown contaminants.

The reaction of phosgene with secondary amine reagents used for derivatizing isocyanate compounds is well recognized[15] and may occur with all of these reagents. In the case of MAMA reagent, the expected reaction with phosgene would be as follows:



In this experiment, the equivalent response of 100 ppb phosgene in comparison to HDI-MAMA-urea standards is found to be 239 ± 15 ppb TRIG. In line with the reaction scheme illustrated above, phosgene appears to react with MAMA as two TRIG equivalents. Thus phosgene presents a potentially significant positive interference in the determination of TRIG using the MAMA reagent and HPLC-UV technique. Furthermore, the occurrence of phosgene and isocyanates together is likely in the manufacturing process since isocyanates are commonly synthesized by reacting phosgene with the corresponding amine. However, in the isocyanate application industries, such as polyurethane painting and flexible and rigid foaming, as well as in reformulating operations where paint and other surface coatings are manufactured, it is unlikely that phosgene would be present in significant amounts. Further, when the presence of phosgene is

suspected in a sample, it could be easily identified by chromatographic retention time and either analyzed separately or not included in the calculation of TRIG content. Triphosgene has been shown to be an acceptable surrogate standard for phosgene analysis using pyridyl-piperazine, one of the common secondary amine reagents used for isocyanate analysis.[15] It is likely that triphosgene would be an acceptable substitute standard for phosgene when using the MAMA reagent system for TRIG analyses.

CONCLUSIONS

The purpose of this work was to further illustrate the use of MAMA reagent as a chemical label for identification and quantitation of TRIG. Previous work indicated that for MAMA-derivatized isocyanates, the ratio of response of UV absorbance at 245 and 370 nm was relatively constant regardless of the structure of the isocyanate parent material.[14] Coupled with the intense fluorescence of TRIG-MAMA derivatives, this UV-absorbance screening technique proved to be a powerful approach to discriminating unknown TRIG forms from other materials in real samples. In the current work, the method was applied to the assay of six isocyanate containing materials which were based on MDI, HDI, or TDI. These materials ranged from industrial formulations of oligomeric isocyanates to two-component and moisture-curing polyurethane surface coatings. TRIG compounds in these samples were easily identified by HPLC with detection by UV absorbance and fluorescence. Quantitation in comparison to the parent diisocyanate monomer yielded quantitative recoveries of TRIG content as compared to a reference titration procedure. A significant interfering peak from reaction of MAMA with phosgene gas was identified but should be easily corrected after chromatographic separation from the rest of the sample. Overall this method appears to be superior to the MOPIP derivatization

procedure for TRIG which uses HPLC with UV and electrochemical detection. As part of ongoing evaluations of the MAMA technique, we are currently using the method to investigate the nature and behavior of TRIG compounds in laboratory test atmospheres and in actual workplace environments.

ACKNOWLEDGMENT

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HPLC RESOLUTION OF HYDROXYL CARBOXYLIC ACID ENANTIOMERS USING 2-QUINOXALOYL CHLORIDE AS A NEW PRECOLUMN DERIVATIZING AGENT

MALCOLM BRIGHTWELL, MARIA PAWLOWSKA, AND JANUSZ ZUKOWSKI*
SmithKline Beecham Pharmaceuticals
New Frontiers Science Park
Third Avenue
Harlow, Essex CM19 5AW, United Kingdom

ABSTRACT

Chiral α-hydroxy carboxylic acids were reacted using 2-quinoxaloyl chloride to form UV and fluorescent derivatives. Under mild conditions either in aqueous or non-aqueous conditions reaction proceeds quickly and without racemisation. The labelled compounds have been resolved into enantiomers on β-cyclodextrin bonded stationary phase operated with polar organic eluents. The high sensitivity and selectivity of the method has been used for the determination of low levels of enantiomeric impurities in commercial "pure" samples.

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INTRODUCTION

Several hydroxy carboxylic acids have been known to show significant biological activities. Their determination in physiological fluids has been used in metabolism studies and in the identification of several disorders [1-3]. The majority of diagnostically useful organic acids are optically active. Thus the enantiomeric ratio determination of these compounds may provide more detailed information on biological processes and disorders in living organism. Previously several studies concerning the enantioseparation of organic acids were reported. However, these separations are not yet routine and an accurate, precise and sensitive method is still needed [4-7].

This paper presents the applicability of 2-quinoxaloyl chloride having a highly absorptive and fluorescence aromatic moiety as a new pre-column derivatisation reagent for α-hydroxy carboxylic acids. The resulting conjugates were optically resolved on β-cyclodextrin bonded stationary phase using non-aqueous polar eluents.

EXPERIMENTAL

A HPLC system consisting of a pump (Waters 600), UV detector (Programmable Absorbance Detector 785A, ABI),

integrator (HP3390A, HP) and a Rheodyne injection valve with a 250x4.6 mm Cyclobond I 2000, 5 μm, column (Technicol, Stockport, UK) was used.

Mobile phase was prepared from HPLC grade acetonitrile, methanol, triethylamine and glacial acetic acid (Fisons, UK). Flow rate was 1 ml/min with the column kept at room temperature throughout the studies. UV detection was carried out at 315 nm.

Derivatising reagent, 2-quinoxaloyl chloride, was purchased from Aldrich and α-hydroxy carboxylic acids (free or as a salt) were supplied by Aldrich and Sigma.

Approximately 0.5 mg of acid and 2-quinoxaloyl chloride was reacted in 2 ml vial containing 1 ml of acetonitrile and one drop of triethylamine for 30 min at room temperature. In the case of poor solubility of acid a few drops of water were added or the reaction was performed in 50% aqueous acetonitrile. Reaction mixture was diluted five folds with acetonitrile and 5 μl was injected into a column.

RESULTS

Derivatisation Chemistry

The structure of 2-quinoxaloyl chloride and the derivatisation reaction are shown in FIGURE 1. The reaction is

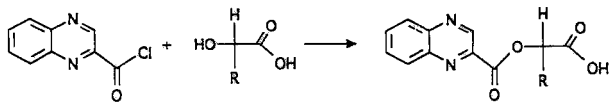

FIGURE 1. Derivatisation chemistry

fast and proceeds under mild conditions (see Experimental) at room temperature either in aqueous or non-aqueous solutions.
As can be seen the labelling with the tagged agent introduces not only an aromatic moiety for easy UV or fluorescent detections but also provides two nitrogen atoms which can interact with the secondary hydroxy groups at the mouth of the cyclodextrin cavity and may contribute to the overall retention and the steric discrimination of the resultant conjugates.

Retention Behaviour

The labelled compounds have favourable chromatographic proprieties and can be easily resolved into enantiomers on β-cyclodextrin bonded silica columns operated with non-aqueous polar organic mobile phases. TABLE 1 lists the retention parameters of derivatised α-hydroxy carboxylic acids obtained under optimal conditions. The neat acetonitrile with small amounts of triethylamine and acetic acid as modifiers has been used as a mobile phase for the enantioseparation of

TABLE 1

Separation Data for Racemic Mixtures of α -Hydroxy Carboxylic Acids (R-CH(OH)-COOH) on β -Cyclodextrin Column operated with Polar Organic Eluents

Acid name	R	k'_1	α	R_S	Eluent
Lactic	$-CH_3$	3.88	1.16	2.41	A
α-Hydroxybutyric	$-CH_2-CH_3$	3.72	1.17	2.49	A
α-Hydroxyvaleric	$-(CH_2)_2-CH_3$	3.61	1.17	2.40	A
α-Hydroxyisovaleric	$-CH-(CH_3)_2$	3.77	1.15	2.16	A
α-Hydroxycaproic	$-(CH_2)_3-CH_3$	3.77	1.15	2.16	A
α-Hydroxyisocaproic	$-CH_2-CH-(CH_3)_2$	4.04	1.13	2.10	A
α-Hydroxycaprylic	$-(CH_2)_5-CH_3$	3.61	1.14	2.01	A
α-Hydroxycapric	$-(CH_2)_7-CH_3$	3.50	1.14	2.02	A
α-Hydroxylauric	$-(CH_2)_9-CH_3$	3.44	1.14	1.98	A
α-Hydroxymyristic	$-(CH_2)_{11}-CH_3$	3.33	1.13	1.95	A
α-Hydroxypalmitic	$-(CH_2)_{13}-CH_3$	3.20	1.14	1.95	A
α-Hydroxystrearic	$-(CH_2)_{15}-CH_3$	3.01	1.13	1.86	A
α-Hydroxyarachidic	$-(CH_2)_{17}-CH_3$	2.82	1.12	1.72	A
α-Hydroxybehenic	$-(CH_2)_{19}-CH_3$	2.53	1.11	1.46	A
α-Hydroxyhexacosanoic	$-(CH_2)_{23}-CH_3$	2.23	1.12	1.34	A
Hexahydromandelic	$-C_6H_{11}$	1.94	1.46	4.03	B
Malic	$-CH_2-COOH$	3.40	1.11	1.58	B

Eluent:

A - acetonitrile + triethylamine + acetic acid, 1000 + 5 + 2.5, v/v/v

B - acetonitrile + methanol + triethylamine + acetic acid, 750 + 250 + 10 + 5, v/v/v/v

homologous alkyl α-hydroxy carboxylic acids. The elution of hexahydromandelic and malic acids in a reasonable time required the addition of as much as 25% (v/v) of methanol to the mobile phase. The L enantiomers were eluted prior to the D enantiomers in all cases.

As can be seen from these data the k' values of the solutes examined decrease in the order of increasing hydrophobicity of the side chain, R, on the chiral carbon atom. FIGURES 2 and 3 show the change of chromatographic parameters with increasing carbon number in the straight side chain. The results clearly indicate that the increasing length of the alkyl side chain influences the chromatographic separation in two ways: it decreases the retardation as well as the ratio of mass transfer distribution between mobile and stationary phases, which results in increasing plate height. Both factors decrease the separation of α-hydroxy carboxylic acid homologues in spite of nearly unchanged chiral discrimination, α, exhibited by the stationary phase.

The data presented above are in a good agreement with the model of chiral discrimination in water - free systems. Briefly, it has been postulated that in non-aqueous systems the inclusion complex formation is suppressed as the cyclodextrin cavity is largely occupied by the acetonitrile molecules. The chiral recognition on native cyclodextrin bonded phases arises from stereoselective hydrogen bondings

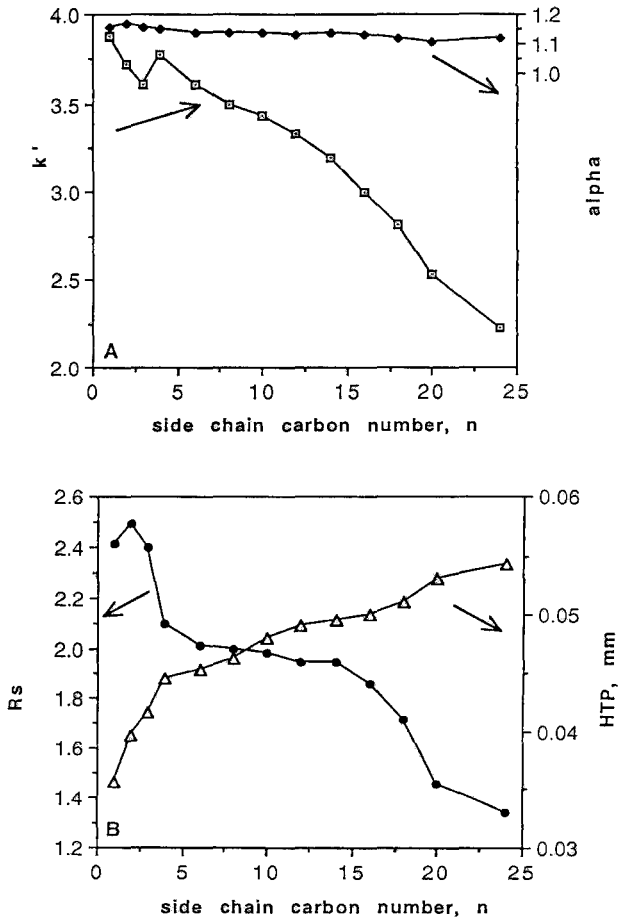

FIGURE 2. Change in chromatographic enantioseparation parameters of homologues α-hydroxy carboxylic acids with increasing carbon number (n) in the straight alkyl chain on the chiral centre. Eluent: acetonitrile + triethylamine + acetic acid, 1000 + 5 + 2.5, v/v/v.

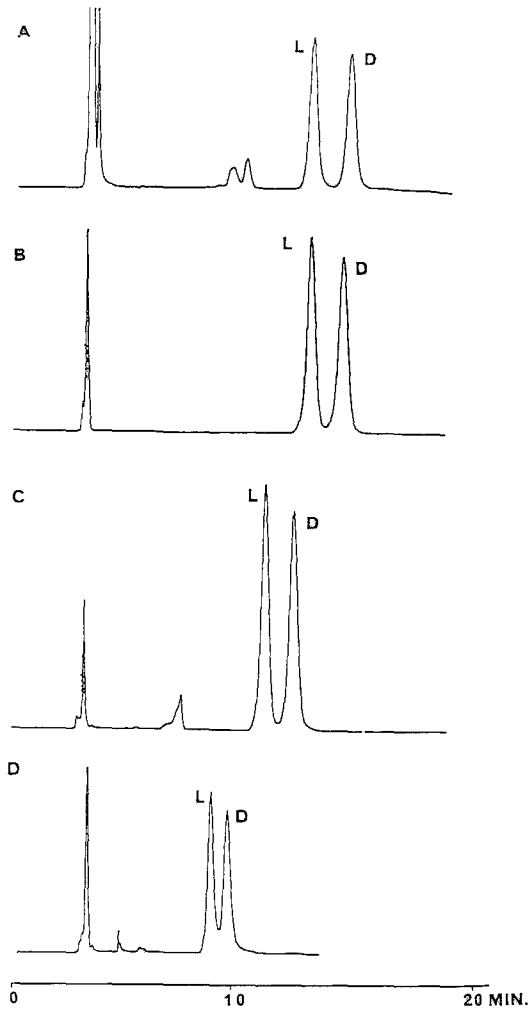

FIGURE 3. Chromatograms representing the change in enantioseparations of selected α-hydroxy carboxylic acids with the increasing hydrophobicity of the straight alkyl side chain on the chiral centre. Eluent as in FIG. 2.
Test compounds: (A) - lactic acid, (B) - caprilic acid, (C) - palmitic acid, (D) - hydroxyhexacosanoic acid.

between analyte and the secondary hydroxyl groups at the mouth of the cyclodextrin cavity [8-10].

This is further supported by the retention behaviour observed in the current study. Nearly unchanged enantioselectivity exhibited by the stationary phase towards the homologues carboxylic acids as well as the high symmetry of the peaks and the same elution order for all analytes investigated lead to the assumption that the chiral discrimination is caused by the same type of stereointeraction e.g. by hydrogen bondings between the hydroxyl groups of the β-cyclodextrin and the polar groups in the functionalised hydroxy carboxylic acids. The increasing length of the alkyl substituent, R, tends to decrease the strength of the hydrogen bonding complexation between the stationary phase and the solute owing to the increasing hydrophobicity of the later, but only slightly influences the stereoselectivity, (see TABLE 1 and FIGURE. 2A), which indicates that the straight aliphatic chain on the chiral carbon atom does not cause any significant hindrance for the stereoselective bonding formation. The influence of the side chain structure on the enantioseparation of racemic α-hydroxy carboxylic acids is presented in FIGURES 4 and 5. As can be seen in FIGURE 4 the enantiomers of α-hydroxycaproic acid with a straight alkyl group are eluted faster from the column than their isomers with a branched side chain due to their higher hydrophobicity. However, the bulkiness of the side chain influences the stereorecognition. In

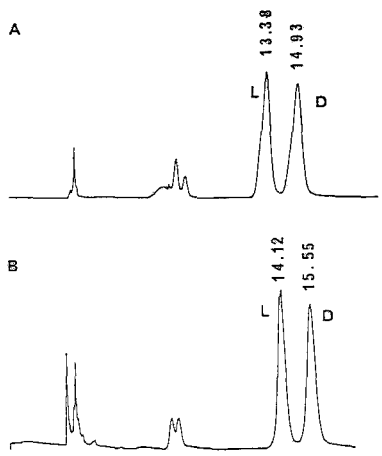

FIGURE 4. Chromatograms representing the enantioseparations of α-hydroxycaproic (A) and α-hydroxyisocaproic (B) acids. Chromatographic conditions as in FIG. 2.

spite of the longer retardation the chiral discrimination of the cyclodextrin phase towards the iso-caproic acid enantiomers is lower, probably due to the steric hindrance of the branched side chain. The same trend has been found in the separation of α-hydroxyvaleric and α-hydroxyisovaleric acids racemates (see TABLE 1). This effect is more pronounced (see FIGURE 5) under different mobile phase conditions, when the polar organic eluent contains significant amounts of methanol (10% v/v) as the third modifier. In this case the stereorecognition is affected not only by the analyte structure but also by competitive adsorption of methanol molecules. The influence of the solute structure on the chiral discrimination on the

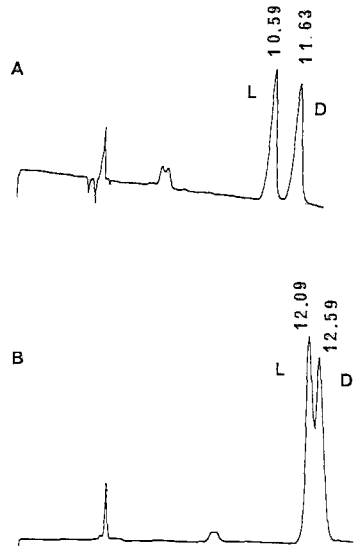

FIGURE 5. Enantioseparation of α–hydroxyvaleric (A) and α-hydroxyisovaleric (B) acids obtained on β-cyclodextrin stationary phase operated with eluent consisting of acetonitrile + methanol + triethylamine + acetic acid, 900 + 100 + 6 + 0.4, v/v/v/v.

native cyclodextrin in the water free system is also exemplified in FIGURE 6. As can be seen the change of the position of the hydroxy group in butyric acid significantly influences both the retardation and the enantioselectivity; in contrast to the excellent base line separation of α-hydroxybutyric acid, β-hydroxybutyric acid enantiomers can be only slightly recognized under the same chromatographic conditions.

The data presented above indicate that in the case of native cyclodextrin, with its homogenous (in energies)

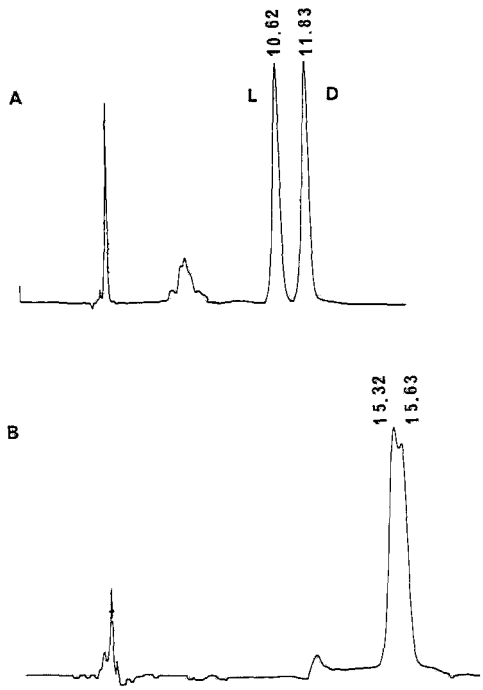

FIGURE 6. Influence of the solute structure on the chiral discrimination of native β -cyclodextrin stationary phase. Test compounds: α-hydroxybutyric acid (A), β–hydroxybutyric acid (B). Eluent: acetonitrile + methanol + triethylamine + acetic acid, 900 + 100 + 6 + 0.4, v/v/v/v.

adsorption sites, retardation and selectivity are mainly determined by the number and configuration of the groups in the analyte suitable for hydrogen bond formation with the secondary hydroxy groups of the cyclodextrin stationary phase. Note, that in the case of malic acid (see TABLE 1) containing two carboxylic groups significant amounts of methanol as well

TABLE 2
Optical Purity of Commercial Samples of L- and D-α-Hydroxy Carboxylic Acids

Acid Name	Source	Content of opposite enantiomer, %	Eluent	Standard deviation (n=4)
D-Lactic	Sigma	1.4	A	0.02
L-Lactic	Sigma	0.1	A	0.03
L-α-Hydroxyisovaleric	Fluka	-	A	-
L-α-Hydroxyisocaproic	Sigma	-	A	-
D-Malic	Aldrich	3.5	B	0.05
L-Malic	Aldrich	14.7	B	0.10
(+)-Hexahydromandelic	Aldrich	1.1	B	0.04
(-)-Hexahydromandelic	Aldrich	0.6	B	0.09

Eluent:
A - acetonitrile + triethylamine + acetic acid, 1000 + 5 + 2.5, v/v/v
B - acetonitrile + methanol + triethylamine + acetic acid, 750 + 250 + 10 + 5, v/v/v/v

as triethylamine and acetic acid were used to weaken the strength of complexation between the cyclodextrin and the analyte by the competitive adsorption of modifier molecules at the available adsorption (binding) sides. The competitive adsorption of the mobile phase components can be used for the optimisation of separation factors in chromatographic systems consisting of cyclodextrin stationary phase and water free eluents and has been previously used in the separation of many different classes of compounds [11-14].

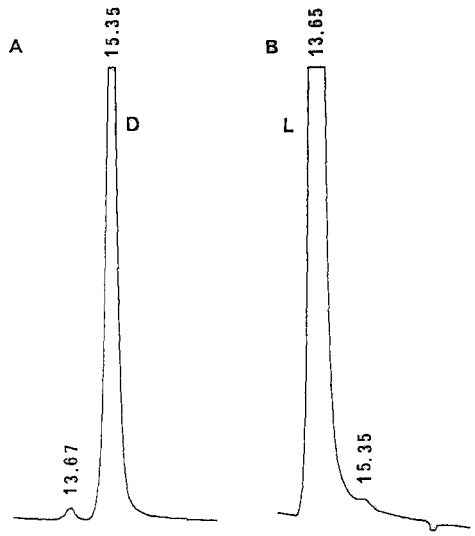

FIGURE 7. Chromatograms used for enantiomeric purity evaluations: D-lactic acid (A), L-lactic acid (B). Conditions as in TABLE 2.

Practical Application of the Method

The labelling with 2-quinoxaloyl chloride converts aliphatic α-hydroxy carboxylic acids into derivatives suitable for sensitive UV detection. The method has been used for determination of enantiomeric purity in a number of commercial samples, the results are presented in TABLE 2. Some level of impurities has been found in most samples investigated. The chromatograms used for the evaluation of optical purity of some commercial samples are shown in FIGURES 7 and 8. As can be seen from the data presented the

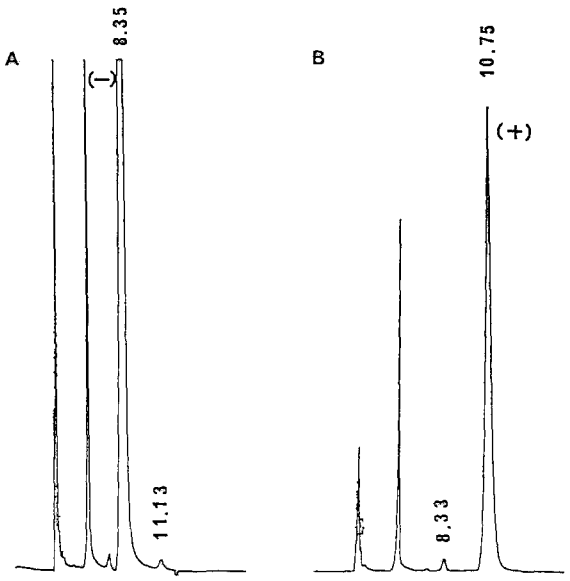

FIGURE 8. Chromatograms used for enantiomeric purity evaluations: (-)-hexahydromandelic acid (A), (+)-hexahydromandelic acid (B). Conditions as in TABLE 2.

procedure developed in this study enabled the determination of enantiomeric composition functionalised α-hydroxy carboxylic acids at the trace level. High selectivity and efficiency of the system as well as high symmetry of the peaks further contribute to the high accuracy and sensitivity of the method. In the case of L-lactic acid as low as 0.1% of the D enantiomer could be determined. Surprisingly high contamination with opposite enantiomer has been found in malic acid "optically pure" commercial samples. On the other hand no contamination has been detected in L-α-hydroxyisovaleric and L-α-hydrohyisocapric acids, which suggest that the derivatisation reaction induced essentially no racemisation.

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INFLUENCE OF THE PHYSICOCHEMICAL PARAMETERS OF PROPARGYLAMINE DERIVATIVES ON THEIR RETENTION ON β-CYCLODEXTRIN POLYMER-COATED SUPPORT

T. CSERHÁTI* AND E. FORGÁCS
Central Research Institute for Chemistry
Hungarian Academy of Sciences
P.O. Box 17
1525 Budapest, Hungary

ABSTRACT

The retention of 17 propargylamine derivatives was determined on a ß-cyclodextrin polymer (ß-CDP)-coated silica column using dioxane-0.05 M K_2HPO_4 (6:4, v/v) as eluent. The inclusion complex formation between the propargylamine derivatives and a water-soluble ß-CD polymer was studied by charge-transfer chromatography carried out on reversed-phase TLC layers. The capacity factors were correlated with the various physicochemical parameters of the solutes. Principal component analysis proved that the hydrophobicity and steric parameters have the highest influence on the retention of propargylamine derivatives in HPLC. The result suggest that the selectivity of the ß-CDP-coated silica support may be different from that of the traditional alkyl bonded reversed-phase supports.

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INTRODUCTION

Cyclodextrins (CDs) are cyclic oligosaccharides which have the ability to form inclusion complexes with many organic and inorganic compounds of various chemical structures (1,2). Cyclodextrins (CDs) and modified CDs have been used in many fields of chromatography (3). They were applied in reversed-phase thin-layer chromatography (RP-TLC) to study the formation of inclusion complexes with barbiturates (4,5) and with chlorophenol derivatives (6,7). CDs influenced the mobility of inorganic ions in isotachophoresis (8), improved the separation of peptides in capillary electrophoresis (9) and enhance the efficiency of enantomeric separation in gas chromatography (10). CDs are used in high performance liquid chromatography either by adding CDs to the eluent (11) or by covalently bonding CDs to the silica surface (12). CDs have been used to improve separation of non chiral compounds (13) and to separate enantiomers both in direct and reversed-phase seoparation mode (14). Silica supports with ß-cyclodextrin polymer (ßCDP) on their surface have been recently prepared, and their retention behavior (15) and their capacity to separate enantiomers have been studied in detail (16).

Propargylamine derivatives are selective inhibitors of B-type monoamine oxidase (17,18), the determination of their lipophilicity (19,20) and their behavior in adsorptive and reversed-phase TLC have been recently reported (21).

The objectives of our work were to study the retention behavior of propargylamine derivatives on

the ß-cyclodextrin polymer coated silica support, to find the physicochemical parameters of solutes accounting for the retention and to compare the various mulivariate mathematical statistical methods for the evaluation of the retention data.

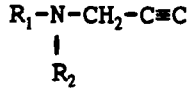
MATERIALS AND METHODS

The chemical structure of monoamine oxidase inhibitory drugs are compiled in Table 1.

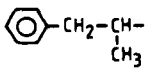
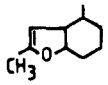
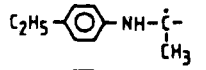
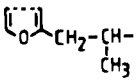
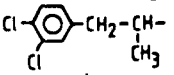
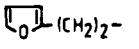
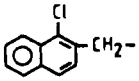
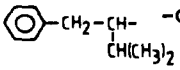
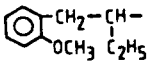
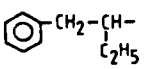
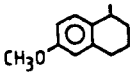
A. Determination of the retention behaviour of drugs by high performance liquid chromatography

The ßCDP coated silica support was prepared at the CYCLOLAB Research and Development Laboratory (Budapest, Hungary). A 25 cm x 4 mm I.D. column was filled in our laboratory with a Shandon analytical HPLC Packing Pump (Pittsburgh, USA) by the procedure normally used for the filling of reversed-phase columns. The HPLC equipment consisted of a Gilson gradient analytical system GILSON Medical Electronics (Villiers-le-Bell, France) with 2 piston pumps (Model 302), Detector (Model 116), Rheodyne injector with 20 μl sample loop (Cotita, California, USA), and a Waters 740 integrator (Milford, Massachusetts, USA). The flow-rate was 0.8 ml/min and the detection wavelength was 240 nm. The eluent was dioxane-0.05 M K_2HPO_4 (6:4 v/v). The drugs were dissolved in the eluent at a concentration of 0.05 mg/ml. The retention time of each compound was determined by three consecutive determinations. The capacity factor and the coefficient of

TABLE 1
Chemical Structures of Monoamine Oxidase Inhibitory Drugs



General structures

No.	R_1	R_2	No.	R_1	R_2
1	(+) 	$-CH_3$	10		$-CH_3$
2	(−)	$-CH_3$			
3		$-CH_3$	11		$-H$
4		$-H$	12		$-CH_3$
5		$-CH_3$	13		$-CH_3$
6		$-CH_3$	14		$-CH_3$
7		$-CH_3$	15		$-CH_3$
8		$-CH_3$	16		$-CH_3$
9		$-C_4H_7$	17		$-CH_3$

variation capacity factor were calculated for each compound.

B. Determination of the interaction between drugs and a water-soluble ß-cyclodextrin polymer by reversed-phase thin-layer chromatography.

