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STUDIES ON NEUROSTEROIDS. II. RETENTION BEHAVIOR OF DERIVATIZED 20-OXOSTEROIDS AND THEIR SULFATES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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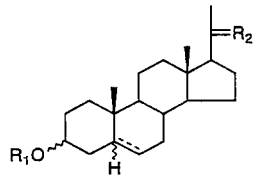
ABSTRACT

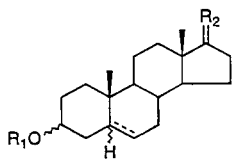
The retention behavior of five 20-oxosteroids (pregnenolone, 3 β - hydroxy- 5 α -pregnan-20-one, 3 β -hydroxy- 5 β - pregnan-20-one, 3 α -hydroxy- 5 α -pregnan-20-one, and 3 α - hydroxy-5 β -pregnan-20-one) and their sulfates derivatized with 4-(*N,N*-dimethylaminosulfonyl)- 7-hydrazino-2,1,3-benzoxadiazole were examined using reversed-phase high-performance liquid chromatography with fluorescence detection. Inclusion chromatography using cyclodextrin as a mobile phase additive was also used for this purpose and found effective in separating the isomeric derivatized 20-oxosteroids and their sulfates.

INTRODUCTION

In the previous paper in this series, we have clarified the retention behavior of five 17-oxosteroids derivatized with a fluorogenic reagent, 5-dimethylamino-1-naphthalenesulfonic hydrazide ($DNSNHNH_2$) or 4- (*N, N* - dimethylaminosulfonyl) - 7 - hydrazino- 2, 1, 3 - benzoxadiazole (DBDH) using reversed-phase HPLC and found that inclusion chromatography using cyclodextrin (CD) as a mobile phase additive was effective for the separation of these derivatives [1]. These data are helpful in establishing the method of determination of 17-oxosteroids (kinds of neurosteroids found in the mammalian brain) using HPLC with fluorescence detection as described in the previous paper [1]. The derivatization rate of 17-oxosteroids with DBDH was higher than that with $DNSNHNH_2$ (data not shown), and several 20-oxosteroids and sulfates of 17- and 20-oxosteroids were also found in the mammalian brain [2]. These data prompted us to clarify the retention behavior of 20-oxosteroids and their sulfates derivatized with DBDH.

In this paper, the retention behavior of five 20-oxosteroids [pregnenolone (I a), 3 β -hydroxy-5 α -pregnan-20-one (II a), 3 α - hydroxy-5 α -pregnan-20-one (III a), 3 α - hydroxy-5 β - pregnan-20-one (IV a), and 3 β -hydroxy-5 β -pregnan-20-one (V a)] derivatized with DBDH is examined using reversed-phase HPLC with fluorescence detection. Those of 20- and 17-oxosteroid sulfates (I - X b) derivatized with DBDH are also examined (Fig. 1). Inclusion chromatography using cyclodextrin (CD) as a mobile phase additive is also used for this purpose.

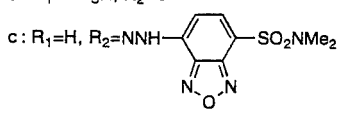




I : 3β-OR_1, Δ5
II : 3β-OR_1, 5α-H
III : 3α-OR_1, 5α-H
IV : 3α-OR_1, 5β-H
V : 3β-OR_1, 5β-H

VI : 3β-OR_1, Δ5
VII : 3β-OR_1, 5α-H
VIII : 3α-OR_1, 5α-H
IX : 3α-OR_1, 5β-H
X : 3β-OR_1, 5β-H

a : R_1=H, R_2=O
b : R_1=SO_3H, R_2=O



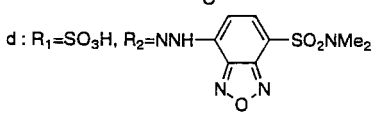


Figure 1. Structures of examined oxosteroids and their derivatives

MATERIALS AND METHODS

Materials

β- and γ-CDs were kindly supplied by Nihon Shokuhin Kako (Tokyo, Japan). Heptakis-(2,6-di-O-methyl)-β-CD (Me-β-CD; 10.5 methyl residues/mol) was prepared and donated by Kao (Tokyo). 20-Oxo- (I-Va) and 17-oxo-steroids (VI-Xa) were kindly donated by Teikoku Hormone Mfg. (Tokyo). The sulfates (I-Xb) of the above oxosteroids were synthesized in our

laboratory in the usual way using a chlorosulfonic acid/pyridine complex. DBDH was purchased from Tokyo Kasei Kogyo (Tokyo).

Derivatization Procedure

The derivatization of the oxosteroids (I - X a) and their sulfates (I - X b) with DBDH has been done using previously described procedures [1,3] to give the corresponding derivatives (I - X c,d).

Apparatus

HPLC was carried out using a JASCO TRI ROTAR chromatograph equipped with an Hitachi F-1000 fluorescence (FL: λ ex. 450 nm, λ em. 550 nm)(Hitachi, Tokyo) detector. A TSKgel ODS 80 TM (5 μm) column (15 cm x 0.46 cm i.d.)(TOSOH, Tokyo) was used at ambient temperature at a flow rate of 1 ml/min, and the void volume was measured with MeOH (λ ex. 280 nm, λ em. 320 nm). The pH of the mobile phase containing KH_2PO_4 was adjusted with H_3PO_4.

RESULTS AND DISCUSSION

Retention Behavior of DBD-20-oxosteroids

The separation of five DBD-20-oxosteroids (I-Vc) *via* reversed-phase HPLC using MeOH or MeCN as an organic modifier was examined, but satisfactory separation has not been done as shown in Fig. 2a,b. These data prompted us to try inclusion chromatography using CD as the mobile phase additive for the separation of these derivatives. The effect of γ - and Me-β-CD on the relative capacity factor (Rk') of these derivatives is shown in Fig. 3. The former host compound was more effective than the

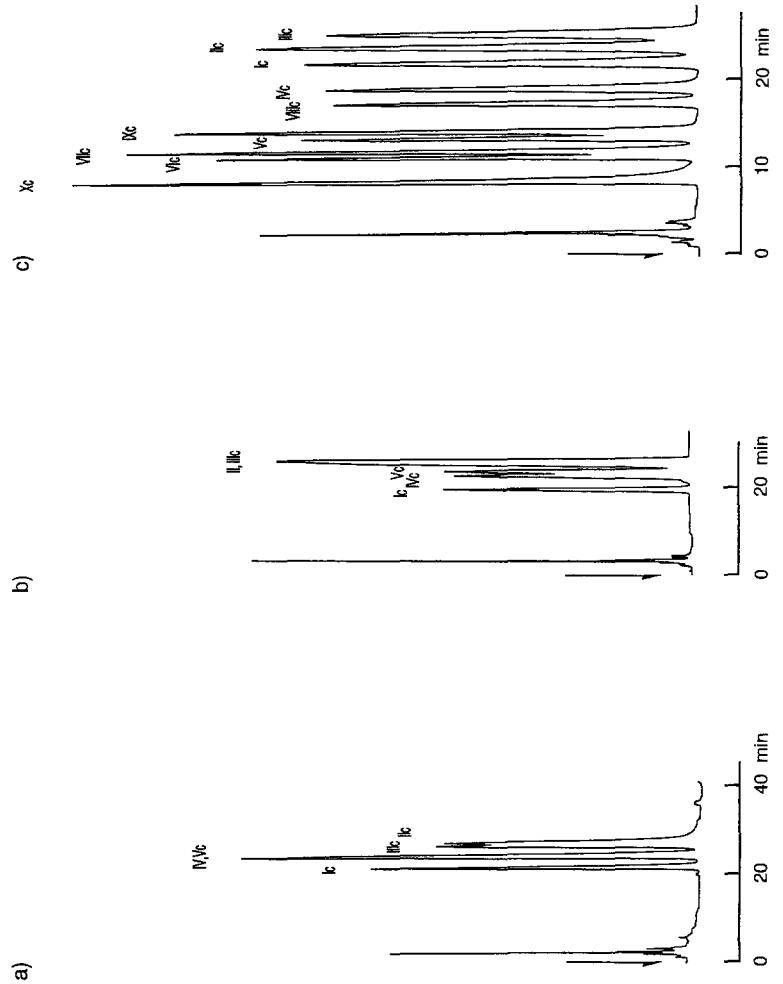


Figure 2. Separation of DBD-20-oxosteroids
Conditions: mobile phase, a) MeOH-H_2O (7:2)
b) MeCN-H_2O (2:1) c) MeOH-H_2O (3:1) containing γ-CD (4mM).

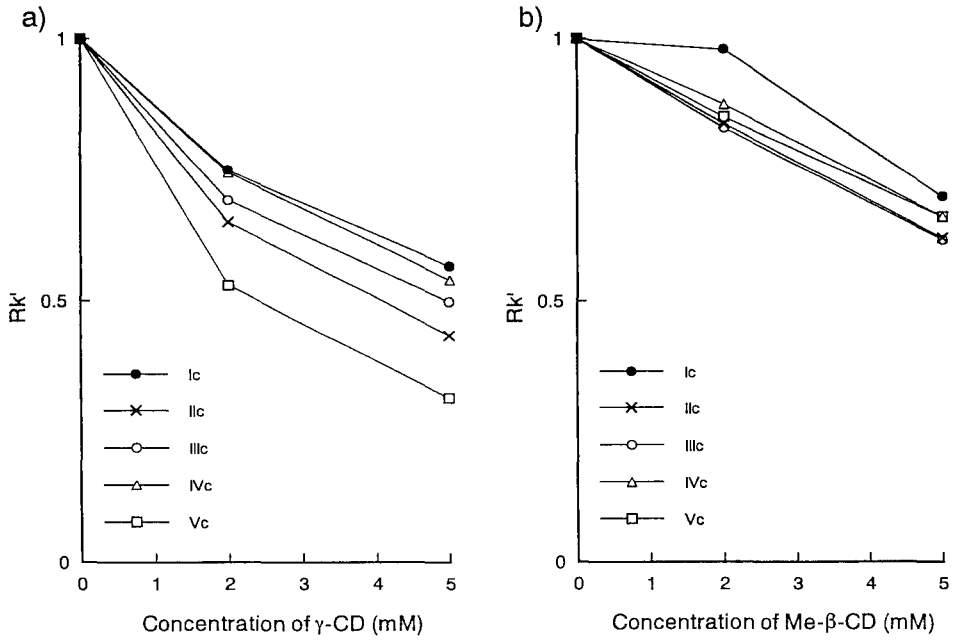


Figure 3. Effect of CD on the retention of DBD-20-oxosteroids
Conditions: mobile phase, MeOH-H_2O (a; 7:2 b; 4:1) containing CD as indicated. The k' values obtained without CD, taken as 1.0 for the calculation of Rk' value, are as follows: a) Ⅰc 12.8 Ⅱc 14.1 Ⅲc 15.9 Ⅳc 15.3 Ⅴc 13.5; b) Ⅰc 8.8 Ⅱc 9.6 Ⅲc 11.1 Ⅳc 10.8 Ⅴc 9.5. t_0 1.7 min.

latter, and the Rk' of Vc (3 β, 5 β -isomer) was decreased more by the addition of γ - CD in the mobile phase (Fig. 3a). These data are compatible with the previously obtained data on cardiac steroids [4] and derivatized 17-oxosteroids [1], that is, γ - CD is remarkably more effective than the other CD in decreasing the k' values of compounds having an A/B cis ring junction and a 3 β -hydroxy group. Satisfactory separation of DBD-20-oxosteroids together with DBD-17-oxosteroids was done by the addition of γ -CD in the

mobile phase using MeOH as an organic modifier, and the chromatogram is shown in Fig. 2c.

Retention Behavior of DBD-20-oxosteroid Sulfates

The use of MeOH or MeCN as an organic modifier together with 0.25% KH_2PO_4 (pH 3.0) as an ion suppressor each gave a single symmetrical peak but did not give satisfactory separation of the five DBD-20-oxosteroid sulfates as shown in Fig. 4a, b. The effect of γ - and Me-β-CD on the Rk' of these derivatives is shown in Table 1. The latter host compound was more effective than the former, and the Rk' of IVd (3α, 5β-isomer) was decreased more by the addition of this host compound in the mobile phase. The same phenomena were also observed when MeOH was used as an organic modifier (data not shown). These data are incompatible with those obtained with unconjugated derivatized 17- or 20-oxo-steroids as shown above. The complete separation of five derivatives was then obtained as shown in the chromatogram (Fig. 4c). All of the above data prompted us to examine the retention behavior of DBD-17-oxosteroid sulfates (Table 2). Me-β-CD was more effective than γ - CD, and the Rk' of IXd (3α, 5β - isomer) was decreased more by the addition of Me-β-CD in the mobile phase. These phenonena are compatible with those obtained with DBD-20-oxosteroid sulfates. Satisfactory separation is shown in Fig. 5.

Conclusions

In order to establish the determination method for neurosteroids, the chromatographic behavior of derivatized 20-oxosteroids and their sulfates has been examined with reversed-phase HPLC

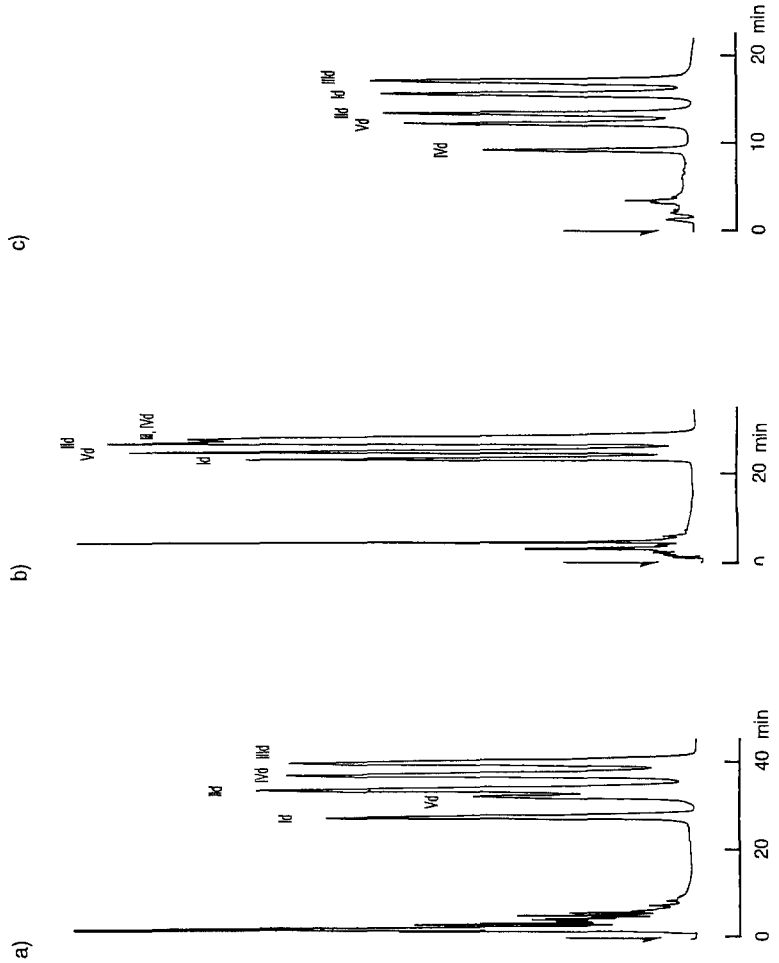


Figure 4. Separation of DBD-20-oxosteroid sulfates
Conditions: mobile phase, a) MeOH-0.25% KH_2PO_4 (pH 3.0) (5:2)
b) MeCN-0.25% KH_2PO_4 (pH 3.0) (9:10) c)MeCN-0.25% KH_2PO_4
(pH 3.0) (9:10) containing Me- β -CD (2 mM).

Table 1. Effect of CD on the Rk' value of DBD-20-oxosteroid sulfates

		Rk' [a]			
		γ - CD [b]		Me-β-CD	
	k' [c]	2 mM	4 mM	2 mM	5 mM
Id	10.2	0.89	0.79	0.71	0.47
IId	11.9	0.80	0.66	0.52	0.29
IIId	12.2	0.84	0.72	0.66	0.41
IVd	12.2	0.62	0.41	0.34	0.17
Vd	11.1	0.63	0.45	0.51	0.28

Conditions: mobile phase, MeCN-0.25% KH_2PO_4 (pH 3.0) (1:1) containing CD as indicated.
a) The k ' value obtained without CD was taken as 1.0 . b) Due to its solubility, the experiment with 5 mM has not been done. c) The k ' value obtained without CD. t_0 1.4 min.

Table 2. Effect of CD on the Rk' value of DBD-17-oxosteroid sulfates

		Rk' [a]			
		γ - CD [b]		Me-β-CD	
	k' [c]	2 mM	3 mM	2 mM	5 mM
VId	10.8	0.94	0.81	0.73	0.48
VIId	12.5	0.86	0.70	0.52	0.31
VIIId	15.9	0.92	0.76	0.66	0.42
IXd	16.9	0.71	0.53	0.39	0.21
Xd	12.9	0.69	0.52	0.55	0.32

Conditions: mobile phase, MeCN-0.25% KH_2PO_4 (pH 3.0) (4: 5) containing CD as indicated.
a) The k ' value obtained without CD was taken as 1.0 . b) Due to its solubility, the experiment with 5 mM has not been done. c) The k ' value obtained without CD. to 1.8 min.

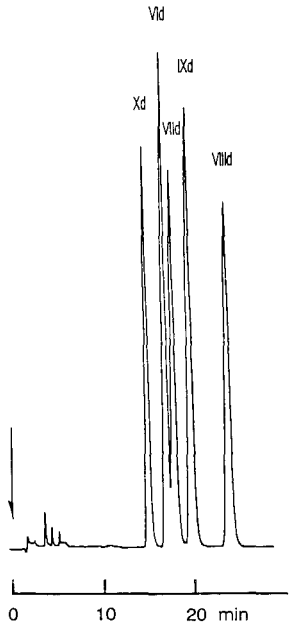


Figure 5. Separation of DBD-17-oxosteroid sulfates
Conditions: mobile phase, MeCN-0.25% KH_2PO_4 (pH 3.0) (4:5) containing γ -CD (2 mM).

including inclusion chromatography. The addition of a host compound in the mobile phase was effective in separating these isomers, and these derivatized oxosteroids or their sulfates were satisfactorily separated by this method. Among the host compounds used, γ - CD and Me- β -CD are more effective than the other CD in decreasing the k' value of the derivatized oxosteroids and their sulfates, respectively. Among these compounds, oxosteroids having the 3 β -, 5 β - configuration and the sulfates having the 3 α -, 5 β - configuration are most affected in their retention behavior by the addition of γ - CD and Me- β -CD, respectively. These data

indicate that the functional group at the 3 position may play an important role in the inclusion phenomenon. The development of the determination of neurosteroids in the mammalian brain is now under investigation in our laboratory, and the details will be reported in the near future.

ACKNOWLEDGEMENTS

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SEPARATION AND DETERMINATION OF STEVIA SWEETENERS BY CAPILLARY ELECTROPHORESIS AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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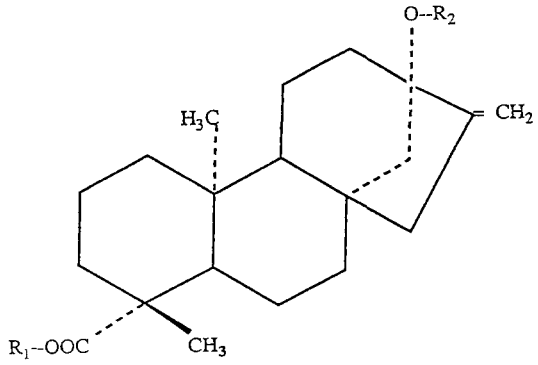
ABSTRACT

In this paper, the separation of steviol glycosides in stevia sweeteners including stevioside, rebaudioside A, rebaudioside C and dulcoside A by capillary electrophoresis and high performance liquid chromatography was investigated. A simple and efficient capillary electrophoretic method was developed. The results were compared with those obtained by HPLC. The individual steviol glycosides were obtained by HPLC fraction collection, and peaks in the electropherograms of the sweetener samples from Chinese refining factories were identified by comparing with those of individual steviol glycosides. The method was applied to the determination of real samples.

INTRODUCTION

Stevia sweeteners, extracted from the leaves of the plant Stevia rebaudiana, are commonly used as natural sweeteners in beverages, foods and medicines [1]. They contain eight steviol glycosides, in which stevioside (SS) has been shown



Name	R_1*	R_2*
1. Dulcoside A	glc	glc-rham
2. Stevioside	glc	glc-glc
3. Rebaudicoside C	glc	glc-rham \ glc
4. Rebaudicoside A	glc	glc-glc \glc

*glc, α-D-glucopyranosyl; rham, β-L-rhamnopyranosyl

Fig.1. Names and structures of steviol glycosides

to be the principal one, and rebaudioside A (RA), rebaudioside C (RC) and dulcoside A (DA) are the other main constituents. Their structures are shown in Figure 1 [2].

Various methods have been reported for the determination of stevia sweeteners, including gas liquid chromatography (GLC), thin layer chromatography (TLC), droplet countercurrent chromatography (DCCC), high performance liquid chromatography (HPLC), colorimetry and enzymatic determination [2]. Comparing with other methods, HPLC analytical methods are simple, rapid and accurate. However, in HPLC analysis of stevia sweeteners, a

special separation column must be used and the column is easily contaminated by the impurities of the real samples.

This paper described an efficient and simple method for determining stevia sweeteners by capillary electrophoresis (CE). It was evaluated by studying the effect of different separation conditions on migration time, selectivity and resolution. The method was applied in the separation and quantitation of real products. The results were compared with those obtained by HPLC.

MATERIALS AND METHODS

Reagents and materials

Stevioside standard was purchased from Wako (Osaka, Japan). Samples of stevia sweeteners were provided by Beijing Tianan Stevia Sweetener Products Company (Beijing, P.R.China). Pure stevioside was also obtained from the stevia sweeteners by methanol recrystallization, and other steviol glycosides (RA, RC and DA) were obtained from the stevia sweetener products by HPLC fraction collection and identified by HPLC.

Acetonitrile (HPLC grade) was purchased from BDH (Poole, England). Sodium tetraborate (analytical grade) was supplied by Fluka (Buchs, Switzerland). Water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare buffers for CE, mobile phase for HPLC and sample solutions.

Apparatus and conditions

CE was carried out on a commercial and a laboratory-built CE system. For

the laboratory-built CE system, a Spellman 30-kV power supply was used (Plainview, New York, USA). A fused-silica capillary tube of 50 cm effective length and 50 μm I.D. (Polymicro Technologies, Phoenix, AZ,USA) was used as the separation column. The peaks were detected by a Micro-UVis20 detector (Carlo Erba, Milan, Italy) with wavelength set at 210 nm. The window for the on-column detection cell was made by removing a small section of the polyimide coating on the fused silica capillary. Data processing was performed on a Shimadzu (Kyoto, Japan) Chromatopac C-R6A instrument. Samples were injected into the capillary by gravity feed with injection time of 20 seconds and injection height of 10 cm. The commercial system was a HP3D CE system (Hewlett Packard, Palo Alto, CA, USA), equipped with the same capillary, samples were injected into the capillary by pressure (30 mbar, 20 seconds). The buffer solution contained acetonitrile and sodium tetraborate solution. Other conditions were described where necessary in the next.

HPLC was carried out on a Shimadzu LC-9A system (Shimadzu Seisakusho, Kyoto, Japan), equipped with a normal phase LiChrospher NH_2 (5 μm) column (250 * 4 mm I.D.) (Merck, Darmstadt, Germany) and a Shimadzu SPD-M6A detector operated 210 nm. Data processing was performed through a computer by the SPD-M6A program. The HPLC mobile phase was prepared by mixing acetonitrile and Millipore water (80/20, V/V), and the flow rate was 0.8 ml/min.

Sample preparation

Samples of stevia sweeteners (100 mg) were dissolved in a 10 ml volumetric flask with Millipore water. The solution was diluted with the HPLC mobile phase to obtain the final concentration of 2 mg/ml. The solution were passed through a SPE C_8 cartridge (Whatman) before HPLC and CE analysis.

Solution for linearity response

Seven concentrations of stevioside, which ranged from 0.01 to 2.00 mg/ml, were prepared by dissolving and diluting in the HPLC mobile phase.

Isolation of individual glycosides from the stevia sweeteners

Stevia sweeteners (20 g) were dissolved in Millipore water (10 ml), and stevioside was recrystallized from the solution by adding methanol (60 ml). The solution was evaporated to dryness and the residue was redissolved in the HPLC mobile phase to get the final concentration of 5mg/ml. After treatment with a SPE C_8 cartridge, the solution was injected into HPLC system and the fractions of the steviol glycosides were collected. The collected fractions were evaporated to dryness and dissolved in HPLC mobile phase separately. Finally, the purity of the collected individual compounds was tested by HPLC.

RESULTS AND DISCUSSION

Fraction Collection by HPLC

Stevia sweeteners mainly contain four steviol glycosides, including SS, RA, RC and DA. Only stevioside (SS) standard could be obtained commercially. Other individual compounds were isolated from stevia sweeteners by HPLC fraction collection using a Lichrospher NH_2 column and the separation conditions given in ref. 3. The chromatograms of the mixture of stevia sweeteners and the individual steviol glycosides isolated from them are shown

in Fig. 2. It can be observed that the collected fractions of each of the individual compounds did not contain significant amounts of the other steviol glycosides present in the original mixture. These fractions were used in subsequent CE experiments for the purpose of peak identification.

Optimization of CE Conditions

The four steviol glycosides are similar in structure in that a steviol aglycone is connected at C-4 and C-13 to mono, di, trisaccharides consisting of glucose and/or rhamnose residues (see Figure 1). They are neutral compounds and usually cannot be separated electrophoretically. But in borate buffer systems, these compounds were transformed into negatively charged borate complexes [4], which could be partially separated in CE. Furthermore, by adding acetonitrile to the buffer solution, the separation was further improved.

The enhancement in selectivity could be attributed to differences in the stability of borate complexes, and in the solubilities of the steviol compounds in the acetonitrile/buffer mixture. The compounds which form more stable borate complexes and dissolved less well in the acetonitrile/buffer solution would be expected to exhibit a higher electrophoretic flow. Since the electrophoretic flow for negatively charged species would be in opposite direction to that of the electroosmotic flow, the compounds which form more stable complexes would be expected to have lower net migration rate and to migrate out later than those forming less stable complexes [4]. The difference in stability of the complexes therefore permitted the separation of the stevia sweeteners in electrophoresis. It is worth noting that differences in the stability of the borate complexes have been utilized previously in CE separation of carbohydrates [5-7] and catecholamines [8] to enhance selectivity.

Instead of acetonitrile, it was found that other organic modifiers, such as methanol and n-propanol also improved the resolution. However, these

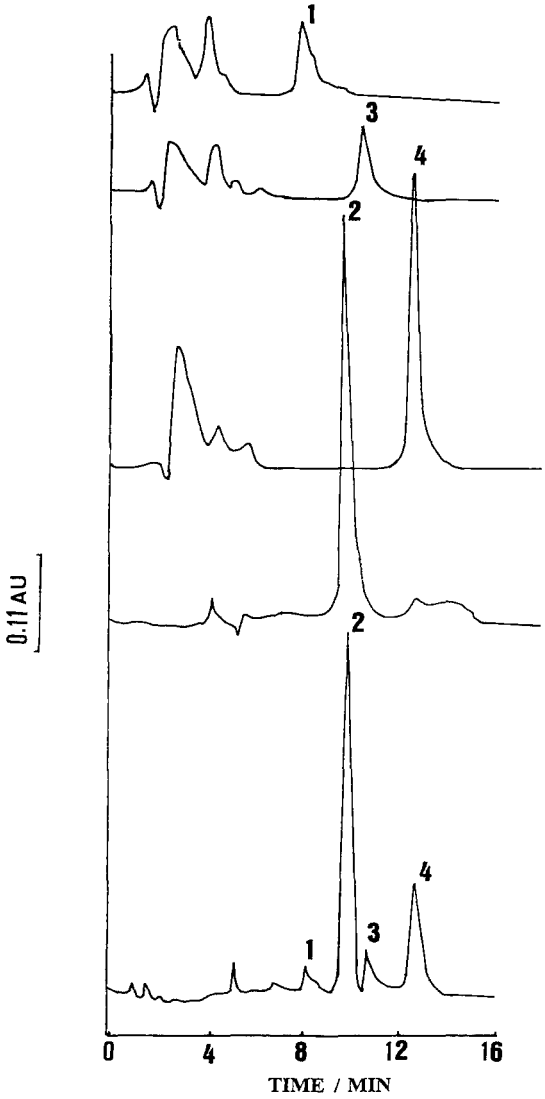


Fig.2. Liquid chromatograms of stevia sweeteners and the individual steviol glycosides obtained by fraction collection. Conditions: Column, Lichrospher NH_2 (5 μm) (250*4 mm I.D.); mobile phase, CH_3CN/H_2O (80/20, V/V); flow rate, 0.8 ml/min; UV (210 nm); att, 0.6; temperature, ambient. Peaks, 1 = DA; 2 = SS; 3 = RC; 4 = RA.

alternative modifiers reduced the electroosmotic flow significantly at the same time, and gave much longer analysis times than those for acetonitrile. Similar observations have been made previously in other CE separations utilizing organic modifiers as buffer additives [9]. Consequently, acetonitrile was used in all subsequent experiments as organic modifier.

In order to obtain the optimal conditions for CE separation, the effects of different experimental parameters on resolution were investigated. The experimental parameters which were studied included borate concentration, pH, acetonitrile concentration and voltage. The criteria used for selecting the optimal conditions were: 1. The resolution between any adjacent pair of peaks would be greater than unity, and 2. The capacity factors would be less than 0.7 (migration times less than 18 minutes).

The concentration of borate was varied from 10 to 60 mM. The migration times and resolution obtained are shown in Fig. 3 and Table 1, respectively. It was found that increasing borate concentration gave better separation but longer migration times for all of the stevia sweeteners. This result was expected owing to the increased concentrations of borate complexes and the higher electrophoretic mobilities at higher borate concentrations [4]. A concentration of 50 mM borate provided satisfactory separation ($R > 1$) within a relatively short analysis times (ca. 15 minutes) and hence was selected for further experiments.

The effect of pH on the migration times and resolution of the stevia sweeteners are shown in Fig. 4 and Table 2, respectively. It was note that increasing pH resulted in better separation, although the migration times would be significantly longer at higher pH. It was found that pH 9.30 provided satisfactory separation and relatively short migration times.

The effect of varying the concentration of acetonitrile is shown in Fig. 5 and Table 3. At higher concentrations of acetonitrile, the resolution improved albeit at the expense of longer analysis time. It was found that at an acetonitrile concentration of 45%, the migration times and the resolution satisfied the criteria stated above.

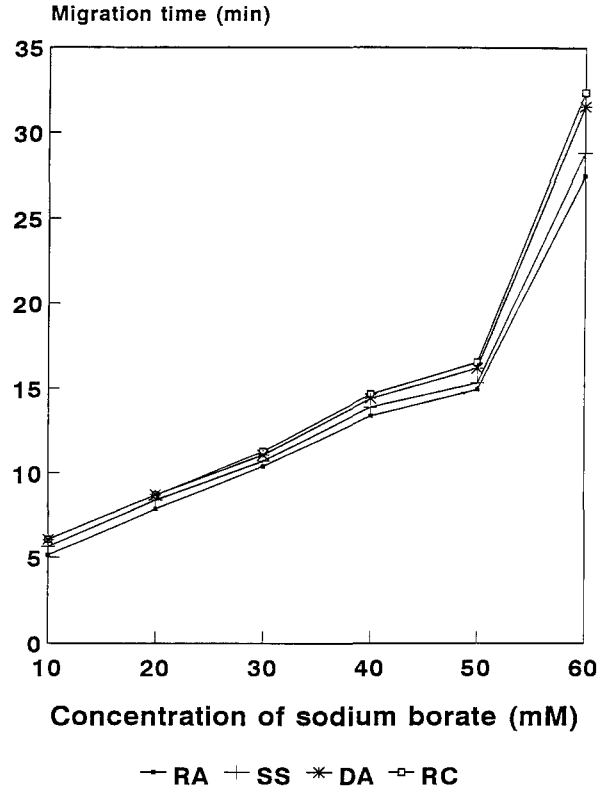


Fig.3. Influence of borate concentration on migration times of stevia sweeteners in CE system. Conditions: Buffer, sodium tetraborate (pH 9.3)/ acetonitrile (55/45, V/V), borate concentration ranging from 10 to 50 mM; UV detection at 210 nm; voltage,16.5 kV; temperature, ambient.

TABLE 1
Effect of Borate Concentration on Resolution

Concentration (mM)	10	20	30	40	50	60
Resolution (SS/RA)	*	0.64	0.88	1.01	1.04	2.12
Resolution (DA/SS)	0.32	0.51	1.06	1.29	2.24	2.78
Resolution (RC/DA)	*	*	0.73	0.79	1.13	2.20

* Peaks overlapping

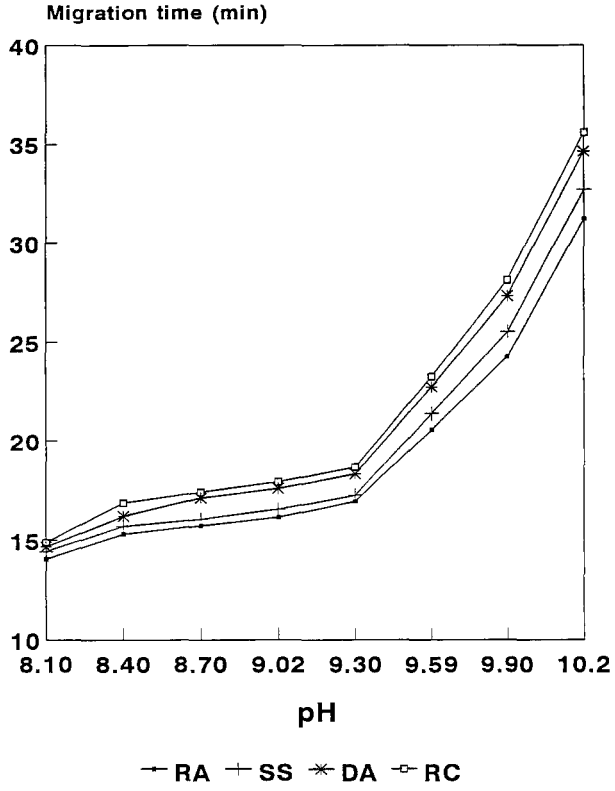


Fig.4. Influence of pH of buffer on migration times of stevia sweeteners in CE system. Voltage, 15 kV; buffer, 50 mM sodium tetraborate/acetonitrile, pH ranging from 8.1 to 10.2; other conditions as in Fig. 3.

