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DETERMINATION OF ERYTHROCYTE PORPHYRINS BY EPI-ILLUMINATION FLUORESCENCE MICROSCOPE WITH CAPILLARY ELECTROPHORESIS

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ABSTRACT

A micellar electrokinetic capillary chromatographic (MEKC) method is described for the simultaneous determination of zinc protoporphyrin (ZnPP) and protoporphyrin (PP) in whole blood. Reproducibility of migration times and peak areas of ZnPP and PP have been shown to improve significantly with the use of an internal standard.

Erythrocyte porphyrins were extracted from whole blood with N,N-dimethylformamide (DMF), followed by sample cleanup and preconcentration on C-18 cartridges. Analytical recoveries of the porphyrins were better than 88%. An epi-illumination fluorescence microscope was used for the detection of these porphyrins. Limits of detection were found to be 50 nM and 8 nM for ZnPP and PP, respectively.

INTRODUCTION

Naturally occurring porphyrins are intermediate metabolites of heme biosynthesis. Disturbances in the biosynthesis, caused by inborn or acquired defects of the corresponding enzymes, give rise to a family of disease called porphyria. Depending on the break of the metabolic pathway, different porphyrins are accumulated in body fluids and tissues. Certain diseases such as lead intoxication and iron deficiency anemia[1] result in protoporphyrin (PP) accumulating in the red blood cells as the Zn-protoporphyrin chelate (ZnPP), while in the case of erythropoietic protoporphyria, the unchelated PP accumulates[2]. Hence, erythrocyte porphyrin profile is an important diagnostic tool for the detection of such hematological disorders.

Separation and quantitative analysis of porphyrins extracted from human excreta and biological tissues have been conventionally performed by high performance liquid chromatography (HPLC) with fluorescence detection[3-5]. Different organic solvents have been used for the extraction of ZnPP and PP in whole blood[6-12]. Solvent systems which included mineral acid in the extracting solvent dissociate Zn from ZnPP[6-8], and separate extraction procedures were required for ZnPP and PP[8]. Other solvent systems such as acetone-DMSO mixture[9], aqueous acetone[10], and aqueous ethanol[12] have successfully been used for the simultaneous extraction of ZnPP and PP from whole blood. To date, there has been no usage of N,N-dimethylformamide (DMF) as the extracting solvent. In this paper, a method employing DMF as an extracting solvent for erythrocyte porphyrins and micellar electrokinetic capillary chromatography (MEKC) coupled with epi-illumination fluorescence microscope detection for their separation was described.

EXPERIMENTAL

Materials

Protoporphyrin IX (PP) and 3-cyclohexylamino-1-propanesulfonic acid (CAPS) were obtained from Sigma (St. Louis, MO, USA). Mesoporphyrin IX (MP) was from Porphyrin Products (Logan, UT, USA), and zinc protoporphyrin IX (ZnPP) from Aldrich (Milwaukee, WI, USA). Sodium dodecyl sulphate (SDS) was purchased from Fluka (Buchs, Switzerland), and HPLC-grade DMF was from BDH (Poole, England). All other chemicals used

were of analytical-reagent grade. The electrophoresis buffer solutions were prepared with Milli-Q treated water (Millipore, Bedford, MA, USA) and filtered through a 0.45 μm membrane (Whatman, Arbor Technologies, Ann Arbor, MI, USA). Disposable solid phase extraction cartridges used were Sep-Pak Light C-18 cartridges and Sep-Pak Plus C-18 cartridges containing 120 mg and 330 mg, respectively, of 90 μm sorbent (Waters Associates, Milford, MA, USA). Disposable syringes were used to deliver liquids through the extraction cartridges.

Capillary Electrophoresis

CE was performed on 65 μm i.d. fused silica capillaries from Polymicro Technologies (Phoenix, AZ, USA). The detector system was similar to that described previously[13]. It consisted of a Nikon Labophot-2A episcopic fluorescence microscope (Nikon Corp., Tokyo, Japan) with a 100 W high pressure mercury lamp as the excitation source. Wavelength selection was accomplished through a filter block comprising of an excitation filter, a dichroic mirror, and a barrier filter. Excitation wavelength of 405 nm was selected with an interference filter (10 nm bandwidth, CVI Laser Corp., Albuquerque, NM, USA). The dichroic mirror reflects radiation below 560 nm and refracts radiation above 580 nm, and the barrier filter transmits radiation longer than 590 nm. A silicon photodetector (Model HC220-01, Hamamatsu Corp., Japan) was employed for the detection of fluorescence signal. Data acquisition was performed on a Hewlett Packard 3390A integrator (Palo Alto, CA, USA). Sample injection was performed hydrodynamically at a height difference of 10 cm for 15 s, corresponding to an injected sample volume of approximately 10 nL. Separations were accomplished at ambient temperature using a Spellman RHR30 high voltage power supply (Plainview, NY, USA).