Silica plates with fluorescence indicator (Silcoplat UV_{254}, Kavalier, Brno, Czech Republic) were impregnated with n-hexane:paraffin oil 95:5 v/v by overnight predevelopment. Eluents were dioxane:water mixtures, the dioxane concentration varying between 35-60 vol% in steps of 5 vol%. To determine the strength of interaction between the drugs and ßCD, a water-soluble ß-CD polymer (further SCDP) was added to the eluent. Its concentration in the eluent varied between 0-20 mg/ml. The SCDP was prepared by crosslinking ß-CD monomers with butylene glycol bis(epoxypropyl ether) in aqueous alkaline solution (ßCD content 66.04%). The SCDP was purchased from CYCLOLAB Research and Development Laboratory (Budapest, Hungary). It has to be emphasized that ßCDP and SCDP were prepared with different process of polymerization, therefore their capacity to form inclusion complexes may be different. After development the plates were dried at 105^0C, and the spots were detected under UV light and with iodine vapour. Each determination was run in quadruplicate. The R_M values were calculated by $R_M = \log (1/R_f - 1)$. The dependence of R_M value on the eluent composition was calculated by

$$R_M = R_{M0} + b_1.C_1 + b_2.C_2 \qquad (1)$$

where R_M = actual R_M value of a compound determined at a given dioxane and SCDP concentrations; R_{M0} = R_M value

of a compound exrapolated to zero dioxane and SCDP concentrations (best estimation of molecular lipophilicity); b_1 = decrease in the R_M value caused by a 1% increase in the dioxane concentration in the eluent (related to the specific hydrophobic surface area of drugs); b_2 = decrease in the R_M value caused by 1mg/ml change in the concentration of SCDP (indicator of the strength of drug-SCDP complex); C_1 and C_2 = dioxane and SCDP concentrations, respectively.

C. Calculation of relationships between retention behavior and physicochemical parameters of propargylamine derivatives.

Principal component analysis (PCA) (22): The capacity factors of drugs determined on ßCDP column, their physicochemical and retention parameters in TLC system were the variables and the monoamine oxidase drugs were the observations. The physicochemical parameters included in the calculation were: π = Hansch - Fujita's substituent constant characterizing hydrophobicity; H - Ac and H - Do = indicator variables for proton acceptor and proton donor properties, respectively; M - RE = molar refractivity; F and R = Swain - Lupton's electronic parameters characterizing the inductive and resonance effect, respectively; σ = Hammett's constant, characterizing the electron-withdrawing power of the substituent; Es = Taft's constant, characterizing steric effects of the substituent; B_1 and B_4 = Sterimol width parameters determined by distance of substituents at their maximum point perpendicular to attachement. The limit of the variance explained was set to 99%. The two-dimensional non-linear map of PC loadings and variables (23) and

the cluster analysis of the original data matrix and the PC loadings and variables was also calculated (24). The inclusion of both nonlinear mapping technique and cluster analysis in the evaluation was motivated by the consideration that each of them are theoretically similar, they calculate and visualize the relative distances between the members of data matrix.

RESULTS AND DISCUSSION

The log k' values and the coefficients of the variation (standard deviations expressed in percent of the mean value) are listed in Table 2. The log k' values are different suggesting that the drugs can be sucessfully separated on this column by an appropriate mixture of dioxane-water. The coefficients of variation are low indicating the good reproducibility of the retention time on ßCDP column.

B. Reversed-phase thin-layer chromatography

The parameters of eq.1. are compiled in Table 3. Compounds 1 and 2 were omitted from the calculations because they exhibited elongated spots in the eluents resulting in the inaccurate determination of their retention.

Propargylamine derivatives did not form inclusion complexes with SCDP in the presence of dioxane (b_2 value in eq.1. was never significant), however, the ring structures in the drug molecules can easily fit into the CD cavity. This finding can be explained by the supposition that the bulky dioxane molecule also enter the CD cavity and inhibits competitively the interac-

TABLE 2.
Retention of Propargylamine Derivatives on ß-Cyclodexdextrin Polymer-Coated Silica Column. Eluent: Dioxane--50 mM K_2HPO_4 (6:4, v/v). Numbers Refer to Propargylamine Derivatives in TABLE 1.

No.	log k' Mean	Coefficent of variation %
1.	-0.462	0.53
2.	-0.456	0.22
3.	-0.430	0.88
4.	-1.617	0.82
5.	-0.153	0.47
6.	-0.168	0.25
7.	-0.276	0.64
8.	-0.428	0.21
9.	0.017	0.85
10.	-0.386	0.56
11.	-0.625	0.69
12.	-0.246	0.75
13.	-0.211	0.42
14.	-0.680	0.45
15.	-0.300	0.42
16.	-0.415	0.59
17.	-0.287	0.38

tion between the drugs and the complex forming centers of SCDP. Eq.1. fits well to the experimental data, the significance level being over 99.9% (see calculated F values) in each instance. The ratio of variance explained by the independent variables varied between 63 and 96 % (see r^2 values).

Both lipophilicity values (R_{M0}) and the specific hydrophobic surface areas (b_1) of the drugs differ considerably, indicating that these parameters can be separately included in future quantitative structure activity relationship calculations.

The parameters of PCA are compiled in Table 4.

TABLE 3.
Relationship Between the R_M Value of Propargylamine Derivatives and the Concentration of Dioxane (C_1) and Water-Soluble ß-Cyclodextrin Polymer (C_2) in the Eluent. Number Refer to Propargylamine Derivatives in TABLE 1. (b_2 never was significant).

$$R_M = R_{M0} + b_1.C_1 + b_2.C_2$$

Parameter	Compound no.				
	3	4	5	6	7
n	17	17	17	17	17
R_{M0}	1.61	1.29	1.61	2.90	2.83
$-b_1.10^2$	3.05	3.40	3.48	4.76	4.35
$s_{b1}.10^3$	2.72	3.06	3.05	2.96	2.29
$F_{calc.}$	125.99	125.63	130.67	258.31	362.41
r^2	0.8936	0.8933	0.8970	0.9451	0.9603
	8	9	10	11	12
n	17	16	18	18	9
R_{M0}	2.36	3.72	1.50	1.84	3.55
$-b_1.10^2$	4.03	4.73	2.85	3.90	6.33
$s_{b1}.10^3$	2.51	4.00	4.02	5.19	6.80
$F_{calc.}$	257.63	140.28	50.13	56.42	86.72
r^2	0.9450	0.9093	0.7580	0.7790	0.9253
	13	14	15	16	17
n	18	18	18	18	18
R_{M0}	2.28	0.91	2.40	1.76	2.09
$-b_1.10^2$	3.43	2.50	3.73	3.18	3.30
$s_{b1}.10^3$	4.81	4.81	4.35	4.07	4.65
$F_{calc.}$	50.75	26.98	73.41	61.37	50.39
r^2	0.7603	0.6277	0.8210	0.7932	0.7590

Five principal components explain the majority of variance indicating that the 13 original variables can be substituted by 5 background (abstract) variables with only 7% loss of information. Unfortunately, PCA does not prove the existence of such background variables as concrete physicochemical entities, but only indicates their mathematical possibility. The log k'

TABLE 4.
Similarities and Dissimilarities Between the Physicochemical Parameters of Propargylamine Derivatives and their Retention on ß-Cyclodextrin Polymer Coated Silica Column. Results of Principal Component Analysis.

No. of component	Eigenvalue	Variance explained %	Sum of variance explained %
1	5.74	44.13	44.13
2	2.52	19.40	63.53
3	2.39	18.38	81.91
4	0.87	6.69	88.60
5	0.56	4.29	92.89

Parameters	Principal component loadings No of principal component 1	2	3	4	5
log k'	0.68	0.38	-0.01	0.28	0.22
R_{M0}	0.89	-0.10	0.13	0.18	0.28
b_1	0.72	-0.42	0.40	0.20	0.23
π	0.88	0.30	-0.27	-0.06	0.01
H-Ac	-0.77	0.03	0.49	-0.25	0.16
H-Do	-0.60	0.18	0.34	0.63	-0.19
M-RE	0.78	0.53	-0.04	0.00	-0.28
F	0.44	-0.64	0.57	-0.11	-0.14
R	0.51	-0.28	-0.76	0.15	0.03
σ	0.49	-0.69	0.49	0.04	-0.11
Es	-0.81	0.14	0.08	0.33	0.27
B_1	0.43	0.59	0.61	0.11	-0.24
B_4	0.30	0.67	0.51	-0.30	0.25

values - together with the measured and calculated hydrophobicity values, the Taft constant characterizing the steric effect of substituents and electronic parameters of drugs - have high loading in the first PC indicating the marked influence of these parameters on the mode of retention of the ß-CDP support. It can be assumed the interactions of solutes with the CD cavity can exert a considerable influence on the retention behaviour of solutes on ßCDP column. These interac-

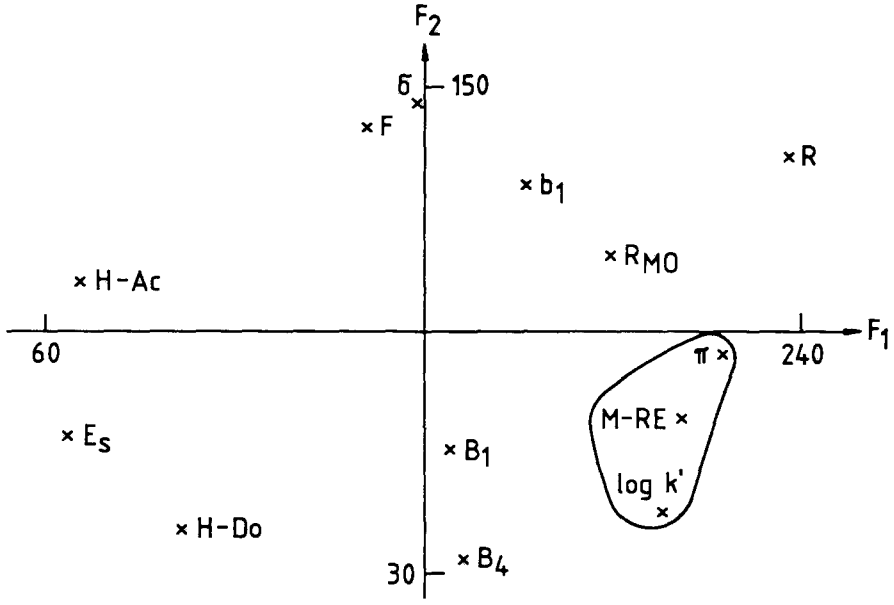


Figure 1. Similarities and dissimilarities between the physicochemical parameters and log k' of propargylamine derivatives on ß-cyclodextrin polymer coated column. Two dimensional nonlinear map of principal component loadings. Number of iterations: 162; max.error: $3.83.10^{-2}$. For symbols see MATERIALS AND METHODS.

tions are determined by the size of the guest molecules and their lipophilicity. The steric parameters define the capacity of the guest molecule to enter in the CD cavity and the lipophilicity of the guest molecule determines the strength of interactions with the hydrophobic inner surface of the CD cavity. It is probable that, as a consequence of polymerization, access to the sites of inclusion complex formation is hindered to various degrees. Furthermore, secondary

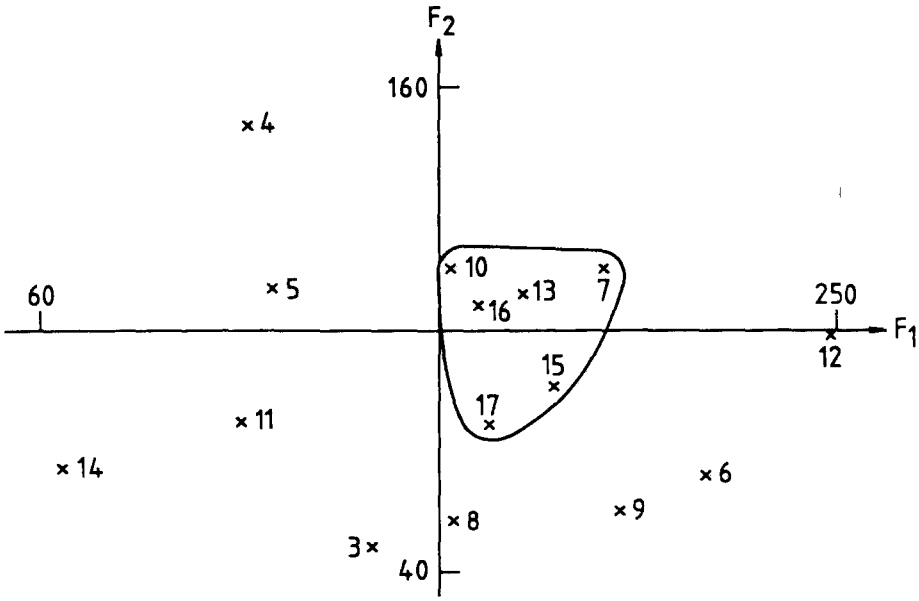


Figure 2. Similarities and dissimilarities between the propargylamine derivatives on ß-cyclodextrin polymer coated column. Two dimensional nonlinear map of principal component variables. Number of iterations: 124; max.error: $3.07.10^{-2}$. Numbers refer to propargylamine derivatives in Table 1.

cavities of different dimensions are formed between the polymer network. These dimensions are at least commensurable with the dimensions of ß-CD cavities, resulting in different inclusion copmlex formation and hence in different retention characteristics. The retention of solutes is probably determined by the interplay of the various binding forces discussed above.

The distribution of variables on the two-dimensional nonlinear map of PC loadings supports our previous assumptions (Fig.1) that both steric and hydro-

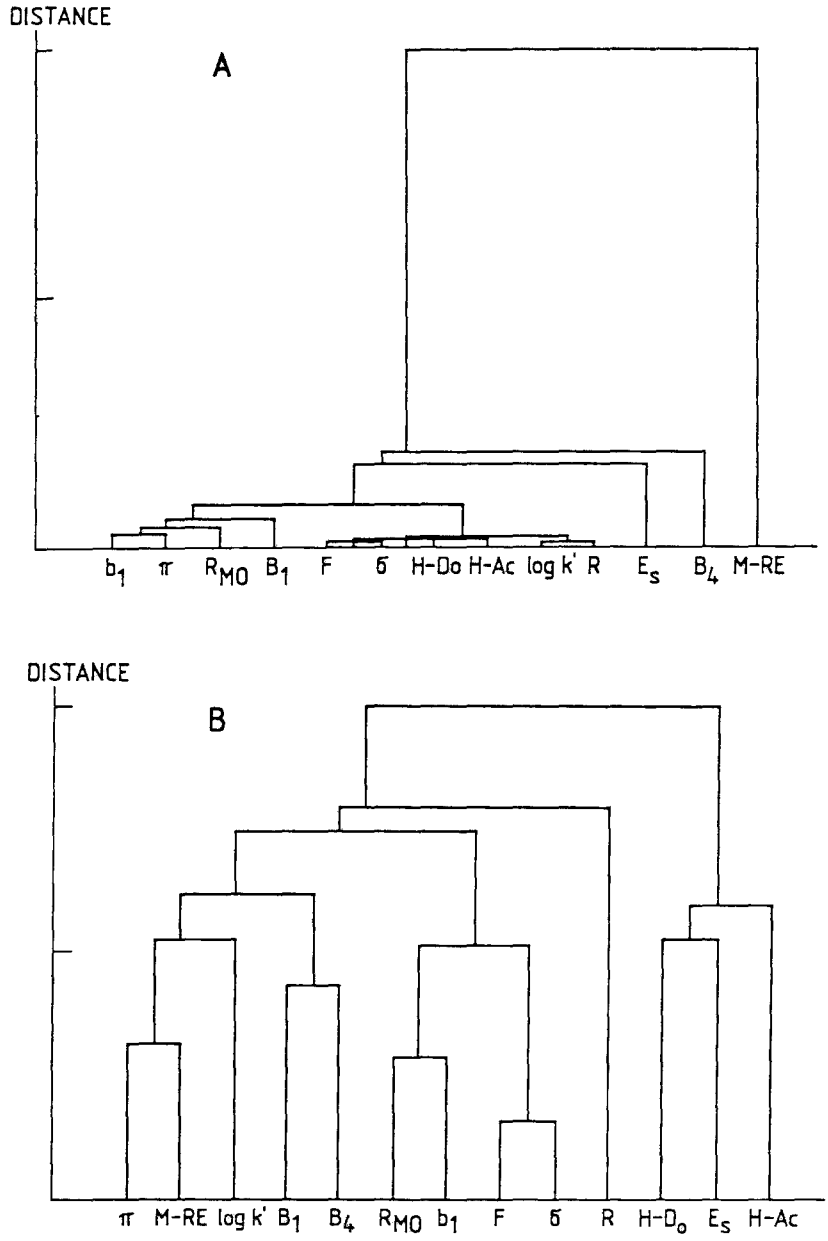


Figure 3. Similarities and dissimilarities between the physicochemical parameters and log k' of propargylamine derivatives on ß-cyclodextrin polymer coated column. Cluster dendograms calculated from the original data matrix (A) and from the principal component loadings (B). For symbols see MATERIALS AND METHODS.

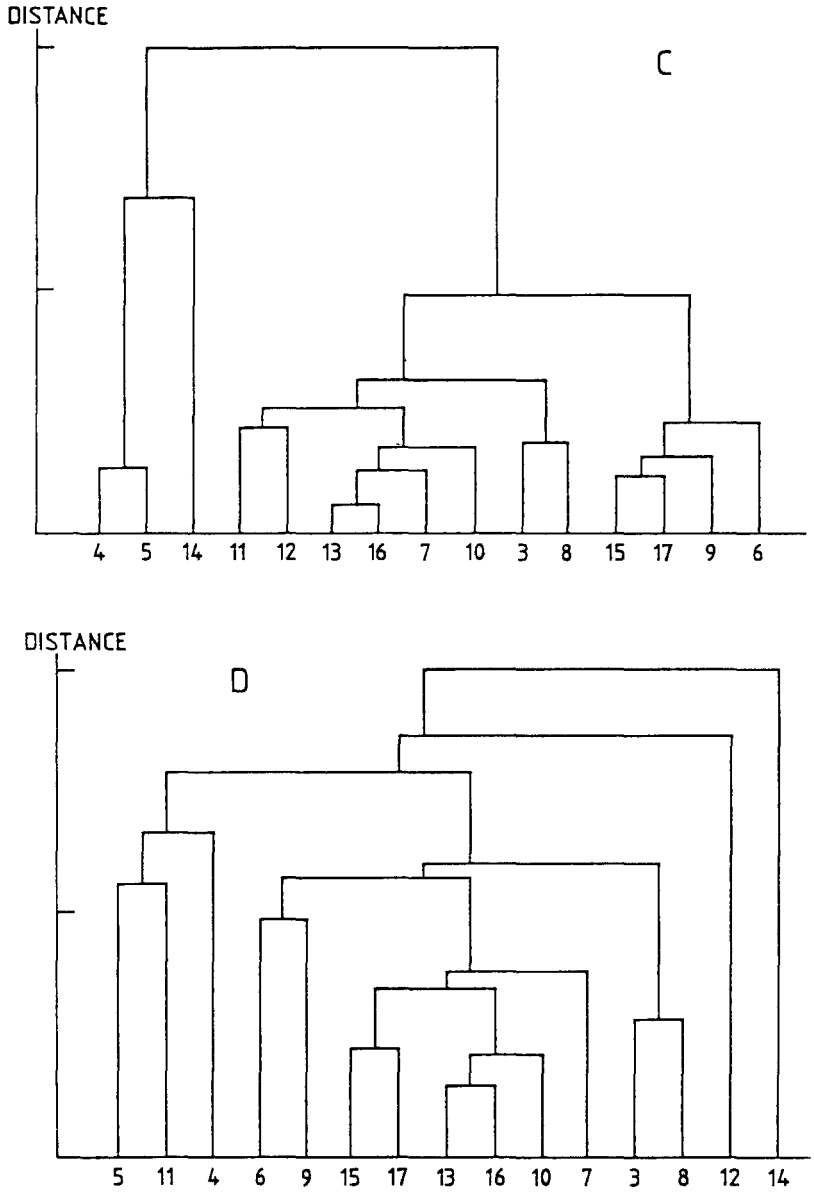


Figure 4. Similarities and dissimilarities between the propargylamine derivatives on ß-cyclodextrin polymer coated column. Cluster dendograms calculated from the original data matrix (C) and from the principal component variables (D). Numbers refer to propargylamine derivatives in Table 1.

phobic parameters influence the retention of drugs on the ßCDP column. The log k' values form a distinct cluster with the molar refraction (related to the bulkiness of the drug) and the calculated lipophilicity. This finding indicates again the mixed retention mechanism of the ßCDP support. The solutes with condensed ring structures are near to each other on the two-dimensional nonlinear map of principal component variables (Fig.2) suggesting again the importance of steric conditions in the retention.

The dendograms of cluster analysis calculated from the original data matrix and from the principal component loadings are shown in Fig 3. The information content of the clusters are different indicating the influence of PCA on the visualization of the results. The dendograms of cluster analysis calculated from the original data matrix and from the principal component variables are shown in Fig 4. These dendograms also show that the application of PCA modifies the distribution of variables. Due to its higher dimensionality we strongly advocate the application of the two-dimensional nonlinear mapping technique instead of cluster analysis. We assume that the two dimensional nonlinear map may contain more information than the one dimensional structure of clusters.

We have to stress that the conclusions discussed above are not the results of theoretical considerations and hence are valid only for this special data set. A generalization of these conclusion can lead to severe misinterpretation.

This work was supported by the grant OTKA 2670.

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DETERMINATION OF 4-HYDROXYTERTATOLOL STEREOISOMERS IN RAT AND HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

T. LAVE[1]*†, C. EFTHYMIOPOULOS[1]‡, D. CHRISTMANN[2], J. C. KOFFEL[1], AND L. JUNG[1]

[1]Département de Chimie Thérapeutique
Faculté de Pharmacie 74 Route du Rhin
67400 Illkirch-Graffenstaden, France
[2]Clinique médicale A
Hospices civils 1 place de l'Hôpital
67000 Strasbourg, France

ABSTRACT

A stereospecific high-performance liquid chromatographic method for the simultaneous determination of the four stereoisomers of 4-hydroxytertatolol, an active metabolite of tertatolol, in human and rat urine is described. The method is based on solid-phase extraction of urine followed by derivatization with S(+)-naphtylethylisocyanate. The four stereoisomers were resolved by reversed phase high-performance liquid chromatography and detected by fluorescence ($\lambda_{excitation}$ = 210 nm, no cutoff emission filter). The analytical method is suitable for the simultaneous determination of the four stereoisomers of 4-hydroxytertatolol in rat and human urine down to 22.0 ng/ml. Preliminary results in rat suggest stereoselective hydroxylation of tertatolol enantiomers.

* To whom correspondence should be addressed.
† Present address: Hoffmann La Roche Ltd., Preclinical Pharmacokinetics, grenzacherstrasse 124, CH-4002 Basel, Switzerland.
‡ Present address: Glaxo Group Research Ltd., Greenford Road, Greenford, Middlesex, United Kingdom.

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INTRODUCTION

Tertatolol (fig. 1) (± - (hydroxy - 2' - tertbutylamino- 3' -propyloxy) -8 - thiochromane , hydrochloride) is a powerful , long acting and non cardioselective beta-blocker without partial agonistic activity (1). Tertatolol differs from other beta-blockers in that it increases renal blood flow in hypertensive and normotensive patients (2). In human and in rat, about 5 % of the oral administered dose of tertatolol is converted to 4-hydroxytertatolol (fig. 1), (± - (hydroxy - 2' - tertbutylamino- 3' -propyloxy) -8 - hydroxy -4 - thiochromane , acetat). There are two asymmetric carbons in this metabolite, making four stereoisomers to be separated. The beta-adrenoreceptor antagonist activity of this hydroxylated metabolite is closely related to that of tertatolol but 4-hydroxytertatolol is devoid of any significant renal vasodilator effect (3).

Analytical method allowing the determination of the two enantiomers of tertatolol and the simultaneous determination of the sum of both enantiomers as well as the sum of the four stereoisomers of 4-hydroxytertatolol has already been developed (4, 5). In order to evaluate the pharmacokinetics of 4-hydroxytertatolol stereoisomers and the substrate and product stereoselectivity of the metabolism of tertatolol, a stereoselective analytical method allowing the quantitative determination of the four stereoisomers of 4-hydroxytertatolol was needed.

We report here an HPLC assay for the simultaneous determination of the four stereoisomers of 4-hydroxytertatolol in rat and human urine. The method involves derivatization with an optically pure fluorescent reagent [S(+)-naphtylethylisocyanate (S(+)-NEI)], which has already been used for the determination of tertatolol enantiomers (4), and fluorimetric detection to allow quantification of the four stereoisomers in the nanogram range.

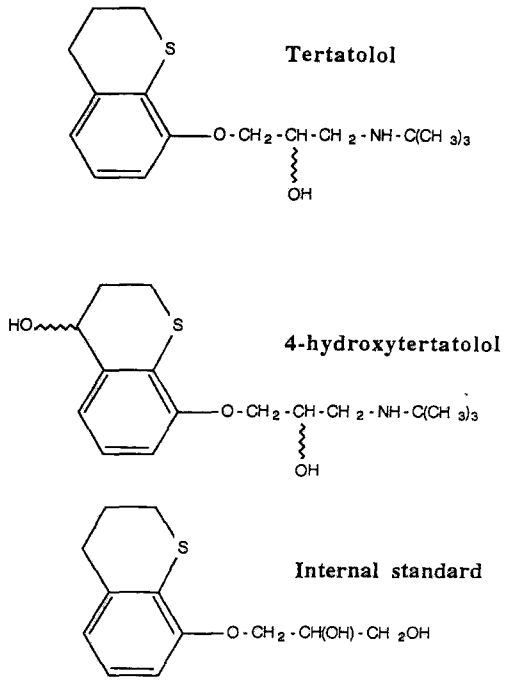

Fig. 1. Structures of tertatolol, 4-hydroxytertatolol and the internal standard. The wavy bonds indicate the presence of both configurations at each asymetric center.

MATERIAL AND METHODS

Chemicals and reagents

4-hydroxytertatolol and the four stereoisomers, as acetate salts, were purchased from Institut de Recherches Internationales Servier (Suresnes, France). The chemical purity were higher than 99% and 95% for racemic 4-hydroxytertatolol and the four stereoisomers, respectively. The optical purity of the four stereoisomers was higher than 90%. The four stereoisomers are designated (1, -) OHT, (2, -)

OHT for the two stereoisomers coming from (-)-tertatolol and (2, +) OHT and (1, +) OHT for the two stereoisomers coming from (+)-tertatolol ; as the configuration of the carbon in position 4 is unknown, the two possible configurations were arbitrarily designated 1 and 2 (fig. 1). Racemic (thiochromannyl-8 oxy)-3 propanediol-2,3, purchased from Institut de Recherches Internationales Servier (Suresnes, France) was used as internal standard (fig. 1). Its chemical purity was higher than 99.5 %. S(+)-NEI was purchased from Aldrich. The chemical purity of this reagent was higher than 99% and the optical purity higher than 99.5%. A working solution of 0.03% S(+)-NEI in dichloromethane was prepared. Other solvents and reagents used (diethylether, dichloromethane stabilized with methyl-2-butene, acetonitrile, 1M sodium hydroxide, *tert.*-butylamine) were analytical-grade products from Merck (Darmstadt, Germany).

HPLC instrumentation and conditions

A Beckmann Gold HPLC system was used, consisting of a 340 organizer injector, two 112 pumps and an analogical interface. The system was equipped with an HPLC column (5 µm Licrospher, 15 cm x 4.6 mm I.D., Merck, Darmstadt, Germany). The detector was a Kratos FS970 fluorimeter which was operated at an excitation wavelength of 210 nm. No cutoff filter was used. The stereoisomers were eluted in a gradient of acetonitrile-water (10:90, v/v to 40:60, v/v in 15 min). The mobile phase was pumped through the column at a flow rate of 1.5 ml/min. Chromatography was carried out at room temperature (≈ 20°C).

Extraction

In a 10-ml tube, 100 ng of internal standard as an alcoholic solution were added. After evaporation of the organic solvent, 0.5 ml of rat urine or 1.00 ml of human

urine and 100 µl of 1 *M* sodium hydroxide were added. The mixture was shaken on a vortex mixer for 1 min, then transferred to an Extrelut® (Merck, Darmstadt, Germany) column and extracted with 6 ml of diethylether. The ether collected was evaporated under a stream of dry nitrogen.

Derivatization procedure

A 100-µl aliquot of dichloromethane and 10 µl of 0.03% S(+)-NEI in dichloromethane were added to the residue. The tube was shaken during 1 min. After reaction at room temperature for 12 h, 10 µl of *tert.*-butylamine was added and the mixture evaporated to dryness under a light nitrogen stream. The residue was redissolved in 20 µl of acetonitrile and shaken on a vortex mixer for 1 min. A 20-µl aliquot of the sample was injected into the HPLC column for 4-hydroxytertatolol stereoisomer quantification.

Standard solutions

A solution of internal standard (10 ng/µl) in ethanol was prepared. A 10-µl aliquot of this solution was added to each sample as internal standard.

Solutions of the individual stereoisomers of 4-hydroxytertatolol, as their acetate salts, were prepared to a concentration of 1 ng/ml (of the free base) in ethanol.

The stereoisomers and the internal standard in ethanolic solutions were stable for several months at -20°C.

Calibration curves

Calibration curves were established by spiking blank urine with various quantities of the individual stereoisomer of 4-hydroxytertatolol and 100 ng/ml internal standard. The prepared urine standards contained 22.0 - 440.0 ng/ml of each stereoisomer.

Extraction recovery

In order to study the recovery of each stereoisomer of 4-hydroxytertatolol from urine, 1 ml of blank urine with known amounts of each stereoisomer was extracted as described above. The internal standard was added after the extraction procedure of the samples.