TABLE 2
Effect of pH of Buffer on Resolution

pH	8.10	8.40	8.70	9.02	9.30	9.59	9.90	10.20
Resolution(SS/RA)	0.32	0.54	0.67	0.78	1.07	1.28	1.50	2.02
Resolution(DA/SS)	0.52	0.57	0.93	1.54	2.36	2.75	3.25	3.54
Resolution(RC/DA)	0.45	0.63	0.87	1.00	1.19	1.15	1.77	1.80

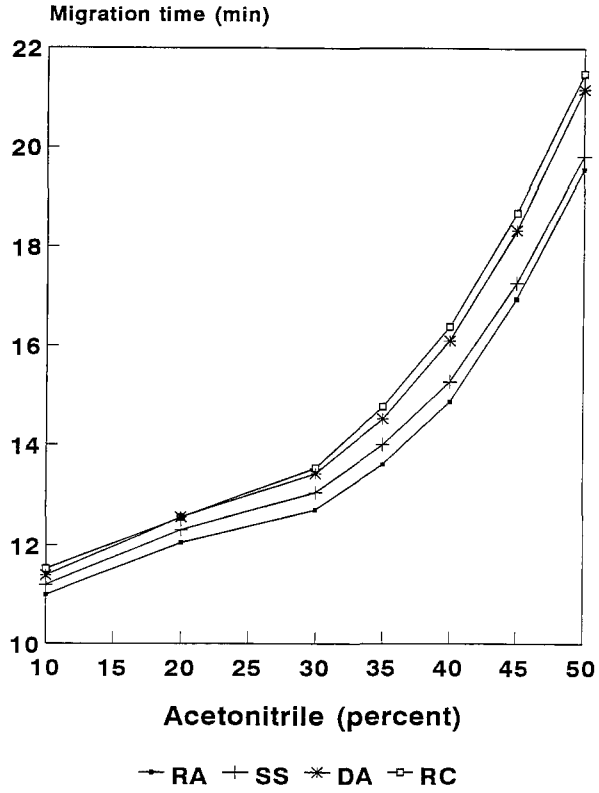


Fig.5. Influence of acetonitrile concentration on migration times of stevia sweeteners in CE system. Voltage, 15 kV; buffer, 50 mM sodium tetraborate/acetonitrile, acetonitrile concentration ranging from 10 % to 50 %; other conditions as in Fig. 3.

TABLE 3
Effect of Acetonitrile Concentration on Resolution

Concentration (%)	10	20	30	35	40	45	50
Resolution(SS/RA)	0.30	0.45	0.60	0.65	0.84	1.07	1.64
Resolution(DA/SS)	0.35	0.50	0.91	1.09	1.68	2.36	3.27
Resolution(RC/DA)	*	*	0.43	0.59	0.70	1.19	1.73

* Peaks overlapping

At higher applied voltages, the electroosmotic flow increased. Consequently shorter migration times would be obtained. The results of migration times and resolution for different voltages are shown in Fig. 6 and Table 4. It was noted from Fig.6 and Table 4 that although at higher voltages, shorter migration times were obtained, the resolutions between adjacent pairs of peaks were less than unity in some cases. Therefore, an applied voltage of 16.5 kV was chosen for subsequent experiments since it gave satisfactory separation and short migration times for all the peaks.

In summary, the optimum separation conditions based on the criteria chosen were determined as: applied voltage of 16.5 kV, a buffer solution containing 50mM sodium tetraborate (pH 9.3) and 55% acetonitrile (ratio of acetonitrile/buffer = 55/45, V/V). The electropherograms of stevia sweeteners obtained using these conditions are shown in Fig. 7. The peaks in the electropherograms were identified by comparing their capacity factors with those of the individual compounds, as shown in Table 5. It was noted that the migration order of stevia sweeteners in CE was very different from that in HPLC, as expected on the basis of different separation mechanisms in the two methods.

Determination of the purity of stevioside

Calibration graphs of HPLC and CE methods (peak-area ratio, y, vs concentration, x mg/ml) were constructed in the range 0.010-2.000 mg/ml for stevioside (The detection limit of the CE system was 0.010 mg/ml). The regression equations of the curves and their correlation coefficients were calculated as following:

HPLC: $y = 148.91x - 1.36$ $r = 0.9996$

CE: $y = 66795.68x - 2370.38$ $r = 0.9936$

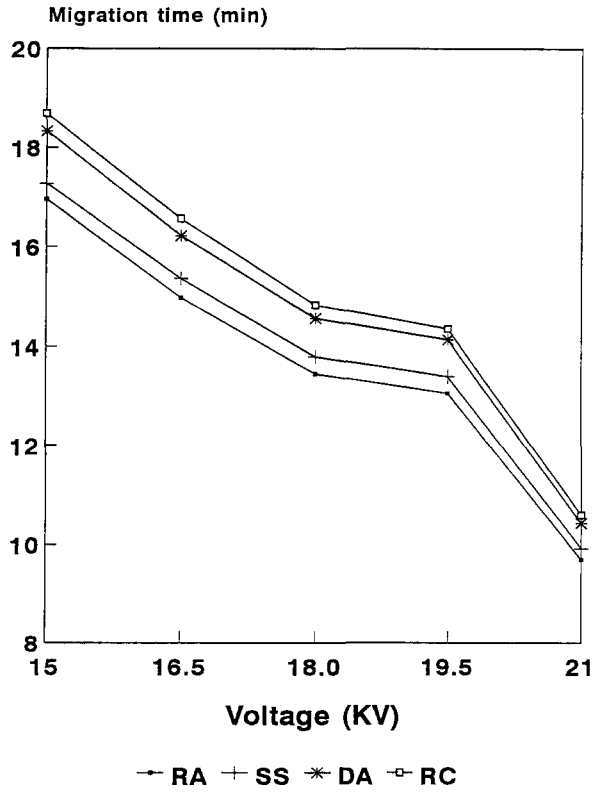


Fig.6. Influence of voltage on migration times of stevia sweeteners in CE system. Buffer, 50 mM sodium tetraborate/acetonitrile; voltage ranging from 15 to 21 kV; other conditions as in Fig. 3.

TABLE 4
Effect of Voltage on Resolution

Voltage (KV)	15	16.5	18	19.5	21
Resolution (SS/RA)	1.07	1.04	0.98	0.80	0.84
Resolution (DA/SS)	2.36	2.24	2.18	2.09	1.58
Resolution (RC/DA)	1.19	1.13	1.02	0.84	0.81

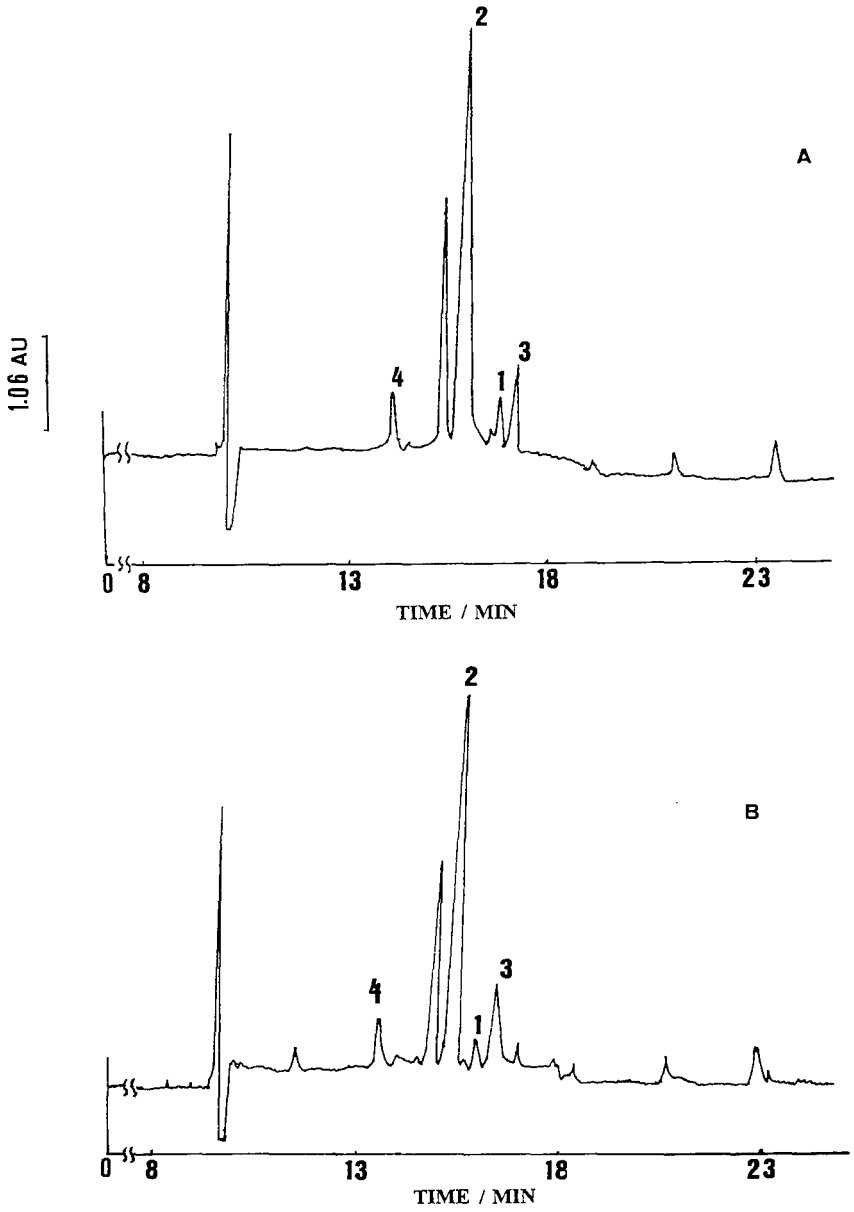


Fig.7. Capillary electropherograms of stevia sweeteners (A: sample 1, B: sample 2). Buffer, 50 mM sodium tetraborate/acetontrile (55/45, V/V); other conditions as in Fig. 3. Peak numbers as in Fig. 2.

TABLE 5
Capacity Factors of the Peaks in Capillary Electropherogram of Stevia Sweeteners (n=4)

	RA	SS	DA	RC
Individual glycosides	0.366±0.002	0.385±0.004	0.439±0.003	0.450±0.003
Sample 1	0.363±0.001	0.398±0.001	0.441±0.001	0.458±0.002
Sample 2	0.363±0.001	0.401±0.002	0.442±0.001	0.461±0.001

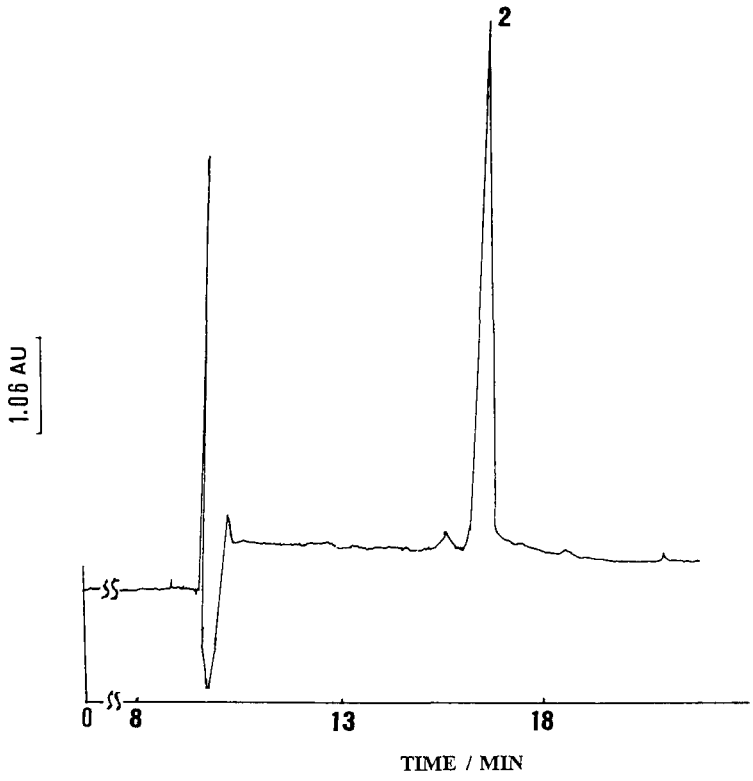


Fig.8. Capillary electropherogram of recrystallized stevioside. Conditions as in Fig. 7.

TABLE 6
Purity (%) of Stevioside in Real Samples (n=4)

	Sample 1	Sample 2	Recrystallized
HPLC	46.5±0.9	45.3±0.6	91.2±1.2
CE	46.2±1.3	45.8±1.8	91.8±1.8

The stevia sweeteners products and recrystallized stevioside were analyzed by capillary electrophoresis using the optimum conditions. The results are illustrated in Figure 7 and Figure 8. By substituting the area ratios of these peaks for y in the above equations, the content of stevioside in the samples were obtained as shown in Table 6. It was found that the results obtained by CE and HPLC were in very good agreement.

CONCLUSION

In this study, the separation and determination of stevia sweeteners by capillary electrophoresis was successfully demonstrated. Optimum conditions for the separation of the sweeteners were obtained. The results were consistent with those obtained using HPLC. Although the CE method has the advantage that it required only very small amounts of samples and little solvent usage, the HPLC method could be used successfully to obtain individual steviol glycosides by fraction collection. Both methods allowed simple, rapid and accurate analysis of the four steviol glycosides. Since the separation mechanisms are different in the two methods, the CE method can be used as an alternative analytical procedure to HPLC when the amount of sample available is small, or in an orthogonal manner to provide additional information.

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STRUCTURAL PROPERTIES GOVERNING RETENTION MECHANISMS ON RP-HPLC STATIONARY PHASES USED FOR LIPOPHILICITY MEASUREMENTS

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ABSTRACT

In this study, the retention mechanisms on the Supelcosil LC-ABZ stationary phase were analyzed by linear solvation free-energy relationships (LSERs). In a first phase, a set of 60 compounds was selected by cluster analysis from a large set (253) compounds of known van der Waals volume (V_w), polarity/polarizability (π^*), H-bond donating acidity (α) and H-bond acceptor basicity (β). The capacity factors log k' at 40% methanol and the log k_w values were shown to be highly correlated with octanol/water partition coefficients (log P_{oct}) and to contain pratically the same structural information, as assessed by LSERs. Test sets of model compounds and dipeptides were used to

validate the log k_W/log P and log k'/log P relations. For anions and zwitterions, specific interactions with the stationary phase occur which produce systematic deviations in the log k/log P relations.

INTRODUCTION

Lipophilicity is a molecular property of drugs and other xenobiotics of significance mainly because of its intimate relation with biological activity, as unambiguously demonstrated in many quantitative structure-activity relationship (QSAR) studies [1]. Lipophilicity is conventionally expressed as partition coefficients in immiscible organic/aqueous biphasic systems such as octanol/water (log P_{oct}), alkane/water or chloroform/water.

The shake-flask or centrifugal partition chromatography methods generally used to measure partition coefficients reveal their limitations mainly for highly lipophilic drugs and environmental toxins. Several studies have demonstrated that capacity factors derived from reversed-phase HPLC (RP-HPLC) (either isocratic log k' using methanol/water as mobile phase or log k_W as extrapolated to 100% water) offer a good alternative for determining the lipophilicity of compounds with log $P_{oct} > 3$ [2-5].

The stationary phases most frequently used to simulate the octanol/water-saturated phase are lipophilic alkylsilanes (e.g. octadecylsilane, ODS) bound to silica. However, two main sources of problems exist with alkylsilanes, namely silanophilic interactions which cause peak tailing and overestimation of lipophilicity, and breakdown of stationary phase at pH above 7.5.

Being devoid of reactive silanol groups and stable over a wide range of pH, the polymer-based lipophilic stationary phases such as octadecylpolyvinyl-alcohol copolymer (ODP) and polystyrene-divinylbenzene copolymer (PLRP-S) offer an alternative to assess molecular lipophilicity [6-8]. However, the relatively longer retention times in the PLRP-S column relative to the ODS column make it less interesting from a practical point of view. Furthermore, the more flexible polymeric support implies some limitations on the hydrodynamic pressure applied to the polymer-based columns.

In RP-HPLC, both partitioning and adsorption mechanisms are implicated in the retention on ODS and ODP phases. For the resemblance of these phases to octanol/water systems where solute adsorption is lacking, the principal retention mechanism in RP-HPLC must be a partitioning mechanism reflecting not only hydrophobic or solvophobic interactions between small nonpolar solutes and the mobile phase, but also polar interactions of solutes with the solvated layer(s) of stationary phases [9,10]. To unravel the structural determinants governing the retention of solutes in RP-HPLC, a powerful approach uses linear solvation free-energy relationships (LSERs) based on the solvatochromic parameters.

Indeed, LSERs were extensively used by Taft, Kamlet, Abraham and co-workers [11-14] to factorize some given molecular properties (S_p) of neutral organic solutes in terms of structural parameters such as the calculated molecular volume (V/100) and the so-called solvatochromic parameters (dipolarity/polarizability π^*, hydrogen-bond donor acidity α and hydrogen-bond acceptor basicity β). The linear equation 1 reflects a

solvation model constructed with two factors, namely the endoergic creation of a cavity in the solvent (as reflected by the V/100 term accounting for solvophobic/hydrophobic and dispersive forces) and the introduction of the solute in the cavity which leads to exoergic polar interactions (as reflected by the π^*, α and β terms).

$$S_p = v \bullet {}^{V}\!/_{100} + p \bullet \pi^* + a \bullet \alpha + b \bullet \beta + c \qquad 1$$

In this equation, v, p, a and b are the regression coefficients which reflect the relative contribution of each solute parameter to S_p. This approach has been applied to evaluate and identify the intermolecular interaction forces underlying the partitioning mechanisms of solutes in various organic/aqueous biphasic systems as described by the equations in Table 1 [12,15].

To be similar to solvent/water partition coefficients [16], RP-HPLC retention indices must correlate with log P values, while the LSER equations must reflect a comparable balance (comparable coefficients) between the structural parameters. However, stationary phases in RP-HPLC are of a complex nature, being composed of nonpolar alkyl chains, unreacted residual polar groups, significant amounts of organic modifier (only methanol is concerned in this study), and adsorbed water. Therefore, the very nature of the stationary phase and hence the retention behavior of solutes will change with the composition of methanol/water mobile phases, leading to different LSER equations [8,17]. The equations in Table 2 allow to select the RP-HPLC indices most similar to those of log P_{oct}. However, quantitative comparison is rendered difficult because the sets of compounds used to generate the equations in Tables 1 and 2 are limited and not always comparable.

TABLE 1.

Solvatochromic analysis of partition coefficients in organic solvent/water biphasic systems.

S_p[a)]	v	p	a	b	c[b)]	n	r^2	s	F	Ref
log P_{oct}	5.35 ± 0.05	-1.04 ± 0.04	0.10 ± 0.04	-3.84 ± 0.05	0.32 ± 0.04	245	0.99	0.13	-	[12]
log P_{oct}	5.83 ± 0.53	-0.74 ± 0.31	-0.15 ± 0.23	-3.51 ± 0.38	-0.02 ± 0.34	78	0.92	0.30	249	[15]
log P_{dee}	5.54 ± 0.81	0.02 ± 0.45	-0.20 ± 0.41	-3.83 ± 0.82	-0.34 ± 0.57	44	0.90	0.33	76	[15]
log P_{ba}	6.34 ± 0.80	0.91 ± 0.60	0.30 ± 0.53	-3.87 ± 1.82	-1.52 ± 1.12	26	0.97	0.26	122	[15]
log P_{chf}	6.00 ± 0.69	-0.14 ± 0.40	-2.99 ± 0.27	-3.17 ± 0.49	-0.18 ± 0.43	60	0.95	0.30	221	[15]
log P_{hep}	6.78 ± 0.69	-1.02 ± 0.39	-3.54 ± 0.30	-5.35 ± 0.50	-0.06 ± 0.43	75	0.95	0.36	438	[15]

a) Organic solvent: oct = 1-octanol; dee = diethylether; ba = n-butyl acetate; chf = chloroform; hep = heptane.

b) The regression coefficients are those of equation 1; 95 % confidence limits are given.

TABLE 2.

Solvatochromic analysis of RP-HPLC capacity factors.

S_p [a]	v	p	a	b	c [b]	n	r^2	s	F	Ref
$\log k_w^{DB}$	5.58 ± 0.88	-0.56 ± 0.51	-0.53 ± 0.30	-2.28 ± 0.52	-1.27 ± 0.47	71	0.86	0.34	79	[17]
$\log k_w^{ODS}$	4.34 ± 1.77	-0.33 ± 0.96	-0.34 ± 0.59	-2.96 ± 0.95	0.59 ± 0.93	34	0.76	0.38	23	[17]
$\log k_w^{OS}$	6.21 ± 0.61	-1.01 ± 0.50	0.85 ± 0.24	-3.10 ± 0.26	-0.87 ± 0.39	43	0.96	0.15	-	[8]
$\log k_w^{ODP}$	5.37 ± 2.06	-1.11 ± 1.33	-0.40 ± 1.12	-3.49 ± 1.10	0.19 ± 0.88	32	0.87	0.49	34	[17]
$\log k_w^{ODP}$	5.16 ± 0.86	-0.17 ± 0.45	-0.27 ± 0.40	-3.20 ± 0.49	-0.26 ± 0.40	40	0.98	0.22	-	[8]
$\log k_w^{SB}$	6.38 ± 1.05	-1.07 ± 0.66	0.72 ± 0.29	-3.12 ± 0.37	-0.86 ± 0.54	30	0.93	0.16	23	[17]
$\log k_w^{IN}$	6.24 ± 0.88	-0.43 ± 0.52	0.02 ± 0.26	-2.67 ± 0.40	-0.68 ± 0.58	42	0.88	0.20	66	[17]

a) stationary phase: DB = DeltaBond™ C_8; ODS = octadecylsilane; OS = octylsilane; ODP = octadecyl polyvinyl-alcohol copolymer; SB = RP select B; IN = Inertsil C_8.

b) See Table 1.

The resulting statistical artifacts may in turn mask or exaggerate the structural information. Thus, a set of structurally diverse, well-balanced compounds is imperatively needed in order to draw sound conclusions from a solvatochromic analysis of lipophilicity indices.

In the present study, our first goal was to select by cluster analysis (CA) an optimal set of compounds covering a wide and regular range in the structural parameters needed to perform LSER analyses. The octanol/water partition coefficients (log P_{oct}) were also included in the parameter hyperspace used in the cluster analysis. Then, the retention of this optimal set of compounds was measured in a novel ODS stationary phase (Supelcosil LC-ABZ column) using methanol/water mixtures as mobile phases. We were particularly interested in this novel stationary phase because of its electrostatic coating avoiding silanophilic interactions and diminishing specific polar interactions. The LSER approach was used to compare the RP-HPLC indices at different mobile phase compositions with the partition coefficients (log P_{oct}). In order to test the predictive power of the LSER equations, we used a test set of neutral compounds not included in the optimal set. To determine the practical limitations of the new Supelcosil LC-ABZ phase, the retention of other polar solutes, namely cyclodipeptides and zwitterionic dipeptides, was also examined.

MATERIALS and METHODS

Solutes

All compounds were obtained from commercial sources (Merck, Darmstadt, Germany; Fluka, Buchs, Switzerland; Janssen, Beerse,

Belgium; Aldrich, Steinheim, Germany) and in the highest available purity. Analytical grade methanol was purchased from Romil Chemical (GB), 3-morpholinopropane sulfonic acid (MPS) from Merck and HPLC grade methanol from Machler (Basel, Switzerland). Deionized water was used throughout.

Multivariate Statistical Analysis

The LSER models were generated by multivariate regression using both the QSAR module in the Sybyl software (Tripos Associates, St-Louis, MO, USA) and the TSAR program (Oxford Molecular, Oxford, GB). To express molecular volumes, we used van der Waals volumes (V_w) calculated with the standard software MOLSV (QCPE N° 509) and the atomic radii of Gavezzotti [18], instead of tabulated V/100 volume parameters. The geometries used to generate van der Waals volumes were optimized with the Tripos force field including an electrostatic term calculated with a dielectric constant $\varepsilon = 1$ until the gradient norm was less than 0.001 kcal • $Å^{-1}$. For the 253 compounds in the starting set, the correlation between the two volume parameters is excellent ($r^2 = 0.988$), and the molecular modeling approach to generate molecular volumes is adequate even for complex compounds.

Selection of an Optimal Set of Compounds Using Cluster Analysis

253 compounds with known solvatochromic parameters (V_w, π^*, α and β) and known octanol/water partition coefficients (log P_{oct}) [12] were selected as a starting set and analyzed by cluster analysis in order to obtain maximal structural and property diversity with a

minimum number of compounds. The TSAR software was used to perform cluster analysis with a single-link hierarchical clustering algorithm based on weighted mean distances generated from data transformed by standardization using the mean and the standard deviation.

The visual inspection of the dendogram produced by cluster analysis of the initial set of 253 compounds allows the identification of 78 pairs of most similar compounds. For each pair, one compound was eliminated based on the random criterion of a higher ranking number. Three similar runs were then performed allowing the elimination of, respectively, 57, 12 and 26 compounds. The optimal set (in terms of cluster analysis) was thus composed of 80 compounds (Tables 3 and 4).

However, due to the non-availability of some compounds (**66-70**), the poor solubility of others (**71-80**) in mobile phases and to experimental problems associated with basic compounds (**62-64**) and with DMSO (**61**), the set was reduced to 60 compounds (Table 3).

In order to determine the performance of the selection process, an independent series of 18 compounds (**81-98**) was used as a test set (Table 6).

Measurement of Capacity Factors (log k')

The chromatograph (Kontron MT1) was equipped with a MSI T-660 Auto-sampler, an HPLC pump model 420, a column oven 480, an oven controller 480 and an UV/Vis detector model 430 with variable wavelength (all from Kontron, Switzerland). For the UV inactive compounds, a Refractive Index (RI) detector (Erma refractometer,

TABLE 3. Investigated compounds in the optimal set.

N°	Solutes	V_W	π^*	α	$\beta^{a)}$	log P_{oct}	log k_W	log k'_{40}
1	n-C_5H_{12}	98.8	-0.08	0.00	0.00	3.39	3.22	-
2	CH_2Cl_2	58.4	0.82	0.10	0.13	1.15	0.94	-0.30
3	$CHCl_3$	72.6	0.58	0.10	0.20	1.94	1.80	0.71
4	CCl_4	87.4	0.28	0.10	0.00	2.63	2.43	1.16
5	CH_2ClCH_2Cl	75.4	0.81	0.10	0.00	1.48	1.16	0.40
6	$CHCl_2CHCl_2$	105.2	0.95	0.10	0.00	2.39	2.16	0.99
7	1-C_4H_9Cl	94.9	0.39	0.10	0.00	2.64	2.17	0.99
8	$(C_2H_5)_2O$	88.0	0.27	0.47	0.00	0.89	0.71	-0.08
9	(n-$C_3H_7)_2O$	124.4	0.27	0.46	0.00	2.03	1.72	0.72
10	CH_3COOCH_3	71.4	0.60	0.42	0.00	0.18	0.09	-0.47
11	$CH_3COOC_2H_5$	88.9	0.55	0.45	0.00	0.73	0.67	-
12	$CH_3COOC_4H_9$-n	124.0	0.51	0.45	0.00	1.82	1.91	0.68
13	CH_3CN	46.7	0.75	0.31	0.09	-0.34	-0.45	-0.74
14	CH_3CH_2CN	64.4	0.70	0.31	0.00	0.10	0.00	-0.53
15	CH_3-CO-N$(CH_3)_2$	92.3	0.88	0.76	0.00	-0.77	-0.44	-0.98
16	CH_3-CO-N$(C_2H_5)_2$	127.9	0.84	0.78	0.00	0.34	0.59	-0.28
17	C_2H_5OH	52.9	0.40	0.45	0.33	-0.25	-0.44	-0.71
18	n-C_3H_7OH	70.1	0.40	0.45	0.33	0.28	0.02	-0.37
19	$(CH_3)_3COH$	86.7	0.40	0.57	0.32	0.36	0.46	-0.20
20	n-$C_5H_{11}OH$	104.9	0.40	0.45	0.33	1.40	1.39	0.40
21	$CH_3CH_2C(CH_3)_2OH$	105.0	0.40	0.57	0.32	0.93	0.98	0.15
22	1-$C_6H_{13}OH$	122.0	0.40	0.45	0.33	2.03	1.73	0.68
23	HCOOH	36.8	0.65	0.38	0.65	-0.54	-0.39	-0.68
24	CH_3COOH	53.6	0.60	0.45	0.56	-0.24	-0.39	-0.67
25	n-C_3H_7COOH	88.6	0.56	0.45	0.56	0.79	0.63	-0.06
26	n-C_4H_9COOH	106.2	0.54	0.45	0.56	1.39	1.30	0.33
27	n-$C_4H_9NO_2$	99.7	0.76	0.25	0.00	1.47	1.28	0.37
28	Tetrahydrofuran	77.5	0.58	0.55	0.00	0.46	0.06	-0.57
29	$C_6H_5CH_3$	104.4	0.55	0.11	0.00	2.69	2.43	1.11
30	C_6H_5-CO-CH_3	122.5	0.90	0.49	0.04	1.58	1.57	0.46

TABLE 3 continued

N°	Solutes	V_W	π^*	α	β[a)]	log P_{oct}	log k_W	log k'_{40}
31	$C_6H_5NO_2$	105.4	1.01	0.30	0.00	1.85	1.74	0.65
32	$C_6H_5OCH_3$	112.5	0.73	0.32	0.00	2.11	1.77	0.75
33	$C_6H_5COOC_2H_5$	147.6	0.74	0.41	0.00	2.64	2.30	1.07
34	C_6H_5-CO-C_2H_5	139.7	0.88	0.49	0.00	2.20	1.98	0.78
35	$C_6H_5COOCH_2C_6H_5$	205.8	1.32	0.50	0.00	3.97	3.38	-
36	2-$ClC_6H_4NO_2$	120.5	1.11	0.26	0.00	2.24	2.18	0.98
37	$C_6H_5CH_2CN$	123.2	1.34	0.41	0.00	1.56	1.49	0.4
38	$C_6H_5CH_2$-CO-CH_3	139.2	1.30	0.58	0.00	1.44	1.39	0.37
39	$C_6H_5CH_2CH_2$-O-CO-CH_3	165.8	1.14	0.55	0.00	2.30	2.20	0.89
40	Pyridine	82.5	0.87	0.44	0.00	0.65	0.48	-0.26
41	Acridine	175.1	1.02	0.44	0.00	3.40	2.74	1.32
42	1-Naphthalenecarboxylic acid	158.4	0.84	0.40	0.59	3.10	3.27	1.57
43	2-Naphthylamine	146.2	0.83	0.50	0.35	2.28	2.09	0.87
44	$C_6H_5NH_2$	99.4	0.73	0.50	0.26	0.90	1.04	0.06
45	$C_6H_5NHC_2H_5$	133.6	0.82	0.47	0.17	2.16	1.79	0.76
46	2-Cl-$C_6H_4NH_2$	113.9	0.83	0.40	0.25	1.91	1.85	0.70
47	2-NH_2-C_6H_4-C_6H_5	175.0	1.32	0.60	0.26	2.84	2.60	1.22
48	4,4'-$(NH_2)_2$-Biphenyl	187.7	1.46	1.00	0.62	1.34	1.44	0.29
49	4-NO_2-C_6H_4-NH_2	118.8	1.25	0.48	0.42	1.39	1.67	0.52
50	C_6H_5OH	93.9	0.72	0.33	0.61	1.49	1.26	0.37
51	3-Cl-C_6H_4OH	109.6	0.77	0.23	0.69	2.49	2.24	0.98
52	3-CH_3-C_6H_4COOH	129.6	0.70	0.41	0.59	2.37	2.10	0.80
53	$C_6H_5CH_2COOH$	128.6	1.19	0.55	0.60	1.46	1.49	0.59
54	3-Cl-$C_6H_4CH_2COOH$	144.1	1.31	0.45	0.62	2.09	2.25	1.15
55	$C_6H_5CH_2CH_2CH_2COOH$	163.0	1.15	0.55	0.55	2.42	2.41	1.07
56	$C_6H_5CH_2OH$	111.1	0.99	0.52	0.39	1.08	1.04	0.19
57	4-Cl-$C_6H_4CH_2OH$	126.5	1.11	0.42	0.40	1.96	1.78	0.71
58	4-NO_2-C_6H_4-OH	113.0	1.15	0.32	0.82	1.92	2.12	0.87
59	1,3-$C_6H_4Cl_2$	116.5	0.75	0.03	0.00	3.48	3.06	1.59
60	Biphenyl	163.5	1.18	0.20	0.00	3.90	3.47	-

a) Taken in ref [12]

Table 4. Unavailable or non-usable compounds in the optimal set.

N°	Solutes	V_W	π^*	α	$\beta^{b)}$	log P_{oct}
61	CH_3SOCH_3	70.8	1.00	0.00	0.76	-1.35
62	$(CH_3)_3N$	76.5	0.16	0.00	0.65	0.22
63	$(C_2H_5)_3N$	127.8	0.14	0.00	0.71	1.36
64	$(n\text{-}C_3H_7)_3N$	178.8	0.14	0.00	0.69	2.79
65	$C_6H_5CH_2N(CH_3)_2$	150.3	0.75	0.00	0.67	1.91
66	$3\text{-}Cl\text{-}C_6H_5OCOCH_3$	144.1	1.19	0.00	0.42	2.32
67	$C_6H_5CH_2COOC_2H_5$	171.7	1.21	0.00	0.68	1.41
68	$C_6H_5OCH_2CON(CH_3)_2$	176.5	1.60	0.00	0.99	0.77
69	$C_6H_5OCH_2CH_2CH_2OCH_3$	164.1	0.86	0.00	0.56	2.70
70	$C_6H_5OC_3H_7\text{-}n$	147.4	0.67	0.00	0.30	3.18
71	Naphthalene	133.7	0.70	0.00	0.15	3.35
72	$1,3,5\text{-}C_6H_3(CH_3)_3$	137.6	0.47	0.00	0.13	3.84
73	$n\text{-}C_9H_{19}COOH$	194.1	0.42	0.55	0.45	4.09
74	$2\text{-}C_{12}H_9Cl$	178.1	1.30	0.00	0.17	4.30
75	$1,2,4,5\text{-}C_6H_2Cl_4$	145.9	0.70	0.00	0.00	4.51
76	$n\text{-}C_7H_{16}$	132.4	-0.02	0.00	0.00	4.66
77	$C_6H_5CH_2CH_2C_6H_5$	196.9	1.10	0.00	0.22	4.80
78	$C_6H(CH_3)_5$	196.9	0.39	0.00	0.17	4.56
79	$2,5\text{-}C_{12}H_8Cl_2$	193.0	1.30	0.00	0.13	5.10
80	$1\text{-}C_{12}H_{25}OH$	225.8	0.42	0.33	0.45	5.13

a) Taken in ref [12]

Tokyo, Japan) was used. Both RI- and UV-detection modes were employed for a number of UV-active compounds to verify the results calculated from RI detection method.

The column was a Supelcosil LC-ABZ (150x4.6 mm ID, Supelco, Bellefonte, Pennsylvania, USA) of 5 μm packing and 100 Å pore size. This ODS stationary phase is pretreated with an electrostatic coating to suppress free silanophilic groups.

For all analyses, the flow rate was set at 1 ml/min, the column oven temperature to 25 ± 1 °C and the detection wavelength was varied according to λ_{max} of analytes. All solutes were dissolved in the mobile phase and the injection volume was 10 µl. The columm dead time (t_0) was defined as the retention time of a non-retained compound (MeOH or uracil) [19]. The isocratic capacity factor log k' was calculated from solute retention time (t_R) using equation 2:

$$\log k' = \log \frac{t_R - t_0}{t_0} \qquad 2$$

Four to five log k' values were measured using methanol/buffer mixtures containing 10-50 % (v/v) methanol for very hydrophilic compounds, 30-70 % (v/v) methanol for compounds of moderate polarity and 50-80 % (v/v) methanol for very lipophilic compounds. The buffer was an MPS (0.02 M) solution of pH = 7.4, except for acidic compounds (pH = 3.0). The log K_w values were thus calculated by extrapolation using linear regression to 100 % water.

RESULTS AND DISCUSSION

Structural Diversity of the Optimal Set of Compounds

The optimal set of 80 compounds derived as described above by cluster analysis of 253 candidates is given in Tables 3 and 4. The dendogram in Fig. 1 shows the similarities in the parameter hyperspace among the selected compounds which are well distributed and without overlap. The population distribution in the optimal set of 80 compounds, in the set of 60 compounds finally used, and in the

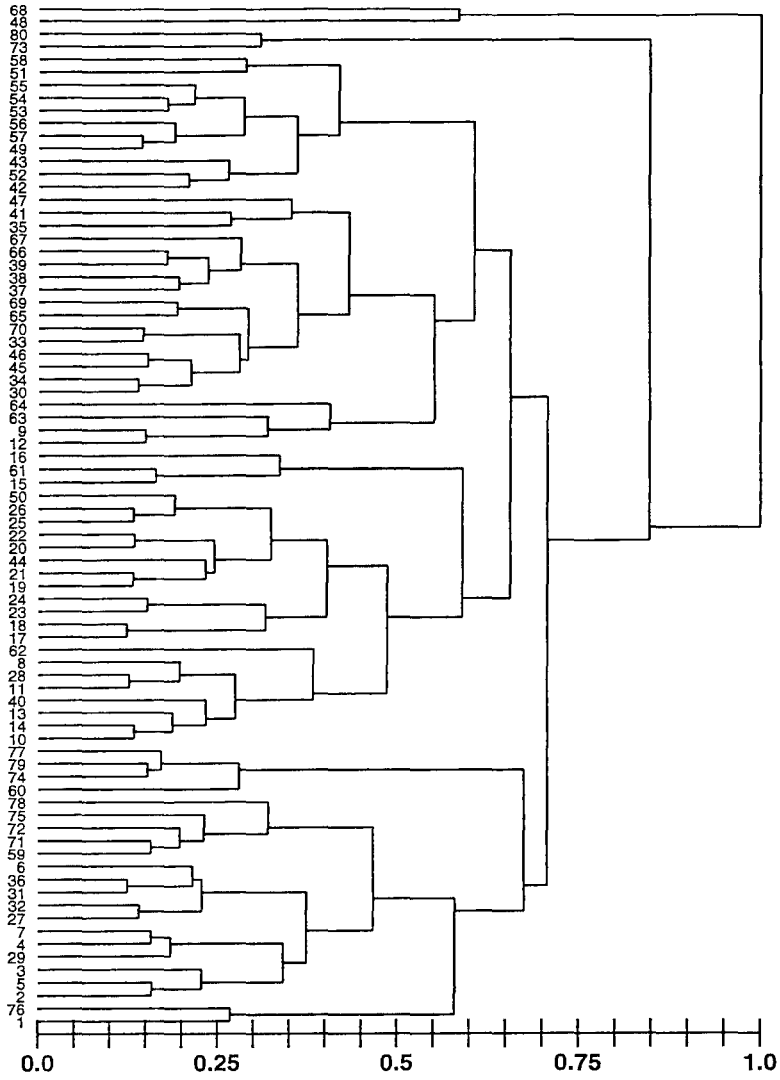

FIGURE 1:
Dendogram illustrating the even distribution in the five-parameter hyperspace of the 80 compounds in the optimal set.

original data were examined for each parameter (Fig. 2). These three distributions are comparable confirming that the selection process by cluster analysis eliminates only compounds with similar properties in the five-parameter space. It should also be noted that no correlation between the four structural parameters (V_w, π^*, α, β) was found in any of the three sets of compounds.

Some interesting features emerge from Figure 2. An approximate Gaussian distribution is observed in the van der Waals volume, ranging from 35 to 205 $Å^3$. In contrast, the population distribution in the set with respect to dipolarity/polarizability is an asymmetric Gaussian curve due to the limited number of alkanes, which have a negligible π^* value and are difficult to measure because of their very long retention and low solubility in the mobile phase. Since all aprotic compounds have a negligible α values, a high population at $\alpha = 0$ is observed. The hydrogen bond acceptor capacity is largely populated for β values between 0.4 and 0.5. This is due to the fact that most alcohols, carboxylic acids and esters have a β value in this range. The log P_{oct} values in the three sets cover the range between -1.3 and 4.0 following a Gaussian distribution centered on log P_{oct} values around 2 (not shown).

Hence an optimal set of compounds has been selected which retains the distribution of properties of the original set and spans as regularly as possible the full property space explored. To the best of our knowledge, such an approach is used here for the first time in order to define an optimal set of compounds for characterizing RP-HPLC phases.

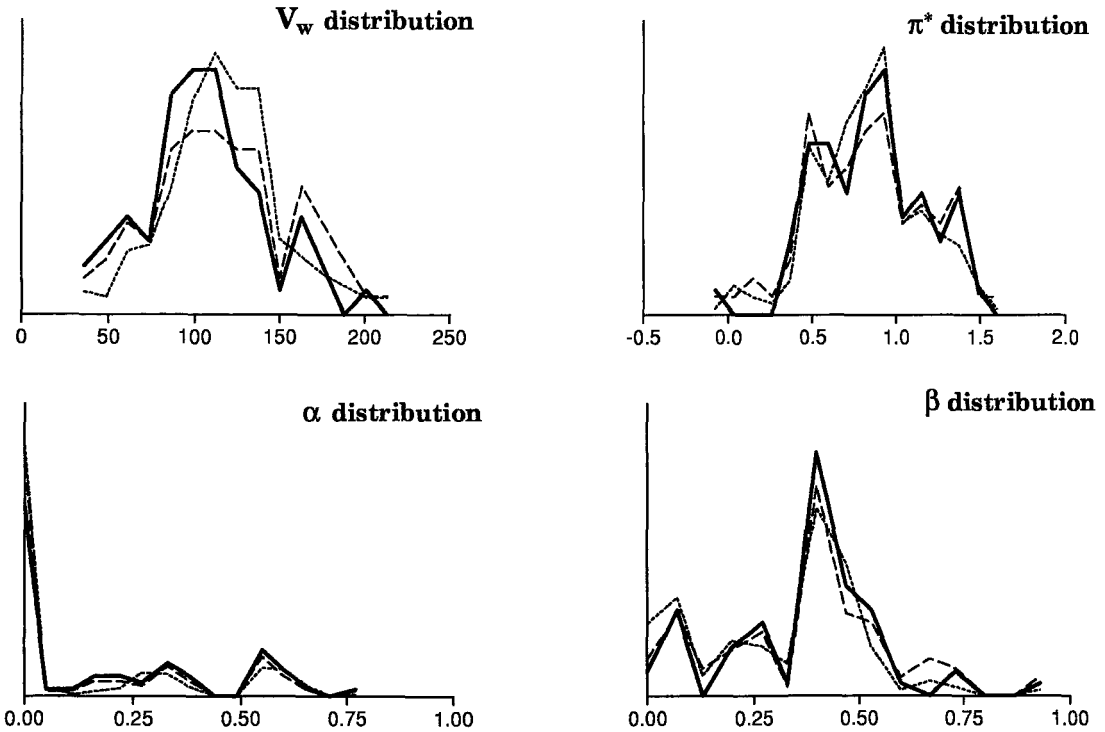

FIGURE 2:
Distribution of compounds in parameter spaces for the starting set (----- 253 compounds), the optimal set (- - - 80 compounds) and the final set (— 60 compounds).

Chromatographic Behavior of Solutes on the LC-ABZ Stationary Phase

With the LC-ABZ stationary phase, the chromatograms of relatively nonpolar solutes such as biphenyl and acridine show very symmetrical peaks compared to other C-18 stationary phases, which is indicative of predominating partitioning mechanisms with little or no adsorption phenomena. For strong hydrogen bond donors such as 1-naphthol or phenol, no "tailing" was observed in the chromatograms even for retentions as long as one hour. Taken together, these results suggest that, for neutral compounds, only partitioning is involved in the retention on the LC-ABZ stationary phase.

The isocratic capacity factors of the 60 selected compounds were determined. Representative results for a 40 % methanol eluent are listed in Table 3. The log k_W values were obtained using linear regression of four or more points (average $r^2 > 0.99$). No parabolic curve (as been with some C-18 stationary phases [20]) was observed for any compound examined.

In contrast to other C-18 stationary phases, the log k_W values of neutral acidic compounds (measured with a mobile phase at pH = 3.0) are different from the log k_W calculated by correcting for ionization the log k_W values of the anions (measured with a mobile phase at pH = 7.4). This observation suggests that, for anionic compounds, the partioning mechanism in the LC-ABZ column is perturbed by specific interactions between the stationary phase and charged species. It follows that for this phase, the neutral form of acidic compounds must be measured directly.

It should be also noted that the ABZ column is not suitable for strong basic compounds. Indeed, measuring protonated species and correcting for ionization appears to give yield erratic results, while the unstability of the stationary phase at pH value above 7.4 does not allow the measurement of neutral species.

Retention Mechanisms on the LC-ABZ Stationary Phase

Application of linear solvation free-energy relationships (LSERs) to our optimal set of compounds gives a statistically significant equation 3 which describes the structural properties governing the retention mechanisms on the LC-ABZ stationary phase:

$$\log k_W = 3.05 \bullet 10^{-2}(\pm 0.20 \bullet 10^{-2}) \bullet V_W - 0.48(\pm 0.22) \bullet \pi^* + 0.42(\pm 0.19) \bullet \alpha - 3.73(\pm 0.28) \bullet \beta - 0.12(\pm 0.21) \quad (3)$$

$$n = 60; \quad q^2 = 0.96; \quad r^2 = 0.97; \quad s = 0.19; \quad F = 405$$

In this and the following equations, 95 % confidence limits are in parentheses; q^2 is the cross-validated correlation coefficient [21]. Mager's standardization [22] of equation 3 gives the relative contibutions of each variable to the LSER model, namely 53.2 % for V_W, 7.7 % for π^*, 33.9 % for β and 5.2 % for α. Eqn (3) indicates that V_W and β are the principal structural descriptors contributing to log k_W, while the importance of π^* and α are only marginal.

To compare the RP-HPLC lipophilicity index to the partition coefficient measured in the octanol/water system, the latter was similarly analyzed using the same series of compounds (Eq. 4). This equation and the relative contribution of each variable, namely 52.9 %

for V_w, 9.2 % for π^*, 35.3 % for β and 2.6 % for α, confirm the close analogy between log P_{oct} and log k_w.

$$\log P_{oct} = 3.30 \bullet 10^{-2}(\pm 0.14 \bullet 10^{-2}) \bullet V_w - 0.62(\pm 0.12) \bullet \pi^* + 0.23(\pm 0.16) \bullet \alpha - 4.23(\pm 0.26) \bullet \beta + 0.09(\pm 0.16) \quad (4)$$

$$n = 60; \quad q^2 = 0.98; \quad r^2 = 0.98; \quad s = 0.15; \quad F = 729$$

This similarity is also seen in the relationship between the two lipophilicity descriptors (Eq. 5).

$$\log P_{oct} = 1.07(\pm 0.06) \bullet \log k_w + 0.03(\pm 0.10) \quad (5)$$

$$n = 60; \quad q^2 = 0.97; \quad r^2 = 0.97; \quad s = 0.20; \quad F = 1798$$

Thus, the log k_w derived from the LC-ABZ stationary phase appears as a promising surrogate of log P_{oct} as shown by their similar structural information content.

Information content of isocratic capacity factors

To obtain more rapidly RP-HPLC derived lipophilicity indices and to eliminate the problem of linear versus parabolic extrapolation to log k_w, it is of interest to examine the retention mechanism on the LC-ABZ phase with different mobile phase compositions and the ability of isocratic capacity factors to predict octanol/water partition coefficients. Multilinear equations (not shown) were derived for the six compositions of the mobile phase used to calculare log k_w, but only the relative contribution of each solvatochromic variable is given in Table 5.

At different ratios of methanol/water in the mobile phase, the nature of both mobile and stationnary phases is modified. The decreasing

Table 5. Solvatochromic analysis of isocratic capacity factors

Mobile Phase[a)]	N	$\%V_W$	$\%\pi^*$	$\%\alpha$	$\%\beta$[b)]
30/70	44	53.3	5.7	7.1	33.7
40/60	56	50.6	6.2	6.3	36.9
50/50	51	46.6	8.1	7.1	38.1
60/40	54	44.8	8.0	7.7	39.6
70/30	44	41.8	9.5	8.9	39.8
80/20	27	42.8	11.7	8.8	36.7

a) Composition of mobile phase: % methanol/water (v/v)
b) Relative contribution of each independent variable, based on normalized equations

contribution of V_W with increasing methanol concentration may be due to the more hydrophobic character and lower hydrogen-bond capacity of methanol compared to water. These results further suggest that at high concentrations of methanol in the mobile phase, other polar interactions with the stationary phase not taken into account by the structural descriptors used here become significant. They also suggest a poorer correlation of isocratic capacity factors (log k') with log P_{oct} at high concentrations of methanol in the mobile phase, as indeed seen: 30% methanol, $r^2 = 0.96$; 40% methanol, $r^2 = 0.97$; 50% methanol, $r^2 = 0.96$; 60% methanol, $r^2 = 0.93$; 70% methanol, $r^2 = 0.86$; 80% methanol, $r^2 = 0.72$. Interestingly, a single determination with 40 % methanol in the mobile phase (log k'_{40}) seems sufficient to obtain a good lipophilicity index based on the similary of the regression coefficients in the solvatochromic equations. The interest of log k'_{40} is also seen in equation 6:

$$\log P_{oct} = 1.57(\pm 0.09) \bullet \log k'_{40} + 0.85(\pm 0.06) \quad (6)$$
$$n = 56;\ q^2 = 0.97;\ r^2 = 0.97;\ s = 0.18;\ F = 1707$$

The slope of this linear equation is higher than 1, suggesting that log k'_{40} is also useful to extend the experimental range of measurable lipophilicity.

RP-HPLC of a test set of compounds

To determine the ability of both log k_w and log k'_{40} to be used as a predictor of log P_{oct}, the predictive power of equations 5 and 6 was established on a test set of 18 model molecules (**81-98**) closely related to the compounds used in the optimal set. The experimental results are given in Table 6.

In the lipophilicity range (-1.0 to 4.0) explored by the test set, the two indices log k_w and log k'_{40} give a satisfactory estimation of log P_{oct} as shown by equations 7 and 8. It should be noted that the quality of prediction is better when using log k'_{40} than log k_w presumably due to the uncertainty in the linear extrapolation to 100 % water.

$$\log P_{oct} = 0.99(\pm 0.11) \bullet \log P(\text{est. from } \log k_w) + 0.10(\pm 0.23) \quad (7)$$
$$n = 18;\ q^2 = 0.97;\ r^2 = 0.98;\ s = 0.20;\ F = 615$$

$$\log P_{oct} = 0.99(\pm 0.05) \bullet \log P(\text{est. from } \log k'_{40}) + 0.04(\pm 0.08) \quad (8)$$
$$n = 17;\ q^2 = 0.99;\ r^2 = 0.99;\ s = 0.09;\ F = 2445$$

RP-HPLC of a test set of peptides

The results described above have demonstrated the performance of the Supelcosil LC-ABZ column to estimate the log P of neutral model

Table 6. Test sets

N°	Solute	log P_{oct}	log k_w	log P[a)]	log k'_{40}	log P[b)]
81	1-C_3H_7Cl	2.04	1.53	1.67	0.69	1.93
82	CH_3COCH_3	-0.24	-0.14	-0.11	-0.69	-0.23
83	$HCON(CH_3)_2$	-1.01	-0.88	-0.91	-1.18	-1.0
84	2-C_3H_7OH	0.13	-0.12	-0.1	-0.47	0.11
85	C_6H_6	2.13	1.68	1.83	0.76	2.04
86	C_6H_5CHO	1.48	1.23	1.35	0.32	1.35
87	C_6H_5F	2.27	1.71	1.86	0.83	2.15
88	C_6H_5Cl	2.84	2.36	2.56	1.18	2.70
89	C_6H_5Br	2.99	2.94	3.18	1.29	2.88
90	1-Naphthol	2.91	2.88	3.11	1.41	3.07
91	2-Naphthol	2.81	2.82	3.05	1.33	2.94
92	1-Naphthylamine	2.24	2.08	2.26	0.92	2.30
93	1,3,5-$C_6H_3Cl_3$	4.02	3.54	3.82	-	-
94	2-Cl-C_6H_4OH	2.15	1.92	2.08	0.80	2.11
95	2-F-C_6H_4OH	1.71	1.41	1.54	0.46	1.57
96	2-Br-C_6H_4OH	2.35	2.11	2.29	0.94	2.33
97	4-F-C_6H_4OH	1.77	1.58	1.72	0.57	1.75
98	4-I-C_6H_4OH	2.91	2.61	2.82	1.32	2.93
99	c-Trp-Tyr[c)]	1.05	-	-	0.23	1.21
100	c-Gly-Tyr	-0.69	-	-	-0.86	-0.51
101	c-Gly-Phe	0.05	-	-	-0.38	0.25
102	c-Phe-Phe	1.59	-	-	0.78	2.08
103	c-Phe-Ser	-0.45	-	-	-0.49	0.08
104	c-Ser-Tyr	-1.09	-	-	-1.03	-0.77
105	Leu-Phe	-1.13	-	-	0.00	0.85
106	Trp-Tyr	-1.38	-	-	-0.11	0.68
107	Phe-Phe	-0.99	-	-	0.22	1.20
108	Phe-Leu	-1.24	-	-	0.03	0.89
109	Trp-Phe	-0.52	-	-	0.36	1.42
110	Phe-Tyr	-1.83	-	-	-0.31	0.36
111	Tyr-Leu	-1.81	-	-	-0.45	0.14
112	Met-Leu	-1.92	-	-	-0.51	0.05
113	Leu-Tyr	-1.95	-	-	-0.70	-0.25

a) Partition coefficient predicted by equation (5)

b) Partition coefficient predicted by equation (6)

c) For dipeptides and cyclodipeptides, a pH of 6 was used.

compounds and the necessity to perform measurements on the neutral form of ionized compounds. Due to the limited stability of the stationary phase, the second condition restricts the range of ionizable compounds to those neutral in the pH range between 2 and 7.5. To define the application domain of the LC-ABZ phase, two other classes of compounds were studied, namely six neutral cyclodipeptides (**99 - 104**) and nine zwitterionic dipeptides (**105 - 113**) (Table 6).

Although higher than for the first test set, the mean deviation between the measured log P_{oct} and the log P_{oct} predicted by equation 6 (0.32 ± 0.16) is still acceptable for the neutral cyclodipeptides. However, for the zwitterionic dipeptides, this deviation is much larger (2.01 ± 0.16) indicating either a change in the equilibrium between neutral and zwitterionic species in the 40 % methanol eluent, or specific interactions between charged amino or carboxylate groups and the stationary phase. In the light of results on ionized acids and bases, the latter phenomenon is the most likely. Work is on progress in our laboratory to clarify the retention mechanisms of ionised species on the Supelcosil LC-ABZ column.

Interestingly, in an homogenous series like the dipeptides, the deviation between log P_{oct} and log P predicted is largely constant (2.01 ± 0.16) suggesting that isocratic capacity factors could be used to characterize lipophilicity variations in the series thus giving direct access to hydrophobic substituent constants similar to those obtained from log P_{oct} values.

CONCLUSION

In conclusion, the LC-ABZ stationary phase is shown to retain neutral solutes by a balance of intermolecular forces closely resembling that underlying octanol/water partition coefficient. The isocratic capacity factor at 40% methanol appears even better than log k_w in resembling log P_{oct}. For anions and zwitterions, specific interactions occur with the stationary phase whose nature remains to be established but which affect lipophilicity prediction.

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COMPARISON OF DYNAMIC BEHAVIOR OF C18 HPLC COLUMNS BY STIMULUS-RESPONSE ANALYSIS. I. DETERMINATION OF PECLET NUMBERS

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ABSTRACT

In this study, the dynamic behaviour of C18 HPLC columns is determined by using the stimulus-response technique. Acetonitrile was used as a non-interacting tracer through the C18 HPLC columns (MikroPak® and ELSIsphere®). The system was pulse stimulated with 10 μl of acetonitrile at the flow rates of 1, 2, 3 and 4 ml/min of HPLC grade water. The resulting chromatograms so called response "C" curve is obtained for both columns, separately. The kinetics of that tracer were evaluated in a model study. The model equation was solved using parameter estimation by cybernetic moment technique" and than Peclet numbers, which is the basic indication of the dynamic flow characteristics of the columns, and axial dispersion coefficients, were calculated.

INTRODUCTION

High performance separation techniques like high performance liquid chromatography (HPLC) first gained acceptance mainly for the analysis of high boiling, thermolabile or ionic compounds. Due to the enormous available instrumental diversity, it is possible to adapt the instrumentation of separation parameters almost optimally to the relevant problem. Separations in HPLC systems are based on the interactions of the solute both mobile and stationary phases. However, by changing the column, it is possible to perform many different

separation mechanism including normal phase, reverse phase (RP), ion-pair, and ion-suppression chromatography [1]. Among them reverse phase chromatography (RPC) is the most widely used HPLC technique for the determination of a wide variety of substances that have a great importance in food analyses such as sugars [2], vitamins [3], organic acids [4], anthocyanins [5], carotenoids [6], food colors [7], mycotoxines [8], herbicides [9] and pesticides [10].

RP columns are prepared by reacting the surface silanol groups (Si-OH) of support particles with various reagents such as trimethylsilyl (C1), butylsilyl (C4), octylsilyl (C8), and octadecylsilyl (C18) groups [1]. Columns used in RPC have different characteristics affecting the degree of separation of a compound to be analyzed. Parameters controlling separation in a RP column are column dimensions (length, diameter), type and particle size of column packing material and ratio of carbon loading. End-capping which directly affects retention time of a solute during elution is also an important property of RP columns.

In most of researches carried out by HPLC, only column dimensions and type and the particle size of packing material are used to be indicated. However, it is not possible to explain the reasons of changing results obtained for two different columns having similar properties by using same mobile phase compositions at the same conditions.

Stimulus-response methods are well developed in chemical engineering processes for measuring rate and equilibrium parameters such as mass transfer coefficients, diffusivities and adsorption rate constants [11-13]. The moments of the response curves to pulse inputs have been extensively used in the analysis of packed bed systems [14-17]. The first biological application of this technique was made by us [18-20]. In the present study, a modified bead-column test using stimulus-response approach was employed to investigate the dynamic interaction between the mobile phase, HPLC grade water and the packing material C18. Reverse phase columns packed with C18 beads were subjected to a series of tests by stimulus-response technique utilising actonitrile as a non-interacting tracer with the packing material. The column was pulse stimulated and the response of the column was measured.

In this study, flow characteristics of two different RP C18 colums MicroPak® and ELSIsphere® were investigated by stimulus-response analysis and the response curves were evaluated with moment technique, then the results were reported in terms of Peclet numbers which indicates the dynamic behaviour of the column under different flow conditions.

THEORY

The model equation for a packed column with axial dispersion is [20, 21].

$$D\frac{\delta^2 c}{\delta x^2} - U\frac{\delta c}{\delta x} - \rho_p N_A' = \varepsilon\frac{\delta c}{\delta t} \tag{1}$$

where N_A' is the net flux to surface (mol/g.s).

The boundary and initial conditions are based upon Danckwerts' boundary conditions [21], so that for a Dirac delta function pulse input;

$$\text{Boundary condition I. at } \xi=0\ ; \quad M=J(s)_{\xi=0} - \left[\frac{1}{Pe}\frac{\delta J(s)}{\delta\xi}\right]_{\xi=0} \tag{2}$$

$$\text{Boundary condition II. at } \xi=1\ ; \quad \frac{\delta J(s)}{\delta\xi}=0 \tag{3}$$

where;

M: Amount of tracer injected.

For the inert tracer, substituting

$$N_A' = 0 \tag{4}$$

Eq. (1) was written in the Laplace domain as

$$\frac{1}{Pe}\frac{\delta^2 J}{\delta\xi^2} - \frac{\delta J}{\delta\xi} - \frac{\varepsilon L}{U}\frac{\delta J}{\delta t}=0 \tag{5}$$

Equations (1) to (3) can be solved in the Laplace domain for J=f(x,s). Then using the relation

$$m_n = -1^n \lim(s\Rightarrow 0)\frac{d^n \overline{J}_A}{ds^n} \tag{6}$$

theoretical moment expressions were derived. For the zeroth moment and first absolute moment and second central moment for the column itself, the result is

$$m_0 = M \tag{7}$$

$$M_1 = \frac{m_1}{m_0} = \frac{\varepsilon L}{U} \tag{8}$$

and

$$M_2^{\#} = \frac{m_2}{m_0} - M_1^2 = \frac{2}{Pe} - \frac{2}{Pe^2}[\,1 - e^{-Pe}\,] \tag{9}$$

Experimental values of the zeroth, first and second moments can be determined from the observed response peaks using the following equations [22].

$$m_0^E = \int_0^a C dt \approx \sum_{i=1}^{n} c_{av.i} \Delta t \tag{10}$$

$$m_1^E = \frac{\frac{1}{2}\sum_{i=1}^{n} c_{av.i}(\tau_{i+1} + \tau_i)}{\sum_{i=1}^{n} c_{av.i}} \tag{11}$$

$$m_2^E = \frac{\frac{1}{3}\sum_{i=1}^{n} c_{av.i}\left[\tau_{i+1}^2 + \tau_{i+1}\tau_i + \tau_i^2\right]}{\sum_{i=1}^{n} c_{av.i}} \tag{12}$$

EXPERIMENTAL

High-performance liquid chromatograph: Varian Star model liquid chromatograph was used. It was equipped with a Rheodyne model 7161 six-way

injection valve, 10 μl loop, and a Varian model 9050 variable wavelength UV-VIS detector set at 276 nm and 0.02 AUFS. Varian model 4400 integrator was used with a chart speed of 4 cm/min to record resulting chromatograms.

Columns: Two columns supplied from MicroPak® and ELSIsphere®, made of stainless steel, 150 x 4 mm (id), packed with C18 octadecyl groups (5 μ), operated at ambient temperature, were used.

Mobile phase: Bi-distilled water, filtered through a regenerated cellulose acetate membrane (0.45 μ) and degassed ultrasonically was used as the mobile phase with flow rates of 1, 2, 3, and 4 ml/min.

Tracer: Acetonitrile (Merck) was selected as non-interacting tracer to determine the reference response of the columns.

Stimulus-Response Analysis: The system "pulse" stimulated by adding 10 μl of acetonitrile as inert tracer to water eluent at flow rates of 1, 2, 3, and 4 ml/min to determine the reference response of the columns. The column response, which is the so-called "C curve", was determined by following the absorption of the acetonitrile in the eluent stream. In each case the absorption was detected by a UV-VIS spectrophotometer. All the experiments were carried out at the temperature of 25 ^{0}C. The details of the experimental procedure was given elsewhere [23].

RESULTS AND DISCUSSION

Figure 1. and Figure 2. exemplifies the response "C" curves obtained in the stimulus-response experiments in the case of no-adsorption utilizing acetonitrile for the columns ELSIsphere® and Mikropak®, respectively. In both figure, it is clearly shown that, with the increasing flow rates of the mobile phase, the response of the both column becomes earlier. The area under the "C" curves was calculated and normalized to be unity [24]. In other words, the amount of acetonitrile adsorbed on the C18 surfaces was equal to zero. It was decided that, in this paper, the "C" curves obtained from the reference responses of the both columns were used for determining Peclet number to investigate the effect of axial dispersion.

The observed moments m_0^E, m_1^E and m_2^E were computed by numerical evaluation of the response peaks according to eqns. 10-12.

The Peclet number (Pe_d=Ud_p/D), which reflects the column flow characteristic in the case of no adsorption was calculated from eqn. 9 and the results are given in

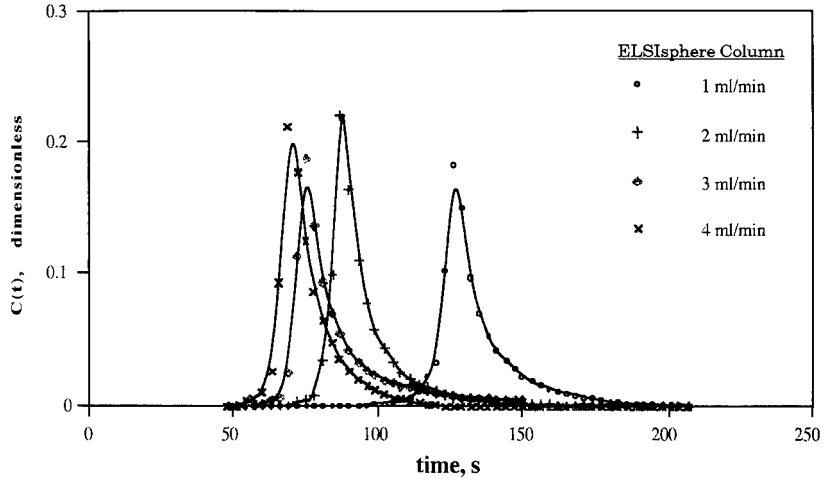

Figure 1. Response "C" curves of pulse-stimulated ELSIsphere® column at different flow rates

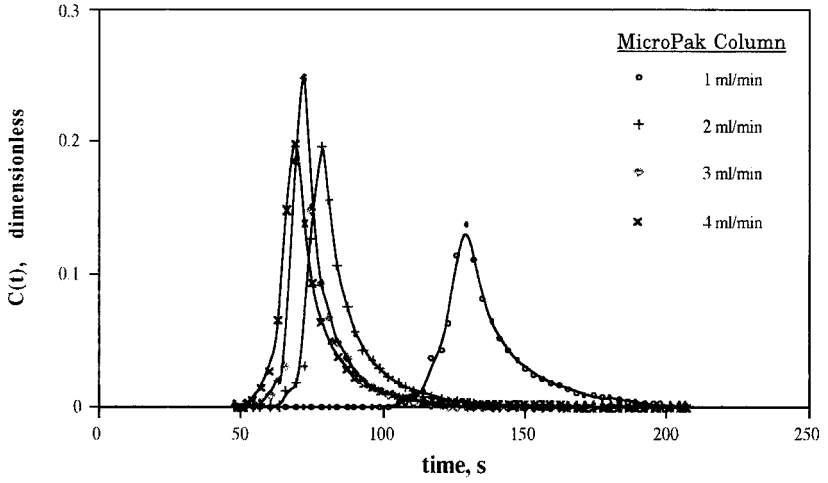

Figure 2. Response "C" curves of pulse-stimulated MikroPak® column at different flow rates

Table 1. Peclet Numbers (N_{Pe}) and Dispersion coefficients (D) of ELSIsphere® and Mikropak® C18 HPLC columns at different flow rates

	ELSIsphere®		Mikropak®	
Column Dimensions				
Length, L (mm)	150		150	
Inner Diameter, ID (mm)	4		4	
Packing Material	Octadecyl (C18)		Octadecyl (C18)	
Particle Size (μ)	5		5	
Flow Rate (ml/min)	N_{Pe}	D,cm^2/s x10^6	N_{Pe}	D,cm^2/s x10^6
1.0	158.8	3.86	141.9	4.12
2.0	109.4	8.07	93.2	10.16
4.0	48.1	22.21	46.6	23.03

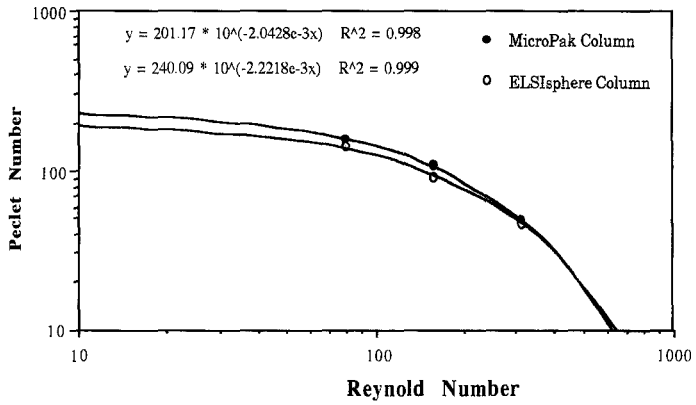

Figure 3. The change of Peclet Number via Reynold Number in C18 HPLC columns

the Table 1. Table 1 also includes the both column characteristics which are planned to be used in our future studies. When the results were compared with the literature, it is observed that, the trend of the curves drawn on the basis of Reynolds number , N_{Re} versus Peclet Number, N_{Pe}, (Figure 3.) are in a very good agreement with the literature [25-26]. It is important to note that calculated Peclet numbers for each column in this stage indicated a large amount of axial dispersion at these Reynolds numbers and the calculated values of axial dispersion coefficients, D, from those Peclet numbers (Table 1) directly characterized the effect of only axial dispersion in the column.