Application to rat urine samples

Male wistar rats (n = 6), eight weeks old and weighing about 250 g, were used. The rats were acclimated to the laboratory conditions one week before the beginning of the study. They had free access to water during the study, but were fasted overnight before dosing and during the study. Racemic tertatolol in aqueous solution was given orally at a dose of 30 mg/kg by gavage. For urine collection, the rats were put in different metabolic cages and urine and feces were collected separately over a 24 h period.

RESULTS AND DISCUSSION

HPLC for the simultaneous determination of tertatolol enantiomers after derivatization with S(+)-NEI, has already been described (4). 4-hydroxytertatolol is an active metabolite of tertatolol with two chiral centers making four possible stereoisomers to be separated. Therefore an analytical procedure using HPLC for the simultaneous quantification of these four stereoisomers was investigated.

In preliminary experiments we attempted a simultaneous separation of tertatolol enantiomers and 4-hydroxytertatolol stereoisomers. Due to the difference in lipophilicity between tertatolol and 4-hydroxytertatolol, it has not been possible to

attain our goal with the octadecyl column used for the separation of tertatolol enantiomers. A cyano column gave better results allowing the simultaneous separation of the two enantiomers of tertatolol and a partial separation of two peaks corresponding to 4-hydroxytertatolol containing (1, -) OHT and (2, -) OHT for the first peak and (2, +) OHT and (1, +) OHT for the second one (fig. 2). The two additional peaks eluting before the two peaks of 4-hydroxytertatolol were corresponding to the tertatolol sulfoxide metabolites. This separation of the stereoisomers of 4-hydroxytertatolol was not sufficient to study the stereoselectivity of hydroxylation of tertatolol enantiomers in position 4. Thus, a procedure similar to that describe for tertatolol enantiomers was developed. The method is based on the extraction of urine by Extrelut®. The extraction yields for the four stereoisomers of 4-hydroxytertatolol was about 98% using diethylether as the extraction solvent. The derivatization of the stereoisomers was carried out with S(+)-NEI at room temperature for 12 h as described for tertatolol enantiomers but with three times lower amounts of reagent in order to avoid chromatographic interferences with the stereoisomers of 4-hydroxytertatolol. Nevertheless, the extent of derivatization of each stereoisomer, determined after analyzing residual 4-hydroxytertatolol by GC/MS (5), was still acceptable (≈ 70%) and close to that obtain for tertatolol enantiomers (4). The internal standard, with a secondary and primary alcoholic functions, did not react with S(+)-NEI in the derivatization conditions.

The separation was achieved by gradient elution with acetonitrile and water. Under the chromatographic conditions described above, the retention times of the four derivatives of 4-hydroxytertatolol were respectively 25.3, 26.1, 27.0 and 28.8 min for (1, -) OHT, (2, -) OHT, (2, +) OHT and (1, +) OHT ; the retention time of the internal standard being about 12 min. The chromatograms of a drug-free rat

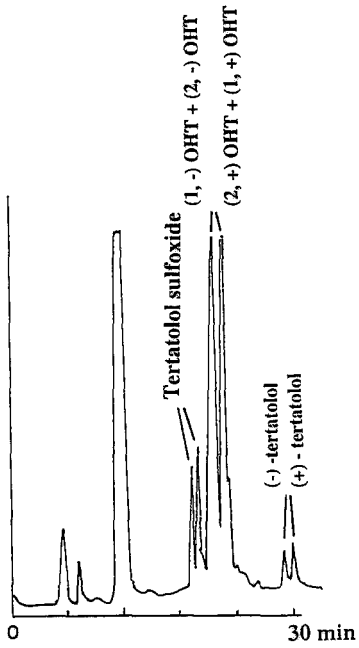

Fig. 2. Separation of the NEI derivatives of tertatolol, 4-hydroxytertatolol and tertatolol sulfoxide on a cyano column (3µm Ultrasphere cyano 7 cm x 4.6 mm i.d., Beckman); mobile phase : acetonitrile-water (10:90, v/v to 35:65, v/v in 30 min, flow rate = 1.5 ml/min). The chromatogram was obtained using pure solutions.

urine sample (0.5 ml) and a sample (0.5 ml), containing 500 ng/ml of 4-hydroxytertatolol, are shown in fig. 3. The extract of the blank urine sample contained no interfering peak.

The accuracy and precision of the method were evaluated from six calibration curves obtained by analysing spiked urine samples. Five different concentrations were used for each calibration curve. The calibration curves were linear ($r > 0.995$) in the range 22.0 - 440.0 ng/ml for the four stereoisomers of 4-hydroxytertatolol.

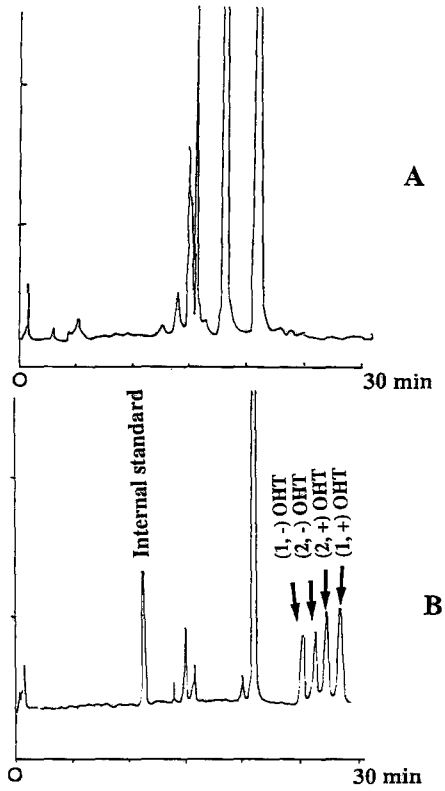

Fig. 3. Chromatograms of extract of drug-free rat urine sample (A) and of 0.5 ml rat urine sample containing 500 ng/ml 4-hydroxytertatolol (B). The samples were processed as described under "Experimental".

The standard curves gave the following equations : $y = (1.58 \pm 0.29).10^{-3}x + (-5.76 \pm 14.8).10^{-3}$ for (1, -) OHT, $y = (2.31 \pm 0.13).10^{-3}x + (-1.79 \pm 0.95).10^{-2}$ for (2, -) OHT, $y = (2.66 \pm 0.17).10^{-3}x + (-1.52 \pm 0.60).10^{-2}$ for (2, +) OHT and $y = (3.85 \pm 0.28).10^{-3}x + (0.12 \pm 0.22).10^{-2}$ for (1, +) OHT. The results of assay precision and accuracy are given in tables I and II for human urine. The small intercept value and the absence of a signal in the analysis of blank samples demonstrated the selectivity of the method.

TABLE 1

Precision (coefficient of variation, C.V.) and accuracy (error, E.R.) of the assay of human urine for (1, -) OHT and (2, -) OHT (n = 6)

Added concentration of each stereoisomer (ng/ml)	(1, -) OHT			(2, -) OHT		
	Calculated (ng/ml)	C.V. (%)	E.R. (%)	Calculated (ng/ml)	C.V. (%)	E.R. (%)
22.0	22.2 ± 0.2	1.0	0.9	21.8 ± 0.9	4.3	0.7
55.0	54.1 ± 3.0	5.6	1.5	53.0 ± 5.8	11.0	3.6
110.0	104.9 ± 4.7	4.5	4.6	111.1 ± 8.2	7.4	1.0
220.0	236.6 ± 16.6	7.0	7.5	206.5 ± 11.8	5.7	6.1
440.0	438.6 ± 18.2	4.0	0.3	436.5 ± 13.4	3.1	0.8

TABLE 2

Precision (coefficient of variation, C.V.) and accuracy (error, E.R.) of the assay of human urine for (1, +) OHT and (2, +) OHT (n = 6)

Added concentration of each stereoisomer (ng/ml)	(1, +) OHT			(2, +) OHT		
	Calculated (ng/ml)	C.V. (%)	E.R. (%)	Calculated (ng/ml)	C.V. (%)	E.R. (%)
22.0	22.4 ± 0.4	1.9	1.7	21.5 ± 1.0	4.8	2.1
55.0	51.9 ± 3.4	6.6	5.5	51.4 ± 8.3	16.1	6.5
110.0	114.5 ± 9.5	8.2	4.1	115.6 ± 8.3	7.2	5.1
220.0	224.8 ± 12.1	5.4	2.1	231.6 ± 29.0	12.5	5.3
440.0	444.6 ± 16.4	3.7	1.0	441.6 ± 19.0	4.3	0.4

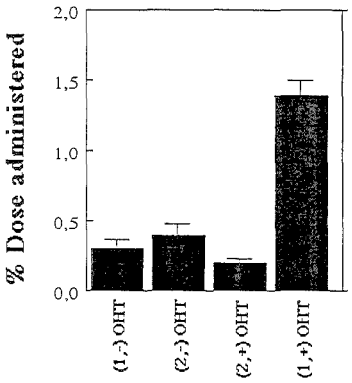

Fig. 4. Stereochemical composition of 4-hydroxytertatolol in rat urine after a single oral dose of 30 mg/kg of racemic tertatolol. The amount of each stereoisomer excreted in urine is expressed as the percentage (mean ± SD, n = 6) of the dose of racemic tertatolol administered.

The method was applied successfully to the simultaneous determination of the four stereoisomers in human and rat urine samples for pharmacokinetic and metabolic studies. The stereochemical composition of 4-hydroxytertatolol in rat urine is presented in fig. 4. As can be seen, the introduction of the hydroxyl group was stereoselective, favouring (1, +)-OHT with a high stereoselectivity [(1, +)-OHT / (2, +)-OHT ≈ 6]. The hydroxylation of (-)-tertatolol was only slightly stereoselective in rats [(1, -)-OHT / (2, -)-OHT ≈ 0.6].

In summary, the analytical method described permits the simultaneous determination of the four stereoisomers of 4-hydroxytertatolol in human and rat urine at concentrations down to 22.0 ng/ml. The method is suitable for pharmacokinetic and metabolic studies, the complete of which will be published later. In a preliminary study, (1, +) OHT seems to be quantitatively the major stereoisomer of 4-hydroxytertatolol in rat urine.

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SIMULTANEOUS PROFILING AND IDENTIFICATION OF CAROTENOIDS, RETINOLS, AND TOCOPHEROLS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY EQUIPPED WITH THREE-DIMENSIONAL PHOTODIODE ARRAY DETECTION

AMI BEN-AMOTZ
Israel Oceanographic & Limnological Research
National Institute of Oceanography
P.O. Box 8030
Haifa 31080, Israel

ABSTRACT

A high performance liquid chromatography (HPLC) system was developed for the simultaneous separation and identification of carotenoids, retinol, its derivatives, and tocopherols. These lipophilic and semilipophilic products were chromatographed on a C18 Vydac column and were monitored and identified using an on-line, three-dimensional photodiode array detector, which permitted profiling on the 200 to 800 nm absorption spectrum of any chromatographic peak in less than 1 sec at a high resolution of 1.2 nm. Identification of peaks and overlapping peaks is conducted by subsequent spectra analysis by the use of a powerful computer station combining Microsoft Windows™ and a chromatography manager. This methodology was successfully applied to extracts of different origins such as plants, sera, tissues and others, memorizing each spectral analysis for processing and comparing within itself or with previously stored data. Quantitative and qualitative processing of the spectral analyses allowed the build-up of a new database of the full absorption profile for each indicated product in comparison to previously published data.

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INTRODUCTION

High pressure liquid chromatography (HPLC) is being employed as the currently most accepted technique for the separation and identification of carotenoids [1]. The HPLC method is most accepted for the elucidation and quantification of carotenoids in prokaryotes, eukaryotes, as well as in any specimen whose contents are under question. Advances in the HPLC method for the separation of carotenoid and retinol mixtures have been summarized and reviewed intensively recently [1, 2]. However, the question of proper peak identification is still open for further analysis. Classical HPLC equipment runs on one to four channels, and the carotenoids are compared to authentic standards by the retention time at the indicated channels. Improved analysis is supported by the use of off-line spectrophotometric techniques such as fluorescence, NMR, mass spectrometry and others.

The recent introduction of the HPLC on-line photodiode array detector permits complete absorption spectrum detection of each chromatographic peak in a very short time of less than 1 sec. The photodiode array detectors owe their spectral superiority to the reverse-optic configuration and the array of photodiodes, each dedicated to a particular band of wavelengths, as well as to the attached computer for data processing [3]. The technology of the photodiode array detectors in the last two years was developed to improve the integrity of the chromatographic results to reach better resolution and sensitivity on the full spectral map through continuous monitoring and acquisition of all UV/Vis wavelengths. The modern, advanced photodiode arrays need a high level of chromatography manager and developed software to give full automation and quantification of the processed, three-dimensional, spectral data.

Since carotenoids possess special characteristic absorption, and because small chemical or structural modifications may slightly alter their spectra, carotenoids are most appropriate candidates for identification by the combined technique of HPLC - photodiode array detection.

In the present study we analyzed and identified a variety of carotenoids, retinols and tocopherols by using a reversed phase HPLC

separation system equipped with a three-dimensional, sensitive photodiode array detector and an upbased chromatogram manager, a system of improved potential for future analysis of lipophilic carotenoids and related products.

MATERIALS AND METHODS

HPLC System

The HPLC System was equipped with a 7725i Rheodyne Injector (Rheodyne Inc., Cotati, CA, USA) on a Waters HPLC system (Millipore, Marlborough, MA, USA), including pumps 501 and 510, a Waters 996 photodiode array detector attached to a Waters Millennium 2010, Version 2.0, Chromatography Manager, on a compatible IBM computer 486 DX2 66 strengthened with Microsoft Windows™ supported by hard disk of IJ, 33 mega RAM and connected to an HP Deskjet 1200C plotter.

Column and Solvents

The column was a Vydac 201 TP 54 stainless steel column of 25 cm×4.6 mm (i.d.) packed with C18 reversed phase material of 5 μm particle size, 300 Å pore size (The Separation Group, Hysperia, CA, USA). The column was protected by a 5 cm C18 ODS (4) guard column (Shimadzu, Kyoto, Japan) and with a small preguard column, Waters Guard-Pak, inserted with a C18 μBondapak cartridge.

Elution was performed at ambient temperature (25±3°C) with an isocratic solvent, methanol: acetonitrile, HPLC grade (9:1, v/v) at a constant flow of 1.0 ml/min. Mobile phase was flushed with nitrogen to avoid air gassing in the solvents. With proper use, the column retained its elution profile for a long period of continuous use. A gradual slight increase of retention time after about 100 injections of sample was reversed by washing the column with methanol: acetonitrile: methylene chloride (8:1:1, v/v/v).

Standards

Synthetic lutein, zeaxanthin, canthaxanthin, β-apo-8-carotenal, β-crypoxanthin, echineone, all-trans α-carotene, all-trans β-carotene, 9,15-dicis β-carotene, all-trans γ-carotene, 15-cis β-carotene and all-trans zeta-carotene were provided by Hoffmann La Roche, Basel, Switzerland. Lutein from alfalfa, lycopene from tomato, α-carotene from carrots, retinol, retinal, 9-cis retinal, 13-cis retinoic acid and α-tocopherol were from Sigma, St. Louis, MO, USA. 9-cis β-carotene, all-trans phytoene, 9-cis phytoene, all-trans and 9-cis phytofluene, violaxanthin and zeaxanthin from *Dunaliella* were self extracted and separated. All standards were kept at -70°C under N_2, dried by a stream of N_2 prior to analysis and injected into the HPLC in methylene chloride.

Carotenoids from *Dunaliella*

Pigments of *Dunaliella* grown for selective induction and accumulation of the necessary carotenoids were extracted from the algal pellet with ethanol/hexane [4, 5]. Carotenoids were pre-separated and pre-purified by using a preparative HPLC column, Vydac 218 TP 1022, a stainless steel C18 column of 10 μm particle size (The Separation Group, Hysperia, CA, USA), followed by dehydration under N_2 and redissolution in methylene chloride for analysis by the analytical HPLC column. *Dunaliella* was used to produce and accumulate the indicated carotenoids and their equivalent stereoisomers [4, 5]. All carotenoids were kept at -70°C under N_2, dried by a stream of N_2 prior to analysis and injected into the HPLC in methylene chloride.

Carotenoids and Vitamins in Human Serum

Five healthy young male humans between the ages of 20 to 25 donated blood after overnight fasting. These men were non-smokers and

were not vegetarians. Blood samples of up to 5 ml were collected and the blood was separated. The serum was stored at -70°C under N_2 until analysis, usually within 7 days. To one ml of serum, 2.5 ml of ethanol were added, and after 5 min of mixing, the vitamins were extracted with 5 ml n-hexane, mixed vigorously and phase separated centrifugally at 2,000×g for 5 min. The upper phase was removed and the water/ethanol phase was extracted a second time with 3 ml n-hexane. The two hexane extracts were combined and evaporated by a stream of N_2. The dried residues were dissolved in a minimal volume of methylene chloride prior to injection.

RESULTS AND DISCUSSION

Column and Solvents

The major aim of this study was to identify and verify the HPLC peaks of various carotenoids and retinols. To reach this goal, one reversed phase column was selected, Vydac 201 TP 54, and one set of solvents (methanol: acetonitrile, 9:1, v/v) was used to elute the samples on this column. The system ran isocratically under constant conditions to allow repetition of many HPLC runs representing only slight modifications in the profile of the chromatograms while retaining the elution time at its initial setting. All HPLC runs were set at a flow rate of 1.0 ml/min at a column pressure of 800±50 psi. The constant pressure was necessary to reach similar elution profiles, while a gradual increase in the column pressure was usually reflected by a plus shift in the retention time, an indication of the necessity of column wash. The two inert solvents, methanol and acetonitrile, with no reactive solvents such as ethyl acetate or ammonium acetate [6], ensured highly satisfactory recovery of all carotenoids and retinoids. The decision to omit more volatile solvents such as dichbromethane or hexane was related to the gradual evaporation of these solvents when stored in the HPLC containers with a flush of N_2. Moreover, the Millennium Maxplot full spectra analysis, as described, requires solvents of very low UV wavelength maxima of which methanol,

λmax = 205 nm, and acetonitrile, λmax = 190 nm, are good choices, while methylene chloride, λmax =233 nm, ethyl acetate, λmax =256 nm, and many other organic solvents may interfere in monitoring and acquisition of the low UV wavelengths between 190 to 250 nm. Under the above preset conditions, the Vydac column showed good results on runs of up to about 30 min. The peak orientation, to achieve ideal integration of valley to valley with no peak profile distortion was best between 0 to 15 min, good between 15 to 20 min, and satisfactory between 20 to 30 min of retention time. Above that time range the orientation of most peaks was gradually distorted, being unsatisfactory for direct peak resolution.

High Resolution Photodiode Array Chromatography

Improved technique of the modern photodiode array detector provides higher resolution and sensitivity of the detection system. This technique gives the full spectral map of each peak through continuous monitoring and acquisition of all UV/VIS wavelengths, which with the Chromatograph Manager provides detailed 3-D graphic covering of the spectrum from 200 to 800 nm. The advanced diodes spectrum techniques improves the optical resolution down to 1.2 nm, a sensitive detection level that permitted minute differences in the spectral shape to achieve compound identification and confirmation. The post-run analysis by the Chromatogram Manager, which combines the power of Microsoft Windows™ and DOS, allows detailed analysis of each peak in the chromatogram for comparison with reference spectra already stored and memorized. Out of many different 3-D chromatographic runs using this advanced detection capability in our laboratory, over the last 12 months, two groups of samples are illustrated: an authentic carotenoid standard, synthetic 15-cis β-carotene along with an extract of human serum. The 3-D graphic profile of the HPLC run of 15-cis β-carotene is illustrated in Fig. 1, covering spectra from 250 to 600 nm at 1.2 nm resolution. The colored 3-D cube can be rotated in any direction and angle to allow a direct view of the spectrum from any side, with a clear side or topographic view of the

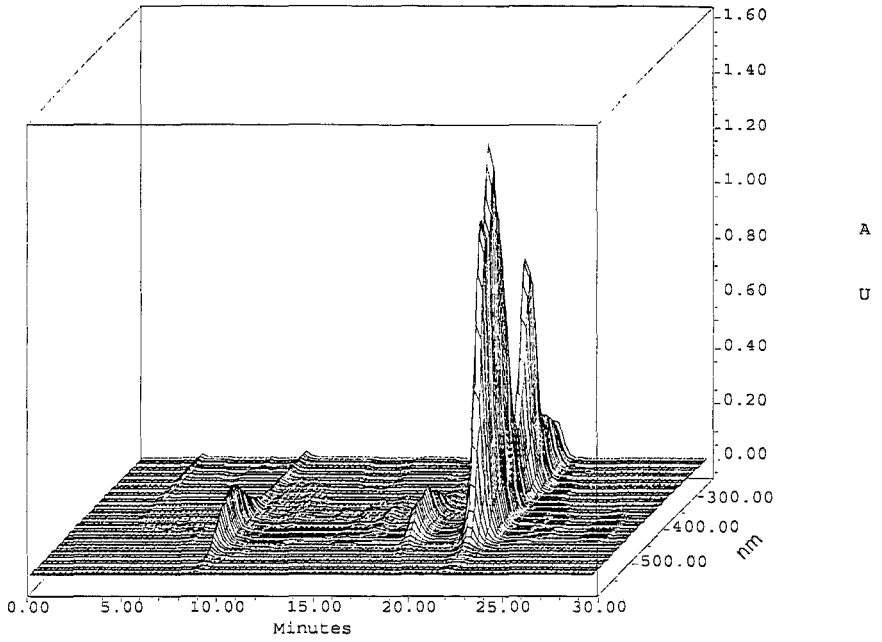

FIGURE 1. 3-D graphic profile of 15-cis β-carotene on C18 reversed phase column. Eluent was methanol: acetonitrile (9:1, v/v) at a flow rate of 1 ml/min and the absorbance was monitored from 250 to 600 nm at a resolution of 1.2 nm.

different components and their absorption intensity. Post-run analysis can be used to identify the different components on the time axis at any selected derivation wavelength channel. The β-carotene peak, at 21.8 min, presenting absorption maxima at 336.6, 450.9 and 470.3 nm, is the 15-cis β-carotene, while the other minor two peaks at 8.6 min, λmax = 298.4, 475.2 nm, and at 18.5 min, λmax = 274.6, 417.0, 450.8, 475.1 were identified as canthaxanthin and all-trans β-carotene, respectively. The content of these contaminant products in the 15-cis β-carotene standard was low, as calculated by the peak areas integration, and did not exceed 5% (w/w).

The wavelength maxima obtained by our HPLC 3-D photodiode array system to many standards was compared to the literature data (Table 1), providing more accurate elution time and absorption maxima for each standard assayed. The absorption maxima illustrated in Table 1 were repeatable for each standard or component, both in chromatography and in absorption spectra. Thus, identification of unknown matching peaks of biological extracts was a relatively simple task by using the acquired memorized database of various standards. This was better illustrated on runs when the retention time shifted aside from its original setting and could not be used solely for peak identification.

Dunaliella bardawil, a unicellular green alga, was already thoroughly studied for its content of carotenoids and other photosynthetic pigments [4, 7]. This species is known for the accumulation of large amounts of all-trans β-carotene and 9-cis β-carotene in a content and ratio related to the light absorbed during one cell cycle. The HPLC 3-D plot of the algal extract showed clear profiles of a few prominent carotenoids (Table 1), with major peaks at 16.5 min, λmax = 436.4, 446.0, 475.2 nm, α-carotene; at 18.5 min, λmax = 417.0, 450.8, 475.1 nm, all-trans β-carotene; and at 20.7 min, λmax = 268.4, 341.5, 436.4, 446.0, 472.7, 9-cis β-carotene. The identical isomeric precursors of β-carotene were also assayed in phytoene-rich *Dunaliella* and are listed in Table 1. The ease of analysis and accuracy in identification of the plant and algal pigments allows the establishment of an HPLC library of "fingerprints" of many pigments known or in question.

The photodiode array - Millennium analysis of human serum is illustrated in Fig. 2. The 3-D plot clearly resolved the different components in the serum extract exhibiting above all two major peaks, both at the hydrophilic side of the elution. Maxplot derivation revealed that these two peaks are retinol, λmax = 323.5 nm at 3.8 min, and α-tocopherol, λmax = 291.3 nm at 6.9 min, closest to γ-tocopherol, λmax = 294.9 at 6.2 min (Table 1). Using the computer software to run the λmax of each observed peak indicated that the major carotenoids in this human serum are the plant origin, hydroxy carotenoid, violaxanthin, λmax = 443.6 nm at 5.3 min, and the fruit origin β-cryptoxanthin, λmax = 450.8 nm at 10.5 min. All other peaks between 4.5 min to the end of the chromatogram,

TABLE 1.
Carotenoids, Retinoids and Tocopherols Assayed by HPLC Photodiode Array in Comparison to Literature Data

Carotenoid and origin	Ret. time (min)	Absorption maxima (nm) Waters 3-D system	Absorption max. (nm) Literature	Ref
β-apo-8-carotenol (synthetic)	9.3	269.9, 465	463	13
canthaxanthin (synthetic)	9.1	475.2	474-478	13
all-trans α-carotene (synthetic)	18.0	269.9, 331.9, 431.5, 441.2, 467.0	422, 442, 471	19
all-trans α-carotene (carrots)	16.5	269.9, 436.4, 446.0, 475.2	--	
all-trans β-carotene (synthetic)	18.5	275.4, 417.0, 450.8, 475.1	429, 449, 475	19
9-cis β-carotene (*Dunaliella*)	20.7	268.4, 341.5, 436.4, 446.0, 472.7	445	20
15-cis β-carotene (synthetic)	21.8	336.6, 450.9, 470.3	448	20
9,15-dicis β-carotene (synthetic)	19.7	284.2, 336.6, 446.0, 465.2	--	
all-trans γ-carotene (synthetic)	21.4	280.3, 441.0, 463.0, 490.2	437, 460, 490	13
all-trans ζ-carotene (*Dunaliella*)	18.3	236.2, 378.0, 399.6, 424.1	295, 377, 398, 422	19
9-cis ζ-carotene (*Dunaliella*)	19.5	236.1, 374.1, 395.2, 420.1	295, 374, 395, 419	19
β-cryptoxanthin (synthetic)	10.5	274.7, 426.8, 450.8, 480.1	425, 449-452, 473-8	13
echinenone (synthetic)	11.6	299.4, 465.5	457-461	13
lutein (alfalfa)	5.0	269.9, 331.9, 446.1, 475.2	422, 443, 470	19
lycopene (tomato)	25.3	293.7, 263.9, 446.1, 470.3, 504.4	444, 470, 502	13
all-trans phytoene (*Dunaliella*)	18.9	277.8, 286.1, 296.0	276, 286, 297	13
9-cis phytoene (*Dunaliella*)	18.0	277.8, 286.1, 296.0	276, 284, 294	19
phytofluene, all-trans & 9-cis (*Dun.*)	18.8, 19.2	331.4, 348.2, 368.2	329, 346, 365	19
Violaxanthin (*Dunaliella*)	4.8	267.5, 329.4, 443.6, 471.0	414, 441, 471	13
Zeaxanthin (*Dunaliella*)	5.3	276.1, 425.9, 450.3, 477.3	341, 427, 448, 475	19
Retinoids:				
Retinol (synthetic)	3.8	327.1	--	
Retinol (serum)	3.8	323.5	325	21
9-cis retinol (synthetic)	3.85	322.3	--	
all-trans retinoic acid (synthetic)	3.0	336.6	--	
13-cis retinoic acid (synthetic)	3.2	341.4	--	
Vitamin E:				
α-tocopherol (synthetic)	6.9	291.3	292	22
γ-tocopherol (synthetic)	6.2	294.8	294	22

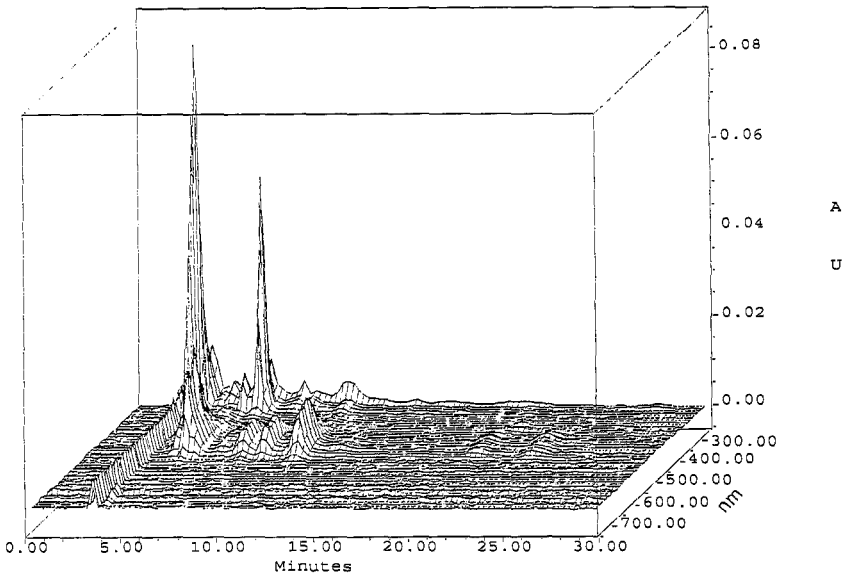

FIGURE 2. 3-D graphic profile of human extract. Separation conditions were as described under Figure 1.

aside from the two tocopherols, were identified as carotenoids, presenting typical absorption maxima around 450 nm. The last observed two peaks at 21.5 min and at 23.5 min were the hydrocarbons all-trans α-carotene and all-trans β-carotene, respectively. β-carotene usually overlapped a small peak of phytofluene clearly detected by the UV absorbing peak of $\lambda max = 286.1$ nm. Lycopene isomers, which elute on the Vydac column after the cis stereoisomers of β-carotene [8, 9] were not detected at the indicated chromatogram at any sensitivity. Attempts to search for lycopene in serum prior to α-carotene [6] were negative. The standard lycopene of tomato extract which was used in our study exhibited a clear peak at 25.3 min with $\lambda max = 470.3$ nm. Lycopene standard and lycopene in the serum extracts were always eluted late after α-carotene and β-

carotene. The HPLC profile of the presented human serum was similar to many other human sera extracts which were analyzed by our photodiode array and the variations were mainly in the composition and content of the hydroxy and keto carotenoids and less in the profile of the hydrocarbon carotenoids. The list of the different polar carotenoids is similar to that described in a few previous manuscripts [6, 9-20] and include α-cryptoxanthin stereoisomers of lutein and zeaxanthin. α-carotene in the human serum, like the α-carotene in the alga *Dunaliella*, resembles the authentic standard α-carotene from carrots, λmax = 446.0 nm, and was significantly different from the synthetic α-carotene, λmax = 441.2 nm. The variation between different batches of standards of α and β-carotene was noted earlier [12]. The photodiode array and the memorized data were very useful when interpreting such minute differences between stereoisomers of carotenoids and between the α and β configuration.