As conclusion, it can be said that, the stimulus-response may be considered as a deft dynamic approach to investigate the flow characteristics of fixed bed systems. The evaluation of 'C' curves by standard and cybernetic moment techniques gives the Peclet numbers and dispersion coefficients of the column, and further in case of utilizing interacting tracers; adsorption or absorption rate constants, affinity, diffusional, and other dynamic behavior of the columns would also be determined. Studies concerning the generalisation of this dynamic test approach are still under investigation [24, 27].

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FRACTIONATION OF LEUKEMIC CELLS IN AQUEOUS TWO-PHASE SYSTEMS USING COUNTERCURRENT CHROMATOGRAPHIC TECHNIQUE

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Abstract

Fractionation of various leukemic cells in aqueous two-phase systems, composed of poly(ethylene glycol) and dextran, was performed in a high speed counter-current chromatography (HSCCC) device with eccentric parallel coil orientation. Cells from different lineages or different culture conditions showed different elution patterns. Due to the instability of the stationary phase and the tendency of cells to concentrate at the phase interfaces, special modifications of HSCCC operation were developed to give satisfactory fractionation, these include optimal timing for sample injection, application of downward concentration gradient of mobile phase fluid and proper rotational speed.

Introduction

Cell fractionation is a valuable tool in chracterization of the heterogeneity within a particular cell population, in order to obtain information of medical and diagnostic interests. Distribution pattern of subpopulations is a sensitive indicator for cell differentiation and development

[1]. Centrifugation and elutriation [2] are methods utilize the differences in cell size and density, and are widely used in separating cells of very different properties. Electrophoresis and immuno-ligand based methods [3] are more specific and are suitable for subpopulation fractionation of cells with subtle different properties. Partition in aqueous two-phase systems provides an alternative to look into small differences in cell surface properties in a population [4-6]. The major controling factor for fractionation in aqueous two-phase systems is surface hydrophobicity, as affected mainly by the composition of cell membrane; charges and cell size also affect partition to a lesser extent. Conventionally, counter-current distribution (CCD) was used exclusively for this type of fractionation [7-9]. An alternative to CCD technique is reported here i.e. high speed counter-current chromatography (HSCCC) [10-14]. HSCCC has been used for separating proteins and small molecules and is simpler to operate and easier to maintain sterility than CCD technique. Also, the flow-through type of operation of HSCCC allows connection to many analytical instruments to assist further analysis of cellular properties. However, there remain some problems in adopting HSCCC to this task. Aqueous two-phase systems are much viscous than organic solvents hence phase separation is slow, this results in poor retention of stationary phase fluid. Also, cells tend to collect at phase boundary due to interfacial tension effect [15,16]. This phenomenon is interfering with the partition of cells into two phases, thus, if time is long enough, most of the cells will be located at the interfaces. Hence, normal chromatographic operation will be possible where cells are retained in the columns with no separation or fractionation. A few approaches were employed here to solve these problems. First, a proper coil orientation was chosen. Eccentric columns were found to be able to handle viscous fluid. Secondly, Interfacial surface tension was reduced by using less concentrated systems, tendancy for cells to collect at the interfaces was thus decreased. Further, to enhance the elution of cells from the columns a downward concentration gradient of the mobile phase was applied to further lowering the interfacial tension to free the cells from the interfaces. Thirdly, to obtain good resolution and to prevent settling of cells, rotational speed of the device and flow rate of mobile phase were optimised.

Material and Methods

Poly(ethylene glycol), MW 6000, was from Sigma, USA. Dextran T500 was from Pharmacia, Sweden. Other chemicals, e.g. sodium chloride, were all of reagent grade, and from Merck, Germany. Aqueous two-phase solution was prepared by mixing ingredients thoroughly at room temperature for at least 24 h. Phases were separated by centrifugation at 500 x g for 20 min and stored separately under 4°C, respectively.

A 3-column-in-series type preparative HSCCC was used (CCC-800, Pharma Tech, Baltimore, USA). This seal-less device used triplet columns containings coils of eccentric/parrallel orientation [12]. Coils are made of a single 0.25 cm inside diameter Teflon tube, wound into double-layer coils of 3 cm diameter. The total capacity of the coils is 420 ml.

Cells derived from several leukemic lines were used in this work, see Table 1. Cells were cultivated in T-flasks to late exponential phase. Harvested cells were washed twice in prewarmed buffer and were resuspended in the top phase of the aqueous two-phase fluid, at 10^8 cells/ml. The dextran-rich bottom phase was used as stationary phase, because of its high viscosity. The columns were first filled with stationary fluid. Then PEG-rich mobile phase fluid was pumped in with columns rotating at specified speed. The flow rate is about 1 ml/min. To save time and material, cell-containing sample of 1 to 3 ml was injected via a sample loop 30 to 45 min after the introduction of mobile phase. A fraction collector (Pharmacia, Sweden) was used to collect cells. Cell number and viability was determined under microscopy for each fraction.

Partition coefficient of cells was determined in single test tube partition experiment, where 0.1 ml of cell suspension was mixed with 2 ml of aqueous two-phase fluid and equilibrated for 12 min at room temperature. Phase separation was achieved without centrifugation. Portions of top phase were taken out for cell enumeration. Due to the interfacial concentrating of cells, partition coefficient was defined as the ratio of cell number in the top phase to that of initial cells.

TABLE 1.
Leukemic Cell Lines Studied.

Cell line	HL60	CTV1	RPMI6410	RPMI8402
Origin*	APL	AMoL	ALL	ALL
Differentiation stage	promyelocyte	monoblast	B-blast	T-blast
Ref.	17	18	19	20

* APL: acute promyelocytic leukemia, AMoL: acute monocytic leukemia, ALL: acute lymphoblastic leukemia.

Results and Discussion

A composition of low polymer content for the aqueous two-phase systems was developed. It consisted of (wt/wt): PEG-6000 4.762%, dextran T-500 4.76%, NaCl 0.174%, Na_2HPO_4 0.426% and NaH_2PO_4 0.360%. The phase composition used in this study is a charge sensitive one [4].

The more viscous bottom phase was chosen to be the stationary phase, because a viscous phase is much more difficult to be expelled by a less viscous phase, and thus a higher stationary phase retention. Also, in preliminary runs, the commonly used coaxial type of columns were found unsuitable for this application. This was due to the high mixing action generated by this type of columns and the difficulty in phase separation of the viscous aqueous two-phase fluid. Eccentric type of columns where tube was wound in smaller coils and without the dynamic mixing flow pattern [13] was found more suitable. Stationary retention was found around 10-30% of the total volume of coils. Contrary to the trend observed in coaxial columns [10], higher rotational speeds gave lower retention ratios in eccentric columns, as shown in Table 2. This may be due to larger pressure head needed to be overcome by the mobile fluid in order to flow through stationary fluid pockets under larger centrifugal force field. This resulted in more vigorous mixing and less retention of the stationary fluid. If we use common chromatogra-

TABLE 2.

Stationary Phase Retention in Eccentric Columns with Aqueous Two-Phase Fluid as Affected by Rotational Speed and Flow Rate S.R.: Stationary phase retention, NTP: no. of theoretical plates. NTP was estimated using trypan blue as model compound and using the relationship between half peak width and NTP [21].

	400 rpm		500 rpm		600 rpm	
	S. R.	NTP	S. R.	NTP	S. R.	NTP
1.0 ml/min	33%	236	28%	349	24%	619
1.5 ml/min	33%	172	27%	294	23%	607
2.0 ml/min	31%	164	22%	312	16%	562

phic theory to estimate the number of theoretical plate [21], we found that despite less stationary retention under higher rotational speeds, the number of theoretical plates was higher than those under lower speeds. This can be attributed to the mixing effect mentioned above. Mobile phase flow rate also affects retention and resolution. A rotational speed of 400 rpm and a mobile phase flow rate of 1 ml/min were chosen in order to prevent cell settling on tube wall when centrifugal force is too large and to avoid excessive pressure (up to 100 psi) when flow rate is high.

Mobile phase was introduced to the columns after stationary phase had already filled all three columns. After the mobile phase front reached the exit, small amount of stationary phase fluid, about 7% of the elution solution, came out with the mobile phase. This bleeding of the stationary phase was significant so the timing of sample injection will be important. Elution pattern of cells was found to be greatly affected by this timing. For example, when the sample was injected early, i.e. 15 min after mobile phase introduction to the columns, cells would concentrated at and around

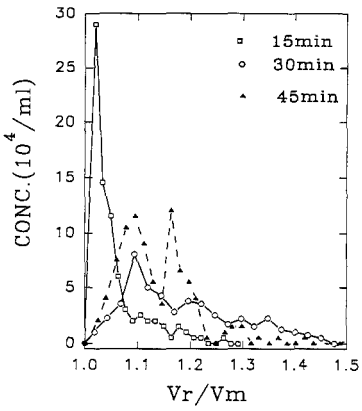

FIGURE 1 Effect of sample injection time relative to mobile phase introduction on the elution pattern of HL-60 cells. Retention volume, Vr, was expressed relative to mobile phase volume, Vm, measured at the moment of sample injection. Only viable cells were counted.

the mobile phase front, and no fractionation was observed. As the sample injection time was delayed, cell elution pattern showed more fractionation, while less cells were eluted (see Fig. 1 for HL-60's results). Some cell lines, e.g. RPMI8402, were found to be totally retained in the columns if sample was injected 45 min after mobile phase introduction (no eluted cell was observed). It is speculated that as mobile phase front moves ahead, it creats a region of unsettling two-phase mixture in its wake where cells do not partition properly. Hence if cells were injected shortly after the front, cells would emerge with the front unfractionated. On the other hand, in region far behind the mobile phase front, the two phases have reached a dynamic balance, the phase boundaries are stable and mixing disturbances are diminishing (this is especially true for the eccentric type columns). Cells bound to the interfaces are therefore difficult to be flushed out by the mobile phase in this region. To obtain fractionation, samples should not be injected too early while application of a downward gradient of the mobile phase should be used to enhance cell elution. Thus

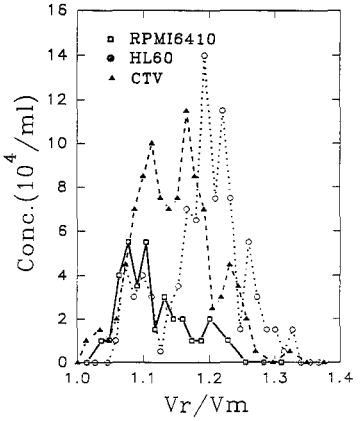

FIGURE 2 Elution patterns of RPMI6410, HL-60 and CTV1 cells. See Fig. 1 for the meaning of Vr and Vm.

cells can be brought out in a gradual, chromatographical mode, when interfacial tension was lowered by the gradient.

As shown in Fig. 2, fractionation of cells was possible using gradient (Fig. 3) method in HSCCC fractionation (only single cell line was used in each run). HL-60 was found to have the most hydrophilic cell surface in the three cell lines tested, since it has the longest retention time. This finding correlated well with the partition coefficients measured by single test tube method. The partition coefficients for the cell lines tested were: 5.43% for Hl-60, 7.50% for CTV1, 8.20% for RPMI6410, and 8.57% for RPMI8402. It is observed that HL-60 has the lowest partition coefficint, i.e. the most hydrophilic one. From Fig. 2, it is noticed that many peaks appeared for a single cell line, signifying certain heterogeneity exists among the population. There seemed to be more of such features (peaks) than observed in CCD, but the signifcance of these peaks are not certain at this time. Also, the recovery of cells was found to vary (data not shown) among different cell lines, further study is needed to clarify this phenomenon.

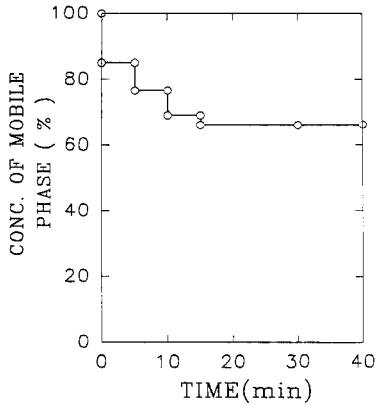

FIGURE 3 The gradient applied to the separation in Figs 2 and 4. Time zero corresponds to the moment of mobile phase introduction. Mobile phase was diluted with buffer solution containing the same amount of salts as in the aqueous two-phase system.

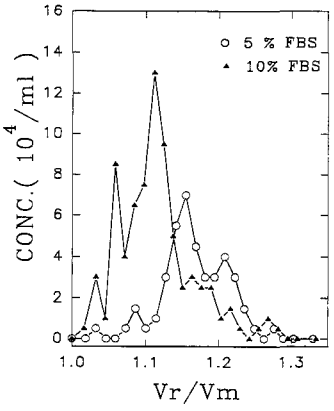

FIGURE 4 Elution patterns of CTV1 cells grown under different FBS levels. See Fig. 1for the meaning of Vr and Vm.

We can readily demonstrate the difference of cell surface properties among cells grown under different levels of fetal bovine serum by this technique. CTV1 cells cultivated under 5% or 10% FBS for 5 days were harvested and fractionated using the gradient method. Cells under 5% FBS were found to be more hydrophilic than those under 10% FBS, see Fig. 4. Cell viability was about the same for these two types of cells, so the different elution patterns reflected shift in cell surface hydrophobicity.

Conclusion

HSCCC was demonstrated to be a potential alternative to the CCD technique commonly employed in the cell fractionation work. Difficulties in handeling aqueous two-phase systems by HSCCC has been addressed. A smaller HSCCC model would be more suitable for cell fractionation since shorter processing time and smaller amount of sample would be required. Further investigation should be worthwhile.

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DETECTION AND CHARACTERIZATION OF PETROLEUM BASED ACCELERANTS IN FIRE DEBRIS BY HPLC

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ABSTRACT

A reverse phase HPLC method has been developed for the detection and characterisation of fire accelerants, viz., petrol (gasoline), kerosene, diesel fuel and their residues, generally encountered in investigations of suspected arson cases. Methanolic solutions of these petroleum products and their residues were analysed on HPLC using C-18 reverse phase column. Mobile phase of Acetonitrile: Water (8:2) at the flow rate of 1 ml/min and UV absorption detection at 275 nm and 285 nm were used. The UV detector at 275 nm and 285 nm gives specificity to detection, as the alkyl naphthalenes (aromatics) predominantly present in these petroleum products/residues have strong, characteristic absorption around 275 and 285 nm. Characteristic patterns of chromatograms coupled with intrachromatographic peak height ratios, generated amongst the peaks of naphthalene, 2-methyl-naphthalene and 2,6-dimethyl-naphthalene identified in the

chromatograms of the petroleum products/residues in question, further adds to the specificity of analysis.

INTRODUCTION

Fire debris from suspected fire and arson cases, all over the world, prominently involve the petroleum products such as petrol (motor gasoline), kerosene and diesel fuel etc. as incendiary materials. A large number of homicidal/suicidal burning cases in the Indian sub-continent have a typical unfortunate, "bride burning" context, resulting due to "dowry disputes", also involve such petroleum-based fire accelerants. In such cases partially burnt/charred clothes, bed sheets, carpets coal, hay etc. from the victim, accused and scene of offence are often referred to Forensic Science Laboratories for the detection and characterisation of these petroleum products/residues. These partly burnt objects, mostly clothes etc., when received in the Laboratories, are generally already exposed to adverse environmental conditions, such as heat, air etc. thereby losing more volatile fractions of these petroleum products. However, higher boiling hydrocarbons remain trapped in the fabric of the clothes or burnt debris.

Earlier, we ourselves and several other workers have reported a variety of physico-chemical and

instrumental methods, such as, normal (1) and derivative (2) Ultraviolet spectrophotometry, Infra-Red spectrophotometry (3-5), NMR (3), TLC (6-9), Gas Chromatography (10-15), GC-MS (16-18), Super-Critical-Fluid-Chromatography (19), Specific-Gravity (20), Aniline point (20) and Distillation range studies (21) etc. for the detection and identification of petroleum products and their residues with varying degree of success.

HPLC would be a powerful complementary technique for the detection and characterisation of petroleum products/residues. Petroleum oil analysis employing liquid chromatography has primarily involved gel permeation and straight phase systems (22-25). Application of reverse-phase liquid chromatography for separation of polyaromatics present in petroleum oils utilising fluorescence detector has been demonstrated (26-29). Methanol extracts were chromatographed on a reverse phase column followed by fluorescene spectroscopic identification of some major peaks (30). The methanol extractables from the API (American Petroleum Institute) fuel oil No.2 standard included two and three fused ring aromatics (naphthalene, 2-methyl-naphthalene and fluorene were identified). A reverse phase liquid chromatographic oil spill identification technique employing dual UV detection

(λ = 210 and 254 nm) was developed using water/methanol gradient for fuel oils and crude oils (31). In the present work a study was undertaken to separate and identify the aromatics/Polycyclic Aromatic Hydrocarbons (PAH) in the methanolic solutions of the most commonly used petroleum products, viz., petrol (motor gasoline), kerosene, diesel fuel and their residues in fire/ arson/burning cases by reverse phase liquid chromatography employing UV detection at specifically chosen wavelengths (λ = 275 and 285 nm.). Pattern recognition of chromatograms at these wavelengths was also useful for the detection and characterisation of these petroleum products.

EXPERIMENTAL

Materials :-

In the present work different standard petrol and diesel samples belonging to different petroleum companies, viz., Hindustan Petroleum, Esso, Indian Oil Corporation, and Bharat Petroleum and different kerosene samples were procured from the market. HPLC grade Acetonitrile, Methanol and Water and AR grade Diethyl ether, Naphthalene, 2-Methyl-naphthalene and 2, 6-Dimethyl-naphthalene; Quick fit standard glass distillation assembly; cloth pieces (30 cms x 30 cms) of varied textile natures were utilised.

Equipment:-

The HPLC system consisted of Thermoseparation products Co. HPLC unit, RP-C-18 column (Lichrospher 100-RP-18, 5 μm, 250 mm Length x 4 mm I.D.) variable wavelength UV detector fitted with Datajet ch-1 integrator.

Chromatographic conditions:-

Mobile phase of Isocratic solvent system comprising of Acetonitrile : Water (80: 20) was used. A flow rate of 1 ml/min at ambient temperature (30° C) was employed throughout the experiment. Detection wavelengths were chosen at 275 nm and 285 nm on the UV absorption detector. Integrator conditions were kept at, Attenuation = 4, Chart speed = 1 cm/min, Threshold = 1.0 and peak width = 0.04.

Preparation of the samples/standards:-

About 30 sq. cms pieces of cloth samples of varied textile natures such as cotton, synthetic (nylon) and terricot etc. were sprayed with different samples of petrol, kerosene and diesel separately. Subsequently they were partially or severely burnt at ambient conditions resulting in either semicharred or charred cloth materials and then extinguished, simulating such materials as received in actual fire/arson cases. These

cloth materials were suitably cut into small pieces and directly extracted with 30 ml (3 x 10 ml) portions of diethyl ether. The cloth pieces for which a clear/colourless ether extract could not be achieved were subjected to steam distillation in a quick-fit glass assembly to collect first 15 ml of distillate followed by extraction with diethyl ether. The ether extract was filtered and evaporated at room temperature to dryness and the residue was dissolved in 3 ml of methanol with vigorous shaking for about 5 minutes and then the methanolic solution was filtered through Whatman-41 filter paper. The clear filtrate thus obtained was utilised for HPLC analysis.

1.0 ml each of fresh petrol, kerosene and diesel fuels (liquids) were separately diluted exactly 100 times with methanol.

25 ml of fresh petrol (gasoline) was evaporated at ambient temperature to 0.5 ml, thus obtaining 2% v/v of residue. This residue was diluted 20 times with methanol.

10 µg/ml of naphthalene (I) and 20 µg/ml each of 2-methyl-naphthalene (II) and 2,6 dimethyl-naphthalene (III) were separately prepared in methanol.

Method:-

10 µl each of the methanolic solutions of fresh petrol, kerosene and diesel fuel samples; evaporated petrol; and also the residues of petrol, kerosene and diesel (prepared from the simulation burning experiment) and standard/reference markers of naphthalenes (I), (II) and (III) prepared as described above were separately injected on HPLC, employing the "Chromatographic conditions" as described above.

RESULTS AND DISCUSSION

Typically characteristic individual patterns of chromatograms were observed for petrol, kerosene and diesel samples, their residues and evaporated petrol samples at 275 and 285 nm wavelengths separately, representative chromatograms are shown in Figures 1 to 4 respectively. Standard reference markers of Naphthalene (I), 2-Methyl-naphthalene (II) and 2,6-Dimethyl-naphthalene (III) were identified as the major/prominent peaks (constituents) at Retention Time (RT) of 4.9, 6.2 and 8.0 $\pm$ 0.1 respectively in the chromatograms for the residues of petrol, kerosene and diesel, evaporated petrol and even in the fresh kerosene and diesel fuel samples both at 275 and 285 nm (Figures 1 to 4). Identification of other minor

FIGURE - 1(a)* : HPLC chromatogram of fresh petrol (1% v/v in methanol) at 275 nm UV-detection.

FIGURE-1(b)* : HPLC Chromatogram of fresh petrol (1% v/v in methanol) at 285 nm UV-detection.

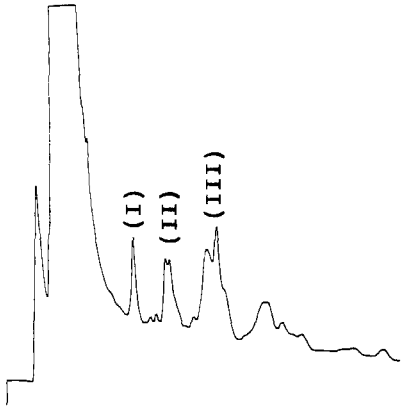

FIGURE -1(c)* : HPLC Chromatogram of petrol residues, from experimental partly burnt clothes , in methanol solvent at 275 nm UV- detection.

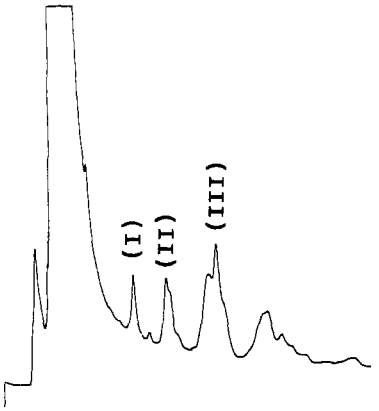

FIGURE-1 (d)* : HPLC Chromatogram of petrol residues, from experimental partly burnt clothes, in methanol solvent at 285 nm UV- detection.

* FIGURES 1 to 4 :- Quantity of injection = 10 µl each. Symbols (I), (II) and (III) marked in the chromatograms represent the peaks for Naphthalene, 2-methyl-naphthalene and 2,6 -Dimethyl-naphthalene respectively. Chromatographic conditions are described in the text.

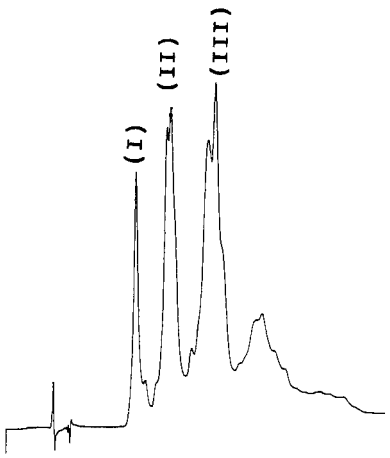

FIGURE-2 (a)* : HPLC Chromatogram of Kerosene (1% v/v in methanol) at 275 nm UV- detection.

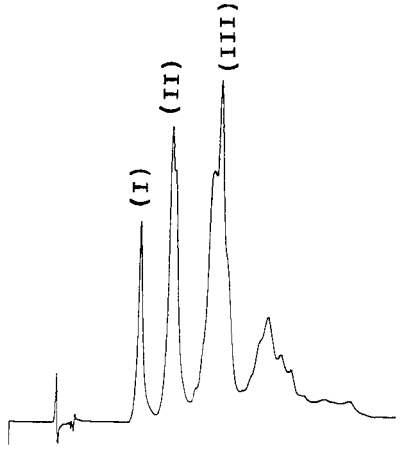

FIGURE - 2(b)* : HPLC Chromatogram of Kerosene (1% v/v in methanol) at 285 nm UV- detection.

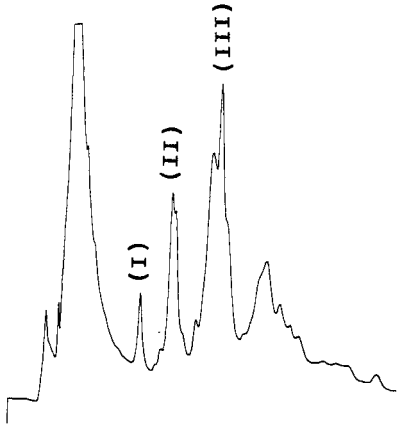

FIGURE - 2 (c)* : HPLC Chromatogram of Kerosene residues from experimental partly burnt clothes, in methanol solvent at 275 nm UV- detection.

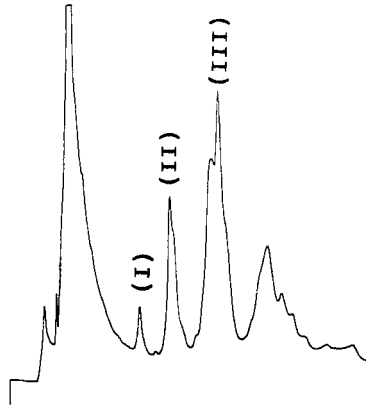

FIGURE - 2 (d)* : HPLC Chromatogram of Kerosene residues from experimental partly burnt clothes , in methanol solvent at 285 nm UV- detection.

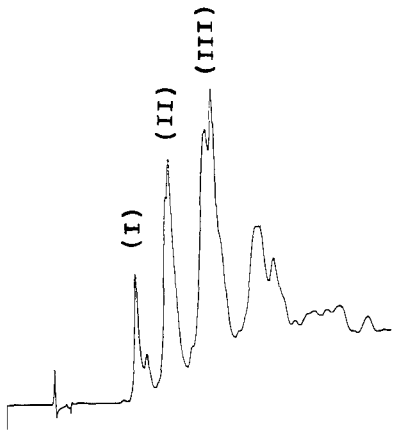

FIGURE - 3 (a)* : HPLC Chromatogram of diesel fuel (1% v/v in methanol) at 275 nm UV- detection.

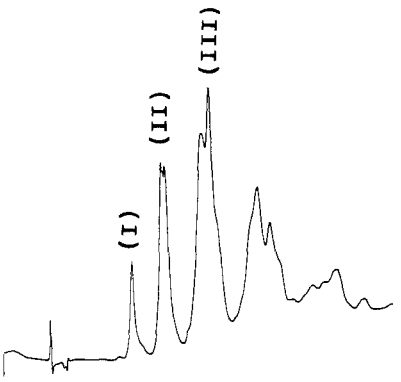

FIGURE - 3 (b)* : HPLC Chromatogram of diesel fuel (1% v/v in methanol) at 285 nm UV- detection.

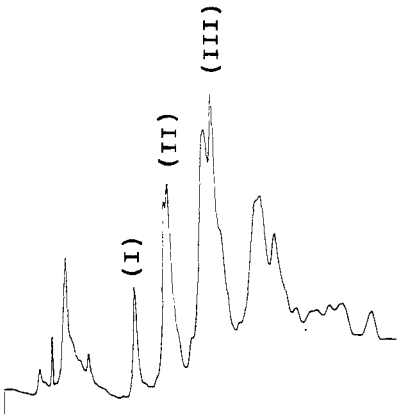

FIGURE - 3 (c)* : HPLC Chromatogram of diesel residues from experimental partly burnt clothes, in methanol solvent at 275 nm UV- detection.

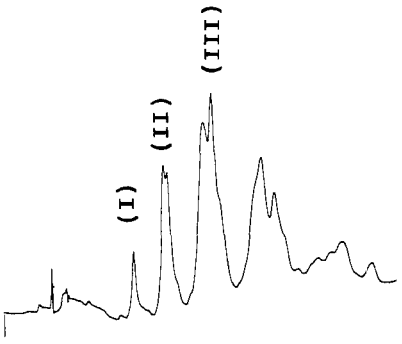

FIGURE - 3 (d)* : HPLC Chromatogram of diesel residues from experimental partly burnt clothes, in methanol solvent at 285 nm UV- detection.

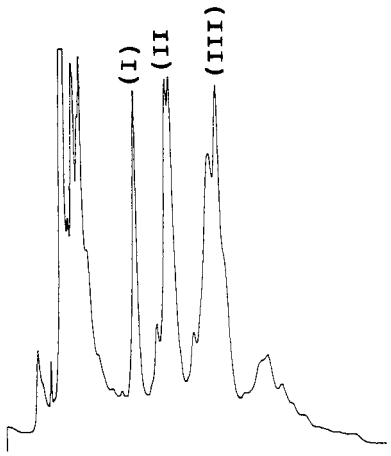

FIGURE - 4 (a)* : HPLC Chromatogram of evaporated - petrol (fresh petrol) evaporated at ambient temperature to obtain 2% v/v of residue and the residue was diluted 20 times with methanol) at 275 nm UV- detection.

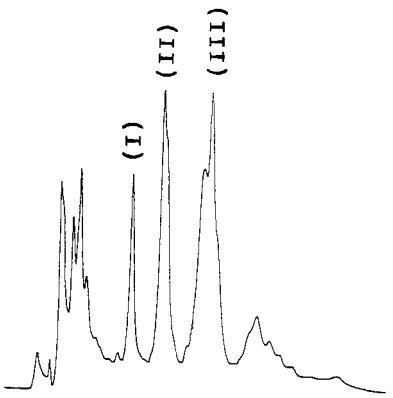

FIGURE - 4 (b)* : HPLC Chromatogram of evaporated - petrol (fresh petrol) evaporated at ambient temperature to obtain 2% v/v of residue and the residue was diluted 20 times with methanol) at 285 nm UV- detection.

peaks/constituents in the chromatograms of these petroleum products/residues could not be done because of the non-availability of other alkyl naphthalenes/ (PAH) etc. as reference markers.

It has been reported earlier by us and other workers also that, naphthalene, alkyl naphthalenes and other aromatics/(PAH) are richly/prominently present in the residues of petrol, kerosene and diesel fuels (from

partly burnt objects), evaported petrol and also even in the fresh kerosene and diesel samples (1,2,6,7,11,16,17,22-24). The present work provides an additional proof in support of above findings.

Apart from characteristic individual pattern recognition of the chromatograms for these petroleum products/residues at 275 nm and 285 nm, it was further attempted to identify/characterise individual petroleum products and their residues with their chromatograms by generating the intrachromatographic ratios of the peak heights of major/prominent peaks pertaining to the naphthalenes (I), (II) and (III) in the following manner:-

(I) $\div$ (II), (I) $\div$ (III) and also (I) $\div$ [(II)+(III)] at 285 nm UV-detection. These ratios were found to be individually characteristic for above mentioned petroleum products and their residues as shown in TABLE-1 and thus could be used for further characterisation/ identification/ confirmation of individual petroleum products and their residues. For finding out these ratios at 285 nm, peak heights were utilised rather than peak areas due to the incomplete separations of bands in 15-20 minutes elution range, utilising optimum chromatographic conditions.

It is revealed from the above Table-1 that the $\pm$ SD values of the ratios of peak heights amongst the

Table : 1. :- Average ($\bar{X}$) with (±) Standard Deviation (SD) values (n=6) of the intrachromatographic ratios of the peak heights of the peaks pertaining to the Naphthalene (I), 2-Methyl- Naphthalene (II) and 2,6-Dimethylnaphathalene (III), which are identified in the HPLC chromatograms of petrol, kerosene and diesel fuel and their residues at 285 nm UV-absorption detection.

Sr. No.	Sample	Average with ± SD values of peak height ratios :-		
		(I)÷(II)	(I)÷(III)	(I)÷[(II)+(III)]
1)	Petrol	1.0509 ± 0.0044	2.5000 ± 0.0048	0.7135 ± 0.0043
2)	Kerosene	0.6914 ± 0.0038	0.6266 ± 0.0037	0.3296 ± 0.0037
3)	Diesel	0.5207 ± 0.0034	0.3971 ± 0.0037	0.2250 ± 0.0036
4)	Petrol residues	0.8301 ± 0.0156	0.5001 ± 0.0153	0.3114 ± 0.0154
5)	Kerosene residues	0.2826 ± 0.0143	0.1725 ± 0.0144	0.1072 ± 0.0146
6)	Diesel residues	0.4412 ± 0.0133	0.3202 ± 0.0139	0.1853 ± 0.0138
7)	Evaporated petrol	0.6783 ± 0.0054	0.6850 ± 0.0051	0.3406 ± 0.0049

peaks (I), (II) and (III) are quite low and thus indicate good precision of assay for all the petroleum products/residues from the present work. Further, it also indicates that amongst them ± SD values for residues of petroleum products from burnt debris are comparatively higher than the ± SD values for the corresponding fresh, original petroleum products and

evaporated petrol sample (leaving a residue of 2% v/v). These higher values of ± SD for said petroleum residues in fire debris may be attributed to the variations in the degree of burning, relationship between these accelerants with various substrate materials and the effects of burning on them. However, even then better characterisation could be achieved by generating and considering all the three ratios as described above, viz., (I)÷(II); (I)÷(III) and (I)÷[(II) + (III)] at 285 nm detection (Table-1), along with the characteristic chromatographic pattern recognition of the individual petroleum products/residues, separately at both 275 nm and 285 nm wavelengths as shown in FIGURES 1 to 4.

Due to volatilisation and combustion composition of original fresh petrol is changed by depleting the petrol of its more volatile (low boiling) components and leaving it enriched in the high boiling point materials (such as alkyl naphthalenes and (PAH) etc.). The corresponding representative chromatograms reveal the amplitudes and, therefore, higher concentration/ enrichment of alkyl naphthalenes and (PAH) in the evaporated petrol (FIGURE-4) and petrol residues [(FIGURE-1 (c) (d)] samples than original fresh petrol [(FIGURE-1 (a) (b)]. These results are in agreement with the GC-MS work reported earlier (16,18).