The introduction of this improved 3-D liquid chromatography detector and the chromatograph manager revealed many peaks of unknown or partially known carotenoids for identification and evaluation. Samples either from plants and human plasma of low content of carotenoids can be detected and analyzed at a fine separation level of cis stereoisomers and of other lipophilic extracted products even when overlapping occurs. The simultaneous analysis of retinol, tocopherols and carotenoids [11, 17] is simple, rapid and accurate. It was of interest to note that the early eluted peak at 3.1 min with absorption maxima varying from 240 nm to 350 nm was always related to the oxidation of the carotenoid studied. We assume that the oxidized carotenoids are relatively polar with early elution properties and shorter absorption maxima. The developed new diode array detection will allow future analysis of different oxidized carotenoids on the polar side of the reversed phase elution column along with better analysis of all other detected peaks of non-oxidized components. Altogether, the developed fine art of HPLC separation by using the modern technique of diodes and computer hardware and software allows the analyzers a wide range of methodologies to identify samples with certainty and reliability.

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DETERMINATION OF AMINO ACIDS BY MICELLAR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PRE-COLUMN DERIVATIZATION WITH *O*-PHTHALALDEHYDE AND N-ACETYL-L-CYSTEINE

M. CATALÁ-ICARDO, M. J. MEDINA-HERNÁNDEZ, AND M. C. GARCÍA ALVAREZ-COQUE*
Departamento de Química Analítica
Universidad de Valencia
C/Dr. Moliner s/n
E-46100 Burjassot, Valencia, Spain

ABSTRACT

Micellar liquid chromatography of proteic primary amino acids with pre-column derivatization with *o*-phthalaldehyde (OPA) and N-acetyl-L-cysteine was studied, using mobile phases containing a short-chain alcohol. The modification of pH gave a large variation of the retention as a result of the protonation of the carboxylate group of amino acids. Maximum resolution and adequate retentions were achieved with a 0.05 M sodium dodecyl sulphate/3% propanol mobile phase at pH 3. The reproducibility was lower than 1.0% at a $1x10^{-4}$ M concentration level and between 0.6 and 2.2% for $1x10^{-6}$ M. The determination of glycine, lysine, methionine and threonine in pharmaceutical formulations gave recoveries, with respect to the values declared by the manufacturers, in the 90-105% range.

* To whom correspondence should be addressed.

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INTRODUCTION

High-performance liquid chromatography of aminoacids with spectrophotometric detection usually requires the formation of derivatives, because of little absorption of ultraviolet light above 210 nm. Precolumn derivatization is usually preferable, because of sensitivity increases. *o*-Pthalaldehyde (OPA) is the derivatization reagent that probably has the best characteristics (1,2). It reacts with primary amino groups, in the presence of a thiol at pH 9.5 and room temperature, to form 1-alkylthio-2-alkyl substituted isoindoles. The derivatives show maximum absorption at 335 nm and are highly fluorescent, with excitation wavelength at 340 nm and emission at 445 nm. Mercaptoethanol has been more extensively used than other thiols for the derivatization of amino acids, however the OPA-mercaptoethanol isoindoles are unstable. It has been demonstrated that N-acetyl-L-cysteine (NAC) improves the stability of isoindoles when used instead of mercaptoethanol (3).

An extensive investigation is being performed in our laboratory on the applicability of Micellar Liquid Chromatography in routine analysis. Procedures for the evaluation of diuretics (4,5), anabolic steroids (6), sulphonamides (7), and beta-blockers (8) in pharmaceuticals, and diuretics (9) and catecholamines (10) in urine, have been developed. The reasons which may impel the analyst to use a micellar solution, instead of a conventional hydroorganic mobile phase, in reversed-phase liquid chromatography, are the lower cost, toxicity and biodegradability of the solvent, the increased sensitivity in the spectrophotometric, fluorimetric and phosphorimetric detection achieved for some compounds (11), the possibility of describing the chromatographic behaviour with simple equations (12), the change in selectivity (13), the performance of elution gradients of surfactant without the need of reequilibration of the column

(14), and finally, the easy solubilization of analytical samples, that avoids the long previous separation steps which are often necessary.

Proteic primary amino acids are found in diverse pharmaceutical formulations, such as vitaminic preparations or appetite stimulants. Cystine, cysteine and methionine are used in formulations indicated in the treatment of alopecia. Finally, some preparations contain an amino acid in the excipient. Thus, glycine is used to increase the rate of dissolution of acetylsalicylic acid, improving its action and stomachic tolerance (15).

In this work, the first investigations on the chromatographic behaviour of the OPA-amino acid derivatives with micellar eluents, are reported. An analytical procedure for the determination of amino acids in pharmaceutical formulations is developed.

MATERIALS AND METHODS

The micellar mobile phases were prepared by mixing aqueous sodium dodecyl sulphate (99%, Merck, Darmstadt, Germany) solutions with an alcohol to obtain the working concentration. The alcohols studied were methanol (HPLC, Panreac, Barcelona, Spain), 1-propanol (analytical reagent, Panreac) and 1-pentanol (analytical reagent, Merck). The pH of the micellar eluent was adjusted with 0.01 M phosphate buffer, prepared with disodium hydrogen phosphate and phosphoric acid (analytical reagent, Panreac).

A 2×10^{-4} M stock solution of the following amino acids were prepared: DL-alanine, L-arginine, L-asparagine, L-glutamic acid, L-glutamine, L-histidine, L-leucine, L-serine, DL-tyrosine, DL-valine (very pure, Scharlau, Barcelona), DL-isoleucine, L-lysine hydrochloride, L-threonine, and DL-tryptophan (very pure, Fluka, Buchs, Switzerland), L-

cysteine, L-methionine and L-phenylalanine (biochemical use, Merck), L-aspartic acid (analytical reagent, Merck), and glycine (pure, Carlo Erba, Rodano, Milano, Italy). Some drops of 6 M HCl was added to dissolve those amino acids which are sparingly soluble at pH 7. The solutions were stored at 4 °C.

The isoindole derivatives were obtained with solutions of $2x10^{-3}$ M *o*-phthalaldehyde (biochemical use, Fluka) and $2x10^{-3}$ M N-acetyl-L-cysteine (very pure, Fluka). The 0.1 M boric-borate buffer of pH 9.5 was prepared with boric acid and NaOH (analytical reagent, Probus). The OPA-NAC reagent was stored at 4° C.

Barnstead nanopure, deionized water (Sybron, Boston, MA, USA) was used throughout. The mobile phase and the solutions injected into the chromatograph were vacuum-filtered through 0.45 μm and 0.22 μm Nylon membranes, respectively (Micron Separations, Westboro, MA, USA).

A Hewlett-Packard (Palo Alto, CA, USA) 8452A diode-array spectrophotometer and a Perkin-Elmer LS50 fluorimeter (Beaconsfield, Buckinghamshire, England) were used.

The chromatographic system included a modular HPLC unit consisting of isocratic pump, automatic injector, UV-visible detector (Hewlett-Packard, Palo Alto, CA, USA, Model 1050), and integrator (Hewlett-Packard, Model 3396A). Data acquisition was performed through the PEAK-96 software (Hewlett Packard, Avondale, PA, USA). The solutions were injected into the chromatograph through a Rheodyne valve (Cotati, CA, USA) with a 20 μl loop. The detection was performed at 336 nm. A Spherisorb ODS-2 analytical C_{18} column (5 μm particle size, 120 x 4.6 mm I.D.) was used, together with a C_{18} precolumn (35 x 4.6 mm I.D.) of similar characteristics (Scharlau, Barcelona, Spain) to saturate the mobile phase with silica. The dead volume was determined by injection of water. The mobile phase flow-rate was 1 ml/min.

The derivatization was performed by mixing an aliquot of the amino acid solution with the OPA-NAC reagent in a 10 ml vial, being the OPA-NAC:amino acid molar ratio ≥ 10. Complete formation of the isoindoles and good reproducibility were achieved by injecting the solutions into the chromatograph one minute after mixing the reagents. The concentration of OPA should not be larger than 10^{-3} M. A more concentrated reagent produced a variation in the retention times for the derivatives of alanine, arginine, cysteine, lysine, methionine and valine. The larger instability of the amino acid isoindoles at an increasing OPA concentration is known.

Pills, capsules and powder were dissolved in 0.05 M SDS, by immersion in an ultrasonic bath. An adequate volume of the drops was taken and diluted with 0.05 M SDS. Other dilutions were made with 0.05 M SDS.

RESULTS

Optimization of experimental conditions

The OPA-NAC derivatives of amino acids showed maximum absorption at 336 nm in a micellar medium at pH 9.5, the molar absorptivities being similar to those obtained in a solution in the absence of surfactant ($\epsilon \approx 6800$) (3). Maximum absorption shifted to ≈ 330 nm in acidic medium. At low pH the emission intensity of the derivatives also shifted to lower wavelengths, from 440 nm to 390 nm, and the emission intensity decreased appreciably. A basic medium is required to form the isoindoles, thus these spectral changes may suggest the formation of other compounds in acidic medium.

The pH of the mobile phase is an important parameter to be controlled when ionizable solutes are eluted in a reversed-phase system with a micellar mobile phase of an ionic surfactant. Therefore, the influence of the pH of the eluent on retention was first studied. It was observed that at pH > 4 the amino acid derivatives were eluted at the beginning of the chromatogram. The variation of the capacity factors with pH is plotted in Figure 1 for some amino acid derivatives. Sigmoidal curves were obtained for all amino acids in the 3-4.5 range. The signal increase corresponds to the protonation of the free carboxylate group of the derivatives. At a higher pH the retention is reduced, due to the increase in the concentration of the anionic form, that is repelled by the negatively charged heads of the surfactant monomers adsorbed on the stationary phase.

The presence of micelles originates a modification in the acid-base properties of solutes, giving rise to shifts in the protonation constants (6,16,17). The extent of the modification depends on the properties of solutes and micelles. The mathematical treatment of the capacity factors (k') vs pH data, at a constant concentration of SDS, allows the determination of the protonation constants (log K) in the micellar medium. Equation [1] was used:

$$\log K = pH + \log \frac{k'_0 - k'}{k' - k'_1} \qquad [1]$$

where k'_1 and k'_0 are the capacity factors of the protonated and unprotonated species, obtained at pH 3 and >4, respectively. Table 1 indicates the protonation constants for a 0.05 M SDS solution. The values of log K were found in the 2.8-4.0 range, whereas the log K for the carboxylate group of free amino acids in a non-micellar medium is in the 1.7-2.7 range, that is, one logarithmic unit lower. In a previous study on the formation and spectrophotometric behaviour of a phthalimidine of

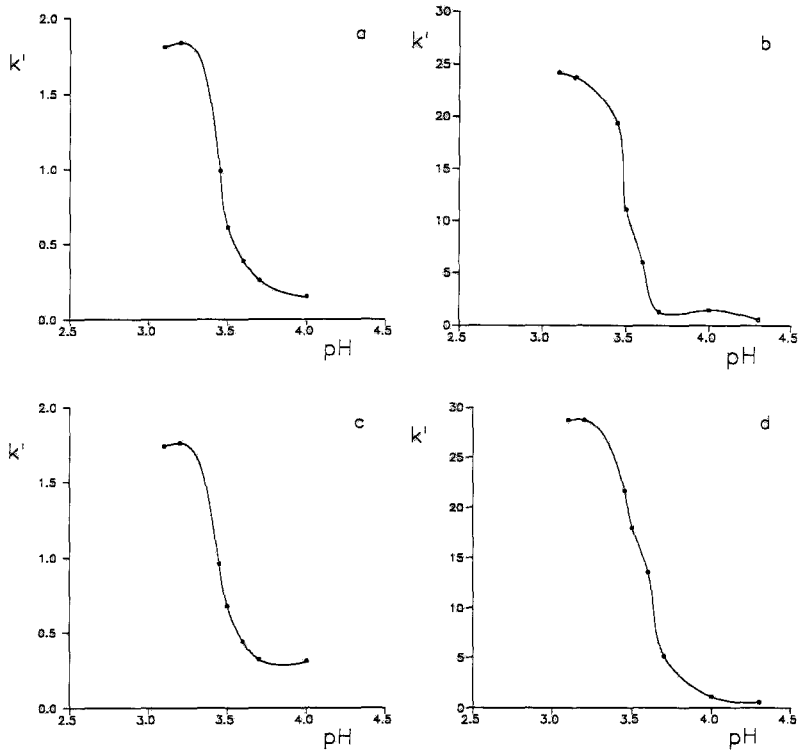

Figure 1. Dependence of the capacity factors on pH with a 0.05 M SDS mobile phase for the OPA-NAC derivatives of: a) aspartic acid; b) phenylalanine; c) glutamine; d) isoleucine.

cystine, obtained by reaction of this amino acid with OPA in a non-micellar basic medium, log K = 3.7 was found for the protonation of the carboxylate group (18).

It was observed that maximum resolution and adequate retention times of the chromatographic peaks of the amino acids was achieved at pH 3. Therefore, the composition of the mobile phase was optimized at this pH. The influence of the concentration of SDS on the retention of the

Table 1. Protonation constants (log K) of the carboxylate group of amino acids

Amino acid	log K	
	Free amino acid[a]	OPA-NAC derivatives[b]
Aspartic acid	1.88	3.1 ± 0.3
Alanine	2.34	3.3 ± 0.2
Arginine	2.17	4.02 ± 0.15
Asparagine	2.02	3.2 ± 0.8
Cysteine	1.71	3.39 ± 0.15
Phenylalanine	1.83	3.5 ± 0.5
Glycine	2.34	3.1 ± 0.2
Glutamine	2.17	3.2 ± 0.2
Histidine	1.82	2.9 ± 0.3
Isoleucine	2.36	3.7 ± 0.2
Leucine	2.36	3.7 ± 0.4
Lysine	2.18	3.29 ± 0.16
Methionine	2.28	2.8 ± 0.5
Serine	2.21	3.1 ± 0.7
Threonine	2.71	3.2 ± 0.4
Tryptophan	2.38	3.2 ± 0.4
Valine	2.32	3.4 ± 0.2

[a] Protonation constants for free amino acids in non-micellar aqueous solution (1)

[b] Protonation constants for OPA-NAC-amino acid derivatives in a 0.05 M SDS solution, obtained in this work.

OPA-NAC derivatives was studied in the 0.05-0.15 M range. The k' values were between 1.9-2.0 (serine and asparagine), and 30.4 (isoleucine) for 0.05 M SDS, and between 1.7 (asparagine) and 13.5 (lysine) for 0.15 M SDS. The amino acid derivatives behaved as binding solutes in a purely micellar mobile phase (without alcohol), decreasing their retention at increasing concentrations of SDS, except the aspartic acid and glutamine derivatives, which presented a non-binding behaviour. The retention of these solutes was almost constant in the 0.05-0.15 M SDS concentration range.

A short-chain alcohol (methanol, propanol and pentanol) was added to the micellar eluent to improve the efficiency of the chromatographic peaks and to achieve adequate retentions. Propanol was selected as the most appropriate. The k' values in a 0.05 M SDS/3% (v/v percentage) propanol mobile phase were between 0.7 (serine and asparagine), and 16.0 (isoleucine).

Analysis of pharmaceutical formulations

A 0.05 M SDS/3% propanol eluent at pH 3 was used to analyze five pharmaceutical formulations found in the spanish market, which contain glycine, lysine, methionine and threonine, together with a number of other components (Table 2 and Figure 2). The formulations were Polivitaendil minerales (vitaminic preparation), Pantobamín and Tres-orix Forte (appetite stimulant), Vincogelatín soluble (dermatological preparation), and Okal, where glycine is present to improve the assimilation and tolerance of acetylsalicylic acid.

The calibration plots, obtained by performing the derivatization of the amino acids by triplicate at five concentrations of each amino acid, were linear in the $4x10^{-5}$ M to $5x10^{-4}$ M range, with correlation coefficients $r > 0.999$. The reproducibility was evaluated from series of

Table 2. Determination of amino acids in pharmaceutical formulations. Contents declared by the manufacturers, found values and reproducibility

Formulation	Composition	Found (mg)	CV (%)
Okal (pills, Puerto Galiano, Madrid)	Glycine (100 mg), acetylsalicylic acid (500 mg), caffeine (30 mg), excipient	Glycine (102.9)	0.8
Pantobamín (drops, Medix, Madrid)	per ml: L-Lysine[a] (62.5 mg), L-threonine (25 mg), cyproheptadine clorhydrate (0.5 mg), L-carnosine (75 mg), thiamine nitrate (5 mg), pyridoxine clorhydrate (2.5 mg), hydroxocobalamine acetate (2.5 μg), dexpanthenol (1.25 mg), riboflavine sodium phosphate (1.25 mg), excipient	L-Lysine (56) L-Threonine (26.3)	0.9 0.8

Polivitaendil minerales (pills, Wasserman, Barcelona)	Glycine (50 mg), retinol (5000 U.I.), ergocalciferol (1000 U.I.), thiamine (10 mg), riboflavine (5 mg), pyridoxine (2.5 mg), cyanocobalamine (5 μg), ascorbic acid (125 mg), vitamin H (50 μg), nicotinamide (10 mg), calcium pantothenate (8 mg), phosphorus (17 mg), calcium (23 mg), iron (10 mg), magnesium (5 mg), manganese (2 mg), copper (1 mg), zinc (2 mg), molybdenum (0.5 mg), fluorine (0.05 mg), iodine (0.1 mg), adenosine triphosphoric acid (3 mg), saccharose (365 mg), excipient	Glycine (47.5)	0.2
Tres-orix Forte (capsules, Prodes, Barcelona)	Lysine[b] (150 mg), cyproheptadine orotate (1.5 mg), carnitine (150 mg), cobamamide (1000 μg), excipient	Lysine (147.5)	0.4
Vincogelatin soluble (powder, Reig Jofré, Barcelona)	Methionine (500 mg), gelatine (6 g), L-cystine (10 mg), calcium pantothenate (100 mg), vitamin B_6 (40 mg), zinc sulphate (60 mg)	Methionine (515)	0.5

[a] The height of the peak at 8 min was measured.

[b] The sum of the heights of the peaks at 3 and 8 min was measured.

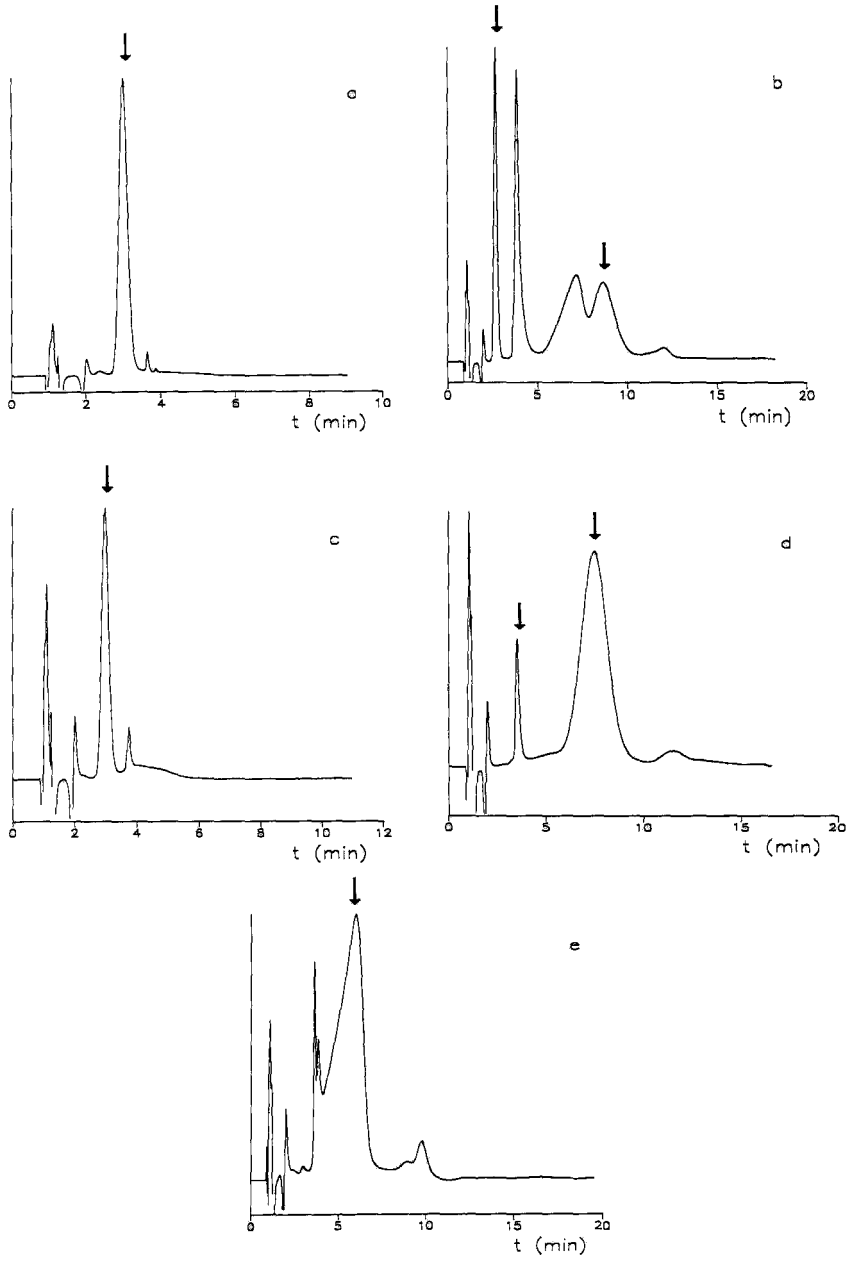

Figure 2. Chromatograms of the formulations: a) Okal (glycine); b) Pantobamín (threonine, 2.5 min, and lysine, 8 min); c) Polivitaendil minerales (glycine); d) Tres-orix Forte (lysine, 3 min and 8 min); e) Vincogelatín soluble (methionine).

five aliquots of the amino acids, which were independently derivatized. The coefficients of variation were lower than 1.0% at a $1x10^{-4}$ M concentration level, and between 0.6 and 2.2% for $1x10^{-6}$ M. The limits of detection were between $1.3x10^{-7}$ M and $6.5x10^{-7}$ M (3s criterium, n = 6).

The amino acid contents were obtained by taking five aliquots of the dissolved formulations, which were derivatized and one minute later injected into the chromatograph. The results were reproducible and the recoveries with respect to the values declared by the manufacturers were in the 90-105% range.

Lysine gave two peaks in the chromatograms at 3 min and 8 min, which indicated the formation of two different derivatives. The determination of lysine in the formulations where this amino acid was found, required the construction of two different calibration curves. In the analysis of Pantobamin, the height of the peak at a higher retention, close to 8 min was measured, due to the presence of other compounds that eluated at retention times close to the peak at 3 min. For Tres-orix Forte, the sum of the heights of the two peaks of lysine gave better results.

ACKNOWLEDGEMENTS

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DETERMINATION OF SAPONINS IN LUPIN SEED (LUPINUS ANGUSTIFOLIUS) USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: COMPARISON WITH A GAS CHROMATOGRAPHIC METHOD

R. G. RUIZ[1], K. R. PRICE[1], M. E. ROSE[2], M. J. C. RHODES[1], AND G. R. FENWICK[1]
[1]*Food Molecular Biochemistry Department*
Institute of Food Research
Norwich Research Park
Colney, Norwich NR4 7UA, United Kingdom
[2]*Department of Chemistry*
The Open University
Milton Keynes MK7 6AA, United Kingdom

ABSTRACT

A quantitative method for the determination of intact saponins in *Lupinus angustifolius* seed by high-performance liquid chromatography (HPLC) is described. The seed was extracted under mild conditions with 70% aqueous ethanol containing 0.01% EDTA and quantified using an internal standard of α-hederin. Soyasaponin VI, also known as soyasaponin βg, a DDMP (2,3 -dihydro - 2,5 - dihydroxy - 6 - methyl - 4*H* - pyran - 4 - one)- conjugated form of soyasaponin I, was the only saponin detected in the lupin seed. The technique is compared with a gas chromatographic method which quantifies the sapogenol resulting from the acid hydrolysis of the saponins.

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INTRODUCTION

Saponins are glycosides with terpenoid or steroid aglycones occurring primarily in plants, which are consumed by animals and man but usually processed industrially or domestically prior to consumption. A wide range of beneficial and deleterious properties has been ascribed to saponins, for example, hemolytic activity, cholesterol-binding ability, bitterness, and fungitoxic activity. However, these properties are better seen as characterizing particular types of saponin rather than being shared by all members of the chemically-complex group of saponins (1).

The quantitative determination of saponins has for a long time posed problems to the analytical chemist (2). A number of chromatographic methods have been used for saponin analysis and especial attention has focused on the use of gas chromatography (3) but it has the limitation that it can only be used for the separation and quantification of the aglycone portion of the saponin (after hydrolysis and suitable derivatization) which involves both the loss of structural information about the glycosidic portion of the molecule and potential loss of material during hydrolysis and derivatization.

Since relatively little is known about the effect of both industrial and domestic processing on the fate of the saponins in food and with recent work demonstrating a relationship between chemical structure and biological activity (4, 5), there is a requirement for analytical methods that can measure the individual saponins as they exist in the food matrix. However, the development of techniques for the analysis of these intact saponins, such as HPLC, has been limited due firstly to the difficulties with the detection of triterpene saponins which do not contain a UV-chromophore and secondly to the lack of appropriate standards. Derivatisation procedures have increased the sensitivity of HPLC methods but suffer from selectivity and stability problems (6). Kudou et al. (7), Yoshiki et al. (8) and Okubo et al. (9) have successfully separated, on

a qualitative basis using HPLC, intact saponins from soybean (*Glycine max*), runner bean (*Phaseolus coccineus*) and American groundnut (*Apios americana*) respectively and the study reported here has further developed this work for the separation and quantification of the intact saponins present in lupin seed (*Lupinus angustifolius*) and compared the results obtained using the conventional GC method for the analysis of saponins.

MATERIALS

Lupin seed used was a commercial Australian variety of *L. angustifolius* called Warrah.

The internal standard used, α-hederin, was purchased from Apin Chemicals Ltd (Abingdon, Oxon, United Kingdom). Saponin βg (soyasaponin VI) was generously donated by Dr. K. Okubo and coworkers of the Faculty of Agriculture, Tohoku University. Soyasaponin I was isolated from soybean.

All solvents used during the extraction process were of analytical-reagent grade and water was purified using a Milli-Q system (Waters Chromatography Division, Millipore Corporation, Milford, USA).

METHODS

High-Performance Liquid Chromatographic Analysis

Extraction procedure

The lupin seed were milled with a coffee grinder (Janke & Kunkel, Ika-Werk). Four grams of the sample and 3 mg of powdered α-hederin were firstly soaked in 70%

aqueous ethanol containing 0.01% EDTA for 10 min and secondly stirred for 1 h at room temperature. The sample was then centrifuged at 8000 rpm for 15 min at 20°C. This extraction process was repeated three more times and the supernatants combined and filtered through a sinter funnel under reduced pressure. The extract was rotary-evaporated to dryness at less than 40°C and dissolved in a mixture of 2 ml of water and 1-butanol (1:1, v/v). After centrifugation, the 1-butanol layer was collected and analyzed by HPLC.

Apparatus

HPLC analyses were performed using a Philips PU 4100 liquid chromatograph coupled to a Philips PU UV 4025 detector and a Gilson 715 data collection system. Separations were performed on a column (25 cm x 4.6 mm ID) packed with Ultratechsphere 5μ C_{18} (HPLC Technology Ltd, Macclesfield, UK).

Chromatographic Conditions

Chromatographic runs were carried out with an acetonitrile-water gradient elution system. Solvents were acetonitrile-acetic acid (1000:0.3 v/v) (Solvent A) and water:acetic acid:EDTA (1000:0.3:0.15 v/v/w) (Solvent B). Solvents were degassed with helium. The gradient was run according to the following programme: 65% A isocratically for 18 min followed by a reduction to 58% A after a further 4 min, held at 58% A for 10 min then changed to 65% A over a further 4 min and finally held at 65% A for 8 min. The flow rate was 0.9 ml/min and detection was monitored by UV absorption at 205 nm.

Gas chromatography and Fast Atom Bombardment - Mass Spectrometry

These techniques are described by Price et al. (3).

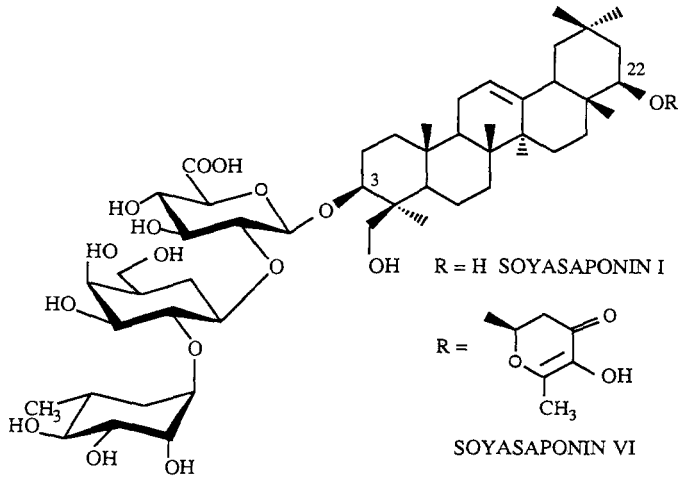

FIGURE 1. Chemical Structures of Soyasaponin I and Soyasaponin VI.