Criterion for selection of wavelengths :-

Among the petroleum hydrocarbons, absorption of radiations in the ultraviolet region is mainly due to aromatics. Naphthalene and alkyl naphthalenes prevalent in the residues of petroleum products could be measured by their characteristic absorption at 225 ± 5 nm (1,2,35-37), 254 nm (31,35), 275 nm (2,35), 285 nm (2,35,37-39) and 319 nm (2,35,37). Amongst them wavelength at 319 nm was not chosen in the present study since it has significantly low absorptivities (log E values) for naphthalenes (C-10 to C-13) in the range of 1.2 to 2.5 (32,34,37) and, therefore, would affect the sensitivity of detection. Further, amongst the other wavelengths the absorption bands at 275 nm and 285 nm would provide both, characteristic as well as strong absorption for the naphthalenes (C-10 to C-13) and would be definately more specific than other wavelengths, e.g., 225 nm or even 254 nm (which shows absorption for all the compounds with monoaromatic/benzene- derivative structures also). Hence, for effectively separating the compounds of interest from the coeluting volatile impurities originating from the pyrolysis of substrate materials commonly encountered in the fire debris, UV-absorption measurements were done at specially chosen 275 and 285 nm wavelengths separately in the present work.

The absorptivities (log E values) at 285 nm of mono-nuclear aromatics, i.e., alkyl benzenes (C-8 to C-12) are less than 1.0 (37,40). However, the absorptivities are more than 2.5 for diphenyls (C-12 to C-13) and more than 3.5 for naphthalenes (C-10 to C-13) at 285 nm (37,40). Hence UV-absorption measurements at 285 nm would be most useful for the determination of total naphthalenes in the petroleum products. In fact, absorption at 285 nm is the basis of ASTM method D-1840 for the estimation of total naphthalenes in the jet fuels (38). Therefore, the ratios of peak heights amongst the naphthalenes (I), (II) and (III) were generated at 285 nm only (Table-1) in the present work.

The present method when applied to actual and simulated exhibits gave satisfactory results without intereference from commonly encountered impurities in such cases, such as, vegetable oils, body fats etc.. Other commonly encountered impurities would not show characteristic patterns of chromatograms as shown in FUGURES 1 to 4 along with characteristic peak height ratios as generated and shown in Table-1. The known negative blanks did not show characteristic peaks. At the same time, however, we acknowledge that much work remains to be done in this area before we clearly understand the relationship between various accelerants, substrate meterials and effects of fire or pyrolysis on them.

Thus the above HPLC method/approach is simple, rapid, sensitive and selective for the detection and characterisation of petroleum products/residues in day-to-day Forensic work. It could also be useful to the quality control/testing laboratories of the petroleum refineries and petro-chemicals/ (PAH) aromatic chemical industries etc.

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LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF NONYL PHENOL SURFACTANT PRESENT IN THE COMMERCIAL AND SPRAY FORMULATIONS OF AMINOCARB (MATACIL®) INSECTICIDE

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ABSTRACT

A high-performance liquid chromatographic method, using a Partisil® ODS-2 column, a mobile phase consisting of 95 % methanol in water and UV detection at 278 nm, has been developed for the determination of *p*-nonyl phenol surfactant present in the commercial formulations and spray-mixes of aminocarb insecticide. Nonyl phenol content in five commercial formulations and four spray-mixes was analysed with good reproducibility after removing the insecticide by alkaline hydrolysis and extracting the surfactant with *n*-heptane. Minimum quantification limit for the analyte was 0.03 μg in 10-μL injection volume. The method is flexible and should be applicable to analyse a variety of nonionic ethoxylated nonyl phenol surfactants present in many industrial and consumer products.

INTRODUCTION

Surface active agents, or surfactants, are amphiphilic compounds containing two structurally distinct parts, one of which is a water-soluble, polar, hydrophilic head and the other, a water-insoluble, apolar, hydrophobic hydrocarbon tail. They are commonly used in pesticide formulations as spray adjuvants [1]. Surfactants can improve the characteristics of the spray solutions in a variety of ways, *viz.*, as spray-modifiers, compatibility agents, spreaders of spray droplets on foliar surfaces and as penetrators of pesticide molecules through plant cuticles, which often act as barriers to the molecules [2-5].

Nonyl phenol and its ethoxylated products are extensively used as nonionic surfactants in various commercial products and as primary solvents in pesticide formulations. The base material, nonyl phenol [RC_6H_4OH; R, the side chain (C_9H_{19}) is primarily *para* substituted, consisting of isomeric branched nonyl radicals], is a pale yellow viscous liquid (MW, 225; MF, $C_{15}H_{24}O$; η, 563 cp at 20°C; v.p., 2 mm Hg at 130°C; d_4^{20}, 0.950; b.p., 293-297°C; n_D^{20}, 1.513; and f.p., −10°C) with mild phenolic odor, practically insoluble in water and dilute aqueous sodium hydroxide but soluble in organic solvents, and is not rapidly biodegradable. The addition of nonyl phenol to pesticide formulations not only modified their properties, but also enhanced the spreading and cuticular penetration of the active ingredient (AI) [2-5]. During broadcast application of insecticide spray-mixes over forest areas using aircraft, the presence of non-evaporators like nonyl phenol in the spray-mixes greatly improved the targetability and deposition of droplets on foliar surfaces, thus enhancing the efficiency of spraying [6].

Aminocarb (4-dimethylamino-*m*-tolyl N-methyl carbamate), marketed commercially as an oil-soluble formulation under the trade name Matacil® 1.8D by Chemagro Limited

(Toronto, Ontario), is used extensively as an insecticide in Canada since 1973 to control the spruce budworm, *Choristoneura fumiferana* (Clem.) larvae, a destructive defoliator of spruce-fir forests of eastern North America [7]. The commercial formulation, Matacil 1.8D, is an oil-soluble concentrate (OSC) consisting of 19.5 % AI (weight %), 30.0 % insecticide diluent-585 (ID-585)(Shell Canada Ltd., Toronto, Ontario), an aliphatic hydrocarbon fraction obtained during petroleum distillation, and 50.5 % nonyl phenol (Rohm and Haas Canada Ltd., West Hill, Ontario) [8,9]. The spray-mixes used in aerial application were prepared by diluting the commercial formulation with ID-585. Nonyl phenol is reported to be toxic to juvenile Atlantic salmon, *Salmo salar* L. [10].

The amount of nonyl phenol present in Matacil 1.8D and in spray-mixes has a considerable effect on the quality and stability of the formulation, and on the overall biological consequences of the insecticide. Therefore, to assure and to maintain adequate quality control, it has become necessary to analyse and quantify the nonyl phenol content in the commercial formulation, Matacil 1.8D, and in the spray-mixes after necessary treatments to remove the AI and other ingredients present in them. Analysis of nonyl phenol in forestry formulations has not been reported earlier in the literature. However, an HPLC method to quantify nonyl phenol residues in forestry matrices was published [11]. Attempts to form volatile and readily detectable fluorobutyrl [12,13] and dinitrophenyl [14] derivatives with the phenolic group and analyse them by GLC-ECD were not successful due to steric hindrance of the bulky nonyl group [11]. Similarly, GLC-FID methods using packed columns containing solid support Chromosorb W-HP coated with OV-1, OV-17, SE-30 etc., were tried. However, the FID was found to be insensitive, and when higher concentrations were injected, the peak

symmetry and consistency in retention times (RTs) were lost. These trials indicated that under the experimental conditions used, the GLC methods using either ECD or FID were not successful and they did not provide unambiguous identification and quantification of nonyl phenol present in the aminocarb formulations and spray-mixes. Therefore, the objective of the present study was to develop a simple, reliable and rapid reverse phase HPLC method to monitor nonyl phenol concentrations found in the commercial formulations of aminocarb (Matacil 1.8D) and their spray-mixes used in the forest insect control programs in Canada.

MATERIAL AND METHODS

Nonyl Phenol Standard

Nonyl phenol standard containing 92 % of the *p*-isomer was supplied courtesy of Rohm and Haas Company, Spring House, PA, USA. The listed impurities in the sample were: *o*-isomer, *ca* 3 %; 2,4-dinonyl phenol, *ca* 3 %; and other structural isomers, *ca* 2 %.

Stock and Standard Solutions

A stock solution of nonyl phenol containing 1.00 mg/mL was prepared by accurately weighing 100.0 mg of the nonyl phenol standard and dissolving it in 100 mL of HPLC-grade methanol. The standard solutions, ranging in concentration from 1.0 µg/mL to 100 µg/mL, were prepared by serial dilution of the stock solution with methanol. All solutions were stored in a refrigerator at 4°C.

Formulations and Spray-Mixes

The five commercial formulations of aminocarb (Matacil 1.8D)(labelled as OSC 1 to 5) and the four spray-mixes (labelled as SM 1 to 4) used in the study were supplied by Chemagro Ltd., Toronto, Ontario, Canada.

Solvents

All solvents and water used in the study were HPLC-grade, supplied by Caledon Laboratories (Georgetown, Ontario) and were tested for their spectral purity prior to use. They were filtered using Millipore® 0.20-μm filters and degassed prior to use in the HPLC.

HPLC Instrumentation

The liquid chromatograph used was a Hewlett-Packard (HP) model 1084 B, fitted with a UV variable wavelength (190-600 nm) detector, interfaced with a variable volume Rheodyne® injector equipped with 10 to 100 μL loops and an autosampler (HP 79842). The computing facilities in the instrument included a microprocessor and an electronic integrator linked to an LC terminal (HP 79850 B) to provide the area, area %, retention time (RT) etc., for each chromatographic peak. Full description of the instrument is given in an earlier publication [15]. The operating parameters were as follows:

Column:	Whatman Partisil® PXS 10/25 ODS-2
Column pressure:	3.5×10^3 kPa
Mobile phase:	95 % methanol in water at 1 mL/min
Oven temperature:	40°C
UV detector wavelength:	278 nm
Injection volume:	10 μL

The instrument was calibrated daily before the analysis of formulations by injecting, in triplicate, 10-μL volumes containing 0.01 to 1.0 μg of nonyl phenol standard and recording the detector response. Calibration curves were prepared daily, before and after sample analysis, by plotting the average peak area (y-axis) against the mass (μg) of nonyl phenol injected (x-axis) to confirm the stability and response of the instrument. Quantitation was done by external standardization based on peak area.

The nonyl phenol standard, under the experimental conditions used, gave a major peak (peak A, *p*-isomer) with RT = 5.5 ± 0.1 min accounting for 92 % of the peak area and two minor peaks, one with RT = 5.1 ± 0.1 min (peak B) and the other with RT = 4.2 ± 0.1 min (peak C) both in total amounting to 5 % of the peak area. Another minor, somewhat broad peak with RT = 7.8 ± 0.3 min (peak D) accounted for about 3 % of the peak area. From the compositions of the nonyl phenol standard used in this study, it is apparent that peak A corresponded to the *p*-isomer of nonyl phenol, peaks B and C could be the *o*- and other structural isomers of the analyte, and peak D with long RT could be inferred as that of 2,4-dinonyl phenol.

Separation of Nonyl Phenol from Formulations and Spray-Mixes

The commercial formulations (Matacil 1.8D) and the spray-mixes were shaken well and aliquots of each (500 μg of formulation and 1.5 mg of spray-mix) were weighed accurately into separate ground-glass stoppered Erlenmeyer flasks (150-mL). Fifty mL of aqueous methanolic (5 %) sodium hydroxide (0.10 M) containing 1 % sodium chloride was added to each flask, the flasks were then placed in a Blue M MagniWhirl® constant temperature water-bath set at 40 ± 1°C and shaken at medium speed for 4 h to hydrolyse the aminocarb

insecticide. The alkaline solution corresponding to each sample was transferred quantitatively to a 150-mL separatory funnel and partitioned thrice, using each time 50 mL of *n*-heptane. The heptane layers were pooled, passed through a column of anhydrous sodium sulfate (20 g) to remove the moisture, flash-evaporated at 35°C to near dryness and the residues were then dissolved in methanol. The methanolic solution was filtered (Millipore 0.20 μm filter) to remove particulates, transferred to a stoppered graduated centrifuge tube and the volume was adjusted by concentration under a stream of dry nitrogen (Meyer N - EVAP®) for HPLC analysis. Methanolic solution of each sample was injected (10 μL), in triplicate, into the liquid chromatograph, the average peak area was calculated and the nonyl phenol concentration was then computed from the prepared calibration curve.

RESULTS AND DISCUSSION

Linearity of UV Detector

The purity of the nonyl phenol (*p*-isomer) standard received from the manufacturer to calibrate the UV detector was not high. It contained only 92 % of the *p*-isomer admixed with positional and structural isomers as well as other impurities amounting to about 8 %. Prior to the analysis of commercial formulations and spray-mixes, the linearity of the UV detector response to *p*-nonyl phenol, was checked by injecting 10-μL aliquots of each standard solution at least thrice. Results were plotted as average peak area *versus* concentration of *p*-nonyl phenol in the standard. The curve was linear in the concentration range of 0.03 μg/10 μL to 1.0 μg/10 μL and passed through the origin ($r = 0.996$). Also, the reproducibility of peak area measurement for the concentration range was > 95 %. The standard deviation

(SD) in the peak area measurement for each individual concentration in this range was nearly equal to its mean value. However, the situations were not the same for the concentrations below 0.03 μg/10 μL (0.01 and 0.02 μg/10 μL). The points not only did not fall on the linear curve but also the SD values were high, indicating the poor detector response at low concentrations. Considering the structural characteristics of the analyte, this is understandable because, apart from the π-electrons in the aryl ring and the lone pair of *p*-electrons on the phenolic oxygen, there is no strong UV absorbing chromophoric group in the molecule. Therefore, at low analyte concentrations we could expect poor detector response, leading to errors in the measurement.

From the observed linearity over the concentration range studied (0.03 to 1.0 μg/10 μL), the limit of quantification (LOQ) for the *p*-nonyl phenol was established conservatively as 0.03 μg. On the other hand, the limit of detection (LOD) could reach as low as 0.01 μg; however, below the 0.03 μg level the detector response would be highly variable and in accordance, the associated signal-to-noise ratio would also be high.

HPLC Chromatogram of the Standard

The problems associated with analysing surfactants are that they occur as mixtures differing in molar masses and structures, and are often mixed with low levels of impurities or contaminants resulting from the manufacturing process [16]. Even the so-called "chemically pure" material usually contained detectable levels of isomeric and other contaminants as impurities; this is true in the case of nonyl phenol also. Unless proper chromatographic resolution is attained, it is very likely that we may over-estimate the concentration of the interested analyte. Despite these problems, under the present

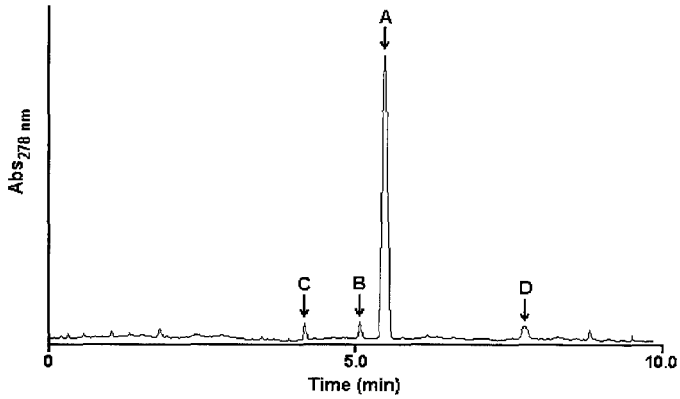

Figure 1. Chromatographic trace of nonyl phenol standard after injecting 10-μL volume containing 50 μg nonyl phenol/mL. Peak A, *p*-nonyl phenol (RT = 5.5 min); peaks B (RT = 5.1 min) and C (RT = 4.2 min), o- and structural isomers; and peak D, 2,4-dinonyl phenol (RT = 7.8 min).

experimental conditions used, we were successful at isolating and resolving the chromatographic peak corresponding to the *p*-nonyl phenol from other isomers and impurities in the standard.

A typical chromatogram of the nonyl phenol standard, determined by injecting 0.5 μg in 10 μL onto the HPLC column, is shown in Figure 1. The chromatographic peak A with RT = 5.5 min, corresponding to the *p*-nonyl phenol component, is well resolved and symmetrical. The two minor peaks, B (RT = 5.1 min) and C (RT = 4.2 min), are also well resolved and symmetrical and these two are assumed to correspond to the *o*-isomer (peak B) and a structural isomer (peak C) whose composition and geometry are unknown. Similarly, the broad peak (peak D) with RT = 7.8 min is presumed to belong to the impurity 2,4-dinonyl phenol [9], although the exact composition of this impurity was not revealed to the author by the manufacturer.

Nonyl Phenol in the Formulations and Spray-Mixes

The five commercial formulations (Matacil 1.8D)(OSC 1 to OSC 5) contained on average 49.5 % (range 47.1 to 51.2 %) of *p*-nonyl phenol, compared to the manufacturer's value of 50.5 %. The four spray-mixes (SM 1 to SM 4) contained on average 15.9 % (range 14.4 to 17.2 %) of the material compared to the expected value of 16.8 %. Both sets of data show good agreement between the expected and measured values, indicating the usefulness of the method reported in this paper to analyse the *p*-nonyl phenol component present in the commercial formulations and spray-mixes of aminocarb insecticide.

Typical chromatograms obtained for the commercial formulation (OSC 1) and the spray-mix (SM 1) are shown in Figures 2 and 3, respectively. In addition to the four distinct peaks (A to D) observed in the standard (Figure 1), these chromatograms contained a number of additional peaks, large and small, indicating the presence of UV absorbing impurities in the solvent, ID-585, as well as in the technical aminocarb used for preparing the commercial formulation (Matacil 1.8D) and the spray-mixes. The latter were prepared by diluting the commercial formulation with ID-585, roughly in the ratio of 1:2. However, no interference peak masked the peak A, which corresponded to the *p*-isomer of nonyl phenol. This is also true for the peaks B and C. On the other hand, examination of Figures 2 and 3 show that an interference peak appeared as a shoulder to peak D and its size increased while analysing the spray-mix, because it had higher concentration of ID-585 compared to the commercial formulation. This clearly indicated that a UV absorbing impurity was present in ID-585 which co-eluted with nearly similar RT as the 2,4-dinonyl phenol (peak D). Apart from this, the distinct separation of peak A, corresponding to the *p*-nonyl phenol, clearly indicated that the extraction and separation procedures used in the study and the

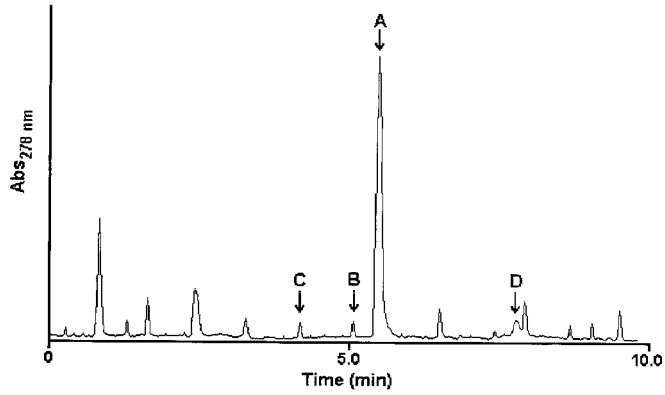

Figure 2. Chromatographic trace of 10-µL injection of Matacil® 1.8D (OSC 1) extract. For peak definitions and retention times, refer to Figure 1.

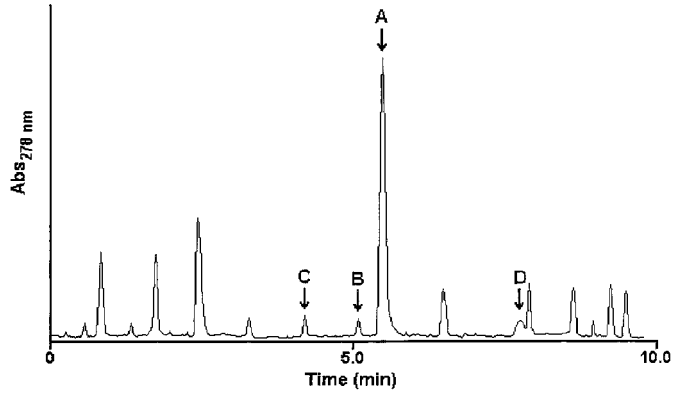

Figure 3. Chromatographic trace of 10-µL injection of Matacil® 1.8D (OSC 1) spray-mix (SM 1) extract. For peak definitions and retention times, refer to Figure 1.

instrumental parameters chosen are excellent, and are ideally suited to analyse and quantify the *p*-nonyl phenol component present in the commercial formulations (Matacil 1.8D) and their spray-mixes used in the forest insect control programs in Canada.

CONCLUSIONS

In recent years, quality assurance and quality control of pesticide formulations used in forest spray applications have become mandatory requirements. Therefore, it is necessary not only to know the exact amount of active ingredient present in the formulation but also to analyse and report the concentrations of potentially toxic adjuvants present in the formulation. This requirement came because some of the emulsifiers used previously in the spray formulations were reported to enhance viral activity in mice and caused Reye's syndrome in children [17]. Today, HPLC methods are used extensively in formulation analysis because they are fairly simple to perform without much cumbersome derivatization and provide rapid and reliable results. The method reported in this paper presents a rapid, accurate procedure for determining the *p*-nonyl phenol in aminocarb formulations and, after necessary modifications in the methodology, has potential for application to analysis of various ethoxylated alkylphenols present in many industrial and consumer products.

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A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF RAPAMYCIN (SIROLIMUS) IN RAT SERUM, PLASMA, AND BLOOD AND IN MONKEY SERUM*

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ABSTRACT

A high performance liquid chromatographic (HPLC) method was developed for the quantitation of rapamycin, an immunosuppressive agent, in biological specimens. The method employs a 15 cm Supelco LC-18 column (5 μm) interconnected to a 25 cm Supelco LC-18 column (5 μm). The mobile phase is a methanol/water gradient system. The flow rate is 0.51 ml/min and detection is by ultraviolet (UV) absorption at 276 nm. The method was validated for its specificity, precision, linearity and sensitivity in rat serum. Endogenous compounds in rat serum did not interfere with the detection of rapamycin or the internal standard (β-estradiol-3-benzoate). Based on a 1.0 ml serum sample, the assay was linear from 5 to 500 ng/ml. The intra-day coefficients of variation were below 10% and independent of concentration. Inter-day precision values ranged from 5.5 to 13.6%, the difference being independent of concentration. The specificity, linearity and sensitivity of the method was also demonstrated in cynomolgus monkey serum, rat plasma and hemolyzed rat whole blood. In each case, the method was specific, with no endogenous interferences. Furthermore, the method showed no interference from two newly reported rapamycin degradation products.

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*This work was presented at the North New Jersey ACS Drug Metabolism Discussion Group Annual Symposium, Somerset, NJ, September, 1990.
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INTRODUCTION

Rapamycin (Figure 1a), an antitumor and antifungal agent isolated from the fungus *Streptomyces hygroscopicus* is under development as an immunosuppressant (1-2). Rapamycin has been found to have potent immunosuppressive activity while exhibiting little toxicity in primates (3-4). In order to carry out pharmacokinetic and drug disposition studies, we developed and validated a specific high performance liquid chromatographic method for the analysis of rapamycin in rat serum, plasma and whole blood and monkey serum.

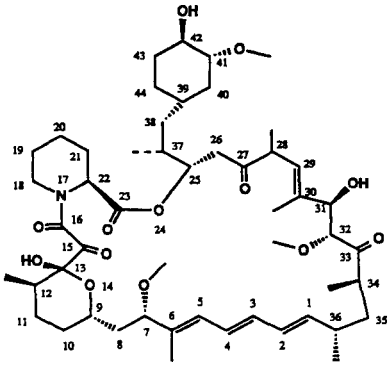
EXPERIMENTAL

Chemicals and Reagents

Rapamycin was obtained from Wyeth-Ayerst Research, Rouses Point, NY. A stock solution was prepared by dissolving 10.45 mg of rapamycin in 50 ml of methanol. Standard solutions were made by diluting the stock solution with methanol to obtain rapamycin concentrations of 10, 5, 2, 1, 0.5, 0.2 and 0.1 μg/ml. The internal standard (I.S.,β-estradiol-3-benzoate) was purchased from Aldrich (Milwaukee, Wisconsin). An I.S. stock solution was prepared by dissolving 18.0 mg of β-estradiol-3-benzoate in 50 ml methanol. Working I.S. solution was prepared by diluting 1.0 ml of stock solution to 50 ml with methanol to give a final concentration of 7.2 μg/ml. Two rapamycin degradation products A and B (Figures 1b and 1c) were prepared in house (5). HPLC grade methanol and water were obtained from EM Science (Gibbstown, NJ). Aluminum oxide (Neutral, Brockmann Activity Grade I) was purchased from J.T. Baker (Phillispsburg, NJ). Isopropyl ether was purchased from Fluka Chemical (Ronkonkoma, NY). Isopropyl ether was purified before each analysis by passing it through neutral alumina (Brockmann activity grade I). Before use, purity was checked by HPLC as described in the HPLC analysis procedure.

Instrumentation

The HPLC system consisted of a Waters Model 600E pump (Waters Associates, Milford, MA), a Waters WISP Model 712 autosampler, a Waters programmable multiwavelength detector set at 276 nm and a Hewlett Packard Model 3390A integrator (Hewlett-Packard, Avondale, PA). Separation of rapamycin was achieved using a Supelcosil LC-18 column (150 mm x 4.6 mm i.d., 5 μm particle size) interconnected to a Supelcosil LC-18 column (250 mm x 4.6 mm i.d., 5 μm particle size) and a Supelguard LC-18



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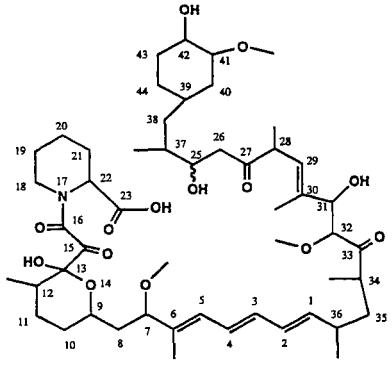

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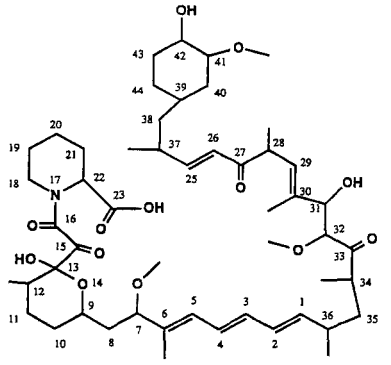

1c

FIGURE 1 Chemical Structures of Rapamycin (1a), Rapamycin Degradation Product A (1b) and B (1c)

TABLE 1

HPLC Gradient Program for the Analysis of Rapamycin[a]

Time (min)	Mobile Phase (%)	
	Methanol	Water
Initial	87	13
25	92	8
36	92	8
40	87	13
55	87	13

[a]Linear gradient was used between each time point and the flow rate was 0.51 ml/min.

precolumn with 2 cm x 4.6 mm Supelcosil cartridge purchased from Supelco (Bellefonte, PA).

Extraction of Rapamycin from Biological Fluids

Fifty µl of methanol were added to 1.0 ml of the experimental sample in a 16 x 125 mm disposable screw-cap culture tube and mixed by vortex. Fifty µl of the working standards were added to the control serum samples and mixed by vortex to prepare the standard curve. Next, 50 µl of I.S. working solution (β-estradiol-3-benzoate) were added to each tube and mixed by vortex. Two and a half ml of purified isopropyl ether were added and the tubes were vortexed, shaken for 15 min and then centrifuged at 2300 rpm for 10 min. The isopropyl ether phase was transferred to a clean conical screw-cap tube and evaporated to dryness under a stream of nitrogen gas.

HPLC Analysis

The mobile phase was composed of methanol and water. An HPLC gradient mobile phase was used for chromatography (Table 1). The isopropyl ether extract was reconstituted in 100 µl of mobile phase [methanol/water (87:13)] and 60 µl was injected onto the HPLC column. The column was maintained at room temperature and the detection wavelength was 276 nm.

Specificity

The specificity of the method was evaluated with regard to interference due to the presence of endogenous substances in the extract of control rat serum. Ten random individual control sera and a control serum spiked with rapamycin and internal standard were processed by the method described above and analyzed. Specificity testing was also performed in four control monkey serum, two rat whole blood and four rat plasma, respectively. Since recently, two degradation products of rapamycin were identified (5), the chromatographic properties of these compounds were also measured using the HPLC system described above to characterize the specificity of the method.

Linearity, Precision and Accuracy

The linearity, minimum and maximum quantifiable concentrations, precision and accuracy of the method were evaluated in both intra-and inter-day studies. Intra-day data were obtained by analysis of four replicates of 5, 10, 25, 50, 100, 250 and 500 ng/ml concentrations of rapamycin in 1.0 ml of control rat serum. Inter-day data were obtained by analysis of five replicates of spiked 5, 50 and 500 ng/ml serum on three separate days using analytical standards independently prepared on each day of analysis. The linearity of the method was also performed in rat plasma and whole blood and monkey serum.

Calculations

Rapamycin concentrations in ng/ml were obtained from the regression line relating rapamycin/internal standard peak area ratios to the compound concentrations calculated using unweighted linear regression in a Hewlett-Packard Model 85 calculator or an IBM compatible computer.

RESULTS

HPLC Analysis

Typical HPLC chromatograms for control serum, control serum spiked with rapamycin and internal standard (β-estradiol-3-benzoate) and a serum sample from a rat given

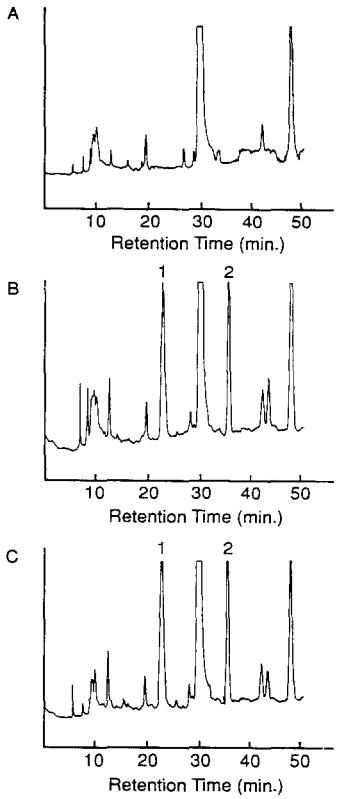

FIGURE 2 Chromatograms of Control Rat Serum (A), Control Rat Serum Spiked with Rapamycin and β-estradiol-3-benzoate (B) and a Serum Sample from a Rat given Rapamycin (C). 1, Rapamycin; 2, β-estradiol-3-benzoate.

rapamycin intravenously are presented in Figure 2. Under the conditions specified, the retention times for rapamycin and β-estradiol-3-benzoate were 23.4 and 36.8 min., respectively. No endogenous interfering substances were detected in the extracts of ten random control rat serum samples. The endogenous interfering substances were not found in the control rat plasma, whole blood and monkey serum as well. The assay of rapamycin was not interferred by the two degradation products (A and B) of rapamycin; A and B showed very similar retention times in the chromatogram (Figure 3).

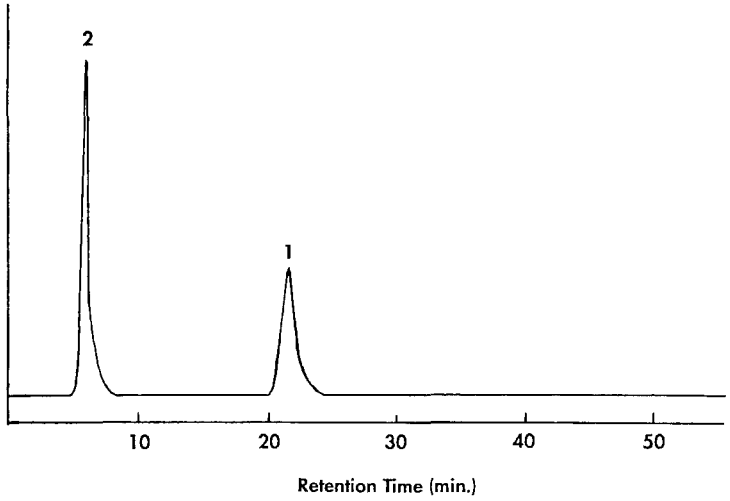

FIGURE 3 Chromatograms of Rapamycin and its Degradation Products A and B. 1. Rapamycin; 2. Degradation Products A and B.

Linearity and Sensitivity

The linearity of the peak area ratio to the concentration of rapamycin was calculated using linear regression analysis. The method was linear for concentrations between 5 and 500 ng/ml in rat serum and whole blood and monkey serum with a correlation coefficient (R^2) of >0.99. For the rat plasma, it was linear from 10 to 500 ng/ml.

Precision and Accuracy

The intra-day precision of the method was assessed by calculating the coefficient of variation for replicate samples (N=4) at 5, 10, 25, 50, 100 (N=3), 250 and 500 ng/ml. The coefficients of variation were 2.7, 7.5, 3.3, 0.6, 5.2, 9.8 and 4.9%, respectively, and were independent of concentration. Analytical bias, based on the percent difference between found and theoretical concentrations, was within ±13% and was not concentration dependent. The coefficients of variation in the inter-day precision study were 5.5, 8.4 and 13.6% at 500, 50 and 5 ng/ml, respectively, the differences being independent of concentration.

DISCUSSION

An HPLC method for the quantitation of rapamycin in rat serum has been developed and validated. The method is specific with no endogenous interferences. The assay is linear in the range of 5-500 ng/ml with the minimum quantifiable concentration of 5 ng/ml. It is important with this method to check the isopropyl ether, the mobile phase and control sera for possible interfering peaks before use.

This method has also been validated for use in the measurement of rapamycin in monkey serum, rat whole blood and rat plasma. In each case, the method was shown to be specific, with no endogenous interferences. For monkey serum and rat whole blood, the method was linear from 5 to 500 ng/ml. For rat plasma, it was linear from 10 to 500 ng/ml. These results indicate that this method may be applied to the analysis of rapamycin in these matrices.

Yatscoff et al (6) reported an HPLC method to measure rapamycin in whole blood. Desmethyl rapamycin was used as the internal standard. In our method, the more readily available internal standard, β-estradiol-3-benzoate has been shown to provide satisfactory results.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF CORTISOL, CORTISONE, AND THEIR 20-REDUCED METABOLITES IN PERFUSION MEDIA

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ABSTRACT

A reversed-phase liquid chromatographic assay to quantitate cortisol, cortisone and their respective 20α- and 20ß-dihydro reduced metabolites in tissue culture media from *in vitro* perfusions of the human placental lobule is described. The internal standard used in this assay was 6α-methyl-prednisolone. Steroids were extracted from the perfusion medium using Sep-Pak reversed-phase cartridges with the average recoveries of each

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steroid at 150 and 600 nmol/L ranging from 84.4 to 99.1% and 85.6 to 93.5% respectively. The separation was achieved by using two C_{18} columns linked in series at 40°C with a mobile phase of methanol/water (53/47 v/v) and a flow rate of 1.1 mL/min. The eluant was monitored by UV absorption at 242 nm. The assay was linear for each steroid to a concentration of 750 nmol/L with a lower detectable limit of 5 nmol/L. Intra-assay coefficients of variation were measured at 150 and 750 nmol/L with ranges of 4.0% (cortisone) to 5.5% (cortisol) and 2.8% (cortisol) to 4.0% (cortisone and 20α-dihydrocortisone) respectively. Inter-assay coefficients of variation were 6.0 (20α-dihydrocortisone) to 9.6% (cortisone) and 5.8 (20α-dihydrocortisol and cortisone) to 6.9% (20ß-dihydrocortisone) at these concentrations respectively. With this method prednisolone coelutes with cortisol however no other interferences, from endogenous steroids or drugs which may be used in pregnancy, were found.

INTRODUCTION

Human placental tissue contains significant concentrations of 11ß-hydroxysteroid dehydrogenase [1], an enzyme which catalyses the conversion of cortisol (11ß,17α,21-trihydroxypregn-4-ene-3,20-dione) to cortisone and may provide a protective function to limit the maternofetal transfer of maternal cortisol [2]. We have demonstrated that a related corticosteroid, prednisolone, is metabolised by the human placenta not only to prednisone, but also to 20α-dihydroprednisone, 20ß-dihydroprednisone and 20ß-dihydroprednisolone [3]. In that study a reversed-phase high performance liquid chromatography (HPLC) assay was used which quantitated prednisolone, prednisone and the related 20α- and 20ß- reduced metabolites [4].

A number of liquid chromatographic methods for the determination of corticosteroids in biological fluids have been reported using both reversed-phase and normal-phase modes of analysis. Reversed-phase

HPLC has been used to determine cortisol concentrations in serum [5–7] and plasma extracts [8,9] and normal–phase HPLC has been employed to assay prednisolone, prednisone and cortisol in human plasma with dexamethasone as the internal standard [10]. Blasco, Lopez–Bernal and Turnbull [11] described a reversed–phase HPLC method for the separation of cortisol and cortisone following incubation of cortisol with placental homogenates as a measure of the activity of 11ß–hydroxysteroid dehydrogenase. Other studies of placental corticosteroid metabolism have used Sephadex LH–20 columns or paper chromatography, which are relatively insensitive, for qualitative analysis [12,13] to identify metabolism at the 11 position in a series of corticosteroids incubated with placental minces and in *in vitro* tissue perfusions respectively. The only reported separation of cortisol, cortisone and their corresponding 20–reduced metabolites has been in human urine using a combination of reversed phase chromatography and gradient elution [14].

The isocratic HPLC assay described here was developed to separate cortisol, cortisone and their related 20α– and 20ß–reduced metabolites in order to investigate further the metabolism of cortisol in the *in vitro* perfused human placental lobule.

MATERIALS AND METHODS

Chemicals and Reagents

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

(Rheodyne Inc., California, USA) fitted with a 100 μL sample loop and a Kortec K95 variable wavelength UV detector set at 242 nm. Two Brownlee Spheri-5 RP-18 columns (250 x 4.6 mm), linked in series and a RP-18 newguard precolumn (15 x 3.2 mm, Brownlee Labs., San Jose, CA, USA) were used for the separation. Column temperature was set at 40°C with a TC1900 Temperature Controller (ICI Instruments, Sydney, Australia).

The mobile phase was 53% (v/v) methanol (HPLC grade, Mallinckrodt, Paris, KY, USA) in reagent grade water, filtered under vacuum through a 0.45 μm nylon membrane (Alltech Associates, Sydney, Australia). The mobile phase flow rate for the analyses was 1.1 mL/min. C_{18} Sep-Pak cartridges were purchased from Millipore (Brisbane, Australia). Cortisol, cortisone, their respective 20-reduced metabolites and 6α-methylprednisolone were obtained from Sigma Chemical Company (St. Louis, MO., USA). All validation studies used perfusate from a placenta not perfused with cortisol or prednisolone as substrate. The perfusion fluid was tissue culture medium M199 (Sigma) with glucose (2 g/L), heparin (25 IU/mL), gentamicin (100 mg/L, David Bull Labs., Melbourne, Australia) and dextran (7.5 g/L, Sigma).

Standard Separations

Stock solutions (2 mg/mL) of each steroid were prepared in methanol. A working mixture was prepared by combining aliquots (100 μL) of each stock solution and diluting to 10 mL with methanol to give a final concentration of 20 μg/mL for each steroid. A 10 μL aliquot of this solution was then analysed by HPLC.

Sample preparation

A solid-phase technique was used to extract the steroids from the perfusion media [16]. The C_{18} reversed-phase cartridges were washed with methanol (10 mL), followed by water (10 mL). The perfusion samples (3 mL) were loaded on the cartridges, the cartridge washed with water (3 x 10 mL) and the steroids eluted with methanol (5 mL). The solvent was evaporated (35°C) with nitrogen, the residue reconstituted in mobile phase (100 μL) and an aliquot (50 μL) injected for analysis. Glassware used during the extraction procedure was silanised.

Linearity

Increasing volumes of the working mixture, together with a constant amount (500 ng) of the internal standard, 6α-methylprednisolone, were added to perfusate (3 mL) to prepare concentrations of each steroid in the range of 150 to 750 nmol/L. The samples were prepared in duplicate for extraction. Mean peak height ratios (steroid : internal standard) were then determined and plotted as a function of increasing steroid concentration.

Recovery

The recovery of each steroid was determined at 150 and 600 nmol/L. Samples of perfusate (3 mL) at each concentration were prepared in duplicate and extracted using reversed phase cartridges. A constant amount (500 ng) of internal standard, 6α-methylprednisolone, was added to each

methanol eluate prior to evaporation under nitrogen. The recoveries were calculated by comparison of the peak height ratios (steroid : internal standard) of the samples with those of non-extracted standards of the same concentrations.

Specificity

A range of endogenous and exogenous steroids and drugs which may be used therapeutically in pregnancy were investigated for interference with quantitation of the steroids being analysed in this assay.

Precision studies

Precision studies were conducted at two concentrations, 150 and 750 nmol/L. Samples of perfusate (3 mL) were spiked with internal standard (500 ng) and with aliquots of the working solution, and were extracted and analysed as described above. Intra-day coefficients of variation were calculated from a set of five separate extractions repeated at each concentration and the inter-day coefficients of variation from corresponding sets of analyses repeated over five days.

Lower limit of detection

This value was determined for each steroid by determining the concentration at which the ratio of sample response to background was 2.0.

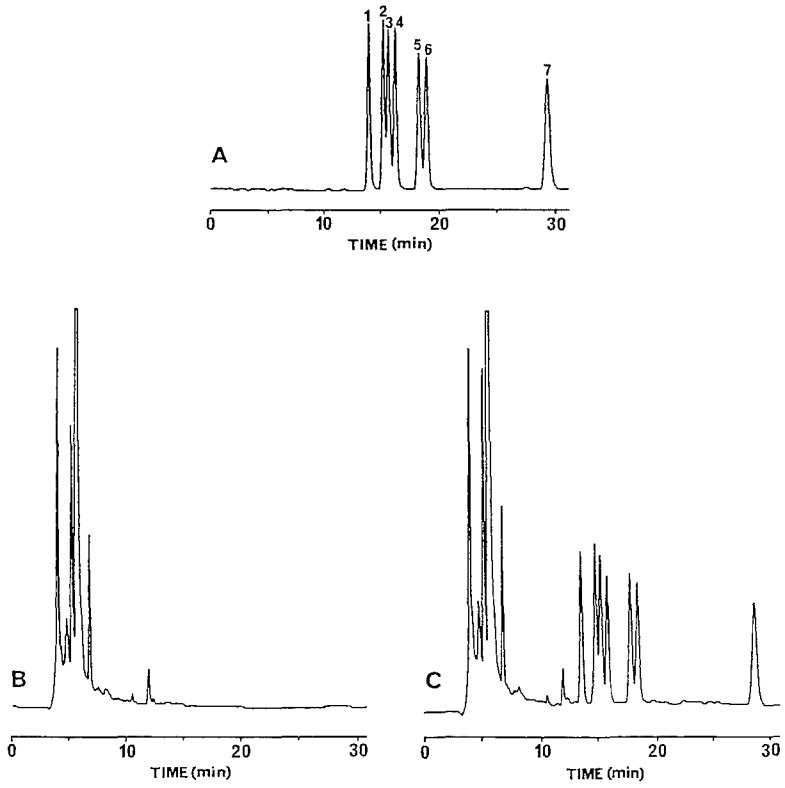

FIGURE 1. HPLC separation of (A) standard steroid mixture with internal standard, (B) extracted blank perfusate and (C) perfusate containing standard steroids and internal standard. Peaks: 1 = 20α–dihydrocortisone, 2 = 20ß–dihydrocortisone, 3 = 20α–dihydrocortisol, 4 = cortisone, 5 = 20ß–dihydrocortisol, 6 = cortisol and 7 = 6α–methylprednisolone (internal standard).

RESULTS

Typical examples of the separation of standards, extracts of blank perfusate and perfusate containing the standard steroids are shown in Figure 1. Although not all the steroids were base–line resolved, accurate

TABLE 1

Recoveries of Steroids from Perfusion Medium

Steroid	Recovery (%)		
	150 nmol/L	600 nmol/L	Mean
Cortisol	86.6	93.5	90.1
Cortisone	99.1	85.6	92.4
20α–Dihydrocortisol	84.4	93.4	88.9
20ß–Dihydrocortisol	95.1	90.7	92.9
20α–Dihydrocortisone	90.6	92.2	91.4
20ß–Dihydrocortisone	94.2	90.3	92.3

quantitation was obtained. The assay was linear for all the steroids in the range 0 – 750 nmol/L with a typical calibration graph for cortisol giving a regression of $y = 1.79 \times 10^{-2} + 1.30 \times 10^{-3}x$, $r = 0.981$, where y is the peak–height ratio, x the concentration of steroid (nmol/L) and r is the correlation coefficient. Mean recoveries of each steroid, using the solid–phase extraction procedure at concentrations of 150 and 600 nmol/L, ranged from 88.9% for 20α–dihydrocortisone to 92.9% for 20ß–dihydrocortisone (Table 1).

Precision studies at concentrations of 150 and 750 nmol/L showed the assay to be reproducible for all steroids under study with intra–assay coefficients of variation ranging from 4.0 (cortisone) to 5.5% (cortisol) and from 2.8 (cortisol) to 4.0 (cortisone and 20α–dihydrocortisone) for the two concentrations respectively. The corresponding inter–assay coefficients of variation varied from 6.0 (20α–dihydrocortisone) to 9.6% (cortisone) and 5.8 (cortisol and 20α–dihydrocortisol) to 6.9% (20ß–dihydrocortisone)

TABLE 2

Coefficients of Variation for Steroids at Two Concentrations

Steroid	Coefficient of variation (%)			
	Intra–assay		Inter–assay	
	150 nmol/L	750 nmol/L	150 nmol/L	750 nmol/L
Cortisol	5.5	2.8	8.5	6.0
Cortisone	4.0	4.0	9.6	5.8
20α–Dihydrocortisol	5.2	2.9	8.5	5.8
20ß–Dihydrocortisol	5.0	3.9	8.8	6.7
20α–Dihydrocortisone	4.3	4.0	6.0	6.6
20ß–Dihydrocortisone	5.2	3.0	9.4	6.9

TABLE 3

Compounds Tested for Interference in this Assay

Steroids	Other drugs
Prednisone	Bupivicaine
Prednisolone	Carbamazepine
Progesterone	Clonazepam
17α–Hydroxyprogesterone	Diazepam
Estradiol	Hyoscine
Estriol	Methimazole
Betamethasone	Paracetamol
Dexamethasone	Phenobarbitone
Testosterone	Ritodrine
Dehydroepiandrosterone	Salbutamol
Cholesterol	

(Table 2). The lower limit of detection observed was 5 nmol/L for each of the steroids. No interference was found from any of the steroids or drugs listed in Table 3 except for prednisolone which coeluted with cortisol.

DISCUSSION

We have developed a specific and reproducible HPLC assay to separate cortisol, cortisone and the 20–reduced metabolites of these compounds in samples from *in vitro* perfusions of the human placenta. Other assays have been described for the resolution of metabolites of prednisolone [4] in tissue culture medium from perfusions in placental tissue and for the metabolism of corticosteroids in adrenal cell cultures [15]. There was no interference from endogenous compounds originating from the perfused tissue, as measured by analysis of 6 hour perfusate samples from perfusions in which cortisol or prednisolone had not been used as substrates. Potential interferences from this source were minimised by removing any blood remaining in the tissue before the tissue culture medium was recirculated through the placental lobule. The only exogenous compound which interfered was prednisolone which co–eluted with cortisol. The partition coefficients of the metabolites of cortisol are similar and two C_{18} reversed phase columns linked in series were required to effect the separation. A column temperature of 40°C was used to lower the back pressure and reduce the retention times.

Although baseline separation of two sets of steroids (20ß–dihydrocortisone, 20α–dihydrocortisol and cortisone; 20ß–dihydrocortisol and cortisol) was not achieved, the resolution was sufficient for quantitation

of these components. Use of other modifiers and solvents in the mobile phase did not improve the resolution.

A high recovery of each of the steroids was achieved using reversed phase cartridges for extraction of the perfusion matrix. At a concentration of 150 nmol/L, the recoveries were in the range 84.4 to 99.1% whereas at 600 nmol/L the corresponding recoveries ranged from 85.6 to 93.5%. These recoveries are comparable with those of McBride *et al.* [9] who extracted 99.3 to 108% of cortisol from plasma, Dawson *et al.* [16] who obtained up to 81% of cortisol, cortisone and corticosterone from plasma and Cannell *et al.* [17] who obtained a 98% recovery of cortisol and cortisone from plasma using the same reversed-phase extraction technique. This isocratic assay is simpler to perform than the gradient elution HPLC method of Schoneshofer *et al.* [14] and will be applied to the separation of metabolites following *in vitro* perfusions of placental tissue with cortisol as substrate.

ACKNOWLEDGMENTS

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STABILITY ANALYSIS OF THREE UV-FILTERS USING HPLC

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ABSTRACT

UV-absorbing substances are widely used in sunscreens to filter out ultraviolet radiation in sunlight. These substances need to be stable in sunscreens in order to obtain the expected protection during the shelf-life of the products.

A conveniant HPLC method for the separation and quantification of three common UV-filters in a sunscreen emulsion is presented. Using this method the stability of benzophenone-3, butylmethoxy dibenzoylmethane and octyl methoxycinnamate was determined. Calibration curves for the three UV-filters showed linear response in the interval 0.03 to 30 µg. The absolute recovery of the spiked sample was close to 100 %. The shelf life data indicate good stability of the filters.

INTRODUCTION

Sunlight have both beneficial and harmful effects on human skin. While being necessary for the initial steps in the formation of vitamin D [1], it may also cause various damages to the skin, such as skin cancer and photoaging [2,3]. The increase in the incidence of skin cancer in western countries appears to be related to excessive exposure to sunlight [2,3]. These harmful effects are especially due to the light of low wavelengths in the UV-portion of the solar radiation spectra.

Skin has several barriers of protection against the adverse effects of solar radiation [4]. This defense system may however not be adequate for all individuals and situations. In order to prevent photodamage, caucasians are recommended to use sunscreens for

outdoor activities in sunny climates if clothing or other sun protection cannot be used [5].

Sunscreens contain UV-filters which prevent the transmission of deleterious UV-radiation into the skin, either by absorbing the energy or reflecting it [4]. There are a number of formulations on the market. These contain various combinations and concentrations of UV-filters, emollients, emulsifiers, preservatives etc in order to provide different sun protection factors and cosmetic properties [4]. Some products adhere more efficiently to skin and hence give protection also after swimming or sweating.

In Europe sunscreens are classified as cosmetics, whereas in the USA they are OTC drugs. Cosmetic having a durability of less than 30 months should be labelled with a date stamp according to directives in the EC. In order to ensure adequate protection after storage the stability of the UV-filters in the product need to be determined. The aim of this study was to investigate experimental conditions on HPLC for the analysis of three common UV-filters in a sunscreen and to determine the stability of the filters in an ordinary sun lotion.

MATERIALS AND METHODS

Materials

Benzophenone-3 (Merck AG, Germany) and butylmethoxy dibenzoylmethane and octyl methoxycinnamate (Givaudan, Switzerland) was used without any further purification (see Table I for their full names). The compounds were weighed (accuracy of 0.01 mg) and dissolved in ethanol (99.5 % from Kemetyl AB, Sweden). A Shimadzu UV-260 double beam spectrophotometer was used to record the absorbance spectra of the test compounds. The main ingredients in the lotion were silicones, acrylic acid copolymer, PVP/triacontene copolymer, cetostearyl alcohol, sorbitan oleate, water and preservatives (imidazolinid urea) with a final pH of 5.

Equipment

An Hitachi L-6200 ternary solvent pump was used for the generation of gradients while samples were injected with a Hitachi AS-4000 auto sampler. The separation was achieved on a C8 column (4.6 x 200 mm, 5 μm, Beckman Ultrasphere). The column was termostated to 25 ^{o}C in a Hitachi T-6300 column oven. The samples were detected using a Hewlett Packard 1040A diode-array detector coupled to a Hewlett Packard 9000 series 300 computer with a Hewlett Packard 9144 tape unit for back-up. The detector performance is checked with a reference filter containing holmium oxide. Primary

TABLE I.
UV-filters Investigated.

Trade name	Chemical name	CTFA name	Stated purity (min %)
Eusolex 4360	2-hydroxy-4-methoxybenzophenone	Benzophenone-3	99.5
Parsol 1789	4-tertbutyl-4′-methoxy-dibenzoylmethane	Butylmethoxy dibenzoylmethane	95
Parsol MCX	2-ethylhexyl-p-methoxycinnamate	Octyl methoxycinnamate	98

analysis of the data was achieved on a Hewlett Packard 9000 computer using the Hewlett Packard software HP 79997 A Rev 3.21. Water equivalent to HPLC quality was obtained from a Millipore water purification unit. Methanol of HPLC-quality ("Lichrosolv") and acetic acid (p.a.) were from Merck AG, Germany.

Extraction of UV-filters and stability test

The above mentioned lotion without UV-filters (vehicle) was used to test the extractability of the UV-filters. Since all UV-filters are freely soluble in alcohols we used ethanol as a solvent for their extraction. The vehicle was weighed into measuring flasks and UV-filters (dissolved in ethanol) and 20 ml of ethanol (heated to 60 ^{o}C) added. The contents were mixed on a magnetic stirrer for 30 min at room temperature. The solution was made up to 25 ml and stirred for a further five minutes. An aliquot was centrifuged at 14,500 g for five minutes and the supernatant used for HPLC analysis.

The stability of the UV-filters was tested both in ethanol and in the sun lotion. UV-filters were dissolved in ethanol and kept in capped test tubes under air and was stored in the dark at room temperature, cold (approx. 4 ^{o}C) and in a freezer (approx - 20 ^{o}C). One additional sample was stored at room temperature under normal light conditions on a lab bench (i.e. dark at night). Aliquots were analyzed on HPLC after one, two, seven and twenty eight days. Two batches of the sun lotion were stored at 40^{o}C. After 30 and 90 days the concentration of the UV-filters was determined.

RESULTS AND DISCUSSION

Absorbance spectra and method of analyses of the UV-filters

The absorbance spectra of the three UV-filters were analyzed between 200 and 420 nm (Figure 1). The spectrum of benzophenone-3 showed minima at 230, 263 and 309 nm and maxima at 207, 242, 287 and 326 nm. Butylmethoxy dibenzoylmethane had minima at 217, 249 and 301 nm and maxima at 206, 233, 267, 286 and 358 nm while octyl methoxycinnamate had minima at 215 and 247 nm and maxima at 212, 227 and 309 nm. Since at 325 nm, the three compounds had nearly equal absorbance, on weight basis, this wavelength was choosen subsequently for routine monitoring of the compounds on HPLC. The sensitivity at this wavelength is lower, (benzophenone-3 - 33 %; butylmethoxy dibenzoylmethane - 52 %; octyl methoxycinnamate - 35 % lower) compared to measurement at the wavelength with maximal absorbance in each case.

The standard separation system used a C8 column with a flow rate of 1.0 ml/min with a gradient from 80 % to 100 % methanol during 10 min (the other phase consisting of 1

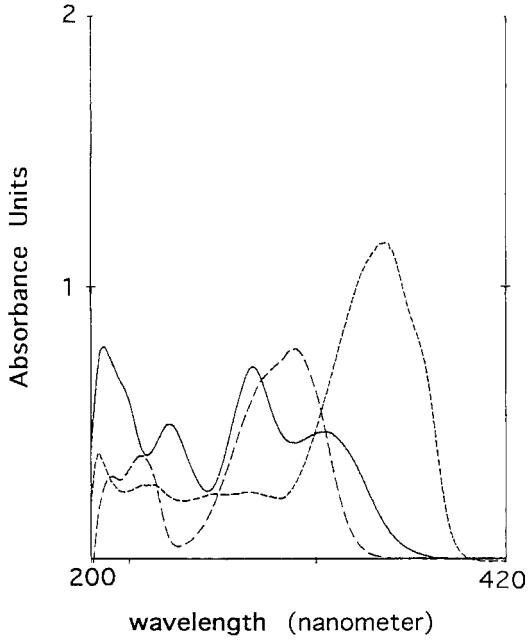

FIGURE 1. Absorbance spectras of UV-filters. Benzophenone-3 (——), butylmethoxy dibenzoylmethane (- - - -) and octyl methoxycinnamate (– – –) were dissolved in ethanol and analyzed at concentrations of 10 mg m^{-3}.

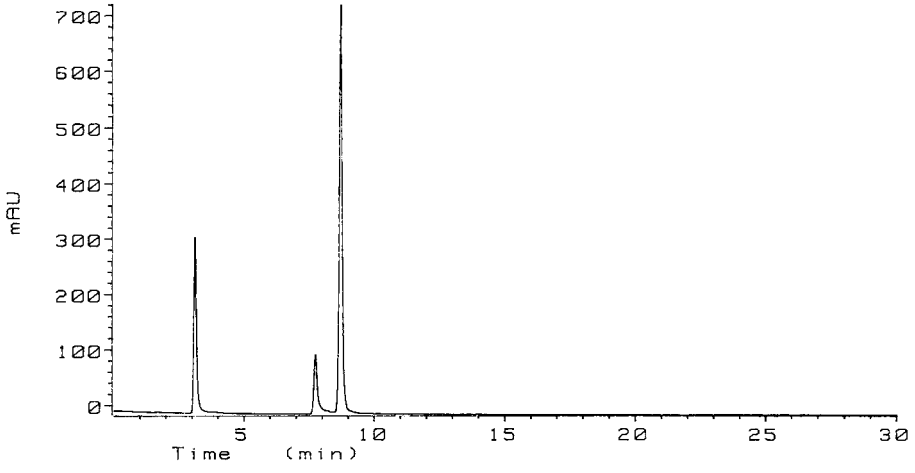

FIGURE 2. Chromatogram of UV-filters on HPLC. A mixture of UV-filters equivalent to 1 µg of each compound was analyzed by HPLC. Benzophenone-3 (3.1 min), butylmethoxy dibenzoylmethane (7.7 min) and octyl methoxycinnamate (8.7 min) are eluting with increasing amounts of methanol in the gradient.

% acetic acid in water). The column is washed for 2 min with methanol and then equilibrated with 80 % methanol for at least 4 min. A typical chromatogram is shown in Figure 2. The reproducibility of the system is high as shown in the small day-to-day drift in retention time (Table II). The carry-over of the compounds were tested at several different concentrations. At high levels of UV-filters ($\geq$ 5 µg) this effect was at a level of 0.18 % or less. At levels used for routine analysis, however, no carry-over was detected in our system.

Purity and standard curves of UV-filters for quantitative analysis

When high amounts of the UV-filters ($\geq$ 30 µg) were analyzed by HPLC some new peaks were found in addition to the main peak in each case. Furthermore, impurities in the main peak of butylmethoxy dibenzoylmethane was found as judged from spectral analysis. The purities were determined to be 98 - 99 % (Table III), which is in accordance with that specified (Table I).

Different amounts of UV-filters (0.03 - 30 µg) were dissolved in ethanol and injected on the HPLC where the area of the peaks at 325 nm were measured. A linear relationship was found throughout the concentration interval analyzed (Figure 3). The detector could not record accurate spectra of the peak maximum at the highest concentrations used of UV-filters due to the high optical density. No useful spectra could be recorded at the lowest concentrations analyzed although the peaks could be clearly distinguished in the chromatograms. To obtain accurate peak purity tests of the peaks one must compensate for base line drift in the case of the butylmethoxy dibenzoylmethane peak to compensate for its tailing (probably due to a tautomeric shift). In the routine analysis vials containing a standard concentration of the three UV-filters were used to test the accuracy of the instruments.

Stability of UV-filters

In ethanol benzophenone-3 and butylmethoxy dibenzoylmethane were stable throughout the whole time period (about one month) as judged from the amounts recovered and spectral analysis of the peak (Figure 4). In the case of octyl methoxycinnamate, however, a new peak with slighly lower retention was found in all samples upon storage. A dramatic increase in amount of this new peak was found in the sample stored in the presence of light at room temperature (Figures 4). Analysis of the spectra for the new peak and octyl methoxycinnamate showed a high degree of similarity. The absolute and relative intensity of the maxima at short wavelengths increased and a

TABLE II.

Reproducibility in the Analysis of UV-filters.

UV-filter	Retention time (min)					
	A	C.V. (%)	B	C.V. (%)	C	C.V. (%)
Benzophenone 3	3.0467 ± 0.0211	0.69	3.0227 ± 0.0238	0.79	3.0084 ± 0.0133	0.44
Butylmethoxy dibenzoylmethane	7.7178 ± 0.0498	0.65	7.6092 ± 0.0282	0.37	7.5838 ± 0.0286	0.38
Octyl methoxycinnamate	8.6992 ± 0.0436	0.50	8.6072 ± 0.0265	0.31	8.5672 ± 0.0428	0.50

Values shown (means ± standard deviations) from six different samples analyzed in duplicate in three independent experiments (A - C).

TABLE III.

Purity of UV-filters Analyzed by HPLC.

Approximately 30 µg of each compound were analyzed by HPLC. The relative quantitation is made relative to the peak area at 325 nm.

UV-filter	retention time (min)	area	%	comment#
Benzophenone 3	2.992	29,458	98.4	
	3.710	488.47	1.6	> 1 compound
	7.4			not integrated
			Σ 100.0	
	3.508	45.16	0.14	
Butylmethoxy	3.859	302.80	0.92	> 1 compound
dibenzoylmethane	7.845	32,438	98.4	impurity in the tail ?
	11.145	185.63	0.56	
			Σ 100.02	
	3.0			not integrated
	3.597	50.27	0.16	
Octyl methoxy-	7.755	199.93	0.66	
cinnamate	8.229	30,161	99.1	
	9.641	28.08	0.09	
			Σ 100.01	

Some of the peaks seem to contain more than one peak judging from the symmetry of the peak and/or spektral/isogram analysis. Non-integrated peaks contained only a few area units.

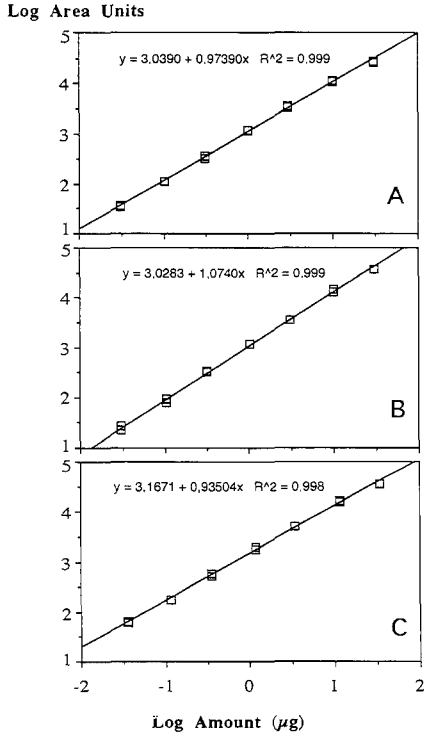

FIGURE 3. Calibration curves for UV-filters. Different amounts of Benzophenone-3 (A), butylmethoxy dibenzoylmethane (B) and octyl methoxycinnamate (C) (0.03 - 30 μg) dissolved in ethanol were analyzed in duplicate by HPLC. Quantitation was performed based on area counts at 325 nm.

blue shift in wave length maximum around 309 nm was found in the new peak. This new compound is most probably due to cis/trans isomerization of octyl methoxycinnamate. Such an isomerization has been demonstrated upon irradiation of cinnamate sun screens [6]. Similar results have been reported after irradiation of UV-filters with ultraviolet light up to five minutes [7]. In that study a blue shift of octyl methoxycinnamate of 2.3 nm was found in the peak maximum at 309 nm which probably may be explained by the dual contribution of octyl methoxycinnamate and the new peak detected in our analysis. This fact points out the advantage of analyzing the effects on the UV-filters through a combination of separation and physico-chemical characterization. No significant effects

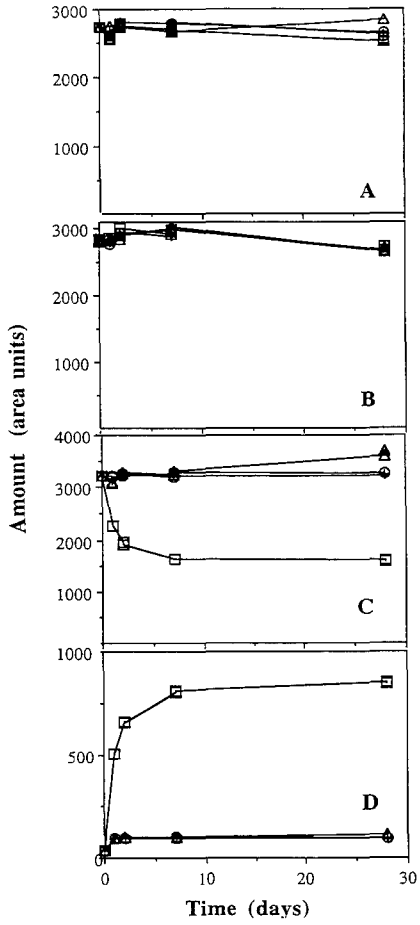

FIGURE 4. Stability of UV-filters. UV-filters were dissolved in ethanol and stored dark at - 20°C (O), at 4 °C (+) and at room temperature (18 - 20°C)(Δ) or in day light at room temperature ( ). Benzophenone-3 (A), butylmethoxy dibenzoylmethane (B), octyl methoxycinnamate (C) and the new peak derived from octyl methoxycinnamate (D).

TABLE IV.

Recovery of UV-filters From Vehicle Lotion.

A lotion without UV-filters was used. Results from four different experiments with duplicate samples (concentrations of UV-filters around 0.25, 0.5 and 1 % on a weight basis) are shown as means ± standard deviations.

UV-filter	% recovery		
	0.25 %	0.5 %	1 %
Benzophenone 3	102 ±4.6	103 ± 1.3	104 ± 1.1
Butylmethoxy dibenzoylmethane	100 ± 4.2	102 ± 0.4	100 ± 1.0
Octyl methoxycinnamate	102 ± 4.7	102 ± 1.1	100 ± 4.6

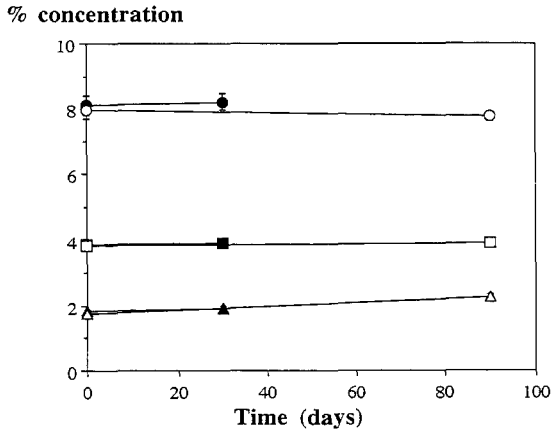

FIGURE 5. Concentration of UV-filters in a sun lotion after storage at 40°C for different times. Different batches of a sun lotion containing Benzophenone-3 ( ), butylmethoxy dibenzoylmethane (Δ) and octyl methoxycinnamate (O) were stored at 40°C for 30 (filled symbols) or 90 (open symbols) days. Extraction and analysis of UV-filters were performed as described in Materials and Methods. Values are shown as means and S.D. of six analysis of each batch and time pooint (C.V. 1.9-4.4 %).

were reported in the UV-spectra of benzophenone-3 and butylmethoxy dibenzoylmethane after irradiation [6], which is in accordance with the results in the present study.

Extractions of the UV-filters with ethanol from sun lotion was found to be effective and the absolute recovery was close to 100 % at several different concentrations (Table IV). No other compounds interfered with the analysis of the UV-filters (neither at 325 nm nor using spectral analysis from 200 - 420 nm). No significant change in concentration of the UV-filters in the lotion was found during storage for 30 or 90 days at 40°C (Figure 5).

CONCLUSIONS

A simple and convenient method for the separation of three UV-filters on reversed phase HPLC is described. The peak areas of the UV-filters have linear responses over a wide concentration range (10^4). The sensitivity in detection can be increased with 30 - 50 % if time programmed wave length changes are performed instead of detection at 325 nm. One should be careful with exposure to light in the case of octyl methoxycinnamate. The UV-filters were found to be stable for at least three months at 40°C in the tested sun lotion. This indicate adequate sunscreening properties of the lotion during storage for 2-3 years at room temperatture.