RESULTS AND DISCUSSION

HPLC analyses of soyasaponin I (SSI) and soyasaponin VI (SSVI), also known as soyasaponin βg (see figure 1), which are the saponins known to be present in *L. angustifolius* seed (10), together with the internal standard α-hederin revealed well resolved peaks whose retention times were 16.0, 28.3 and 32.3 min respectively (see figure 2).

Due to the relative instability of SSVI and in order to check its purity, data from the GC analysis of soyasapogenol B, which resulted from the hydrolysis of 1 mg of each of SSI and SSVI, showed SSVI to be 30% pure.

Peak-area ratio calibrations with the internal standard (HPLC) were then constructed for both SSI and SSVI with the purity of SSVI calculated from GC

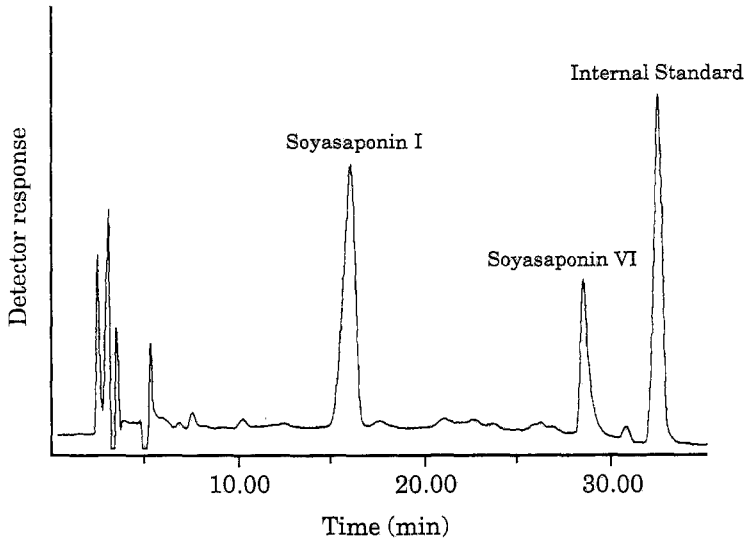

FIGURE 2. Chromatogram of a Mixture of Soyasaponin I, Soyasaponin VI, and α-Hederin.

measurements being included in these calculations. The equation constant, correlation coefficient, weight range and limit of detection for each saponin are illustrated in Table 1. The calibration plots showed good linearity for both SSI and SSVI (figure 3).

Fast atom bombardment-mass spectrometry (FAB-MS) of the seed extract containing the intact saponins after exhaustive hot solvent extraction as described in Price et al. (3), showed the presence of SSI, with a molecular weight of 942, and SSVI, with a molecular weight of 1068 (figure 1). The two saponins were calculated to be in roughly equimolar proportions. The total saponin content of the lupin seed was estimated to be 385 mg/kg by using a combination of the quantification of the acid released sapogenol by GC analysis and the ratio of sapogenol/saponin from the FAB mass spectral measurements.

TABLE 1

Chromatographic Constants for HPLC Analysis

Saponin	equation y = a + bx		r	Range (µg)	Internal Standard (µg)	Limit of Detection (µg)
SS1	-4.9228e-3	3.1573e-2	0.998	0 - 50	30	3.2
SSVI	-1.4656e-2	3.6615e-2	1.000	0 - 48.3	30	1.5

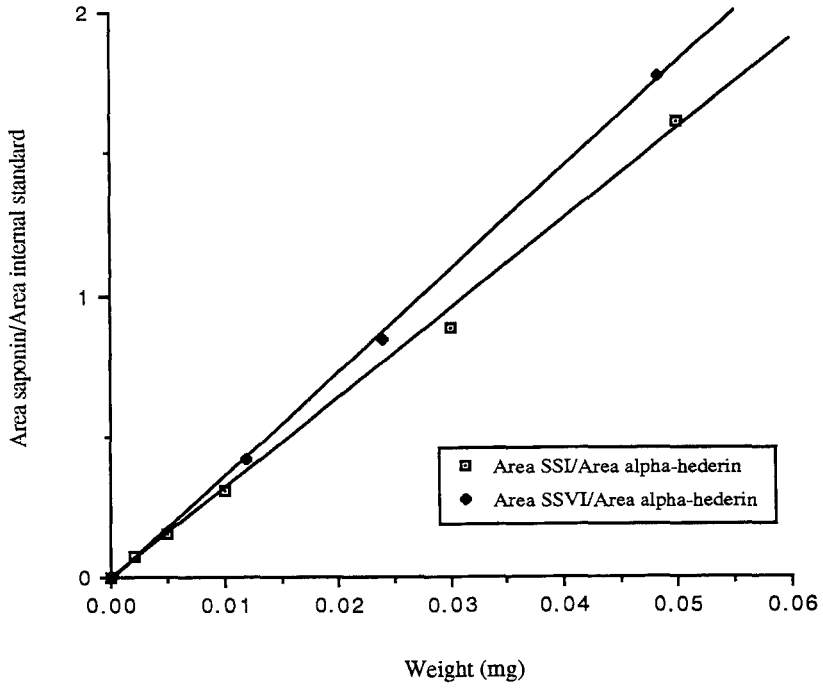

FIGURE 3. Calibration curves for Soyasaponin I and Soyasaponin VI.

In contrast the chromatogram resulting from the analysis of the lupin seed extract by HPLC following the mild extraction conditions described here is shown in figure 4. Retention time comparisons relative to the internal standard a-hederin and confirmation by co-chromatography of the reference saponins showed the presence of only SSVI. SSI was not detected. This finding is in agreement with Massiot et al. (11) who isolated SSVI from alfalfa and characterized it as the natural precursor of SSI and Kudou et al. (7) who postulated that these DDMP-conjugated saponins were, in fact, the genuine saponins in the case of native soybean seeds whilst soyasaponins I-V, the saponins normally associated with soya following conventional exhaustive hot solvent

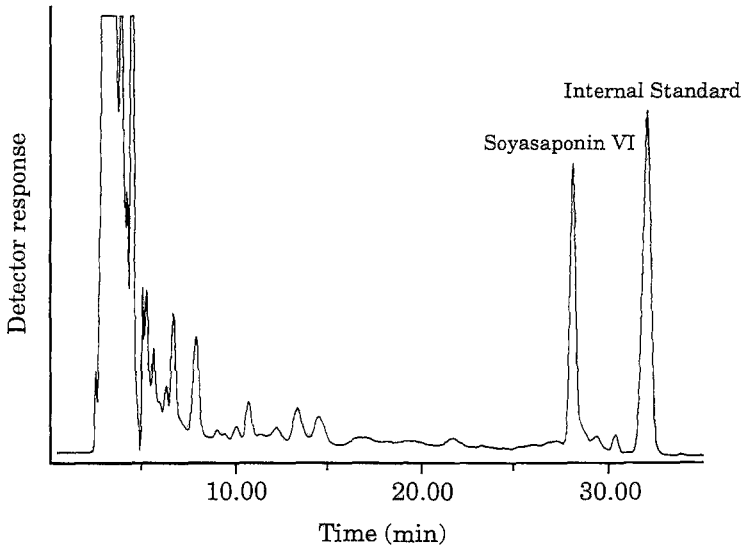

FIGURE 4. Chromatogram of *Lupinus Angustifolius* Seed Saponins (Soyasaponin VI) with Internal Standard (α-Hederin).

extraction, were in fact artifacts derived form degradation of the DDMP saponins during the extraction or concentration processes.

The saponin content of the lupin seed was estimated to be 450 mg/kg using the HPLC technique. This value was 14% higher than the one obtained from the GC technique, which suggests that saponin losses occurred during some of the stages involved in the latter method. Since the internal standard used for the GC analysis is not glycosidic in nature it cannot be added during the extraction, cleanup or hydrolysis stages and it is likely therefore that these would be the stages where saponin loss could occur. The mild extraction technique used here was confirmed to be quantitative by subsequent exhaustive hot solvent extraction and HPLC analysis of the extract in which neither SSI or SSVI were detected.

The HPLC method described here provides the quantification of intact, underivatised individual saponins in legumes with the use of a suitable internal standard and a proven mild quantitative extraction technique. It will therefore enable quantitative studies to be made for the first time on the effect of different kinds of processing not only on the total saponin content but also on the true saponin composition. These data will, in turn, allow the fate of the individual saponins, some of which have been shown to be relatively unstable (12) to be determined. Since some of the biological properties reported for saponins are now recognised to be dependent on their particular chemical structures (13), this type of information will allow further understanding of not only the relationship between chemical structure and bioactivity but also the impact of processing on that bioactivity in relation to both human and animal health.

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ANALYSIS OF GINKOLIDES AND BIOBALIDES BY CAPILLARY ELECTROPHORESIS

STUART A. OEHRLE
Waters Corporation
34 Maple Street
Milford, Massachusetts 01757

ABSTRACT

A method has been developed to analyze for biobalide, ginkolide A and ginkolide B by capillary electrophoresis (CE). Analysis was accomplished by using a phosphate and sodium dodecyl sulfate (SDS) buffer with direct UV detection at 185nm. Run times of less than eighteen minutes for the compounds of interest was possible.

INTRODUCTION

Interest in analyzing for ginkolides is due to the fact that they possess unique pharmacological properties.[1] Ginkolides occur naturally in leaves of the tree *Ginko biloba* and up until now have been analyzed for exclusively by HPLC with either UV or refractive index (RI) detection.[1-3] Capillary electrophoresis (CE) was investigated as a possible alternative or confirmatory method for these compounds. Figure 1 shows the structures of the compounds under investigation. The purpose of this paper is to report on a preliminary method that has been developed to analyze for these compounds.

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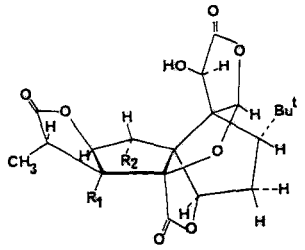
GINKOLIDES



	R_1	R_2
Ginkolide A	OH	H
Ginkolide B	OH	OH

BIOBALIDE

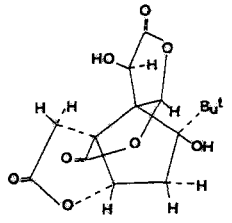


Figure 1. Structure of terpenes from *Ginko biloba* that were investigated.

EXPERIMENTAL

Chemicals

Standards of biobalide, ginkolide A, and ginkolide B were obtained from Sigma (St. Louis, MO) and were used as is. A mix of various terpenes was provided by a producer of terpene mixes and used as received. Phosphate and sodium dodecyl sulfate (SDS) was obtained from Waters Corporation (Milford, MA). High purity water was obtained from a Milli-Q water system (Millipore, Bedford, MA). Methanol used in dissolving the samples and standards was of HPLC grade.

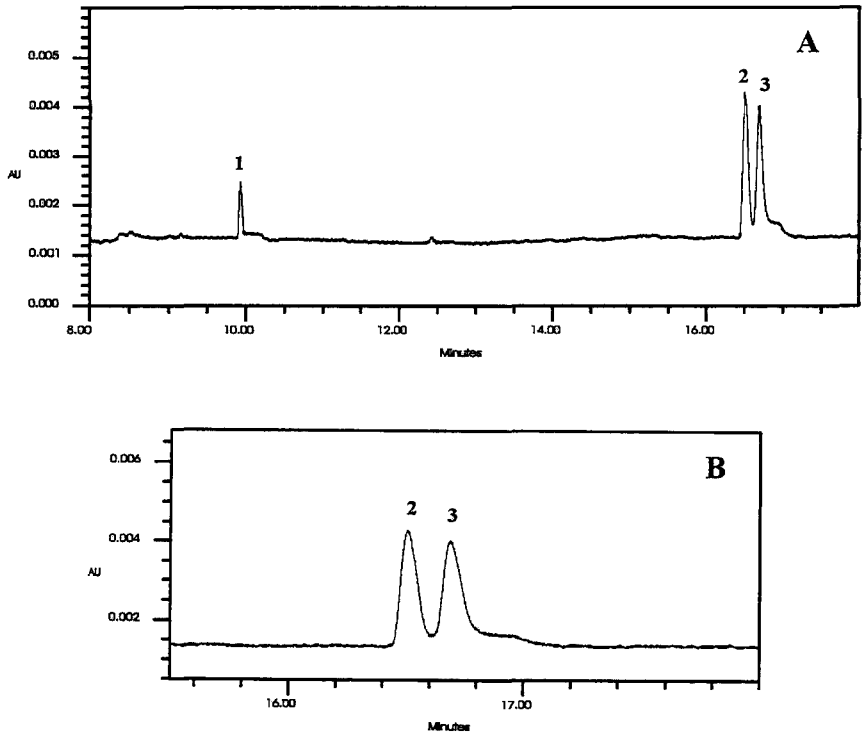


Figure 2. Electropherogram of terpene standards (A) with a close-up of ginkolide A and B separation shown in figure 2B. Peaks: **1**: biobalide; **2**: ginkolide A; **3**: ginkolide B. Amounts of each are 0.12 mg/mL. Conditions as stated in text.

Buffers and Solutions

A standards solution (1.0 mg/mL) of all ginkolides and biobalide was prepared by dissolving them in methanol. Prior to CE analysis the standard solution was diluted in the running buffer resulting in a mix with a concentration of approximately 0.12 mg/mL of each compound. The terpene mix was prepared in a similar manner with a final concentration of the mix being 0.11 mg/mL. The buffer used for the final CE analysis was a mixture of phosphate and SDS at a concentration of 25 mM and 90 mM respectively.

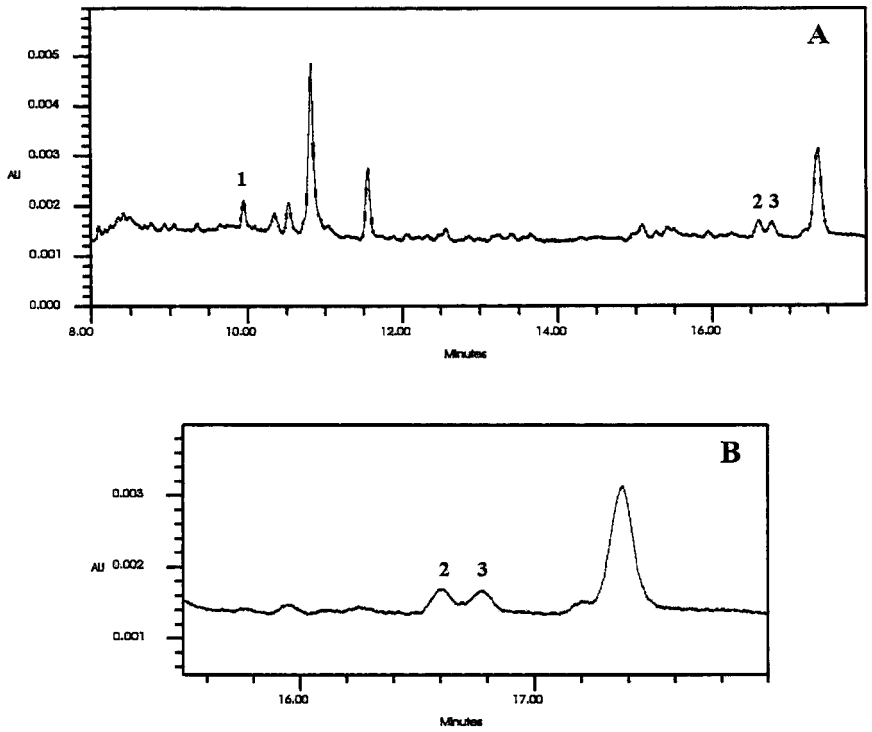


Figure 3. Electropherogram of terpene mix at a concentration of 0.11 mg/mL (A) with a close-up of ginkolide A and B separation shown in figure 3B. Peaks: **1**: biobalide; **2**: ginkolide A; **3**: ginkolide B. Conditions as stated in text.

Instrumentation

The capillary electrophoresis (CE) system used was a Quanta 4000e (Waters Corporation, Milford, MA) with a positive power supply. A Hg lamp was used for direct UV detection at 185 nm. Accusep polyimide fused-silica capillaries of dimension 60 cm X 75 um I.D. were used throughout and obtained from Waters.

Data acquisition and control of the CE was carried out with a Waters Millennium 2010 chromatography manager. The detector time constant was set at 0.3 seconds and the data collection rate was 5 points/sec. The temperature inside the CE was maintained at 30 C.

RESULTS

Due to the compounds low absorbance, 185 nm was chosen as the wavelength to monitor. Initial work using a borate, boric acid, and SDS buffer proved unsuccessful for separating these compounds. A phosphate, and SDS buffer was investigated with a reasonable separation accomplished at 25 mM phosphate and 90 mM SDS. Figure 2 is an electropherogram of the standard mix. Due to the high concentration of components a sampling time of only 5 seconds was used. Figure 2A is a close-up of the ginkolide A and B separation showing good resolution between them. Using this method a terpene sample was analyzed. Figure 3 is an electropherogram of the separation with the compounds of interest identified. In this case the sampling time was raised to 15 seconds.

CONCLUSIONS

As shown in the previous examples, analysis for biobalide, ginkolide A and ginkolide B can be done by CE. Further, CE offers an alternative method for analyzing these compounds with relatively fast run times and rood resolution.

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IDENTIFICATION OF WESTERN MEDICINES AS ADULTERANTS IN CHINESE HERBAL MEDICINES USING A BROAD-SPECTRUM DRUG SCREENING HPLC SYSTEM

S. JASON LAI[1], STEVEN R. BINDER[1], HERBERT ESSIEN[1], AND KUO-CHING WEN[2]

[1]*Clinical Systems Division*
Bio-Rad Laboratories
4000 Alfred Nobel Drive
Hercules, California 94547
[2]*Division of Drug Pharmacognosy*
National Laboratories of Foods and Drugs
Department of Health, Executive Yuan
161-2 Kuen Yang Street
Nankang, Taipei, Taiwan
Republic of China

ABSTRACT

Analysis of herbal medicines is a difficult task because of the complexity and variety of the available formulations. Identification of adulterants in herbal medicines poses an even greater challenge to the laboratories which are required to conduct a routine surveillance program. There is no single broad spectrum screening method which will be able to screen all non-herbal medicine in a single run. However, it is logical to identify a few broad spectrum screening methods in order to cover the most frequently encountered drugs. REMEDi HS, a commercial system, has been evaluated for screening the neutral and basic synthetic medicines as adulterants in the herbal medicines. The system utilizes an on-line sample purification and cleaning procedures, and it requires minimal sample pre-treatment. The library used during this study contained 555 drugs and metabolites. Diazepam, chlordiazepoxide, sulpiride, etenzamide, caffeine and metoclopramide were identified in the five herbal medicines submitted from

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consumer. The latter four medicines have not been reported before as adulterants in herbal medicines. All results were confirmed by GC/MS.

INTRODUCTION

Herbal medicines are rarely considered in analytical toxicology, even though about 70 percent of world population currently use some forms of herbal medicines [1]. Recently, the presence of adulterants in herbal medicines have been reported [2, 3]. For example, several steroid adulterants were reported to be present in herbal medicines in Pakistan[2]. A recent report indicated that an adulterated herbal pill (Chui Fong Tou Ku Wan) was sold in the US, and analysis of the tablets has shown the probable presence of a steroid (prednisone) and a benzodiazepine (diazepam) [3]. The US Food & Drug Administration has traced manufacturer of these illegal pills to Hong Kong [3].

In Taiwan, herbal medicine is subjected to the same routine surveillance as western pharmaceuticals under the jurisdiction of the National Laboratories Of Foods And Drugs (NLFD). The institute, which regulates foods, cosmetics, pharmaceuticals, medical devices and narcotics, also investigates consumer complaints such as the analysis of questionable products. Often, the adulterated herbal medicines are sold under the name of "wonder drug" without any indication of the content and the manufacturing location.

When considering the number of the commonly used western medicines (at least 1000 drugs) and the complexity of herbal medicine, the identification of the adulterants in the herbal medicines poses a great difficulty to laboratories. In addition, since drugs do cross borders, the analyst may be faced with unfamilar ingredients. The most difficult task for the laboratories that conduct routine surveillance of medicine content is to structure a manageable and systematic approach for an effective screening. Because of the great variation of chemical properties, it is not possible to devise a single screening method to cover all medicines. However, it is logical to seek a few complementary methods which can provide an adequate broad screen in a timely fashion. The fewer the methods required, the more effective the surveillance program will be.

Chromatographic methods are desirable because of their ability to identify a large number of drugs in a single analysis. Among various chromatographic techniques, Gas Chromatography (GC)[4-12], Thin Layer Chromatography (TLC) [13-15] and High Performance Liquid Chromatography (HPLC) [16-19] are commonly used for screening drugs. HPLC is a desirable method because it minimizes sample preparation.

A HPLC system equipped with a two-dimensional data analysis capability will provide a more definite identification than retention index alone. Two dimensional data analysis in HPLC combines chromatographic retention indices and spectrometric parameters (such as UV wavelength ratios or full UV spectral matching). Numerous examples of HPLC applications in western medicines can be found for the identifications of antidepressant [20], benzodiazepines [21, 22], neuroleptics [23], laxatives [24] and diuretics [25, 26]. This technique has also been used for analysis of vegetable products, such as the identification of active ingredients in the fruits of Gardenia Jasminoides Ellis and Gardenia Jamsminoides Var. Grandiflora Nakai [27], the analysis of active ingredients of medical plants such as Ginseng [28].

Sample treatment to remove the unwanted sample matrix is a required step in this type of analysis, mainly to eliminate interference. Off-line extraction procedures are commonly used in GC and HPLC drug screening. However, an on-line extraction and purification procedure will permit automation of the analysis process. Previously, we have reported that a HPLC system (REMEDi) for screening over 250 drugs in serum and urine matrix using on-line purification procedure [29-31].

Here we describe the application of an enhanced model of this HPLC system (REMEDi HS) for screening neutral and basic drugs in the adulterated herbal medicines with minimal sample preparation.

MATERIALS AND METHODS

Samples

Five herbal samples were analyzed. Two were in the powder form, and three were in the form of pellets. These five samples submitted as part of rountine consumer inquiries to NLFD.

Sample Preparation For REMEDi HS

A 10 ml of methanol/water solution (50/50, v/v) was mixed with 100 mg herbal medicine (the pellet sample was crushed and grinded before weighing) in a 20 ml sample vial. The solution was vortexed for 30 seconds and mixed on a rocker for 10 minutes.

Fifty μl of the top clear solution was transferred from the 20 ml vial to a microfuge tube. 950 μl 1% saline solution (1 g NaCl in 100 ml D.I. water) and 200 μl of Internal Standard solution were added to the microfuge tube and mixed well by vortexing for 10 - 15 seconds.

The tube was placed in a microfuge and spun for one minute at 9,500 xg. 1.0 ml of the supernatant was used for the analysis.

REMEDi HS & Reagents :

The configuration of the HPLC system (REMEDi) and mobile phase were described in a previous report [29]. In this study, an enhanced system was used (REMEDi HS™, BIO-RAD Laboratories, Hercules, CA, USA). The injection volume has been increased from 0.5 ml to 1.0 ml. Additionally, the light path of the flow cell has been changed from 5 mm to 10 mm. For most of the drugs evaluated, the identification limit was 100 to 300 ng/ml. The library contained 555 drugs and metabolites (software version 4.12). A 486 ALR computer was used.

Figure 1 depicts the analysis steps by REMEDi HS, including on-line sample preparation, analytical separation, scanning UV detection, evaluation of spectral data, retention data & calculation of concentrations (when required) and a comprehensive report. The HPLC system consists of 5 cartridges and 4 switching valves, and it uses 5 reagents. The internal standards used were Ethylnordiazepam (IS1) and Chlorpheniramine (IS2).

GC/MS :

A Hewlett-Packard 5989A GC/MS Engine (Palto Alto, CA, USA) was used for the study. A J&W Scientific (FISONS, Folsom, CA 95630) DB-5

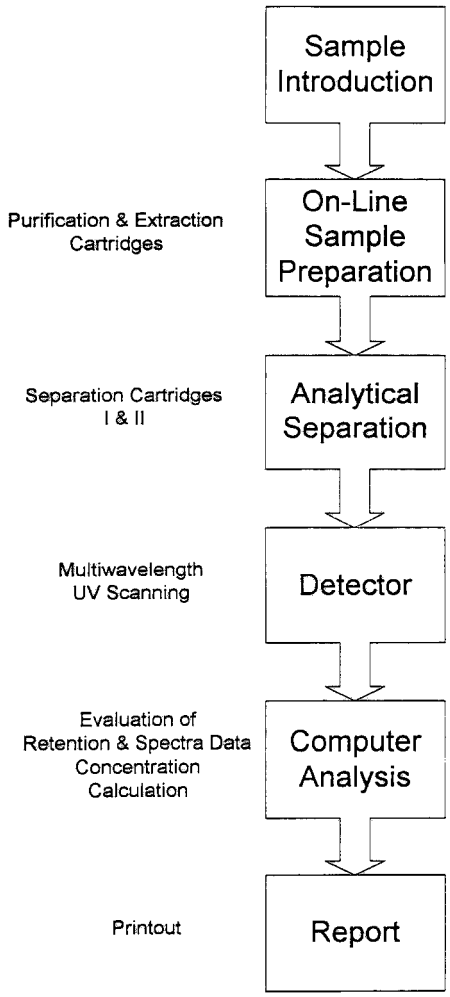

Figure 1. REMEDi HS Schematic.

capillary column (15 m, 0.25 mm I.D., 0.25 microns film) was used. Temperature program was set at 80 ^{o}C for 1 minute, then increased to 280 ^{o}C at a rate of 15 ^{o}C/min, and maintained at 280 ^{o}C for 11 minutes. The injection volume was 2 μl.

Sample Preparation For GC/MS Using Solid Phase Extraction

The 200 mg Clean Screen Extraction Column from United Chemical Technology (Horshma, PA 19044, USA) was used for solid phase extraction for GC/MS. The procedure for "Therapeutic And Abused Drugs In Urine For GC Or GC/MS Confirmation (DRU200DAZ120392)" from United Chemical Technology was used. A sample volume of 2 ml was used. The final volume of extracts was 50 μl in methanol. All organic solvents used in solid phase extraction are GC/MS grade.

RESULTS AND DISCUSSION

Formulation And Analysis Of Chinese Herbal Medicine

Chinese herbal medicines has been used in the Asian region for several thousands of years, and even today it is still the predominant medical practice in this region, including Taiwan, mainland China, Korea, Japan and southeastern Asian countries. The majority of the herbal formula are well documented and provided with a quantitative composition. The early written publications [32, 33] can be traced back as far as two thousand years ago in Chinese Han Dynasty (B.C. 202 - A.D. 220), 113 formulas were recorded in the book of Shang Han Lun and 110 formulas in the book of Chin Kuei Yao Lueh. Since then, the number of recorded formulas grew rapidly. About thirteen hundred years ago during the Chinese Tang Dynasty (A.D. 618 - A.D. 755), there were over 6,000 formulas in the book of Wai Tai Mi Yao; during the Chinese Sung Dynasty (A.D. 960 - A.D. 1279), 30,000 formulas were recorded in the book of Tai Ping Sheng Huei Fang; in Chinese Ming Dynasty (A.D. 1368 - 1644), there were over 60,000 formulas in the book of Pu Chi Fang; and in Chinese Ch'ing Dynasty (A. D. 1644 - 1911), there were over 8,000 formulas in the book of Pen Tsao Kang Mu. The total known herbal formulas exceed 100,000 when counting additional formulas from other medical compendia. Among this

huge number of formulae of Chinese herbal medicines, about 1,200 are commonly used, and 300 of them have been thoroughly analyzed [33]. About 200 of them are presently used in Japan, and 116 are available as over-the-counter medicines by TSUMURA Pharmaceutical(Tokyo, Japan).

In 1888, contemporary scientific investigations began to be conducted on Chinese traditional medicine, and paeonol was first isolated by Nagai from the root bark of Paeonia moutan Sim which is used as a Chinese crude drug under the name of Mu-tan-phi[34, 35]. In the oldest Chinese Materia Medica, Shin-rung Pen T'sao Ching, 365 crude herbal drugs were recorded [34], and recently 60 of them were studied extensively and the chemical structures of the major ingredients were identified [36]. Except for a few herbal formulas containing only a single herbal plant, most of the herbal medicines are multiple ingredient formula, which usually include eight to twelve different herbal plants. The analytical measurement of herbal medicines is a great challenge to most laboratories. Presently, the available published research literature only describes a small portion of the existing herbal medicines.