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LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENOLIC COMPOUNDS IN HOSPITAL DISINFECTANT PRODUCTS

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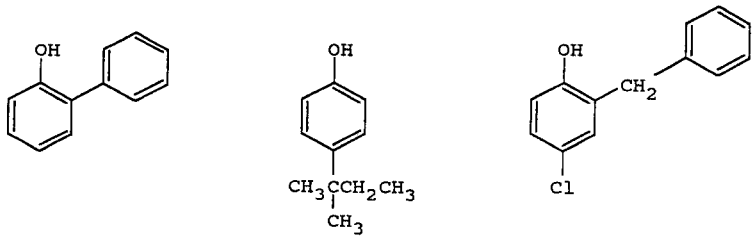
ABSTRACT

A liquid chromatographic method has been developed to quantify phenolic compounds in various hospital disinfectant products. Each product contained two of the following three phenols: o-phenylphenol (OPP), p-tert-amylphenol (PTAP), and o-benzyl-p-chlorophenol (OBPCP). An isocratic mobile phase consisting of methanol and phosphate buffer was used in conjunction with a 5 cm column, which contained 3 micron C-18 packing material, to enact the separation of the phenolic compounds from each other and from product matrix components. Calibration curve data were generated for each phenolic compound to demonstrate linear response over the concentration range of interest. Spiked sample recovery studies were performed to assess the accuracy of the method for the analysis of the phenolic compounds in various product matrices. Finally, photodiode array generated spectra were used to demonstrate the homogeneity of the phenolic compound peaks.

INTRODUCTION

Phenolic compounds are commonly used as the antimicrobial ingredient in hard surface disinfectants which are used in hospitals for general disinfection of noncritical and semicritical areas. The three phenolic compounds (Figure 1) that are used in these types of products are o-phenylphenol (OPP), p-tert-amylphenol (PTAP), and o-benzyl-p-chlorophenol (OBPCP). They exhibit broad-spectrum antimicrobial activity, including gram-negative and gram-positive bacteria. They also exhibit fungicidal, tuberculocidal, and virucidal activity against lipophilic viruses. However, they are not sporicidal and are not used when sterilization is required. These compounds also have a tolerance for organic load and hard water and demonstrate residual activity.(1)

Previous liquid chromatographic studies have involved the analysis of OPP or OPP and OBPCP in conjunction with other phenolic compounds in both normal and reversed-phase chromatographic systems (2 - 7). These studies have determined the amount of OPP in matrices such as urine (2) and immature rat liver microsomal fractions (3). They have also been used to determine the amount of OPP and OBPCP present in cosmetic products when these compounds are used as preservatives (6,7). None of these studies, however, have addressed the liquid chromatographic analysis of OPP, OBPCP, and PTAP in the acidic or alkaline matrices of hospital disinfectant products.



OPP PTAP OBPCP

FIGURE 1. Structures of o-phenylphenol (OPP), p-tert-amylphenol (PTAP), and o-benzyl-p-chlorophenol (OBPCP).

In this study, a fast, simple, high performance liquid chromatography (HPLC) method has been developed which can be used to separate and quantify all three phenolic compounds. The method is applicable for the analysis of the phenolic compounds in three different hospital disinfectant products.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a Varian (Walnut Creek, CA, USA) 9095 autosampler with a 50 μL injection loop, a Varian 9010 gradient pump, and a Varian 9065 photodiode array detector. The data were collected and analyzed with a Varian Star workstation. The HPLC column

used was a YMC (Wilmington, NC, USA) ODS-AQ (5 cm x 4 mm I.D., 3 μm packing) with a 0.5 μm precolumn filter (Upchurch Scientific, Oak Harbor, WA, USA).

Reagents

The mobile phase was prepared with HPLC grade reagents only. Fisher (Pittsburgh, PA, USA) water, methanol, potassium phosphate monobasic dihydrate, and 85% o-phosphoric acid were used. OPP (99+% purity, Aldrich, Milwaukee, WI, USA), PTAP (99+% purity, Aldrich), and OBPCP (95+% purity, ICN, Cleveland, OH, USA, recrystallized to a purity of 99+%) were used to prepare standards and spiked samples. The disinfectant products were produced by Calgon Vestal Laboratories (St. Louis, MO, USA). These products will be identified in this paper as Disinfectant 1 (an acidic product which contains OPP and PTAP), Disinfectant 2 (an alkaline product which contains OPP and OBPCP), and Disinfectant 3, (an alkaline product which contains OPP and PTAP).

Mobile Phase Preparation

The isocratic mobile phase consisted of 55% methanol and 45% phosphate buffer (v/v). The phosphate buffer was prepared by dissolving HPLC grade potassium phosphate monobasic dihydrate in HPLC grade water to produce a 50 mM phosphate solution. The pH was then adjusted to 3.0 by the addition of 85% o-phosphoric acid. The mobile phase was

prepared by combining 55% methanol and 45% buffer (v/v) and then vacuum filtering the mixture through a 0.45 μm nylon filter.

Standard and Sample Preparation

OPP, OBPCP, and PTAP standards were prepared by dissolving appropriate amounts of each phenolic compound in mobile phase. Placebo formulations of the disinfectant products were not available. Spiked samples for recovery experiments were prepared by adding known amounts of the appropriate phenolic compounds to disinfectant products of known phenolic compound concentrations. These samples were then diluted with mobile phase. All standards and samples were syringe filtered with a 0.45 μm Gelman Acrodisc LC13 PVDF filter before being analyzed by HPLC.

RESULTS AND DISCUSSION

The goal of this study was to develop a single HPLC method that would not only separate the three phenolic compounds, but could also be used for the analysis of these compounds in one acidic disinfectant product and two alkaline disinfectant products. If the same chromatographic conditions could be used for all three products, then this would represent a savings in terms of analysis time since no chromatographic change over would be required when switching the analysis from one product to another. It was

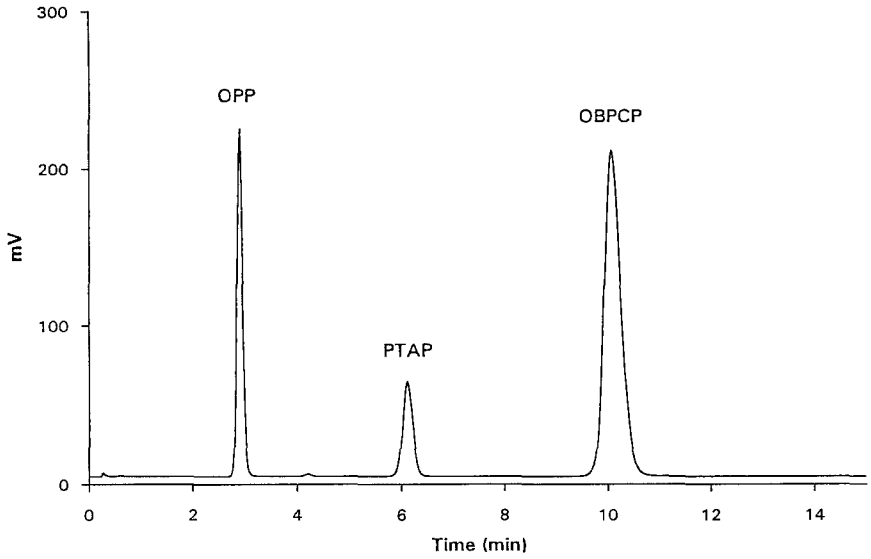


FIGURE 2. Chromatogram of OPP, PTAP, and OBPCP standards.

also important that the final method be isocratic so that it could more easily be transferred to other laboratories. The final chromatographic conditions consisted of an isocratic mobile phase of 55% / 45% (v/v) methanol and 50 mM phosphate buffer at pH 3.0, respectively, a flow rate of 1.5 mL/min, an injection volume of 50 μL, UV detection at 220 nm, ambient column temperature, and an analysis time of 15 minutes.

Calibration Curve Data

Calibration curve data were generated for each of the three phenolic compounds. Two runs (analyses) of each phenolic compound standard

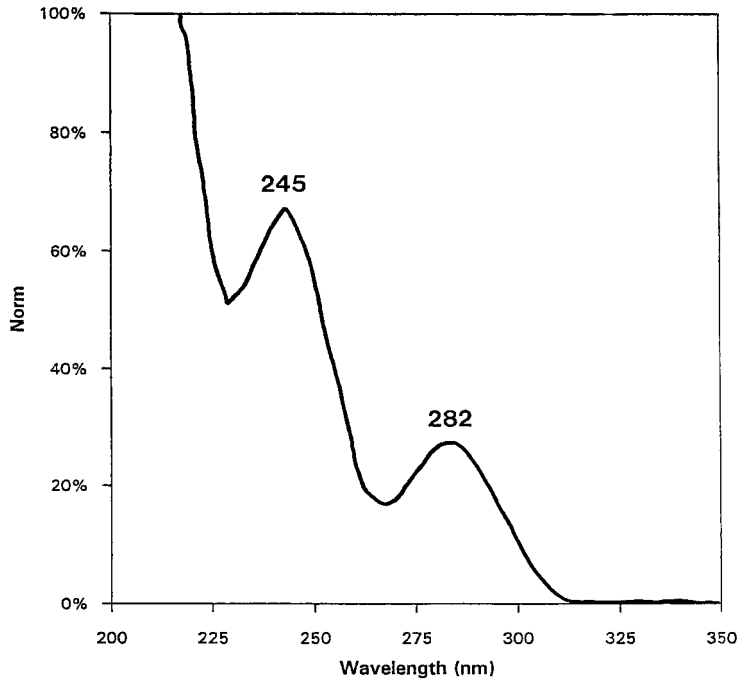


FIGURE 3. Photodiode array detector generated UV spectrum of OPP.

series were performed but no limit of detection or quantitation values were established since this is usually not an issue for this type of product analysis. A typical chromatogram of the standards is found in Figure 2 and the photo diode array generated UV spectra for the phenolic compounds are found in Figures 3, 4, and 5. The Run 1 calibration curves consisted of six OPP standards ranging from 9.85 ppm to 60.7 ppm, six PTAP standards ranging from 11.0 ppm to 59.9 ppm, and five OBPCP standards ranging from 55.0 ppm to 273 ppm. The Run 2 calibration curves consisted

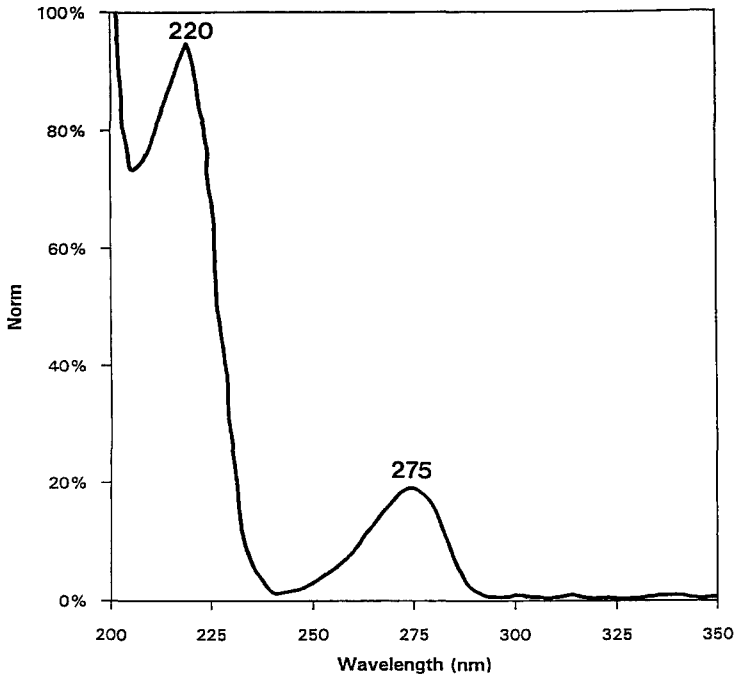


FIGURE 4. Photodiode array detector generated UV spectrum of PTAP.

of six OPP standards ranging from 11.0 ppm to 61.5 ppm, six PTAP standards ranging from 12.0 ppm to 58.4 ppm, and six OBPCP standards ranging from 61.3 ppm to 310 ppm. A linear response was found over each concentration range for all the phenolic compounds. Table 1 contains a summary of the OPP, PTAP, and OBPCP calibration curve data.

Analysis of Formulated Disinfectant Products

In order to assess the accuracy of the assay, spiked samples were prepared for each of the disinfectants and then analyzed for phenolic

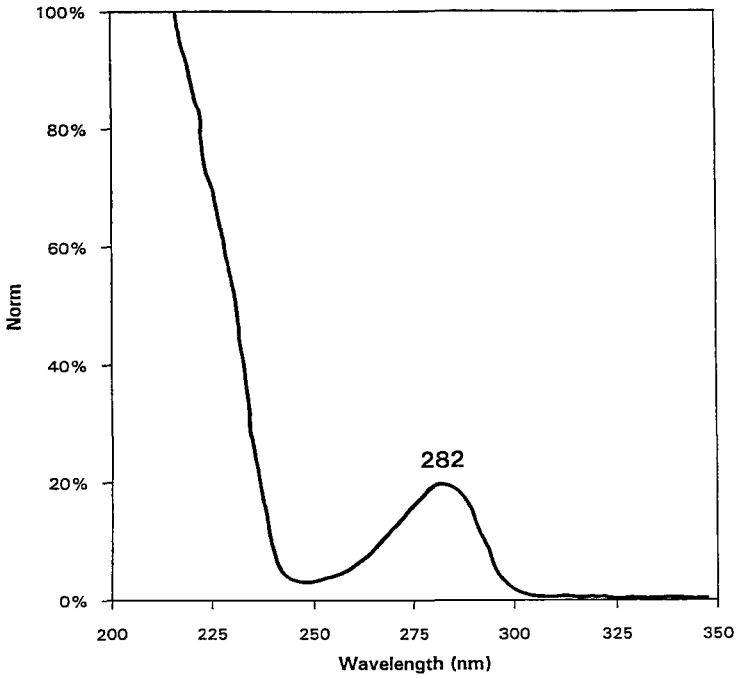


FIGURE 5. Photodiode array detector generated UV spectrum of OBPCP.

TABLE 1. Summary of Calibration Curve Data.

Run	R^2	Slope	y-intercept
OPP-1	0.9999	12555	6551
OPP-2	1.0000	12330	4723
PTAP-1	1.0000	6705	714
PTAP-2	1.0000	6591	-423
OBPCP-1	1.0000	7588	4800
OBPCP-2	1.0000	7419	4695

TABLE 2. Spiked Sample Recovery Data for Disinfectant 1.

Analyte	Theoretical Sample Conc, ppm	Theoretical Spike Conc, ppm	Expt.Total Conc, ppm	Expt. Spike Conc, ppm	% Recovery
OPP	41.93	2.81	44.82	2.89	103
	40.50	5.97	46.52	6.01	101
	40.54	9.05	49.71	9.16	101
	37.78	3.18	40.94	3.16	99.2
	41.43	5.97	47.41	5.98	100
	40.43	9.48	49.85	9.42	99.3
Mean OPP ± Std Dev					100.6 ± 1.4
PTAP	42.06	2.78	44.84	2.77	99.8
	40.64	5.90	46.55	5.92	100
	40.68	8.94	49.73	9.06	101
	37.91	3.14	41.11	3.20	102
	41.56	5.90	47.60	6.03	102
	40.57	9.36	49.95	9.39	100
Mean PTAP ± Std Dev					100.8 ± 1.0

compound content. The spiked sample recovery data are found in Table 2 for Disinfectant 1, in Table 3 for Disinfectant 2, and in Table 4 for Disinfectant 3. Since true placebos were not available, the phenolic compounds were spiked into disinfectant samples that already contained the phenolic compounds. The resultant samples were analyzed in order to generate percent recovery data in the following manner. The "Theoretical

TABLE 3. Spiked Sample Recovery Data for Disinfectant 2.

Analyte	Theoretical Sample Conc, ppm	Theoretical Spike Conc, ppm	Expt.Total Conc, ppm	Expt. Spike Conc, ppm	% Recovery
OPP	23.15	5.52	28.72	5.57	101
	25.55	9.54	35.12	9.57	100
	27.59	3.00	30.61	3.02	101
	25.45	5.54	31.05	5.60	101
	29.24	9.35	38.57	9.32	100
Mean OPP ± Std Dev					101 ± 0.5
OBPCP	118.17	27.81	145.73	27.56	99.1
	130.40	48.05	178.40	48.01	100
	140.81	15.12	156.08	15.26	101
	129.88	27.91	157.97	28.09	101
	149.26	47.09	195.92	46.66	101
Mean OBPCP ± Std Dev					100 ± 0.9

Sample Concentration" value was determined by analyzing the disinfectant for its phenolic compound content. The "Theoretical Spike Concentration" value was determined based upon the amount of the phenolic compound that was added to the disinfectant. The "Experimental Total Concentration" value was determined by analyzing the sample after it had been spiked with the appropriate phenolic compounds. The "Experimental Spike Concentration" value was determined by subtracting the "Theoretical

TABLE 4. Spiked Sample Recovery Data for Disinfectant 3.

Analyte	Theoretical Sample Conc, ppm	Theoretical Spike Conc, ppm	Expt.Total Conc, ppm	Expt. Spike Conc, ppm	% Recovery
OPP	45.27	3.01	48.36	3.09	103
	46.19	5.92	52.13	5.94	100
	48.08	9.66	57.77	9.69	100
	46.58	2.98	49.63	3.04	102
	48.92	5.70	54.57	5.64	98.9
	49.95	9.05	58.93	8.98	99.2
Mean OPP ± Std Dev					101 ± 1.6
PTAP	39.88	2.96	42.89	3.01	102
	40.69	5.81	46.57	5.88	101
	42.36	9.49	52.02	9.67	102
	41.04	2.93	44.02	2.99	102
	43.10	5.59	48.68	5.59	100
	44.00	8.89	53.01	9.01	101
Mean PTAP ± Std Dev					101 ± 0.8

Sample Concentration" value from the "Experimental Total Concentration" value. Finally, the % Recovery value was determined by [(Expt. Spike Conc) (100)] ÷ (Theoretical Spike Conc). Typical mean recovery values of 100% or 101% demonstrated accuracy for the quantitation of relevant phenolic compounds in each product.

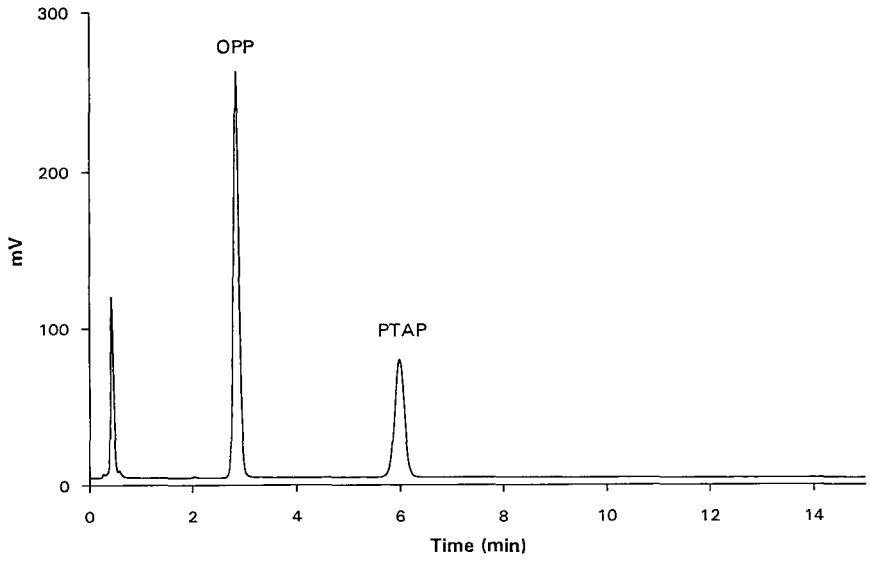


FIGURE 6. Chromatogram of a Disinfectant 1 sample.

The photodiode array detector was used to assess peak homogeneity. Photodiode array generated purity parameter (PuP) data can be used to determine if the individual phenolic compound peaks are homogeneous in each product matrix. For a given wavelength range, the PuP is the average wavelength weighted by the square of the absorbance (8). PuP = $[(\Sigma A_i^2)(\lambda_i)] \div \Sigma A_i^2$, where A_i is absorbance at wavelength λ_i. A series of five spectra were collected across each phenolic compound peak in each disinfectant product matrix in order to assess the homogeneity of each peak. If the peak is homogeneous, then the PuP will remain essentially the same as spectra are collected across the peak. Representative

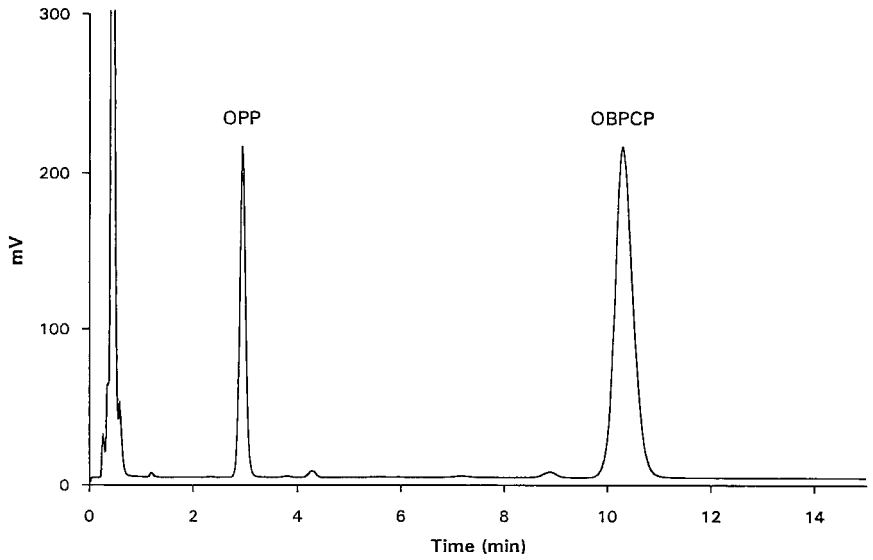


FIGURE 7. Chromatogram of a Disinfectant 2 sample.

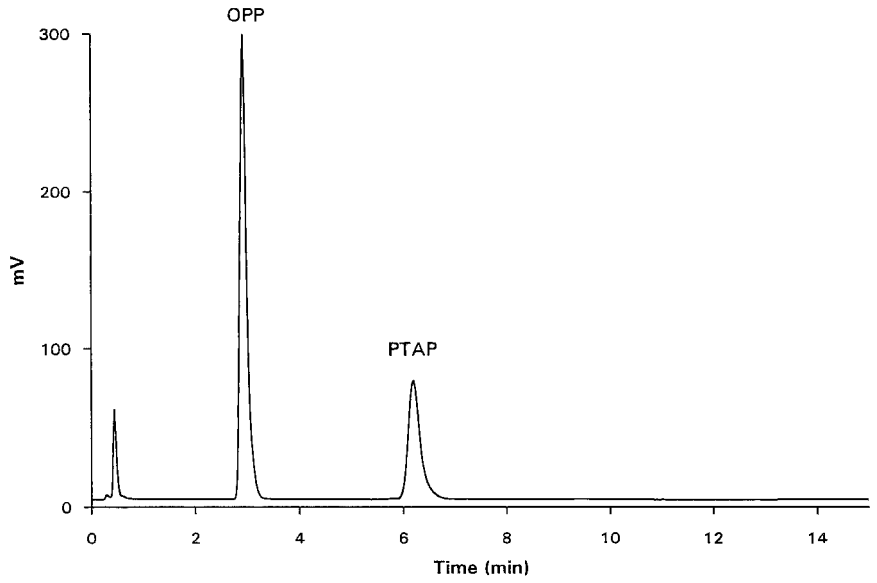


FIGURE 8. Chromatogram of a Disinfectant 3 sample.

TABLE 5. Purity Parameter (PuP) Data for Disinfectant 1.

Spectrum #	OPP PuP (210 to 367 nm)	PTAP PuP (210 to 367 nm)
1	224.34	220.30
2	224.25	220.32
3	224.33	220.32
4	224.17	220.33
5	224.29	220.33
Mean ± Std Dev	224.27 ± 0.07	220.32 ± 0.01

TABLE 6. Purity Parameter (PuP) Data for Disinfectant 2.

Spectrum #	OPP PuP (210 to 367 nm)	OBPCP PuP (210 to 367 nm)
1	224.32	217.60
2	224.14	217.53
3	224.40	217.63
4	224.39	217.61
5	224.07	217.49
Mean ± Std Dev	224.26 ± 0.14	217.57 ± 0.05

TABLE 7. Purity Parameter (PuP) Data for Disinfectant 3.

OPP Spectrum #	OPP PuP (210 to 367 nm)	PTAP PuP (210 to 367 nm)
1	224.04	220.44
2	224.02	220.45
3	223.93	220.41
4	224.02	220.34
5	223.77	220.44
Mean ± Std Dev	223.96 ± 0.11	220.42 ± 0.04

chromatograms for each disinfectant product are found in Figures 6 through 8. The PuP data in Tables 5 though 7 indicate that the individual phenolic compound peaks were homogeneous in each disinfectant product matrix.

CONCLUSION

A simple, fast HPLC method was developed to quantify the phenolic compounds OPP, PTAP, and OBPCP in various hospital disinfectant products. Calibration curve data indicated that the response of each phenolic compound was linear over the concentration ranges of interest. The analysis of spiked recovery samples demonstrated that the method was accurate and photodiode array spectral data indicated that each phenolic compound peak was homogeneous in each disinfectant matrix.

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DETERMINATION OF NORGESTIMATE IN SERUM BY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SUBSEQUENT RADIOIMMUNOASSAY

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ABSTRACT

A method is described for the specific determination of norgestimate in human serum at concentrations of 25 to 1000 pg/mL. The method is based on automated high-performance liquid chromatography and on subsequent radioimmunoassay. Validation of the method included demonstration of low procedural blanks, recoverably of added standard and low intra- and inter-assay variability.

INTRODUCTION

Norgestimate is a progestin synthesized by The R. W. Johnson Pharmaceutical Research Institute at Ortho Pharmaceutical Corporation.

It has been extensively investigated clinically as an oral contraceptive. To measure serum norgestimate concentrations following oral administration, a radioimmunoassay was developed. The measurement of norgestimate in biological fluids by radioimmunoassay alone presented a problem because the antisera employed in the existing assay was not completely specific. To overcome the uncertainties of immunological cross-reactivity and to provide assay specificity, an adjunct HPLC procedure was developed. This communication describes an automated high-performance liquid chromatography/radioimmunoassay (HPLC/RIA) method for the separation and quantitation of norgestimate in human serum.

MATERIALS AND METHODS

Materials and Reagents

Norgestimate(18,19-dinor-17-pregn-4-en-20-yn-3-one,17-(acetyloxy)-13-ethyl,oxine(17α(+)-) was synthesized at The R. W. Johnson Pharmaceutical Research Institute at Ortho Pharmaceutical Corporation (Raritan, NJ). 6,7-^{3}H-Norgestimate (44 Ci/mole) was purchased from Roussel-UCLAF (France) and purified by HPLC prior to use. The following metabolites of norgestimate: 3-keto-norgestimate (18,19-dinor-17-pregn-4-en-20-yn-3-one,17-(acetyloxy)-13-ethyl-(17α)-(+)-),17-deacetyl norgestimate (18,19-dinor-17-pregn-4-en-20-yn-3-one,17-hydroxy-13-ethyl,oxime), and norgestrel (18-19-dinor-17-pregn-4-en-20-yn-3-one,17-hydroxy-13-ethyl,(17α)-(+)-) were synthesized at The R. W. Johnson Pharmaceutical Research Institute at Ortho Pharmaceutical Corporation.

Stock solutions of non-radiolabelled norgestimate were prepared in

ethanol at a concentration of 2 mg/mL. From this stock solution, different concentrations of solutions for RIA standards (ranging from 20 to 1000 μg/mL) were obtained by serial dilution with 0.1 M phosphate buffer (pH 7.4) containing 0.1% bovine gamma-globulin and 0.01% Tween 80.

Dextran-coated charcoal tablets were obtained from Reflex Industries, Inc. (San Diego, CA).

The antiserum for the RIA for norgestimate, prepared by immunizing rabbits against the thyroglobulin conjugate of norgestrel-17-acetate-6-hemisuccinate, was provided by Dr. Delwood Collins at Emory University, Atlanta, Georgia.

HPLC-grade methanol, methyl-t-butyl ether, and ultrapure water were obtained from Burdick and Jackson Laboratories (Muskegon, Mich).

HPLC Equipment

The HPLC equipment employed in this work included a Model L-6200 pump, a Model L-4000 variable wavelength UV detector (Hitachi/EM Science, Cherry Hill, NJ), a Model ISS-100 autosampler (Perkin Elmer, Norwalk, Conn), and a 3357 Lab Automated System (Hewlett Packard, Piscataway, NJ). A Model 201/202 fraction collector fitted with a 3-way slider valve (Gilson, Middleton, WI) was used in conjunction with the HPLC equipment. The column (30 cm x 3.6 mm i.d.; Waters Millipore, Milford, Mass) was prepacked with 10 micron μ-Bondapak C_{18} and protected by a guard column system containing a 1.8 cm cartridge (Brownlee Labs, Santa Clara, CA).

Solvent Extraction and Purification of Serum

In tests of the recoverability of added norgestimate, 10 μL of a methanolic solution containing 50,000 dpm of ^{3}H-norgestimate was added to 1.0 mL portions of blank human female serum. To the resulting solutions was added 3 mL of methyl t-butyl-ether. The extraction was carried out by vortexing for 1 minute and centrifuging at 1500 cpm for 5 minutes. The organic (upper) phase was pipetted into another tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue was then dissolved in a small volume (100 μL) of methanol before application to the HPLC system.

Automated HPLC Separation

In this study, an isocratic system incorporating methanol-water (80:20, v:v) at a flow rate of 1 mL/min was used. Before and during each daily run, the exact collection time for norgestimate was established by applying a mixture containing 20 ng of each of the unlabelled metabolite standards and observing the resulting retention times by UV. Under the conditions of the method, the approximate retention times of the standards were:

norgestimate (both syn and anti-isomers)	-	9.3-10 minutes
3-keto-norgestimate	-	7.7 minutes
deacetylated norgestimate	-	6.4 minutes
norgestrel	-	5.6 minutes

The collection volume for norgestimate was 2.0 mL obtained approximately 9-11 minutes after injection.

The effluent from the HPLC column corresponding to the norgestimate peak was evaporated to dryness at 40 °C under nitrogen. After evaporation, the residue was dissolved in 0.8 mL of gamma-globulin phosphate buffer. The quantity of buffer was selected in accordance with the expected concentration of steroid. This expedient provided results that were within the useful range of the standard curves.

For the RIA, diluted antiserum (1:3500) was added to an equal volume of the corresponding ^{3}H-labeled standard in protein-phosphate buffer and 0.2 mL of the resulting solution (4,000 cpm) was mixed with either sample or standard solution. Aliquots (0.05 mL) of standards in protein-phosphate buffer containing 0, 20, 40, 80, 100, 200, 400, 800, or 1000 pg of norgestimate were assayed in triplicate to establish a standard curve. Samples were assayed singly. In addition, a set of test tubes containing only buffer and ^{3}H-norgestimate was processed similarly in triplicate and the results were used to determine the nonspecifically bound (NSB) fraction. The solutions containing antiserum, ^{3}H-norgestimate or unlabelled norgestimate (sample or standard) were incubated at room temperature for 18 hours. A 40 mg dextran-coated charcoal tablet was then added to each tube and the mixtures were incubated for an additional 1 hour after centrifugation at 4 °C for 1 hour. The supernatant was poured into a liquid scintillation vial containing 15 mL of picofluor-15. Radioactivity was determined in a liquid scintillation spectrometer equipped with both hard copy (paper) and computer disc outputs (10 minute counts). Results were calculated automatically by means of a logit-log RIA data reduction program with appropriate corrections for procedural recoveries and aliquots used.

RESULTS

Figure 1 shows the separation of norgestimate (syn and anti-isomers) and three of its known metabolites. Based on the observed retention times, all of these metabolites were separated completely from norgestimate during the HPLC step. The average overall procedural recovery (extraction and HPLC fractionation) based on monitoring of losses of ^{3}H-norgestimate was determined to be 52 ± 5.2% (N=20).

Procedural blanks for the overall procedure were generally less than 15 pg/mL of serum.

Accuracy of the procedures was assessed by determining in triplicate, the recoveries of at least four different quantities of norgestimate added to 1 mL of serum. The results of such studies done on different days are summarized in Table 1. Plots of the averages of the measured values versus the quantities added yielded regression lines with r^2 values of 0.9981, 0.9967, and 0.9998, respectively. Based on the results of these experiments, the sensitively of the method for norgestimate is 25 pg/mL. Intra-assay variabilities of generally less than 15% were observed from the results of spiking experiments summarized in Table 1. Analysis of the results of these experiments in which blank serum was spiked in triplicate with amounts of norgestimate ranging from 50 to 500 pg/mL yielded inter-assay variabilities of generally less than 15%.

In a separate experiment, blank serum was spiked with 3-keto-norgestimate and norgestimate in the concentration ratios of 1/5, 1/1, and 5/1. The results of this experiment, as shown in Table 2, were quite similar to those in which the serum samples were spiked with norgestimate alone (Table 1), thus attesting to the specificity induced by the HPLC separation.