Identification

The sample solutions of the 5 adulterated herbal medicines had a dark brown color, and they were applied to the system directly with a minimal sample preparation. The REMEDi HS uses an on-line approach to remove the unwanted herbal sample matrix, while allowing analysis of a wide variety of medicines with greatly different chemical properties. The identification is made by a two-dimensional data analysis immediately after the separation. Up to 50 samples can be continuously analyzed, and the whole process is carried out automatically. Figure 2 is a typical chromatogram of an herbal sample by REMEDi HS. Table 1 lists the identified western medicines and their quantities in these five adulterated herbal medicines, including chloridiazepoxide, sulpiride, etenzamide, metoclopramide, diazepam and caffeine. GC/MS confirmed the presence of these six drugs. Their retention times in GC/MS were 6.84 minutes for etenzamide, 8.54 minutes for caffeine, 12.46 minutes for diazepam, 12.96 minutes for chlordiazepoxide, 13.73 minutes for metoclopramide and 16.32 minutes for sulpiride. Sulpiride was analyzed as the trimethylsilyl derivative.

```
DATE     :  03/29/94   TIME:  21:16 hrs   METHOD:  TI.QNT          VOLUME:1000
SAMPLE ID:  BR1621          INJ  #      50870        OPERATOR ID: TI
COMMENTS :                                VIAL #  4
```

PEAKS DETECTED

IDENTITY	NOTES	PEAK#	RT	L-MAX	PEAK-HT	W-L
		1	1.11	269	22953	235
		2	1.29	234	72773	205
		3	1.45	233	29287	205
		4	1.93	UNKNOWN	7592	205
CHLORDIAZEPOXIDE[-s-]	W1	5	2.14	271	49225	205
		6	2.45	UNKNOWN	6530	205
IS1[Nordiazepam,N-ethyl]		7	3.35	233	563829	205
Sulpiride		8	10.06	214	286975	205
IS2[Chlorpheniramine]		9	11.28	229	276098	205

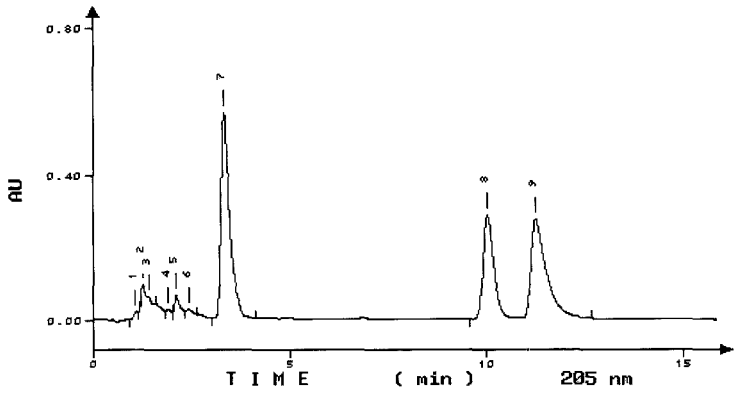

Figure 2. A Chromatogram Of An Adulterated Herbal Medicine Sample. Peak 5 : Chlordiazepoxide; Peak 7 : Internal Standard 1 (Ethyl-nordiazepam); Peak 8 : Sulpiride; Peak 9 : Internal Standard 2 (Chlorpheniramine).

Table-1 Adulterants Found In The Herbal Samples

No.	Form	REMEDi	GC/MS	Concentration	Class
1	Powder	Chlordiazepoxide	Chlordiazepoxide	1.01 mg/g	Tranquilizer
		Sulpiride	Sulpiride	12.16 mg/g	Tranquilizer
2	Pellet	Diazepam	Diazepam	0.33 mg/g	Tranquilizer
		Etenzamide	Etenzamide	10.75 mg/g	Analgesic
3	Powder	Metoclopramide	Metoclopramide	0.38 mg/g	Anti-emetic
4	Pellet	Diazepam	Diazepam	0.53 mg/g	Tranquilizer
		Etenzamide	Etenzamide	5.64 mg/g	Analgesic
		Caffeine	Caffeine	12.38 mg/g	Stimulant
5	Pellet	Diazepam	Diazepam	0.87 mg/g	Tranquilizer

Table-2 Reproducibility At the Detection Limit of REMEDi HS

Drug Name	R.T.	CV%	RRT2	CV%	Pk Ht	CV%	Detection Limit (ng)
Chlordiazepoxide	2.14	0.27	0.082	0.71	19083	2.38	100
Diazepam	2.92	0.20	0.156	0.37	28205	3.44	100
Etenzamide	1.82	0.37	0.037	3.07	292843	8.71	500
Metoclopramide	9.33	0.06	0.833	0.37	20807	7.26	200
Sulpiride	10.18	0.10	0.894	0.06	32128	0.46	300
Caffeine	1.43	<0.001	0.006	<0.001	74747	5.53	500

* Sample matrix is a solution of 1% Saline/Methanol (95/5; v/v). n=3.

Two Dimensional Data Analysis

The identifications of these compounds are made by comparing their retention indices and UV spectrum data. Table 2 lists the reproducibility of the retention data and the peak heights of these six drugs, and their identification limits are also tabulated. For REMEDi, three wavelength ratios are used as well as a similarity factor (SF) which measure the matching fitness of two UV spectra throughout the measured UV range (205 nm to 300 nm).

The figure 3 shows the UV spectrum of these six drugs. Table 3 lists the wavelength ratios for these six drugs.

Quantitation

Calibrators were prepared in 1% saline. Table 3 shows the linearity data of the six adulterants identified in these five herbal samples.

The Adulterants in Herbal Medicines

Among the six adulterants reported here, sulpiride, etenzamide, caffeine and metoclopramide were not reported previously.

Diazepam is one of the well-known adulterants in the herbal medicines [3]. It is a benzodiazepine type of tranquilizer, and it is frequently prescribed for the management of general anxiety disorders, panic disorders, and to provide

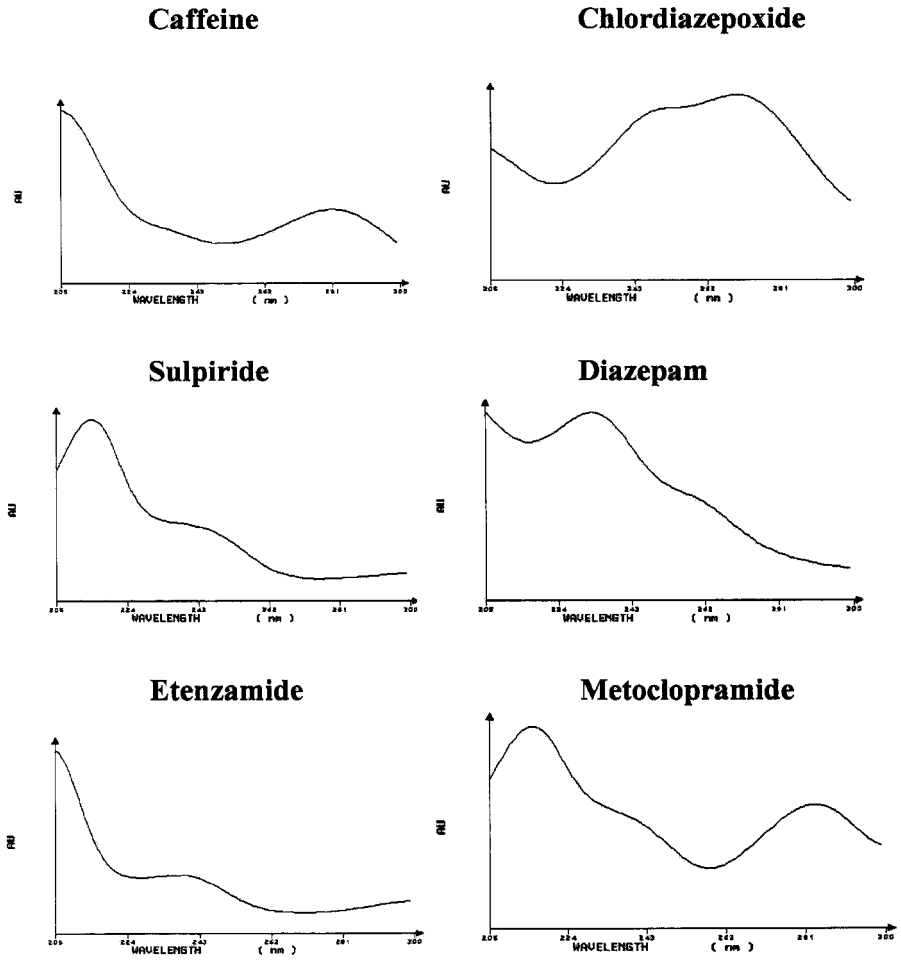

Figure 3. UV Spectra Of Caffeine, Chlordiazepoxide, Sulpiride, Diazepam, Etenzamide, And Metoclopramide.

Table-3

Drug Name	Conc (ug/ml)	Mean Peak Height (n=3)	CV%	Linearity Fit (r value)
Chlordiazepoxide	0.10	19083	2.38	0.999936
	2.00	330479	1.47	
	4.00	652407	0.40	
Diazepam	0.10	28205	3.44	0.999919
	0.50	142351	0.54	
	1.00	278811	0.08	
Etenzamide	0.50	292843	8.71	0.999712
	1.00	505019	5.60	
	1.50	735598	3.87	
Metoclopramide	0.20	20807	7.26	0.999862
	2.50	241525	0.36	
	5.00	481487	0.49	
Sulpiride	0.30	32128	0.46	0.999481
	2.50	255007	0.37	
	5.00	481487	0.36	
Caffeine	0.50	74747	5.53	0.999984
	1.00	155718	6.41	
	5.00	766694	1.18	

* Sample matrix is a solution of 1% Saline/Methanol (95/5; v/v). n=3.

preoperative sedation, light anesthesia, and amnesia, treatment of epileptics, alcohol withdrawal symptoms, skeletal muscle relaxant. Benzodiazepines are easily subject to abuse and development of tolerance and dependence [37].

Chlordiazepoxide is also a benzodiazepine, and it was reported previously in an adulterated chinese herbal medicine [38] . This particular chlordiazepoxide-containing herbal sample also has a high dosage of sulpiride (12.16 mg/g), which is usually administered as an antidepressant, an antipsychotic agent or a digestive aid. The combination of sulpiride and chlordiazepoxide is similar to the formulation of a commercially available neurosis medicine (trade name, Anisum), which contains sulpiride and diazepam.

Sulpiride is reported to have mood elevating properties, and has anti-emetic actions and an effect on gastric secretion. It is commonly used in this region for this purpose, and it has an detection rate of about 1% in a recent study of the patient samples from emergency room in this region [39].

Etenzamide was found in two herbal samples (#2 & #4). Etenzamide, an analgesic agent, is rarely used in the USA, but is often used as a pain killer in Japan and nearby countries. A formulation of western medicine was reported to contain etenzamide (Ethoxybenzamide), acetaminophen, bromvalurea and caffeine, manufactured by Mintong Pharmaceutical (Taiwan). In these two samples, Sample 2 contains diazepam, and Sample 3 contains diazepam and caffeine. Caffeine can increase mental alertness, and is often included in the formulations containing aspirin, acetaminophen, or codeine [40].

Metoclopramide stimulates motility of the upper gastro-intestinal tract and is used for the management of some forms of nausea and vomiting, in gastro-oesophageal reflux, and gastric stasis [41]. In one study conducted in Taiwan, it was detected in 1.2% in the patient samples collected in the emergency department [39].

Most of the adulterants identified in this report belong to the category of pain relievers. The adulterants are usually pure chemical components which can be rapidly absorbed by the human body, and the patient will have the sensation of instant relief. This type of adulterated drug is commonly sold under the name of "wonder drug", or it is added as an "undisclosed" ingredient to a regular herbal medicine by unlawful manufacturers. Because herbal medicine has been integrated with Asian medication history and daily life for many thousands of years, the adulterants in the form of herbal medicine are less likely to be noticed by the patient and they are difficult to detect in the herbal matrix.

CONCLUSION

Six adulterants are identified in herbal medicines, including diazepam, chlordiazepoxide, sulpiride, etenzamide, metoclopramide and caffeine. All results were confirmed by GC/MS. Except diazepam and chlordiazepoxide, the other four drugs were not previously documented. These six drugs belong to four classes of drugs : analgesic(etenzamide), anti-emetic (metoclopramide), xanthine stimulant (caffeine) and tranquilizers (diazepam, chlordiazepoxide & sulpiride). The findings which we report here demonstrate that the identification of each individual drug from the screening method can expedite the confirmation process. Specifically, four out of these six drugs (etenzamide, metoclopramide, sulpiride and caffeine) could not be screened by immunoassays, nor are they routinely analyzed by a single chromatographic method.

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ANALYSIS OF HYGRINE IN COCA LEAVES USING A NOVEL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

ROBERT L. GLASS
Plant Sciences Institute
Agricultural Research Service
U.S. Department of Agriculture
Beltsville, Maryland 20705

ABSTRACT

A novel high-performance liquid chromatographic (HPLC) method is described for the determination of hygrine in leaves of Erythroxylum coca and Erythroxylum novo. The analysis was performed on a strong cation exchange HPLC column with a mobile phase consisting of MeOH: 0.1 M KH_2HPO_4, pH 7 (75%:25%, v/v) and a UV detector set at 220 nm. Recoveries of hygrine averaged about 64% from extracts fortified with 2.5 to 10.0 mg of hygrine per 10 ml of extract. Hygrine contents in leaves of E. coca and E. novo were determined as 0.09 and 0.10% of the dry weight, respectively.

INTRODUCTION

Hygrine (N-methyl-2-acetonyl pyrrolidine) is an alkaloid found in leaves of a variety of plants such as Datura, Hyoscyamus, Nicotiana and Erythroxylum. Recently, this pyrrolidine alkaloid was found to be the precursor of the tropane alkaloids hyoscyamine and scopolamine, both of which are widely used in the preparation of many pharmaceutical products (1,2).

Hygrine in plant tissue has been traditionally analyzed by conventional chromatographic techniques, such as thin-layer chromatography (TLC)(2) and capillary gas chromatography (CGC)(3). In a recent study (4), hygrine from coca extracts was derivatized with heptafluorobutyric anhydride and then analyzed by CGC. Although CGC has been routinely used for the analyses of many tropane alkaloids (3), this method has not been validated for hygrine, in respect to linearity, precision, and recovery of hygrine from coca leaf extracts.

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In the present paper, a modified procedure for extracting hygrine from coca leaves and a high-performance liquid chromatographic (HPLC) method are described. The HPLC method is simple and reliable for routine analysis of hygrine. This analytical HPLC method was developed as part of a project aimed at isolating, via preparative HPLC, some of the unknown tropane alkaloids that are found in coca extracts (6). More than 45 trace-level tropane alkaloids were recently detected in coca extracts using CGC-MS (mass spectrometry) (4).

EXPERIMENTAL SECTION

Chemicals: Methanol, ethanol and chloroform of HPLC grade were purchased from EM Science (Gibbstown, NJ). All other chemicals were of reagent grade or better. Water used to prepare solutions and mobile phases was initially deionized and was subsequently run through a HP Model 661A water purifier (Hewlett-Packard Co., Avondale, PA). This treatment produced extraordinary pure water with a resistivity > 18 Ωm-cm.

Hygrine Synthesis: Hygrine was synthesized by the procedure initially reported by Galinovsky et al. (7) and later modified by Leete et al. (8). N-methyl pyrrolidone (1.0 g) (Aldrich Chem. Co., Milwaukee, WI) dissolved in ethyl ether was partially reduced to the amino-aldehyde by refluxing with lithium aluminum hydride. After the ether in the mixture was removed by rotary evaporation, acetone dicarboxylic acid (1.0 g) (Aldrich Chem. Co.) dissolved in 0.1 M sodium dihydrogen phosphate was added to the mixture. Hygrine was separated and purified by vacuum distillation (10 mm) between 75 and 85^{0}C which gave a colorless oil (0.13 g, 13% yield); density 0.934. The chemical purity of the synthesized hygrine was higher than 90 % as determined by CGC analysis in our laboratory and CGC/MS analysis at the Drug Enforcement Administration Special Testing and Research Laboratory, Mclean, VA.

Standard Solutions: A 55-ul volume of hygrine was dissolved in 50 mL of methanol to give a standard solution of concentration 1.0 mg/mL. Standards ranging in concentrations from 0.5 to 0.05 mg/mL were prepared by serial dilutions using methanol. These standards were stored in amber vials at 0^{0}C.

HPLC Analysis: A Model 8800 ternary gradient HPLC pump (Spectra-Physics, San Jose, CA) was used with a Model 7125 Rheodyne valve (Cotati, CA) fitted with a 5-μl loop. Hygrine was separated on an Adsorbosphere SCX (strong cation exchange) column (25.0 cm x 4.6 mm i.d., 5 μm; Alltech Associates Inc., Deerfield, IL). The SCX column was used without a guard column. The mobile phase consisted of methanol: 0.1 M KH_2PO_4, pH 7.0 (75:25, v/v) delivered isocratically at 1.2 mL/min resulting in a column head pressure of about 1250 psi. Detection was made with a Model UV2000 dual wavelength detector (Thermo Separation Products, Fremont, CA) operated at 220 nm (0.05 AUFS). Chromatographic data were obtained on a Model 427 Integrator (Beckman Instruments, Inc, Fullerton, CA) with a chart speed of 0.5 cm/min and an attenuation of 32 mv full scale.

Calibration Curve: The area counts of the individual peaks and the corresponding concentrations were used to construct the standard

curve for hygrine. The curve followed Beer's law in the range 0.1 to 1.0 mg/mL.

Extraction Procedure: The original procedure for extracting alkaloids from coca leaves (9) was modified for the extraction of hygrine in the present investigation. E. coca and E. novo leaves were collected from plants grown from seeds under greenhouse conditions as described in an earlier alkaloid study in this laboratory (5). Air-dried leaves (0.1-10.0 g) were crushed by hand and were refluxed with 95% ethanol (50-300 ml) for 30 min. The extract was passed through Whatman No. 41 filter paper to remove all particulate matter. The solvent was removed by rotary evaporation at 60°C under vacuum (2-10 mm). The residue was re-dissolved in 50 ml of chloroform and then transferred to a separatory funnel. The chloroform extract was shaken separately with two 25-ml volumes of 1.5% citric acid in water (w/v) which were then combined in a beaker. The aqueous layer was adjusted to pH 5.5 with 1.2 M sodium bicarbonate and was subsequently shaken with two 25-mL aliquots of chloroform for the purpose of removing cocaine and other interfering alkaloids from the aqueous phase. The aqueous layer was then adjusted to pH 7.5 with 1.2 M sodium bicarbonate and to the final pH of 8.8 with 1.0 M sodium hydroxide which was done to keep the final volume near 150 ml. Hygrine and the remaining alkaloids were partitioned into chloroform by mixing the aqueous layer with 50 mL of chloroform in a glass bottle which was shaken for 1.5 hr on a rotary mixer. The mixture was sub- sequently collected in a separatory funnel and was allowed to stand for 15 min. The chloroform layer was reduced to a volume of 1-2 mL on the rotary evaporator. The extract was diluted to a final 10-mL volume with methanol for HPLC analysis.

Fortified Samples: Sub-samples (10 mL) of a crude E. novo leaf extract were fortified with 0.5, 2.5, 5.0 and 10.0 mg of hygrine from the working standard solutions of hygrine (1.0 mg/mL). A minimum of two replicates were made of each fortification level. The crude leaf extract was prepared by refluxing 2.0 g of dry E. novo leaves with 100 mL of 95% ethanolic solution for 30 min as described earlier in the extraction procedure. Recoveries of hygrine from the fortified samples and other quantitative data were analyzed by the Axum statistical program (TriMetrix, Seattle, WA) to determine the 95% confidence intervals.

RESULTS AND DISCUSSION

HPLC Analysis: In the present investigation, the Adsorbosphere SCX (strong cation exchange) column has been found to be efficient column for analyzing hygrine in coca extracts. Both reversed phase chromatography (C_{18} column) and normal phase chromatography (NH_2 column) were efficient in resolving standards of hygrine but were incapable of resolving hygrine in coca extracts (6). The chromatograms in Figure 1 show the resolution of hygrine in (A) a standard solution of hygrine (0.25 mg/mL), (B) an extract fortified with 2.5 mg of hygrine per 10 mL of E. novo extract, and (C) an unfortified E. coca extract. The retention time for hygrine was about 13 min at a flow rate of 1.2 ml/min. The calculated number of theoretical plates (N) was 18,500 plates/m^2. The high pH of the potassium dihydrogen phosphate (pH 7) used in the mobile phase appeared to have adversely affected the column's stability by causing excessive baseline drift.

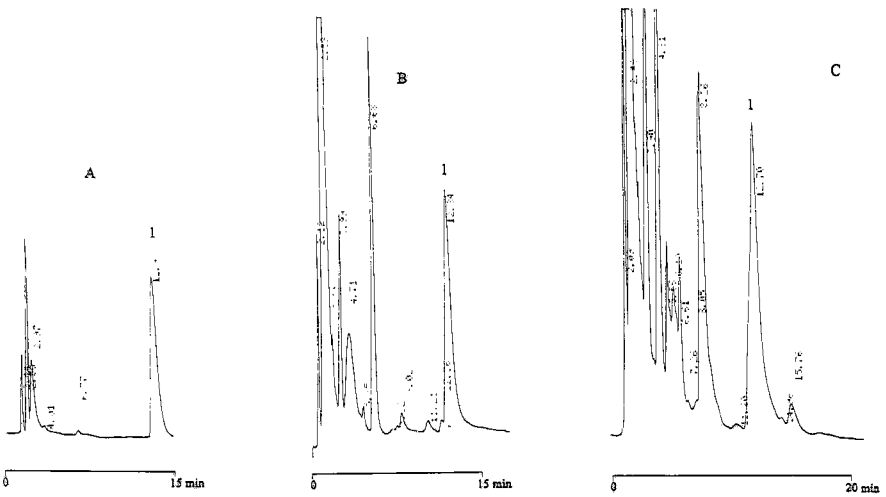

Figure 1. HPLC Chromatograms of hygrine in (A) a standard solution of hygrine (0.25 mg/mL), (B) an extract fortified with 2.5 mg of hygrine per 10 mL of E. coca extract, and (C) an unfortified E. coca extract. Peak 1 is hygrine.

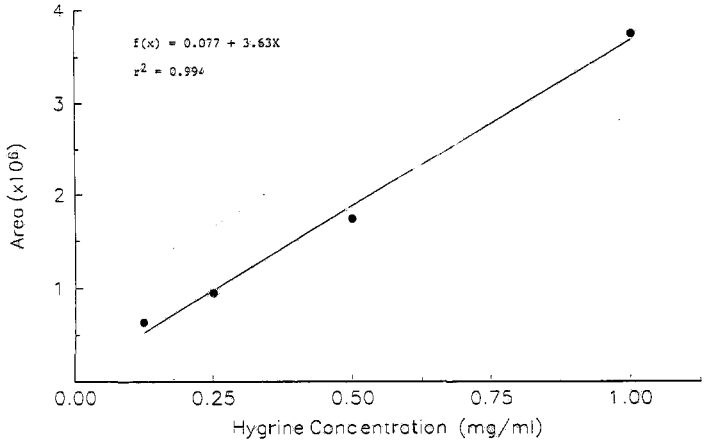

Figure 2. Hygrine standard curve. The dotted lines indicate a 95% confidence interval about the regression line.

TABLE I.

Recoveries (%) of Hygrine from Fortified Coca Extracts[a].

Hygrine Added (mg)		Hygrine Found (mg)	Recovery Content (%)	S.D.	C.V.[b] (%)
0.5	(n = 3)	N.D.[c]	----		
2.5	(n = 2)	1.62	64.80	0.80	1.20
5.0	(n = 3)	3.50	70.87	3.54	4.86
10.0	(n = 3)	5.53	55.27	3.60	6.51
		Means	63.65	2.60	4.19

[a]Hygrine was added to 10-mL aliquots of a working stock extract which was prepared by refluxing 2.0 g of dry E. novo leaves in 100 mL of 95% ethanol. [b]Results are the mean of two or more replicates, standard deviation, and the coefficient of variation (%). [c]Denotes not detectable.

The standard curve for hygrine (Fig. 2) was fitted by the regression equation $f(x) = 0.077 + 3.63x$, with the coefficient of regression (r^2) of 0.994. The detection limit (signal/noise = 3) for hygrine was determined as 0.05 mg/mL, or 250 ng/injection at the 0.05 AUFS sensitivity level.

Analysis of Leaves: Table I shows that the recoveries of hygrine in fortified samples ranged from about 55 to 71% with a mean recovery of 63.7% and a coefficient of variation of 4.19%. Hygrine was below the limit of detection in the 0.5 mg per 10 mL sample. The recoveries of hygrine witnessed here are similar to those observed for cocaine in an earlier study (5). The losses of hygrine witnessed here presumably occurred in the rotary evaporation steps. Significant losses of hygrine occurred when fortified samples went to dryness during rotary evaporation (6).

TABLE II.

Hygrine Contents (%) of Air Dried E. coca and E. novo Leaves.

Species	Avg. Dry Wt.	Hygrine Content (%)	S.D.	C.V. (%)
E. novo	2.00g (n = 2)	0.10	0.01	10.00
E. coca	2.02g (n = 3)	0.06	0.01	16.66
	4.30g (n = 3)	0.05	0.01	20.00
	10.00 (n = 3)	0.17	0.02	11.76
	Mean	0.09	0.013	16.14

[a]Results are the mean of two or more replications, standard deviation, and the coefficient of variation (%).

Table II shows that the hygrine content of air-dried E. coca and E. novo leaves was 0.09 and 0.1 %, respectively. The precision was less for the analysis of the leaf extracts (C.V.=16.14%, n=9) than for the analysis of hygrine in fortified samples (C.V.=4.19 %, n=8). The 0.09% hygrine content found in the present investigation is near the 0.1% hygrine content reported for Colombia E. coca leaves using a CGC method in which hygrine was derivatized with heptafluorobutyric anhydride (4). In a similar study using a CGC method (10), a higher hygrine content of 0.3% was reported for greenhouse cultivated E. coca leaves. Preliminary results obtained from CGC analyses in this laboratory show a higher hygrine content present in coca leaves, i.e., approximately 0.3 % in E. coca and 0.2% in E. novo leaves (6). The variability in the present method apparently masks the difference in the hygrine content of these two plant species. A comparative study of hygrine in E. coca and E. novo using CGC and HPLC analyses will be presented in the near future.

CONCLUSION

The modified extraction procedure and the new HPLC method using the strong cation exchange column proposed in this paper provide a reliable method for quantitating hygrine in coca leaf extracts. The precision of the method is good for hygrine in fortified samples with a C.V. of 4.2% and is acceptable for hygrine

in coca extracts with a C.V. of 16.1%. This HPLC method offers the advantages of being simple and not requiring extensive derivatization of hygrine. Also, this analytical procedure can be easily scaled up to a preparative method for the separation and purification of hygrine and any of the alkaloids that are chromatographed under these reported conditions.

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RETENTION BEHAVIOR OF VITAMIN D AND RELATED COMPOUNDS DURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KAZUTAKE SHIMADA*, KUNIKO MITAMURA, MAKI MIURA, AND ARIKA MIYAMOTO
Faculty of Pharmaceutical Sciences
Kanazawa University
13-1 Takara-machi
Kanazawa 920, Japan

ABSTRACT

The retention behavior of vitamin D_2-D_5 and provitamin D_2-D_5 are examined using high-performance liquid chromatography. Inclusion chromatography using cyclodextrin as the mobile phase additive in reversed-phase high-performance liquid chromatography is also used for this purpose. Reversed-phase high-performance liquid chromatography is more effective than normal-phase high-performance liquid chromatography in separating these analogs. The addition of methyl-β-cyclodextrin to the mobile phase is effective in separating the pair of vitamin-D_2 and -D_3 or provitamin-D_2 and -D_3. The separation of the pair of stereoisomeric Cookson-type derivatives of vitamin-D_2 or -D_3 was also examined and found that normal-phase high-performance liquid chromatography is effective for this purpose.

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INTRODUCTION

In the previous paper of this series, we clarified the retention behavior of conjugated metabolites of vitamin D (D) and related compounds during high-performance liquid chromatography (HPLC) and found that the inclusion chromatography using cyclodextrin (CD) as a mobile phase additive is effective in separating the pairs of fluorescent derivatives of the D_3 and D_2 conjugates or related compounds [1]. Naturally obtained D includes D_2-D_7 , which can be differentiated from one another by the side chain structure of the steroid moiety. Although the separation of sterols by HPLC has been done [2], that of D has not been precisely examined. These data prompted us to examine the retention behavior of D and related compounds during HPLC.

MATERIALS AND METHODS

Materials

Heptakis-(2, 6-di-O-methyl)- β -cyclodextrin (Me- β -CD) was prepared and donated by Kao (Tokyo, Japan). The other CDs (α, β and γ) were donated by Nihon Shokuhin Kako (Tokyo). D_2, D_3 and ergosterol (pro D_2) were purchased from Tokyo Kasei Kogyo (Tokyo). 7-Dehydrocholesterol (pro D_3) was obtained from Wako Pure Chemical Ind. (Osaka, Japan). D_4 and provitamin D_4 (pro D_4) were kindly donated by Dr. Tachibana (Nisshin Flour Milling Co., Saitama, Japan). D_5 and pro D_5 were prepared from β-sitosterol (Funakoshi, Tokyo) in this laboratory in the usual way [3]. The fluorescent Cookson-type reagent, 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1, 2, 4-triazoline-3, 5-dione (MBOTAD), was synthesized in this laboratory as previously described [4]. Preparative thin-layer chromatography (prep. TLC) was done with pre-coated TLC (silica gel HF_{254}, 20 x 20 cm, 0.5 mm: E. Merck AG, Damstadt, Germany).

Apparatus

HPLC was carried out using a Shimadzu LC-6A chromatograph (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-6AV ultraviolet detector (UV). Reversed- (YMC • GEL $C_{8,}$ 5 μm, 15 cm x 0.46 cm i.d.)(YMC, Kyoto) and normal- (Develosil 60-5, 5 μm, 25 cm x 0.46 cm i.d.)(Nomura Chem., Seto, Japan) phase columns were used under ambient conditions at a flow rate of 1 ml/min. ^{1}HNMR spectra were measured with a JNM-EX 270 (270 MHz) spectrometer. $CDCl_3$ was used as the solvent with Me_4Si as internal standard. Chemical shifts and *J*-values are given in ppm and Hz, respectively. The following abbreviations are used: d=doublet and m=multiplet.