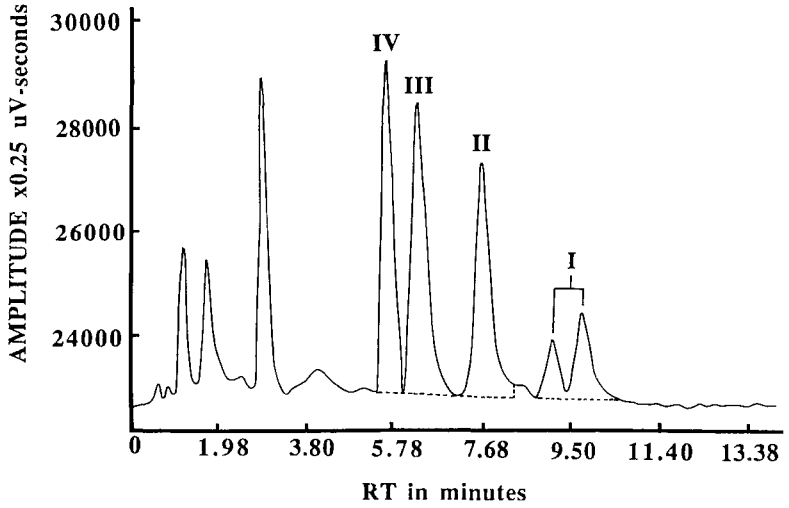

FIGURE 1: HPLC separation of syn and anti norgestimate (I), 3-keto-norgestimate (II), 17-deacetyl norgestimate (III), and norgestrel (IV); data based on UV absorption (254 nm). Column: Waters μBondapak C_{18}, 10 μm, 30 cm x 3.6 mm I.D.; Solvent system: methanol-water (80:20 v/v); Flow rate: 1 mL/min; pressure 1150 p.s.i.

TABLE 1
Recovery of Norgestimate Added to Blank Serum

Run No.	Quantity Added (pg/mL)	Quantity Measured (pg/mL)	Coefficient of Variation (%)	Recovery (%)	Coefficient of Determination (r^2)
1	50	49	21.0	98	0.9981
	100	88	6.5	88	
	250	251	17.1	100	
	500	546	8.2	109	
2	50	42	18.0	84	0.9967
	100	98	6.8	98	
	250	257	11.9	103	
	500	473	14.5	95	
3	25	28	25.0	112	0.9998
	50	51	10.9	102	
	100	89	10.0	89	
	250	242	8.8	97	
	500	499	5.8	100	
	1000	985	19.3	98	

TABLE 2
Recovery of Norgestimate in the Presence of 3-Keto-Norgestimate Added to Blank Serum

Concentration Ratio 3-Keto-Norgestimate	Quantity Added (pg/mL)	Quantity Measured (pg/mL)	Coefficient of Variation (%)	Recovery (%)	Coefficient of Determination (r^2)
1/5	25	24	8.7	96	0.9896
	50	59	0.5	82	
	100	104	3.4	104	
	250	246	0.3	98	
	500	610	9.3	122	
	1000	1021	13.9	102	
1/1	25	22	0.9	88	0.9856
	50	53	10.7	106	
	100	144	15.7	144	
	250	309	0.0	124	
	500	522	25.2	104	
	1000	---	---	---	
5/1	25	17	16.6	68	0.9928
	50	48	11.8	96	
	100	130	11.5	130	
	250	256	8.5	102	
	500	608	16.3	122	
	1000	1420	3.5	142	

DISCUSSION

The use of HPLC to impart specificity to existing RIA methods has been described for both steroids and peptides.[1-14] The method described in this report was developed for use in a bioavailability study of an oral contraceptive formulation containing norgestimate. Since one of the metabolites of norgestimate (3-keto-norgestimate) demonstrated significant cross-reactivity with the norgestimate antiserum, the separation of the two compounds was necessary in order to avoid an over-estimation of norgestimate serum levels. In addition, since the study was conducted in

premenopausal females, it was also necessary to separate any endogenous steroids from norgestimate prior to RIA. Inclusion of an HPLC separation in the procedure eliminates both the possibility of significant interference of one of the possible metabolites and by potentially cross-reactive endogenous steroids in serum that vary considerably during the normal menstrual cycle. Thus, accurate quantification of norgestimate would not be attainable without the HPLC separation step. This method has been recently applied successfully to serum samples obtained at various times after oral administration of tablets containing norgestimate. Using this procedure, nearly 50 samples could be conveniently extracted each day, and the extracts could then be loaded into the HPLC autosampler for overnight separation in preparation for RIA the following day(s).

In an effort to completely automate the method and optimize sample throughput, we are currently attempting at use a robotic work station to perform the extraction step. Thus, the procedure outlined is suitable for many applications involving routine sample analysis.

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LIQUID CHROMATOGRAPHIC ANALYSIS OF THIAMPHENICOL RESIDUES IN MILK

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ABSTRACT

The present paper describes a modified high-performance liquid chromatographic (HPLC) method for the determination of thiamphenicol (THA) residues in spiked milk at levels as low as 30 ppb. Milk spiked with THA was extracted with ethyl acetate. Following addition of hexane, the extract was cleaned up with a silica cartridge. Analysis was performed on a reversed-phase C_{18}, 5 μm, column using water-methanol (70:30) as mobile phase. Recovery was found to range from 68.0 to 90.0 % and precision data suggested that relative standard deviation ranged from 7 to 9.4 %.

INTRODUCTION

Thiamphenicol (THA) is a synthetic broad-spectrum antibiotic, analog of chloramphenicol (CAP). With the prohibition of CAP in food producing animals, since it is known to produce irreversible aplastic anaemia in humans (1), THA appears to be a very viable substitute for CAP. Thiamphenicol is currently marketed in many European countries including France, Italy, Spain, Germany, Belgium and also in Central and

South America, Africa and Asia including Japan. In EEC provisional Maximum Residue Levels (MRLs) have been established for THA residues.

Both gas chromatographic (2-5) as well as high-performance liquid chromatographic methods (6-9) have been described for the determination of THA in tissues and body fluids (plasma, serum, urine). HPLC analysis of body fluids involves either the direct injection of the sample (serum) mixed with methanol, or employs first a solvent extraction of the sample, followed by evaporation and reconstitution in the mobile phase (8). Previously reported HPLC methods for THA determination in serum and plasma when applied to milk yield chromatograms with many interference peaks, as these procedures lack the necessary sample clean up step.

This paper describes an HPLC method particularly suitable for the determination of THA residues in milk. As far as the authors know there are no data available on this subject.

EXPERIMENTAL

Instrumentation

HPLC was carried out on a Gilson system consisting of a Model 802 manometric module, a Model 302 piston pump, a model HM/HPLC dual-beam variable wavelength UV-Vis spectrophotometer set at 224 nm and a Model NI variable-span recorder. A HPLC technology Model TC 831 column oven set at 35 °C permitted temperature regulation. Injections were made on a Hichrom 25X0.46 cm (excel range) column prepacked with Nucleosil 120, C_{18}, 5 μm, through a Rheodyne 7125 sample injector equipped with a 100 μl loop.

HPLC procedure

The mobile phase used was methanol-water (30:70 v/v). The mobile phase, after filtration through a 0.2 μm filter (Nylon-Rainin), was degassed under vacuum and delivered at a flow rate of 1 ml/min.

Chemicals

Thiamphenicol (THA) was obtained from Sigma (St. Louis, MO, USA). Methanol, ethyl acetate, water (all Lichrosolv), n-hexane (analytical grade), citric acid (extra pure) were purchased from Merck (Munchen, FRG).

Stock solution was prepared by weighing accurately and dissolving THA in 10 % v/v methanol in water (0.5 mg/ml). Each day an aliquot of the stock solution was further diluted to give working solutions containing THA, in the range 0.05-1.0 μg/ml.

Silica gel disposable SPE columns (3 ml; 500 mg) were from Analytichem International (Harbor City, USA). Just before use, the column was pretreated by passing 8 ml of ethyl acetate-hexane (2:3).

Extraction procedure

2 g of milk was weighed in a tube containing 0.08 g of citric acid monohydrate. A volume (6 ml) of ethyl acetate was added and the tube was vortexed at moderate speed. After centrifugation at 2300 g, the organic layer was pipetted to another tube. The extraction procedure was repeated with another 4 ml ethyl acetate. After addition of 15 ml of n-hexane to the total extraction solvent, the solution was centrifuged and passed through the pretreated silica gel column at a flow rate of ca 8-10 ml/min. The column was washed with 2 ml of hexane and dried in a stream of nitrogen for 10 min. Thiamphenicol was eluted from the column with 3 ml of methanol (2 ml using vacuum off and 1 ml using vacuum on). The eluate was collected and evaporated in a stream of nitrogen at 30 °C. The residue was dissolved in 1 ml of the mobile phase using Vortex mixer for 30 s. The solution was used for HPLC analysis.

Aliquots of the sample and standard solutions were injected by means of the loop injector (100 μl). Samples were eluted isocratically at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms from standard, blank and spiked milk extracts are shown in Fig. 1. The chromatographic conditions are the same as those proposed by Felice et al. (8) for the determination of THA residues in bovine plasma

Working solutions and milk samples were monitored at 224 nm. THA was eluted in 6.35 min. Regression analysis of the data obtained by running a series of working solutions of THA showed the response to be linear through the range 0.05-1.0 μg/ml ($y=0.16+1.3x$; $r=0.999$; where y represents peak height in mm and x the quantity of the compound injected in nanograms).

Spiking Studies

Recovery experiments were carried out in cow milk spiked at 30, 62.5, 125 and 250 ng/g. In four replicates each amount was added to milk. The results are presented in Table 1. The precision of the method was studied by assaying on each of three different days several milk samples with THA at 250 ppb level. The data are presented in Table 2.

This modified developed method allows the determination of THA residues in milk with a quantitation limit of 30 ppb. This limit can not, unfortunately, compared to, since there are no data in the literature. On the other hand, EEC established the level of 40 μg/kg as provisional MRLs only for bovine and poultry tissues (13).

Sample Pretreatment

Ethyl acetate was used for the extraction of THA from milk as was also the case for the extraction of THA from meat (9). The mixing was

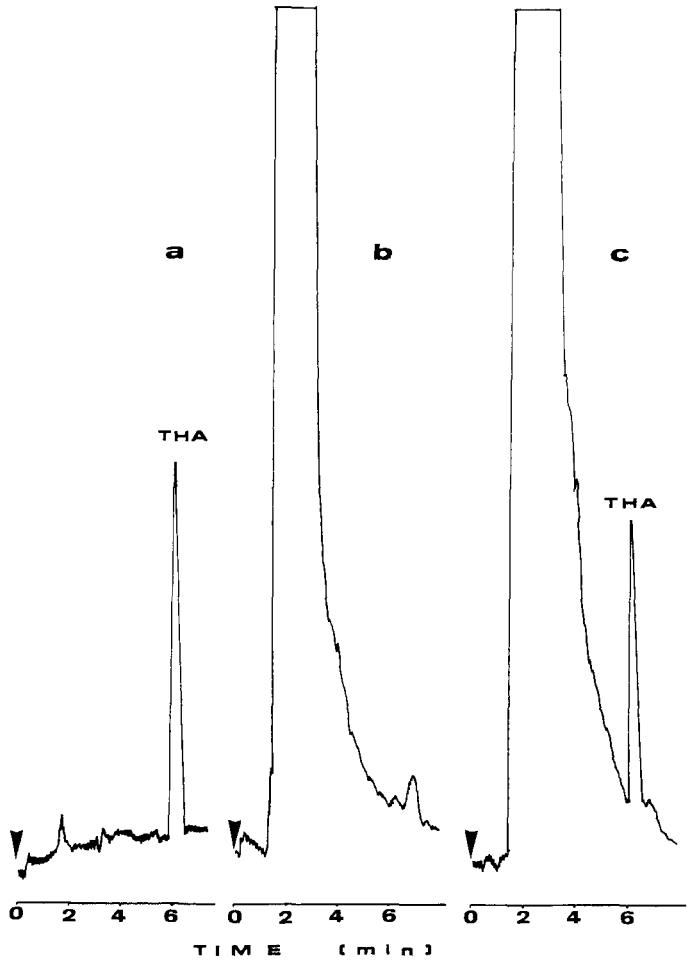

FIGURE 1. Typical chromatograms of (a) a standard solution of thiamphenicol (THA) (0.5 μg/ml), (b) a blank milk sample extract, and (c) a spiked (0.25 μg/g) milk sample extract. Conditions: mobile phase, MeOH–H_2O (30:70 v/v); column, 25X0.46 cm; C_{18} (5 μm); temperature 35 °C; flow rate 1 ml/min; wavelength 224 nm; recorder sensitivity 0.02 AUFS; chart speed 5 mm/min; injection volume 100 μl.

TABLE 1

Recovery Data for THA Analysis in Spiked Milk (n=4)

Thiamphenicol added (ng/g)	Mean Concentraton found ± SD (ng/g)	Mean Recovery %
30	27 ± 1.9	90.0
62.5	48.8 ± 4.4	78.2
125	94.8 ± 18.4	75.8
250	177.4 ± 16.7	70.7

TABLE 2

Precision Data for the Determination of THA in Milk Samples Spiked with 250 ng/g (n=4)

Day	Mean Concentration found ± SD (ng/g)	Rel SD %
1	177.4 ± 16.7	9.4
2	170.2 ± 8.6	5.4
3	171.4 ±12.0	7

performed on a vortex mixer in order to eliminate the emulsion formation observed, sometimes, during the mixing using ultra sonic energy.

The clean up procedure of the ethyl acetate extract was based on that scheme described by Haagsma et al.(10-11) for the determination of CAP in swine tissue and milk. The method comprises addition of hexane to the ethyl acetate extract and SPE using a small silica gel column. Elution was performed with methanol. The modification made concerned the removal of matrix components precipitate from the ethyl acetate-hexane layer. The centrifugation of the ethyl acetate-hexane layer, have been found to give better recovery than the filtration that Haagsma and her co-workers described(10-11). Moreover, in trace analysis, centrifugation should be preferred to filtration, since the sample does not come into contact with filter material that might cause recovery problems (12).

Characterization of the recorded peak was based solely on the retention behaviour of THA

In conclusion, the proposed modified HPLC method is a sensitive, rapid, easy and simple method and it should be useful for the routine analysis.

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SIMPLE AND RAPID METHOD OF ANALYSIS FOR FURAZOLIDONE IN MEAT TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple method for the determination of furazolidone in meat tissues by HPLC using nitrofurazone as internal standard is presented. The samples were extracted with acetonitrile, the organic layer then being separated and evaporated to dryness. The lower limit of quantification was 3 µg/kg with an injection volume of 20 µl, the recovery of furazolidone varying from 97 to 100 %.

INTRODUCTION

Nitrofuran derivatives (e.g. furazolidone, furaltadone, nitrofurazone) are widely used in Europe in the prevention and treatment of gastro-intestinal infections in pigs, calves and poultry caused by E. coli and Salmonella spp.(1). Severe side-effects (e.g. neurotoxicity,growth depression, haemorrhagic

diathesis) have been reported especially in association with furazolidone in food producing animals (2). Nitrofurans are metabolised to a great extent in food-producing animals, and since the demonstration of their mutagenic and (pro) carcinogenic properties, the use of nitrofurans has been strictly regulated in most countries (1-3).
Several analytical methods for the determination of nitrofurans in biological materials based on high-performance liquid chromatography have been published (3-9). The methods are however time-consuming and require the use of large quantities of chemical reagents.
The purpose of the present study was to develop a rapid, simple, and sufficiently sensitive method, for the determination of furazolidone, which required minimal sample manipulation and only small quantities of chemical reagents.

MATERIALS AND METHODS

Materials and Reagents

Samples of cows meat were used.
All chemicals and solvents were of analytical or HPLC grade. Furazolidone and nitrofurazone were purchased from Sigma Co. (St. Louis, MO, USA). Stock solutions (1mg/ml) of furazolidone were prepared by dissolving the compounds in acetonitrile. Stock solutions (1mg/ml) of nitrofurazone were prepared in acetone and ultrasonicated for 1 min. Working standards were prepared by dilution with water. The solutions were stored in the refrigerator.
Spin-X centrifuge filter units from Costar (Cambridge, MA, USA) were also employed.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, an ISS 100 sampling system equipped with a Lauda RMT6 cooler (12°c) from Messgeräte Werk Lauda, (Lauda Köningshafen, Germany), and a LC 235C Diode Array detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at 365 nm. The integration was carried out using the software programme Omega-2 (Perkin-Elmer), wich was operated on an Olivetti M300 personal computer connected to a Bj-330 printer (Canon).

The analytical column (stainless steel, 25 cm x 4,6 mm ID) and guard column (stainless steel, 2 cm x 4,6 mm ID), were packed with 5-µm particles of Supelcosil LC-ABZ (Supelco, Bellefonte, PA, USA).
The mobile phase was a mixture of two solutions, A and B (75:25). Solution A was 0.02 M heptane sulphonate-0.025 M trisodium phosphate, made by dissolving 4.45 g/l 1-heptane sulphonic acid sodium salt (Supelco) and 9.5 g/l trisodium phosphate 12-hydrate (Merck) in ca. 750 ml of water when preparing 1 litre of solution. The pH was then adjusted to 2.5 with 5 M phosphoric acid and the solution made up to volume with water. Solution B was acetonitrile. The mobile phase was degassed with helium before use.
The flow-rate was 1 ml/min. The samples were injected at intervals of 10 min. Aliquots of 20 µl were injected into the column.

Sample pretreatment

The stepwise procedure for pretreatment of tissue samples is shown in Fig. 1.
The tissue sample, 3 g of ground muscle, was weighed into a 50-ml centrifuge tube with a screw cap (NUNC, Roskilde, Denmark). Volumes of 200 µl of internal standard solution (nitrofurazone 1 µg/ml) and 6.8 ml acetonitrile were added. The mixture was homogenized for approx. 6 sec. in an Ultra-Turrax TP 18/2 (Janke & Kunkel KG, Ika Werk, Staufen,F.R.G.), and then centrifuged for 5 min. (5000 rpm.). 6.5ml of the supernatant (corresponding to 1.95 g meat), were transferred into a glass-stoppered centrifuge tube, and 2 ml 5 M NaCl added. The sample was shaken vigorously for 10 s., and centrifuged for 2 min. at 3000 rpm. The upper layer was transferred to another glass-stoppered tube. The organic layer was evaporated to dryness at 43°c under a stream of nitrogen. The dry residue was dissolved in 250 µl 0.02M 1-heptane sulfonic acid sodium salt-0.01M di-sodium hydrogenphosphate-2-hydrate (Ferax, Germany), (the pH was then adjusted to 6.0 with phosphoric acid) and acetonitrile (80:20). After 1 ml of hexane had been added, the sample was again whirlimixed. After centrifugation for 4 min, the hexane layer was discharged. The water-based phase was filtered through a Costar Spin-X centrifuge filter unit (low type) with 0.22 µm cellulose acetate binding by centrifugation for 4 min. at 10000 rpm. (5600g). Aliquots of the filtrate were injected into the HPLC system.

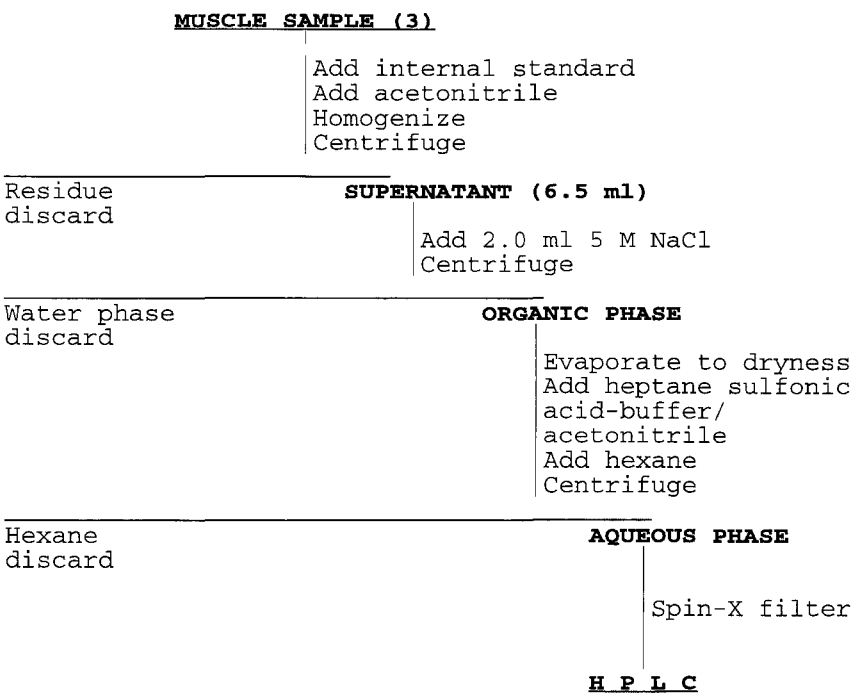

FIGURE 1
Extraction and clean-up procedure for furazolidone and nitrofurazone from meat.

Calibration curves and recovery studies

The calibration curves for furazolidone were obtained by spiking muscle tissue samples with standard solutions and internal standard to yield 2, 3, 5, 10, 20, 50, 100, 200 ng/g of furazolidone and 0.2 µg of nitrofurazone (IS). Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked muscle samples with those of standard solution. The linearity of the standard curves for furazolidone in muscle was tested using peak-height measurements and the internal standard.

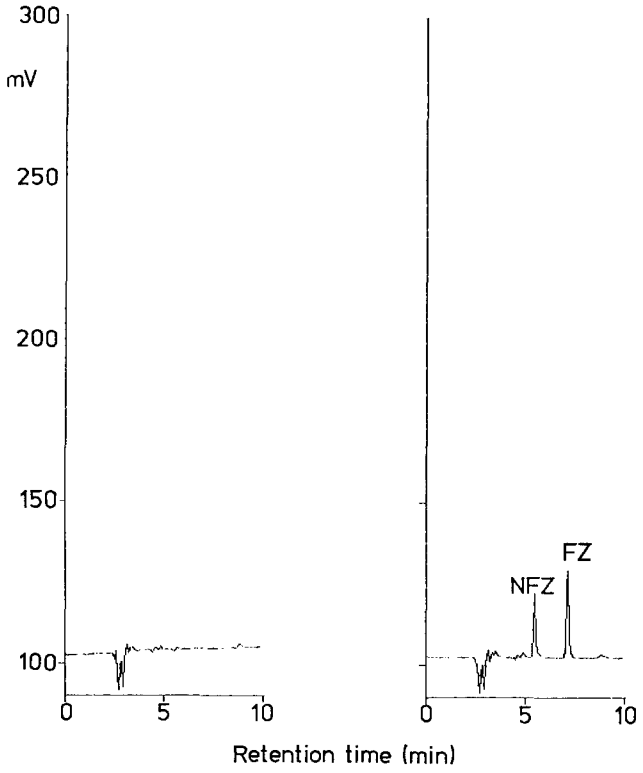

FIGURE 2

Chromatograms of extracts from meat.

A - drug-free meat; B - meat spiked with furazolidone and nitrofurazone.

RESULTS AND DISCUSSION

Chromatograms of clean muscle, and spiked samples are shown in Figure 2. The standard curves were linear in the investigated areas; 2 - 200 ng/g for furazolidone. The linearity of the standard curve was 0.9997 when using the internal standard method. The external standard method of calculation gave a linearity

TABLE 1.

Recovery and repeatability for furazolidone and nitrofurazone from spiked samples of muscle.

Tissue	No. of samples	Amount in spiked samples (ug/g)	Recovery % NTZ. Mean	NTZ. S.D.	FZ. Mean	FZ. S.D.
Meat	8	0.01			100	1.2
(3g)	8	0.1			97	1.7
	8	0.066	90	2.4		
	8	0.066	88	2.5		

NTZ. = nitrofurazone
FZ. = furazolidone
S.D. = standard deviation

coefficient of 0.9995. The precision and recovery for furazolidone and nitrofurazone (internal standard) from muscle were also calculated and are shown in Table 1. The extraction procedures were validated, and showing good recovery of both furazolidone and nitrofurazone. The recovery of furazolidone from muscle tissue varied from 97 to 100 % and that of the internal standard from 88 to 90 %, respectively. The precision of these recovery studies varied from 1.2 to 1.7% for furazolidone and from 2.4 to 2.5% for nitrofurazone. The calculations were also performed without internal standard.

The method was tested under practical conditions by analysing about 60 samples from different animals (cows and pigs). No interference was seen. During analysis, when calibrating the curves, and when performing recovery studies, no special attention was paid to light intensity, a factor considered to be of great significance by other authors.

This study has shown that residues of the antibacterial compound furazolidone in meat may be determined using minimal sample manipulation. The cost of chemicals and the manual work-up procedures is also reduced compared to previously published methods. An experienced technician can carry out sample clean-up of about 18-24 samples per day. The assay shows good precision both when using internal and external standard method. The

limit of quantification was 3 ng/g for furazolidone. The method is robust, sensitive and specific, with good recovery of both substances. The method is demonstrated to be efficient for quantification of residues of drug of furazolidone. Only small amounts of solvents are necessary.

ACKNOWLEDGEMENT

We are grateful to the Agricultural Research Council of Norway for financial support.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(9), 1879-1883 (1995)

THE BOOK CORNER

CARBOHYDRATE ANALYSIS HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS, Edited by Z. El Rassi, Journal of Chromatography Library Volume 58, Elsevier, Amsterdam, The Netherlands, 668 pages, 1995. Price: $242.75.

This book is an excellent reference for every one interested in carbohydrate analysis. The volume discusses all aspects of carbohydrate analysis from sample preparation to separation and detection in an easy to understand manner. The editor, Dr. Z. El Rassi is a leader in this field and should be commended for a job well done. The authors are leaders in their areas. The book is free of errors and well illustrated and referenced, and is a must for all those interested in carbohydrates and glycoconjugates analysis.

Carbohydrates and glycoconjugates are very important biological species involved in many life processes. Because of the structural diversities and the multilateral importance of carbohydrates, the analytical methodologies used to analyze them continue to evolve. High performance liquid chromatography (HPLC) has been extensively used in the separation and isolation of carbohydrate species. More recently, high performance capillary electrophoresis (HPCE), has been explored. The objective of this book is to provide a comprehensive review of carbohydrate analysis by HPLC and HPCE by covering the separation methods for all classes of carbohydrates including mono- and disaccharides; linear and cyclic oligosaccharides; branched oligosaccharides (e.g., glycans); polysaccharides; glycoconjugates (e.g., glycolipids, glycoproteins); carbohydrates in food and beverage; compositional carbohydrates of polysaccharides; carbohydrates in biomass degradation; etc.

The book is well balanced in terms of its content: covers the fundamental aspects of the various modes of HPLC and HPCE that are currently applied to the analysis of carbohydrates; discusses analytical and preparative separations; describes the principles of detection and quantitative determination of carbohydrates by HPLC and HPCE; reviews sample preparations; and provides an ample amount of important applications.

The book is divided into three major parts. The first part, Chapter 1, reviews enzymatic and chemical methods currently utilized in sample preparation.

The second part deals with Analytical and Preparative Separations, and encompasses a series of 8 chapters. Seven of these chapters, Chapters 2 through 8, describe, in detail, the

different HPLC and HPCE systems currently used in analytical separations of carbohydrates and glycoconjugates. An additional chapter, Chapter 9, reviews the various aspects of semi-preparative and preparative HPLC for the isolation of small and large quantities, respectively, of intact and pure carbohydrates and glycoconjugates.

The third part is on The Detection, a topic as important as the separation part. In general, carbohydrates lack chromophores or fluorophores in their structures. This inherent property of carbohydrates causes difficulties in determining these species at low levels. This section contains a series of 8 chapters, Chapters 10 through 17, covering, in detail, the different direct and indirect detection methods that have been introduced for the sensitive detection of carbohydrates. The various detection topics include electrochemical, refractive index, mass spectrometry, light scattering, chiroptical, pre- and post-column derivatization reactions for optical detectors (UV, Vis and fluorescence), post-column enzyme reactors, indirect UV and fluorescence detection, low wavelength UV and other miscellaneous modes of detection. Each of these chapters discusses the basic principles, advantages and limitations, and applications of the particular detection technique. It is highly recommended.

Table of Contents

CENTRIFUGAL PARTITION CHROMATOGRAPHY, Edited by A.P. Foucault, Chromatographic Science Series, Volume 68, Marcel Dekker, Inc., New York, 432 pages, 1994. Price: $150.00.

Modern countercurrent chromatography (CCC) originates from the pioneering studies of Y. Ito et al., who first constructed, in Japan, an apparatus designed to differentiate particles in suspension or solutes in solution in a solvent system subjected to a centrifugal acceleration field. This first machine opened the way in two main directions: one, pursued by Y. Ito in the United states, is based on a wide variety of countercurrent chromatographic apparatuses most of the recent ones using a variable gravity field produced by a two-axis gyration mechanism and rotary seal-free arrangement for the column; the other, pursued by K. Nunogaki in Japan, is based on

the "CPC apparatus" (centrifugal partition chromatographic apparatus) and uses a constant gravity field produced by a single-axis rotation mechanism, and two rotary seal joints for inlet and outlet of the mobile phase.

The historical linkage between countercurrent distribution (Jantzen, Watanabe, Van Dijek, Martin and Synge, Craig and others) and countercurrent chromatography is responsible for the name *countercurrent chromatography* in which a strong gravitational field is used to keep a liquid stationary phase in a steady immobilized state while the mobile phase is pumped through. With technological improvements, the performance of today's instruments is much closer to that of liquid-liquid chromatography using a solid support to hold the liquid stationary phase.

The goal of this volume is to provide a forum for scientists who are already using centrifugal partition chromatographs in their research to share with others their personal knowledge in this specific field of chromatography. This book is devoted exclusively to the CPC apparatus (single-axis rotation mechanism).

CPC and HPLC (high-performance liquid chromatography) are similar in several respects. They share the same fundamental mechanism (partitioning of solutes), the same goal (separation, purification), and the same ancillary equipment (pumps, injectors, detectors).

The book introduces centrifugal partition chromatography (CPC) for any biphasic system - offering in-depth coverage of instrumentation, theory, liquid-liquid partition coefficients, and CPC in organic and inorganic chemistry - and provides over 80 ternary phase diagrams of three-solvent systems that can be applied to virtually all partitioning, separation, and purification situations.

The book is divided into 12 chapters written by international experts from North American, Europe, and Japan. It examines chromatographic properties, illustrates practical operations, and gives examples of CPC solutions to real experimental problems, highlights the distinction between CPC and high-performance liquid chromatography, explains hydrostatic, hydrodynamic, and overall pressure drops, discusses solvent systems, strategies for solvent selection, and the elution mode in CPC, shows how to design solvent systems for CPC of complex organic mixtures, describes carrier-aided CPC for preparative-scale separations and the use of CPC as a multistage liquid-membrane transport system, and much more.

With nearly 800 references, tables, equations, and figures, *Centrifugal Partition Chromatography* is a good resource for analytical and pharmaceutical chemists and biochemists, separation scientists, pharmacologists, and upper-level undergraduate and graduate students in these disciplines.

Table of Contents:

3. **Pressure Drop in Centrifugal Partition Chromatography**, M. J. van Buel, L. A. M. van der Wielen and K. Ch. A. M. Luyben, (51).

4. **Solvent Systems in Centrifugal Partition Chromatography**, A. P. Foucault, (71).

5. **Fractionation of Plant Polyphenols**, T. Okuda, T. Yoshida and T. Hatano, (99).

6. **Centrifugal Partition Chromatography in Assay-Guided Isolation of Natural Products: A Case Study of Immunosuppressive Components of Tripterygium wilfordii**, J. A. Glinski and G. O. Caviness, (133).

7. **Liquid-Liquid Partition Coefficients: The Particular Case of Octanol-Water Coefficients**, A. Berthod, (167).

8. **Centrifugal Partition Chromatography for the Determination of Octanol-Water Partition Coefficients**, S. J. Gluck, E. Martin and M. H. Benko, (199).

9. **Mutual Separation of Lanthanoid Elements by Centrifugal Partition Chromatography**, K. Akiba, (219).

10. **Separator-Aided Centrifugal Partition Chromatography**, T. Araki, (241).

11. **Centrifugal Partition Chromatographic Separations of Metal Ions**, S. Muralidharan and H. Freiser, (317).

12. **Preparative Centrifugal Partition Chromatography**, R. Margraff, (331).

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(9), 1885 (1995)

CALL FOR PAPERS

SIXTH ANNUAL FREDERICK CONFERENCE ON CAPILLARY ELECTROPHORESIS

Hood College
Frederick, Maryland

October 23 - 25, 1995

The Sixth Annual Frederick Conference on Capillary Electrophoresis will include oral and poster presentation by individual conference participants, and optional enrollment in a one-day CE course on October 22, 1995.

Invited speakers will provide an expert overview of the basic aspects and applications of CE and MECC, including instrument and column design, detection, optimization and factors that influence mobility, selectivity, resolution, and the application of CE in the separation of small ions as well as large biomolecules. The emphasis will be on clinical, pharmaceutical and biomedical applications.

Investigators interested in presenting their work for consideration by the Scientific Committee should send a 200-word abstract (accepted as 20 minute talks or poster displays) by Friday, July 14, 1995. Abstracts received after July 14th will be considered for poster presentation.

For further information, contact Margaret L. Fanning, Conference Coordinator, PRI, NCI-FCRDC, P. O. Box B, Frederick, MD 21702-1201, USA; Tel: 301-846-5865; FAX: 301-846-5866.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(9), 1887 (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(9), 1889-1895 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

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

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

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

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

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

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

JUNE 5 - 8: 5th Symposium on Our Environment / 1st Asia-Pacific Workshop on Pesticides, Singapore. Contact: The Secretariat, 5th Symp on our Environment, Chem Dept, National University of Singapore, Kent Ridge, Republic of Singapore 0511.

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

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

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

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

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

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

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

JULY 23 - 27: American Society of Pharmacognosy, 36th Annual Meeting, University of Mississippi, Oxford, Miss. Contact: Russell Cooper, Center for Public Service & Continuing Studies, 14 E.F. Yerby Center, Box 1667, University of Mississippi, University, MS 38677, USA.

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

JUNE 25 - 28: Method Development in HPLC, Virginia Tech, Blacksburg, Virginia. Contact: Dr. H. McNair, Chem Dept, Virginia Tech, Blacksburg, VA 24061-0212, USA

AUGUST 13 - 17: ICFIA'95: International Conference on Flow Injection Analysis, Seattle, Washington. Contact: Prof. G. D. Christian, Dept of Chemistry BG-10, University of Washington, Seattle, WA 98195, USA.

AUGUST 14 - 19: 35th IUPAC Congress, Istanbul, Turkey. Contact: Prof. A. R. Berkem, 35th IUPAC Congress, Halaskargazi Cad. No. 53, D.8, 80230 Istanbul, Turkey.

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

AUGUST 20 - 25: ACS sponsored Symposium on Saponins: Chemistry and Biological Activity, Chicago, Illinois. Contact: G. R. waller, Oklahoma State University, Dept of Chem & Molecular Biology, Stillwater, OK 74078, USA.

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

SEPTEMBER 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.

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

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

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

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

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

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

1996

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

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

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

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

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.

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.

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CONTENTS

(continued on inside back cover)

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