Preparation of MBOTAD Adducts of D_2 and D_3

Derivatization of D_2 or D_3 (each *ca.* 52 μmol) with MBOTAD (*ca.* 82 μmol) was done according to a previously described procedure [4]. The reaction mixture was submitted to prep. TLC using $CHCl_3$-AcOEt (2:1) as the developing solvent and the following zones were extracted with AcOEt: D_2, ^2Rf 0.24 (yield 55.9%), 0.36 (8.8%); D_3, ^2Rf 0.24 (86.1%), 0.19 (12.0%).

RESULTS AND DISCUSSION

Although D includes D_2-D_7 , we have examined the retention behavior of D_2-D_5 and pro D_2-D_5, which are available during HPLC. These can be differentiated from one another by the side chain structure of the steroid moiety as shown in Fig. 1.

Separation of D_2-D_5 or pro D_2-D_5

Initially, efforts were directed at the separation of D_2-D_5 during normal-phase HPLC. Although two solvent systems [CH_2Cl_2-MeOH (200:1), CH_2Cl_2-isopropanol (120:1)] were examined, D_2-D_5 were eluted at the same retention times (t_R 10.00, 9.19 min, respectively).

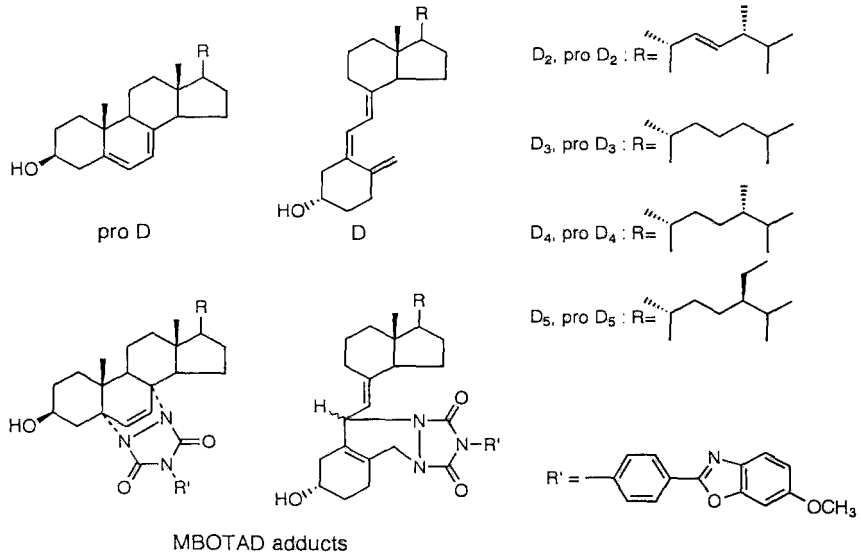

Figure 1. Structures of pro D, D and its MBOTAD adducts

The separation of D_2-D_5 by reversed-phase HPLC using an octylsilyl column and MeOH or MeCN as an organic modifier has been attempted. MeOH was superior to MeCN as an organic modifier, the latter gave an unsymmetrical peak, but D_2 and D_3 could not be separated by both organic modifiers (Fig. 2a). These data prompted us to use inclusion chromatography using CD as the mobile phase additive for this separation. Although the high concentration of Me-β-CD as the host compound (7 mM) was necessary, a satisfactory result was obtained as shown in Fig. 2b. The other CDs (α- and γ-CD) were ineffective while β-CD could not be used at the more than 1 mM because of its sparing solubility in the mobile phase. The former phenomenon can be explained by the cavity size of the host compounds; α-CD was too small and that of γ-CD was too big to include the steroid.

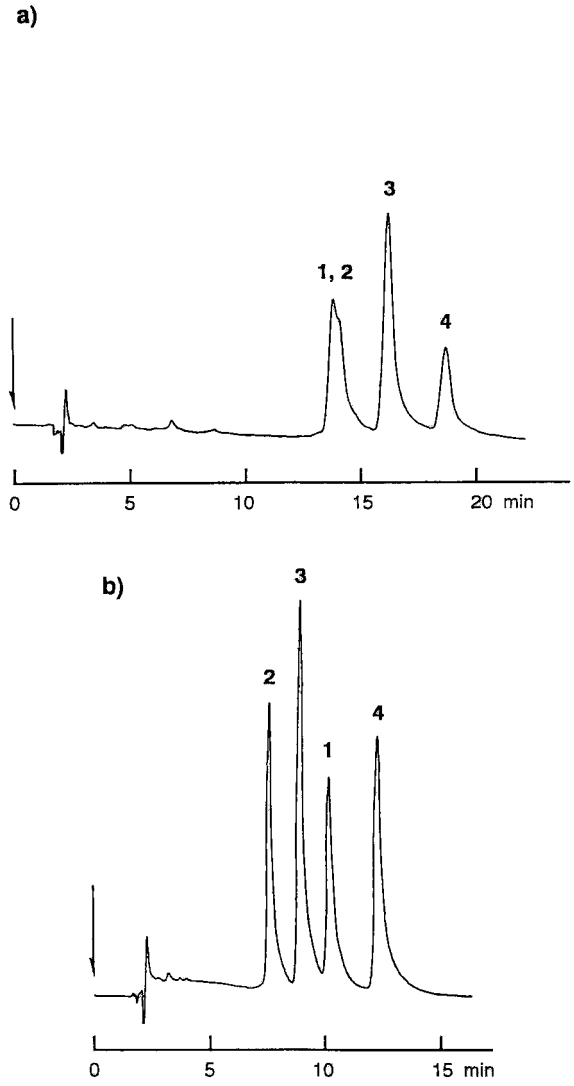

Figure 2. Separation of D_2-D_5

Conditions: mobile phase, MeOH-H_2O (9:1) containing Me-β-CD, a) 0 mM b) 7 mM ; detection, UV 265 nm.

1: D_2 2: D_3 3: D_4 4: D_5

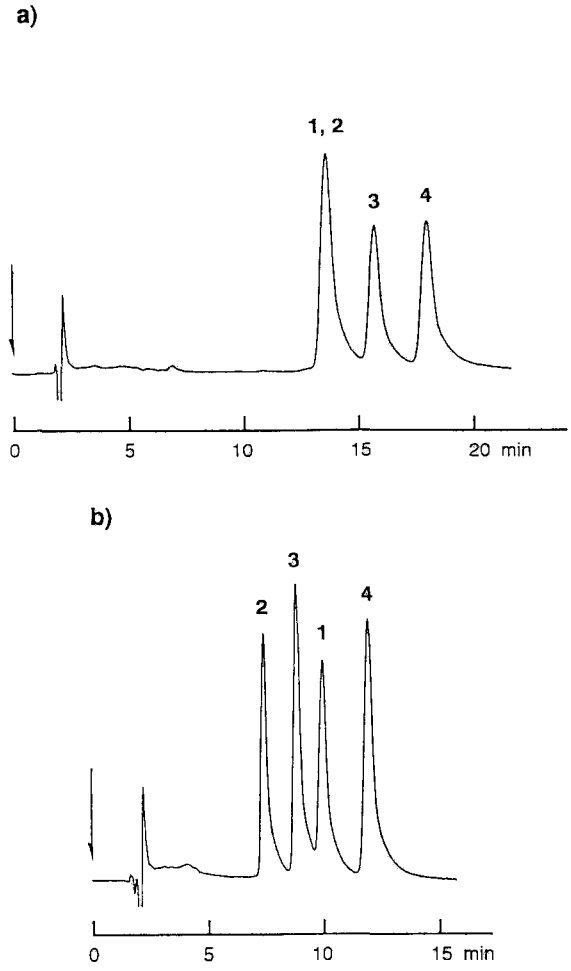

Figure 3. Separation of pro D_2-D_5

Conditions: mobile phase, MeOH-H_2O (9:1) containing Me-β-CD, a) 0 mM b) 7 mM ; detection, UV 280 nm.

1: pro D_2 2: pro D_3 3: pro D_4 4: pro D_5

Table 1. ^{1}HNMR spectra data and separation of stereoisomeric MBOTAD adducts of D_2 or D_3

Adduct		^{1}HNMR		HPLC[a]	
		H-4 α	H-3 α	t_R (min)	Resolution (6*R* vs. 6*S*)
D_2	6*R*	2.51, d (15.6)[b]	4.03, m	16.35	
	6*S*	-[c]	4.13, m	11.14	8.17
D_3	6*R*	2.44, d (15.8)	4.14, m	16.46	
	6*S*	-	4.14, m	11.26	8.16

a) Conditions: CH_2Cl_2-MeOH (60:1), UV (280 nm)

b) *J*-value

c) Superimposed with other methylene protons

Almost the same phenomena as that previously described were observed during the separation of pro D_2-D_5. A satisfactory separation has been obtained by the addition of Me-β-CD (7 mM) to the mobile phase as shown in Fig. 3a,b.

Separation of stereoisomeric MBOTAD adducts of D_2 or D_3

It is well known that the derivatization of D with the Cookson-type reagent produces two stereoisomeric adducts, β - and α -side attacked, in a ratio of approximately 6:1; this is helpful in identifying the desired compounds in biological fluids [5]. Although we have developed the highly sensitive fluorescent Cookson-type reagent, MBOTAD, the stereochemistry of the adducts with D has not been clarified (Fig. 1). Based on the ^{1}HNMR spectra (Table 1) of adducts of D_2 and D_3, the obtained main and minor compounds are elucidated as 6*S* and 6*R* compounds, respectively [4]. These stereoisomers were clearly separated by normal phase HPLC as shown in Table 1. The addition of Me-β-CD to the mobile phase of the reversed-phase HPLC improved the separation of these

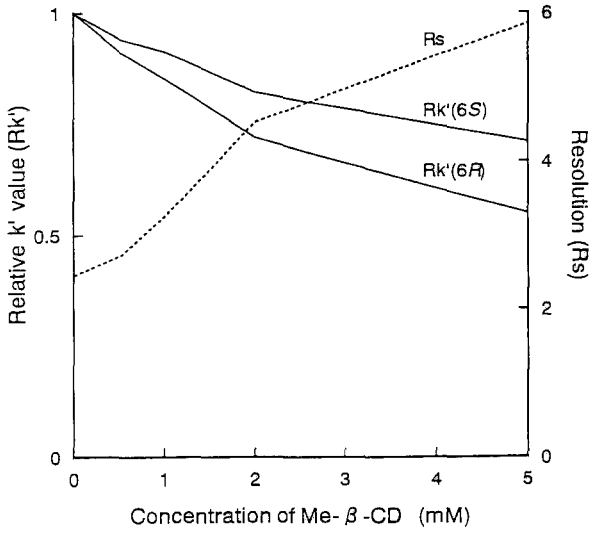

Figure 4. Effect of Me- β -CD on the resolution of the stereoisomeric MBOTAD adducts of D_3
Conditions: mobile phase, MeCN-H_2O (5:1) containing Me- β -CD as indicated; detection, UV 280nm.

stereoisomeric pairs as shown in Fig. 4. The other CDs were not very effective in the separation of the MBOTAD adducts of conjugated D [1].

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DETERMINATION OF CEFODIZIME IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COLUMN-SWITCHING

S. BOMPADRE[1], L. FERRANTE[1], L. LEONE[1]*, M. DE MARTINIS[2], L. GINALDI[2], AND D. QUAGLINO[2]

[1]*Institute of Biomedical Sciences*
University of Ancona
60131 Ancona, Italy
[2]*Department of Internal Medicine*
University of L'Aquila
67100 L'Aquila, Italy

ABSTRACT

A column-switching high-performance liquid chromatographic assay with ultraviolet detection, at 263 nm, was developed to determine cefodizime, a new parenteral cephalosporin, in human plasma. The method is based on pre-column extraction in a closed system allowing direct injection of plasma samples without any sample pretreatment. The method allows direct, rapid, precise, and simple determination of cefodizime in plasma over the range of 1-400 μg/ml using a 100 μl loop and 25 μl of plasma. The detection limit of the assay was 0.1 μg/ml. Recovery for spiked plasma

* To whom the correspondence should be addressed

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samples was near 100% and relative retention time showed good repeteability. An application of the method to study the pharmacokinetics of cefodizime in immunodepressed patients is given.

INTRODUCTION

Cefodizime is a new aminothiazolyl third generation cephalosporin for parenteral use, with a broad antibacterial spectrum and possessing immunomodulating properties (1).

Microbiological and high-performance liquid chromatographic (HPLC) assays have been used to determine cefodizime in biological fluids (2-5). Microbiological methods are slow and give poor precision and specificity (since other antibiotics interfere). Conventional HPLC methods used so far for the determination of drugs are based on time-consuming extraction procedures which increase the potential of introducing a bias in the results. These problems have increased the interest in methods for biological samples not requiring sample preparation. Newer methods used to analyse drugs in biological fluids include direct injection of biological samples by column switching. The use of a column-switching system allows to achieve selectivity from endogenous compounds.

We have developed a pre-column extraction HPLC method allowing direct, rapid and sensitive analysis of cefodizime in plasma. It has been applied to study the kinetics of this drug after intravenous administration to immunodepressed patients.

EXPERIMENTAL

Reagents and chemicals

All chemicals were reagent or analytical grade. Acetonitrile was obtained from E. Merck (Darmstadt, Germany). Acetic acid was purchased from Carlo Erba Farmitalia (Milano, Italy). Sodium 1-heptanesulfonate was supplied by Sigma Chemical Company. Cefodizime was from Hoechst. Analytical grade, filtered water was obtained daily from an Elgastat UHQ PS apparatus (ELGA, High Wycombe, Bucks, England).

Apparatus

The HPLC system consisted of a VARIAN (Walnut Creek, CA, USA) model Vista 5500 HPLC pump, a Spectra Physics model SP-8000B solvent-delivery system, and a Model 166 Programmable ultraviolet detector from

Beckman. The chromatograms were integrated with a System Gold laboratory data system (Beckman, Berkeley, CA, U.S.A.). The injector was a Rheodyne model 7725i manual injection valve equipped with a 100 µl sample loop. The coupled-column system was operated by a pneumatic, six-port, automated switching valve (VALCO Schenkon, Switzerland), controlled by the HPLC system.

Chromatographic conditions

The analytical column was a HP ODS analytical column (20 cm x 0.4 cm I.D., particle size 5 µm) from Hewlett-Packard (Palo Alto, CA, U.S.A.). The extraction column was a 5 cm x 0.4 cm I.D., dry-filled with CN 40 µm silica. The mobile phase 1 consisted of a mixture of 2% acetic acid and 10% acetonitrile in 5 mM sodium 1-heptanesulfonate (pH 3); mobile phase 2 consisted of 2% acetic acid and 27% acetonitrile in 5 mM of sodium 1-heptanesulfonate. Sample aliquots of 100 µl (plasma + mobile phase 1, 1:3, v/v) were injected into the chromatograph. The flow-rate for both columns was set at 1.0 ml/min. The effluent from the analytical column was monitored by UV at a wavelength of 263 nm. The retention time of cefodizime was 7.6 minutes and cycle time of one analysis was 10 minutes. Chromatographic analyses were performed at ambient temperature.

Column switching procedure

The scheme for the switching procedure is shown in Figure 1.

Plasma samples were diluted 1:3, v/v, with mobile phase 1 and a total of 100 µl of the diluted sample was injected, without any sample preparation, directly onto the extraction column. Mobile phase 1, which passed through the column and was directed to waste, was delivered by the pump 1, while the pump 2 delivered the same mobile phase 1 to the analytical column (switching valve at the initial position: Figure 1A). After a flushing period of 1 min to remove matrix components (while cefodizime was retained on the stationary phase) the first column was connected with the analytical column via the switching valve for 2,5 minutes. Cefodizime retained in the precolumn was eluted into the second (analytical) column for quantitation with the mobile phase 1 from the pump 1. At this stage the mobile phase in pump 2 changed from mobile phase 1 to mobile phase 2 (Figure 1B). After 3,5 minutes mobile phase 1 was switched back to pass through the extraction column to prepare it for next sample, while pump 2 maintained the flow of mobile phase 2 through the analytical column where cefodizime was separated and detected by UV (Figure 1C).

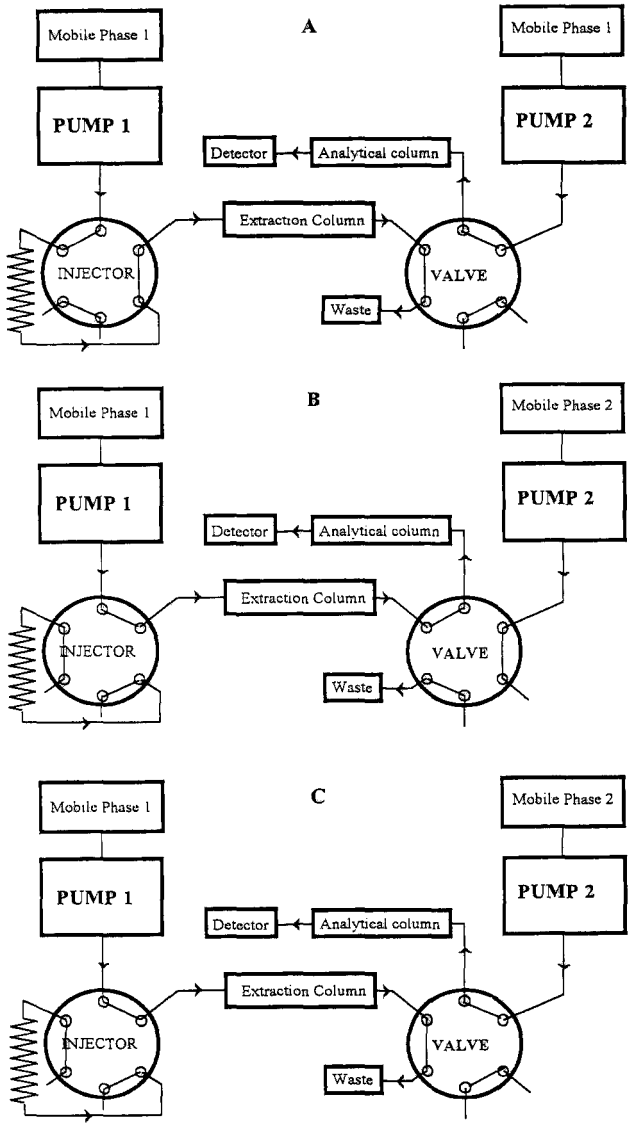

FIGURE 1. Scheme of column switching. A: extraction of injected sample (mobile phase 1 from pump 1 to extract cefodizime and from pump 2 to prepare analytical column). B: elution of the drug from the pre-column into the analytical column with mobile phase 1 from the pump 1. C: separation of the analyte with the mobile phase 2 from pump 2 and conditioning of pre-column with mobile phase 1 from pump 1.

Sample processing

Blood samples were drawn from patients treated with cefodizime by i.v. injection and collected into heparinized glass tubes. Plasma was quickly separated by centrifugation and stored at - 20°C until analysis.

Quantification

Standards for the calibration curve were made by spiking control plasma at the following concentrations: 0, 1, 5, 10, 25, 50, 100, 250, and 400 μg/ml. Each spiked plasma standard was injected eight times. The calibration data of peak-area against the concentration of the drug were fitted to a linear, unweighted, model. The resultant linear regression curves were used to calculate the drug concentrations in the samples.

Recovery

Analytical recoveries were calculated by comparing the peak areas of standards or spiked samples passed through both the extraction and analytical columns with the peak areas of standard of the same concentration injected directly onto the analytical column.

RESULTS AND DISCUSSION

Problems such as time-consuming extraction steps and low recoveries have increased the interest in methods for biological samples not requiring sample preparation. The proposed method is extremely simple since it requires no extraction or clean-up steps and no internal standard. Cefodizime was determined by direct injection of plasma onto a precolumn with sample clean-up in combination with a column-switching method. The cleanup column was connected before the switching valve, and the desired portion was eluted into the second column.

In order to optimize the extraction process 5 cm x 0,4 cm I.D. columns dry packed with different phases were tested. Three kinds of packings (particle size 40 μm) were tentatively used: C2 (ethyl), C8 (octyl), and CN (cyanopropyl) silica phases. Since the selected analytical eluent consisted of a mixture of 2% acetic acid and 27% acetonitrile in 5 mM of sodium heptanesulfonate, experiments were carried out with the same mobile phase containing various acetonitrile concentrations, from 10 to 25%. Using different stationary phases the lower concentration of the organic modifier that ensure a suitable sample clean-up, a good separation of the analyte, and a retention

time as short as possible was investigated. After several experiments the best results were found to be provided by CN packing material and 10% acetonitrile. C8 and C2 required higher concentrations of organic modifier (22% and 18% respectively) in the mobile phase and gave larger peaks. The lenght of the extraction step needed to retain the drug in the column was optimized by studying recoveries after different extraction times.

The connection time of the extraction column to the analytical column was optimized by stepwise reduction until the peak area of cefodizime started to decrease. An optimum yet safe connection time was found to be 2,5 min. A longer connection time increased only the retention time of cefodizime because the extraction eluent did not elute the drug in the analytical column. After the analyte has eluted from the primary column and has been transferred onto the secondary column, the pre-column was back-flushed to remove the components of the matrix that are strongly retained. Therebe, the analytical column is protected from contamination by late-eluting matrix components. The back-flush al. minimize peak-broadening. Figure 2 shows chromatograms obtained after injecting both a blank and a spiked plasma. As can be seen cefodizime peak is well separated from the other detectable

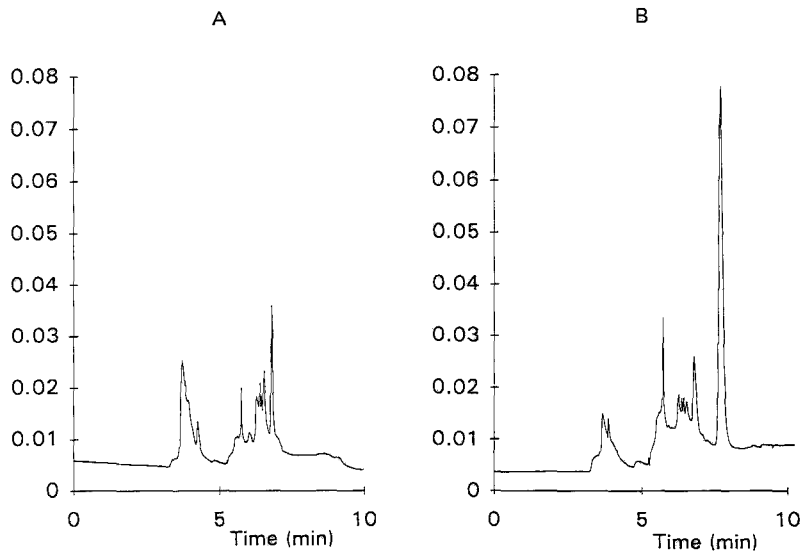

FIGURE 2. Chromatograms of (A) drug-free plasma and (B) plasma spiked with 15 μg/ml of cefodizime.

components in human plasma at the selected wavelenght. The sensitivity of the proposed method can be further improved by increasing the sample volume and it can therefore be used to analyse samples containing very low amounts of cefodizime with acceptable precision and recovery.

Linearity, precision and accuracy

To investigate the linearity of the procedure, blank plasma samples were spiked with amounts varying

TABLE 1

Precision, accuracy and linearity for cefodizime (spiked plasma)

Y=(39,58 ± 0,10) X + (-6,70 ± 3,33)

Nominal Accuracy (%) (µg/ml)	Actual value (Mean ± S.D., n=8) (µg/ml)	Precision (%)	Accuracy (%)
1	1.06 ± 0.04	3.77	6.00
5	4.72 ± 0.22	4.66	-5.60
10	10.39 ± 0.45	4.33	3.90
25	25.34 ± 0.76	3.00	1.36
50	49.1 ± 2.34	4.77	-1.80
100	102.34 ± 2.21	2.16	2.34
250	253.17 ± 4.01	1.58	1.27
400	394.45 ± 4.78	1.21	-1.39

from 1 to 400 µg/ ml of cefodizime (Table 1). The regression line obtained obeyed the equation: y = (39.58 ± 0.10) x + (- 6.70 ± 3.33), the correlation coefficient being r^2 = 0.998. Accuracy was between 1.27 and 6.0 % for the concentration range 1-400 µg/ml. Values for precision (coefficient of variation, C.V.) were between 4,77 and 1,21 % (Table 1).

Recovery and detection limit

Mean recoveries (± S.D.) of the investigated drug from spiked plasma samples (calculated by comparison

with standards dissolved in mobile phase 1) are shown in Table 1. As can be seen, the recovery of cefodizime was found to be 96% in plasma.

Using the criterion of minimum detectability as 3 times the system noise, the detection limit was 0.1 μg/ml using a 100-μl loop.

Application

The method described was applied to determine plasma concentrations of cefodizime after 2 g i.v. administration in 3 male patients. Plasma levels of the drug were fitted to a three compartment open model with elimination from the central compartment by means of an iterative, nonlinear least-squares technique. Plasma levels of cefodizime from a patient and the fitted curve are shown in Figure 3. The main pharmacokinetic parameters (means ± SD) were obtained by standard methods(6) and include: terminal half life ($t_{½\beta}$), area under the plasma concentration time curve (AUC), serum clearance (Cls), volume of distribution of the central compartment V_1), and mean residence time in the central compartment (MRT_1). Our results, summarized in Table 2, are in agreement with those previously reported (7).

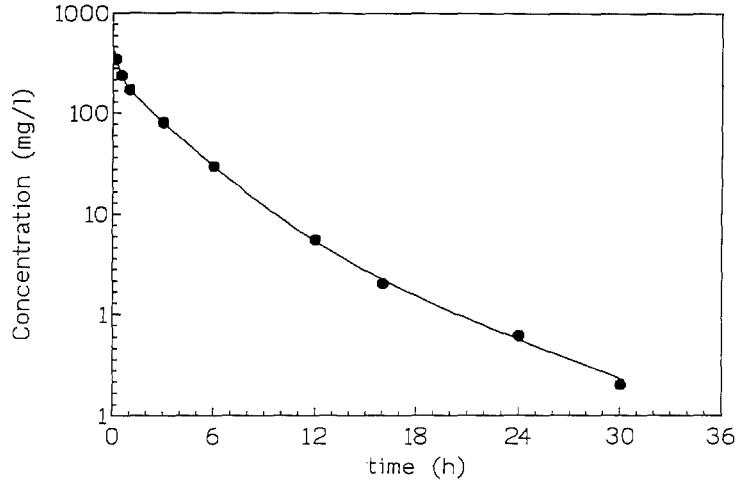

FIGURE 3. Plasma concentration-time curve of cefodizime in a patient after a 2 g i.v. dose.

TABLE 2

Pharmacokinetic parameters of cefodizime after intravenous administration of a 2 g dose (means ± SD).

$t_{1/2\beta}$ (h)	AUC (mg.h/L)	Cls (ml/min)	V_1 (L)	MRT_1 (h)
3.37	756.97	44.04	4.62	1.75
±1.02	±7.11	±0.41	±0.20	±0.09

CONCLUSIONS

In conclusion, a simple, sensitive, selective and validated HPLC method for the analysis of cefodizime in plasma has been developed. The sample preparation of the proposed method is extremely simple since it requires no extraction or clean-up steps, only dilution of the sample and direct injection onto the chromatographic column. Good precision and accuracy in plasma was achieved without the need for an internal standard. The assay is suitable for human pharmacokinetic studies.

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RAPID DETERMINATION OF PRAZOSIN IN PERFUSION MEDIA BY HPLC WITH SOLID PHASE EXTRACTION

A. J. FLETCHER[1], R. S. ADDISON[1]*, R. H. MORTIMER[1,2], AND G. R. CANNELL[1]

[1]*Conjoint Endocrine Laboratory*
Royal Brisbane Hospital
Herston Road
Brisbane, Australia 4029

[2]*Department of Obstetrics and Gynecology*
University of Queensland
Royal Brisbane Hospital, Herston
Brisbane, Australia 4029

ABSTRACT

A method is described for the quantitation of prazosin in tissue culture medium used for *in vitro* perfusion of human placental lobules. Prazosin was extracted using solid phase cartridges and the samples analysed by high performance liquid chromatography. The analysis utilised a C_{18} reversed-phase column maintained at 40°C with quantitation by fluorescence detection. The assay was linear to 100 ng/mL, intra-assay coefficients of variation measured at concentrations of 5 and 50 ng/mL were 5.8 and 6.2% respectively and inter-assay coefficients of variation were 4.9 and 2.7% for the same concentrations. The mean recovery of prazosin was 90.9 and 85.2% from solutions with concentrations of 5 and 50 ng/mL respectively. The minimum detectable limit was 0.1 ng/mL and the internal standard for this assay was propranolol. Analysis of a range of endogenous and

*To whom correspondence should be addressed

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exogenous compounds likely to be present in pregnancy plasma revealed only minor interference from lignocaine.

INTRODUCTION

Prazosin [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl)piperazine], a selective post-synaptic alpha adrenergic antagonist, has been prescribed in pregnancy for the treatment of hypertension (1). This drug lowers peripheral resistance and decreases vasoconstrictor tone present in hypertensive patients.

Prazosin has previously been quantitated by direct spectrofluorimetric analysis of samples (2-5) and by high performance liquid chromatographic (HPLC) assays using either ultraviolet or fluorimetric detection (6-17). The earlier direct fluorimetric measurement assays may be non-specific due to other fluorescent compounds in the matrix such as a reported metabolite of prazosin (13) whereas HPLC based techniques provide the specificity and sensitivity required to measure low concentrations of this drug. The majority of HPLC assays used solvent partitioning from alkaline plasma with ethyl acetate or another organic solvent followed by back extraction into dilute acid (6-8,10,12,13,15). One exception was dilution of plasma with acetonitrile to precipitate protein and analysis of an aliquot of supernatant (16). Solid phase extraction offers an alternative to the use and associated problems of disposal of large volumes of organic solvents as required by conventional liquid-liquid extraction procedures.

An assay for quantitation of prazosin in perfusion media has been developed as part of a study of metabolism and kinetics of maternofetal

transfer of antihypertensives in pregnancy. These studies utilise an *in vitro* perfused placental lobule model (18) with dosing of drug in either the maternal or fetal compartment. In this assay, prazosin and the internal standard (propranolol) are extracted directly from samples of tissue culture media from the perfusions using reversed-phase cartridges.

MATERIALS AND METHODS

Chemicals and Reagents

Prazosin and propranolol (internal standard) were obtained from Sigma Chemical Company (St Louis, MO, USA). Sep-pak cartridges (C_{18}) were obtained from Millipore Corporation (Milford, MA, USA) and methanol was of liquid chromatography standard (FSE, Homebush, Australia). All other chemicals were of analytical reagent grade. The perfusion fluid, as described by Cannell *et al.* (18), consisted of tissue culture medium M199 (Sigma Chemical Company) augmented with glucose (2 g/L), heparin (25 IU/mL), gentamicin (100 mg/L, David Bull Labs., Melbourne, Australia) and dextran (approximate molecular weight = 40000, 7.5 g/L, Sigma Chemical Company). All assay validation studies were conducted using fresh tissue culture media as a sample matrix.

HPLC Instrumentation

The HPLC system consisted of a Kortec model K35D pump (ICI Instruments, Sydney, Australia), a Rheodyne model 7125 injector

(Rheodyne, Cotati, CA, USA) fitted with a 100 μL sample loop and a Hitachi F1000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan). Samples were analysed on an Alltima C_{18} column (5 μm particle size, 250 mm x 4.6 mm ID, Alltech Associates Inc., Deerfield, IL, USA). The column was maintained at 40°C in a model TC1900 column oven obtained from ICI Instruments (Sydney, Australia).

HPLC Analysis

The mobile phase used was methanol (55%, v/v) in 0.05 M phosphate buffer (pH 5.8, final pH = 5.1) and was filtered through a 0.45 μm nylon membrane filter (Alltech). The flow rate was 1 mL/min and the fluorescence detector was set at an excitation wavelength of 280 nm and an emission wavelength of 395 nm.

Sample Extraction

Reversed-phase cartridges were primed by washing with methanol (10 mL) followed by water (10 mL). Internal standard (1 μg) was added to samples (1 mL) which were diluted with water (2 mL) before being applied to reverse-phase cartridges for extraction. The loaded cartridges were washed with water (10 mL) and excess water removed by air aspiration. Prazosin and internal standard were eluted from the cartridges with methanol (5 mL), collected in a silanised glass tube and solvent removed by a gentle stream of nitrogen at 40°C. The residue was reconstituted in mobile phase (50 μL), centrifuged for 5 minutes (700 g) and a 30 μL aliquot analysed by HPLC.

Standard Solutions

Stock solutions of prazosin and propranolol (1 mg/mL) were prepared in methanol. Standard prazosin solutions were diluted in perfusate to provide solutions for other validation studies.

Linearity

A solution of prazosin in perfusate was serially diluted to provide samples with concentrations ranging from 2.5 to 100 ng/mL. Duplicate aliquots (1 mL) were spiked with a constant amount (1 μg) of internal standard and were extracted and analysed as described above. Peak height ratios (prazosin to internal standard) were calculated and plotted as a function of increasing prazosin concentration.

Recovery

The recovery of prazosin was determined at concentrations of 5 and 50 ng/mL. Duplicate samples (1 mL) at each concentration were spiked with internal standard (1 μg), extracted using solid phase cartridges and analysed as described earlier. Peak height ratios (prazosin to internal standard) were determined and compared with those obtained by analysis of a corresponding set of nonextracted standards. The recovery of the internal standard, propranolol, was determined using this procedure at a concentration of 1 μg/mL.

TABLE 1

Drugs Tested for Interference in the Assay for Prazosin

Alcuronium	Hydrocortisone	Phenytoin
Aminophylline	Hyoscine	Prednisolone
Atenolol	Hyoscine-N-Butylbromide	Prednisone
Atropine	Labetolol	Promethazine
Betamethasone	Lignocaine	Propylthiouracil
Bupivicaine	Methimazole	Pyridoxine
Cortisone	Metoclopramide	Ranitidine
Dexamethasone	Noradrenaline	Salbutamol
Diazepam	Phenobarbitone	Verapamil
Diltiazem	L-Phenylephrine	

Specificity

A series of drugs and endogenous compounds (Table 1) were tested for potential interference with the analysis of either prazosin or the internal standard under the conditions of the assay. Stock solutions of each compound to be analysed were prepared in ethanol, the solvent evaporated and the residue dissolved in mobile phase. Retention times were then determined by HPLC analysis. Perfusate samples from six hour recirculating placental perfusion experiments, which did not use prazosin as substrate, were extracted and analysed to determine if any compounds from the tissue culture medium, or endogenous compounds released from the placental tissue during perfusion, would interfere in the assay.

Precision Studies

Intra-assay and inter-assay coefficients of variation were determined for samples with prazosin concentrations of 5 and 50 ng/mL. Five perfusate samples (1 mL) were extracted and analysed for intra-assay precision. This procedure was repeated over 5 days to determine inter-assay precision.

Minimum Detectable Limit

This value was defined as the concentration at which the ratio of sample response to that of the background was 2.0.

Human Placental Perfusion

A peripheral lobule of a term human placenta was perfused in dual recirculating mode as described previously (18). Prazosin was added to the maternal circulation (50 ng/mL final concentration) and samples (1.2 mL) were drawn from both maternal and fetal circulations at regular intervals during the perfusion period (5 hours). These samples were extracted and analysed for prazosin concentration.

RESULTS

HPLC analyses of tissue culture medium from 6 hour placental perfusions are illustrated in Figure 1, with chromatograms from analyses of

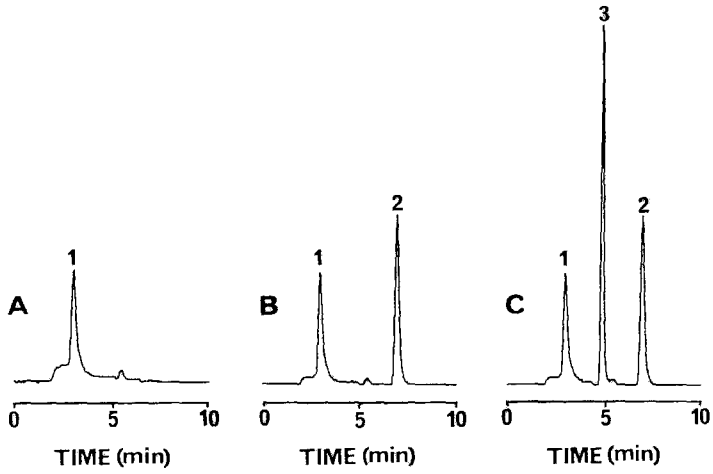

FIGURE 1. HPLC analysis of prazosin in tissue culture medium from 6 hour placental perfusions with (A) blank medium, (B) medium with internal standard and (C) medium with prazosin and internal standard.

blank medium, medium containing internal standard and medium with prazosin and internal standard. Prazosin and the internal standard, propranolol, eluted at 4.9 and 7.1 minutes respectively and were baseline separated under the conditions of the assay.

The mean recoveries of prazosin using solid phase extraction, at concentrations of 5 and 50 ng/mL, were 90.9 and 85.9% respectively, while the recovery of the internal standard, propranolol, was 70.5% at a concentration of 1 μg/mL. The response was linear in the range 2.5 to 100 ng/mL with a typical calibration curve giving an equation of $y = 0.0178x + 0.0573$; $r = 0.995$, where y = peak height ratio of prazosin to internal standard, x = concentration (ng/mL) of prazosin and r = correlation coefficient..

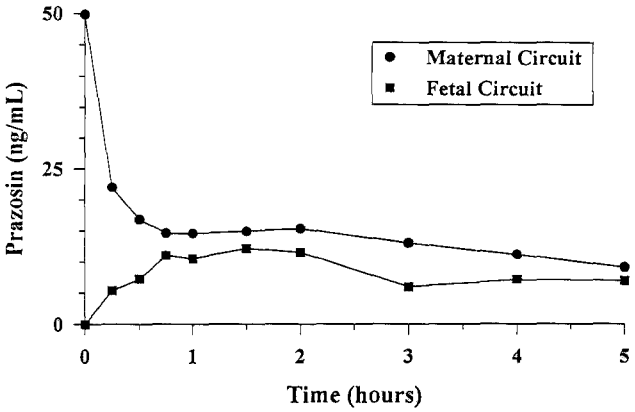

FIGURE 2. Prazosin transfer from the maternal to the fetal compartment following a 5 hour placental perfusion with dosing of prazosin in the maternal compartment.

The assay was reproducible with intra–assay and inter–assay coefficients of variation respectively of 5.8 and 4.9% for samples with a prazosin concentration of 5 ng/mL and 6.2 and 2.7% for samples with a prazosin concentration of 50 ng/mL. The compounds listed in Table 1 were analysed under the conditions of the assay for interference due to coelution with prazosin or the internal standard. No compounds were found which interfered in the assay except for lignocaine which coeluted with prazosin, however the interference was not significant due to the low fluorescence response for lignocaine at therapeutic concentrations, at the wavelengths used in this assay.

The assay was applied to the analysis of prazosin transfer from the maternal to the fetal compartment of a human placental lobule perfused *in vitro* following dosing of prazosin in the maternal circuit. Samples were analysed at intervals during a five hour perfusion and the concentration of prazosin in both circuits is shown in Figure 2.

DISCUSSION

This assay utilises fluorescence detection with an excitation wavelength of 280 nm and emission intensity monitored at 395 nm. These values were chosen by experiment with samples from 6 hour perfusions, with and without prazosin as substrate, to minimise interference and maximise sensitivity for extracts from this tissue culture matrix. The combinations for other assays for prazosin extracted from plasma have generally used excitation and emission wavelengths in the ranges 243 to 258 and 370 to 390 nm respectively (6–18). The limit of detection for the assay was 0.1 ng/mL, which compares favourably with the 0.1–2 ng/mL limits described for other HPLC assays using fluorescence as the detection method (6–10).

Prazosin and propranolol were extracted from the sample matrix using reversed-phase cartridges with reproducible and high mean recoveries for prazosin of 90.9% (5 ng/mL) and 85.9% (50 ng/mL) and 70.5% (1 μg/mL) for propranolol. This technique was rapid and large numbers were able to be processed using an extraction manifold. Prazosin has commonly been extracted with chloroform or ethyl acetate from plasma or serum made basic with potassium or sodium hydroxide. The organic phase is then extracted with dilute sulphuric acid and the drug extracted back into the organic phase and isolated following evaporation of the solvent. These time consuming liquid extraction techniques, with associated problems of disposal of organic solvents, have recovered prazosin with efficiencies in the range 44 to 99% (6–8, 10, 12, 13, 15). Acetonitrile was used in one report for simple protein precipitation of samples with essentially no drug loss, with an aliquot of the supernatant being analysed directly (16). Methanol was used in a similar manner in another report, however no quantitation of recovery was stated by these workers (14). Kelly *et al.* (19), in an assay for ondansetron

enantiomers from plasma using prazosin as the internal standard, employed solid phase extraction using cyanopropyl columns, while Jackman *et al.* (20) used C_{18} columns to extract the prazosin–related α–adrenoreceptor antagonist, doxazosin, from plasma with 75% efficiency.

Prazosin eluted in 6.2 minutes and the internal standard in 7.9 minutes (Figure 1) and the assay was linear in the range 2.5 to 100 ng/mL, thus covering the low concentrations which may be encountered in tissue perfusion experiments designed for kinetic or metabolic studies. Quantitation of prazosin was reproducible with intra–assay and inter–assay co–efficients of variation of 5.8 and 4.9% (5 ng/mL) and 6.2 and 2.7% (50 ng/mL) respectively. No endogenous compounds from either the sample matrix, compounds leached or released from the placental lobule, or other drugs interfered with the assay (Table 1). Although lignocaine coeluted with propranolol, no significant interference was recorded at therapeutic concentrations of this agent. The perfusion fluid collected at the beginning of the perfusion experiments from both the maternal and fetal circulations is discarded (19), thus minimising or eliminating any potential interferences from drugs present in the maternal or fetal blood.

The technique has been applied to a representative perfusion of prazosin in the *in vitro* perfused placental lobule with quantitation of drug levels in both the maternal and fetal circulations following dosing of prazosin in the maternal circulation (Figure 2). Prazosin equilibrated between the maternal and fetal compartments within 45 minutes illustrating that a proportional fraction of therapeutic doses of drug given to the mother would be rapidly transferred across the placenta to the fetal compartment.

CONCLUSION

This assay is simple to perform, sensitive and reproducible and utilizes a rapid solid phase extraction technique, thereby eliminating the need for use and subsequent disposal of organic solvents. The assay is suitable for the study of prazosin disposition in perfusion experiments in placental lobules or other organs.

ACKNOWLEDGMENTS

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(14), 2925 (1995)

ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC
and
HPLC SYSTEM TROUBLESHOOTING

One-Day & Two-Day
In-House Training Courses

The courses, which are offered for presentation at corporate laboratories, are aimed at chemists and technicians who work with HPLC. They cover HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC module and system problems.

The following topics are covered in depth:

- Introduction to HPLC Theory
- Modes of HPLC Separation
- Developing and Controlling Resolution
- Mobile Phase Selection & Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration & Quantitation
- Logical HPLC Troubleshooting

The instructor for the courses, Dr. Jack Cazes, is Editor-in-Chief of the Journal of Liquid Chromatography, of Instrumentation Science & Technology journal, and of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 30 years; he pioneered the development of modern HPLC technology. Dr. Cazes was also Professor-in-Charge of the ACS short course and the ACS audio course on Gel Permeation Chromatography for many years.

Details of these in-house courses may be obtained from Dr. Jack Cazes, Post Office Box 2180, Cherry Hill, NJ 08034-0162, USA; Tel: (609) 424-3505; FAX: (609) 751-8724.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(14), 2927-2932 (1995)

ANNOUNCEMENTS

QUALITY AUDITING
for the
INDUSTRIAL LABORATORY

A One-Day Conference

Monday, Feb. 5, 1996 - Saddle Brook, NJ
Wednesday, Feb. 7, 1996 - Chicago
Friday, Feb. 9, 1996 - San Francisco

This timely conference will provide practical guidance for the quality auditing of chemical and biological laboratories, detailing how to develop and improve quality control and quality assurance programs to meet certification standards and requirements, and to easily pass external-source audits.

The conference is aimed at laboratory directors, administrators, managers, supervisors and analysts, as well as quality auditors responsible for QC and QA at analytical and microbiological laboratories serving pharmaceutical, food, cosmetic and biotechnology industries.

The conference will include

* The Need for QC and QA in the Laboratory
* The Regulated Industries
* Goals of a Laboratory Quality Audit
* Working Knowledge & Audit Documents
* Parameters for Evaluation

* Laboratory Instrumentation: Calibration & Preventable Maintenance
* Final Report, Followup & Frequency of Audits

The lecturers, Donald C. Singer (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) and Ronald P. Upton (Independent Consultant, Newton, NC) are eminently qualified to lead this conference. Mr. Singer is Chairman of the ASQC's Technical Media Committee and he has over 15 years experience in establishing and maintaining laboratory QC practices for various industries; Dr. Upton has had more than 20 years experience in quality control and reliability assurance in the pharmaceutical industry.

Further details may be obtained from Marcel Dekker Professional Conferences, 270 Madison Avenue, New York, NY 10016; Tel.: (800) 203-8782; FAX: (212) 685-4540.

BIOTECHNOLOGY LAW
for the
NON-LAWYER

A One-Day Conference

Monday, Oct. 30, 1995 - San Francisco
Wednesday, Nov. 1, 1995 - Research Triangle
Friday, Nov. 3, 1995 - Boston

These timely and informative conferences will cover the intellectual property aspects of biotechnology law, from initial identification & reporting through licensing. The conferences will clearly and, in *jargon-free language*, explain the rules, regulations and procedures typically encountered by researchers in the development of their innovations.

Designed specifically for the non-legal audience, ***Biotechnology Law for the Non-Lawyer*** is essential for industrial scientists, engineers and managers in R&D facilities who must understand the fundamentals of evolving law relating to developments in the field of biotechnology.

The conferences will include

* Identifying, Evaluating and Reporting Innovative Research Developments
* Choosing the Form of Legal Protection
* Preparing the Patent Application
* Inventorship in the Research Laboratory
* Ownership of Laboratory Discoveries & Work Product
* Patent Infringement in Biotechnology
* Intellectual Property Policies, Research Agreements & Conflicts of Interest
* Technology Transfer and Commercialization

The lecturers, Gale R. Peterson (Cox & Smith, Inc., San Antonio, Texas) and Robert A. Armitage (Vinson & Elkins L.L.P., Washington, DC) are eminently qualified to lead these conferences.

Further details and registration information may be obtained from Marcel Dekker Professional Conferences, 270 Madison Avenue, New York, NY 10016; Tel: (800) 203-8782; FAX: (212) 685-4540.

ISO-9000
PRINCIPLES & PRACTICE

A One-Day Conference

Monday, Jan. 29, 1996 - Los Angeles
Wednesday, Jan. 31, 1996 - Chicago
Friday, Feb 2, 1996 - Saddle Brook, NJ

Stressing the basic principles and application of the International Standards Organization (ISO) 9000 registration, this cutting-edge conference answers the most asked questions regarding the organization, documentation and implementation of a quality assurance system based on the ISO-9000 (ANSI/ASQC) series of quality standards.

This conference is designed specifically for executive officers, directors, administrators and managers in industry who are responsible for selecting and setting a quality standards system that will enable their companies to compete in world markets and achieve quality in a cost-effective manner.

The conference will include

* General Introduction to Quality Standards
* Quality System Requirements
* Third Party Assessments; Implementation Strategy; Documentation Overview
* ISO in the Service, Software & Environmental Industries
* ISO and BS 7750; ISO and Other Quality Systems
* The Future of ISO

The lecturer, Dr. D. H. Stamatis, is President of Contemporary Consultants, Southgate, Michigan. He has over 25 years of experience in management and quality training/consulting in the private sector, government and academia and has written and lectured extensively in the field.

Further details and registration information may be obtained from Marcel Dekker Professional Conferences, 270 Madison Avenue, New York, NY 10016; Tel: (800) 203-8782; FAX: (212) 685-4540.

ACCOUNTING & FINANCE FOR ENGINEERS

A One-Day Conference

Monday, Jan. 8, 1996 - San Francisco
Wednesday, Jan. 10, 1996 - Atlanta
Friday, Jan. 12, 1996 - Saddle Brook, NJ

This premier conference stresses the basic principles and applications of accounting and finance in a manner that is particularly well suited to the needs of non-accountants. Fundamental concepts and techniques of the subject will be discussed in detail.

The conference is aimed at scientists and engineers, in managerial and non-managerial positions who need to understand cost concepts and financial management of the work they do for their companies.

The conference will include

* Understanding Financial Statements
* Cost Concepts
* Cost Behavior
* Cost Accounting
* Cost-Volume-Profit Analysis and Leverage
* Applying the Time Value of Money
* Evaluating Capital Expenditure Projects

The lecturer, Dr. Jae K. Shim, is Professor of Accounting & Finance at the California State University in Long Beach. As an outstanding educator and consultant, he has published numerous articles in academic and professional journals. He has more than thirty college and professional books to his credit, including *Managerial Accounting; Financial Accounting; The Vest-Pocket CPA; The Vest Pocket CFO; Barron's Accounting Handbook; Encyclopedia of Accounting and Finance;* and others.

Further details and registration information may be obtained from Marcel Dekker Professional Conferences, 270 Madison Avenue, New York, NY 10016; Tel.: (800) 203-8782; FAX: (212) 685-4540.

SYSTEM VALIDATION CONCERNS
and the
U.S. FOOD & DRUG ADMINISTRATION

A One-Day Conference

Monday, March 18, 1996 - Philadelphia
Wednesday, March 20, 1996 - Chicago
Friday, March 22, 1996 - San Francisco

Control is the key issue in the regulated world of pharmaceuticals. Validation is the ability to demonstrate that control through the ability to

access appropriate records of tests, procedures and the data underlying results.

This cutting-edge conference is aimed at scientists, engineers, managers, administrators in the pharmaceutical industries who need to understand and comply with government regulation of the industry.

The seminar will focus on

* System Validation Principles
* USFDA Requirements and Guidelines
* Background to the Validation Debate
* Definitions and Terminology
* System Life Cycle for the Regulated System
* Testing the System: Principles, Terms & Strategies
* Case Study

The lecturer, Dr. Sandy Weinberg is Chairman of Weinberg, Spelton & Sax, Inc., and is responsible for Research and Development and Regulatory Affairs Publications. He has worked with a number of world-class companies. Dr. Weinberg is Research Professor of Management Information Systems, Director of the Center for Computing in Health Care and a member of the Institute for Biomedical Engineering & Science at Drexel University. He has authored seven books and more than 100 academic and professional articles, and he writes regular columns for several magazines and journals.

Further details and registration information may be obtained from Marcel Dekker Professional Conferences, 270 Madison Avenue, New York, NY 10016; Tel.: (800) 203-8782; FAX: (212) 685-4540.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(14), 2933-2941 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

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

SEPTEMBER 7 - 8: Engineering & Construction Contracting Conference, Phoenician Resort, Scottsdale, Arizona. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA

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

SEPTEMBER 18 - 21: Safety in Ammonia Plants & Related Facilities, Loews Ventana Canyon, Tucson, Arizona. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

SEPTEMBER 26 - 29: CCPS International Conference on Modelling & Mitigating the Consequences of Accidental Releases of Hazardous Materials, Fairmont Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

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

OCTOBER 23 - 25: 6th Annual Frederick Conference on Capillary Electrophoresis, Hood College, Frederick, Maryland. Contact: Margaret L. Fanning, Conference Coordinator, PRI, NCI-FCRDC, P. O. Box B, Frederick, MD 21702-1201

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

OCTOBER 30: Biotechnology for the Non-Lawyer - A One-Day Seminar, San Francisco, California. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

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

NOVEMBER 1: Biotechnology for the Non-Lawyer - A One-Day Seminar, Research Triangle Park, North Carolina. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

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

NOVEMBER 2: Anachem Symposium, Dearborn, Michigan. Contact: Prof. C. Evans, University of Michigan, Chem Dept, 4807 Chemistry Bldg, Ann Arbor, MI 48109-1055, USA.

NOVEMBER 3: Biotechnology Law for the Non-Lawyer - AS One-Day Seminar, Boston, Massachusetts. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

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

NOVEMBER 12 - 17: AIChE Annual Meeting, Fontainbleu Hotel, Miami Beach, Florida. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

NOVEMBER 14 - 16: Kemia'95: Finnish Chemical Congress, Helsinki Fair Center, Helsinki, Finland. Contact: The Associatioon of Finnish Chemical Societies, Hietaniemenkatu 2, FIN-00100 Helsinki, Finland.

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

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

1996

JANUARY 8: Accounting & Finance for Engineers - A One-Day Seminar, San Francisco, California. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

JANUARY 10: Accounting & Finance for Engineers - A One-Day Seminar, Atlanta, Goergia. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

JANUARY 12: Accounting & Finance for Engineers - A One-Day Seminar, Saddle Brook, New Jersey (New York Area), Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

JANUARY 29: ISO-9000: Principles & Practice - A One-Day Seminar, Los Angeles, California. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

JANUARY 31: ISO-9000: Principles & Practice - A One-Day Seminar, Chicago, Illinois. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

FEBRUARY 2: ISO-9000: Principles & Practice - A One-Day Seminar, Saddle Brook, New Jersey (New York Area). Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

FEBRUARY 5 - 7: PrepTech'96, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. R. Stevenson, 3338 Carlyle Terrace, Lafayette, CA 94549, USA.

FEBRUARY 5 : Quality Auditing for the Industrila Laboratory - A One-Day Seminar, Saddle Brook, New Jersey. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

FEBRUARY 7: Quality Auditing for the Industrial Laboratory - A One-Day Seminar, Chicago, Illinois. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

FEBRUARY 9: Quality Auditing for the Industrial Laboratory - A One Day Seminar, San Francisco, California. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

FEBRUARY 25 - 29: AIChE Spring National Meeting, Sheraton Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

MARCH 3 - 8: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 18: System Validation Concerns and the U. S. Food & Drug Administration, Philadelphia, Pennsylvania. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

MARCH 20: System Validation Concerns and the U. S. Food & Drug Administration - A One-Day Seminar, Schaumburg, Illinois (Chicago Area). Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

MARCH 22: System Validation Concerns and the U. S. Food & Drug Administration - A One-Day Seminar, San Francisco, California. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

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

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

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

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

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San

Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JULY 14 - 18: 5th World Congress of Chemical Engineering, Marriott Hotel, San Diego, California. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 9 - 14: 31st Intersociety Energy Conversion Engineering Conference (co-sponsored with IEEE), Omni Shoreham Hotel, Washington, DC. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 17 - 20: 31st National Heat Transfer Conference, Westin Galleria, Houston, Texas. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

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

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization," Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

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

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

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

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

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

1997

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

APRIL 14 - 19: Genes and Gen Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

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

1998

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

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

1999

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

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

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

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

2001

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

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

2002

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

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

The **Journal of Liquid Chromatography** will publish, at no charge, announcements of interest to scientists in every issue of the journal. To be listed in Meetings & Symposia, we will need to know:

a) Name of the meeting or symposium,

b) Sponsoring organization,

c) When and where it will be held, and

d) Whom to contact for additional details.

Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P.O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

INSTRUCTIONS TO AUTHORS

Journal of Liquid Chromatography is published in the English language for the rapid communication of research in liquid chromatography and its related sciences and technologies.

Directions for Submission

One typewritten manuscript, suitable for direct reproduction, and two (2) clear copies with figures must be submitted. Since the Journal is produced by direct photography of the manuscripts, typing and format instructions must be strictly followed. Non-compliance will result in return of the manuscript to the author and will delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards before mailing.

Manuscripts may also be submitted on **computer diskettes**. A printed manuscript must also be submitted with diskettes because, at the present time, we are experimenting with manuscripts on diskettes. Diskettes must be readable with an IBM-compatible computer (Macintosh or other type not acceptable) and must be formatted with MS-DOS 3.1 or greater. Be sure to indicate the word processing software that was used to prepare the manuscript diskette.

Manuscripts and computer diskettes should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 2180
Cherry Hill, NJ 08034

Reprints

Due to the short production time for papers in this journal, it is essential to order reprints immediately upon receiving notification of acceptance of the manuscript. A reprint order form will be sent to the author with the letter of acceptance for the manuscript. Reprints are available in quantities of 100 and multiples thereof. Twenty (20) free reprints will be included with orders of 100 or more reprints.

Format of the Manuscript

1. The general format of the manuscript should be:
   Title
   Author(s)' names and full addresses
   Abstract
   Text Discussion
   References

2. **Title & Authors:** The entire title should be in capital letters and centered within the width of the typing area, located at least 2 inches (5.1 cm) from the top of the page. This should be followed by 3 lines of space, then by the names and addresses of the authors, also centered, in the following manner:

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN WATER BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

3. **Abstract:** The title **ABSTRACT** should be typed, capitalized and centered, 3 lines below the addresses. This should be followed by a **single-spaced**, concise abstract. Allow 3 lines of space below the abstract before beginning the text of the manuscript.

4. **Text Discussion:** Whenever possible, the text discussion should be divided into major sections such as

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGEMENTS
REFERENCES

These **major headings** should be separated from the text by two lines of space above and one line of space below. Each major heading should be typed in capital letters, centered and underlined.

Secondary headings, if any, should be placed flush with the left margin, underlined and have the first letter of main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. The first word of each **paragraph** within the body of the text should be indented five spaces.

6. **Acknowledgements**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

7. **References** should be numbered consecutively and placed in a separate section at the end of the manuscript. They should be typed single-spaced, with one line space between each reference. Each reference should contain names of all authors (with initials of their first and middle names); do not use *et al.* for a list of authors. Abbreviations of journal titles will follow the American Chemical Society's Chemical Abstracts List of Periodicals. The word **REFERENCES** should be capitalized and centered above the reference list.

Following are acceptable reference formats:

Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18: 1979-1998 (1985)

Book:

1. L. R. Snyder, J. J. Kirkland, Introduction to Modern Liquid Chromatography, John Wiley & Sons, Inc., New York, 1979.

2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page.

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used.

10. Material that cannot be typed, such as Greek symbols, script letters and structural formulae, should be drawn carefully with dark black India ink. Do not use any other color ink.

Additional Typing Instructions

1. The manuscript must be prepared on good quality **white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). The typing area of the first page, including the title and authors, should be 5½ inches wide by 7 inches high (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches high (14 cm x 21.6 cm).

2. The **title, abstract, tables and references** are typed single-spaced. All other text should be typed 1½-line spaced or double line spaced.

3. It is essential to use **dark black** typewriter or printer ribbon so that clean, clear, **solid characters** are produced. Characters produced with a dot/matrix printer are not acceptable, even if they are "near letter quality" or "letter quality." Erasure marks, smudges, hand-drawn corrections and creases are not acceptable.

4. **Tables** should be typed on separate pages, one table to a page. A table may not be longer than one page. If a table is larger than one page, it should be divided into more than one table. The word **TABLE** (capitalized and followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

5. **Figures (drawings, graphs, etc.)** should be professionally drawn in **black** India ink on separate sheets of **white** paper, and should be placed at the end of the text. They should not be inserted in the body of the text. They should not be reduced to a small size. Preferred size for figures is from 5 inches x 7 inches (12.7 cm x 17.8 cm) to 8½ inches by 11 inches (21.6 cm x 27.9 cm). **Photographs** should be professionally prepared *glossy* prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn.

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