New capillaries were rinsed with water for 30 min, followed by 1N NaOH for 10 min, and 0.1N NaOH for another 30 min. After a short wash with water, the capillary was filled with the electrophoresis buffer. When peak broadening or noisy baseline was observed, the capillary was rinsed successively with 1N HCl (2 min), water (2 min), 0.2N NaOH (5 min), water (2 min), and finally stabilized with electrophoresis buffer to restore to its original performance. At the end of the day, the above washing procedure was repeated, except that the NaOH solution was left in the capillary overnight.

Porphyrin Standards

Standard stock solutions of MP, PP and ZnPP were prepared by dissolving approximately 1 mg of each of the respective porphyrin in 1 mL of DMF. The concentrations of the porphyrin solutions were determined from their absorptivity[14]. As the porphyrins are photosensitive, all porphyrin solutions were protected from light whenever possible and were stored at -20°C.

Blood Samples

Whole blood from a healthy subject was treated with disodium ethylenediaminetetraacetate (EDTA) and stored in the dark at -20°C. Spiked blood samples were prepared by adding appropriate volumes (not more than 10 μL) of standard solutions containing ZnPP and PP to 500 μL of whole blood.

Extraction Procedures

Spiked blood (100 μL) was prediluted with 200 μL of 0.5N acetic acid and then extracted with 1500 μL of DMF containing MP as the internal standard. Extraction was achieved with vortex mixing for 1 min and sonicating in an ice-water bath for 5 min. The protein precipitate was packed by centrifugation at 2000*g* for 5 min. The yellowish supernatant was then transferred to a clean polypropylene vial.

Sample cleanup and preconcentration were carried out on a C-18 cartridge. The cartridge was first activated with 2 mL of methanol followed by conditioning with 1 mL of 0.5N acetic acid. The supernatant from the blood extract (1.5 mL) was diluted with 2 mL of 0.5N acetic acid to lower the solvent strength of the sample solution. This solution was loaded onto the cartridge at a flow rate of ca. 3 mL/min, and the cartridge was then washed with 1 mL of water and 1 mL of 20% aqueous DMF. Most of the water remaining in the sorbent was removed by forcing 10 mL of air through the cartridge using a disposable syringe. The retained porphyrins were then slowly eluted with about 0.3 mL (2 x 0.15 mL) of DMF.

RESULTS AND DISCUSSION

Electrophoretic Separations

The separation of the dicarboxylic porphyrins was performed using one set of the optimized conditions achieved employing the overlapping resolution mapping scheme, described in our previous paper[15]. Baseline resolution of the porphyrins was obtained with a 20 mmol/L CAPS buffer (pH 10.8) containing 20 mmol/L SDS and 8% (v/v) DMF, as shown in Fig. 1. From electropherograms of equimolar mixture of ZnPP and PP in DMF, the fluorescence of PP was found to be higher than that of ZnPP (data not shown). In fact, the ratio of the fluorescence peak areas of PP to ZnPP was found to be approximately 3. The difference in their fluorescence is mainly due to the difference in the excitation maximum of ZnPP and PP. In this study, fluorescence detection was selected with an excitation wavelength of 405 nm, which gives a maximum fluorescence for PP , whereas ZnPP emits maximally at an excitation wavelength of 417 nm.

Table 1 shows the migration time reproducibility for the porphyrins. When the capillary was flushed with electrophoresis buffer between runs, the run-to-run relative standard deviations (RSD) for the migration times of ZnPP and PP were more than 1% for 8 consecutive runs. A drift towards longer migration times and a gradual broadening of the porphyrin peaks were also observed. The situation did not improve with replenishment of the electrophoresis buffer at both electrodes. It was believed that the migration drift was, in large part, due to adsorption of the porphyrins onto the capillary wall, as noted by other workers[16,17]. To restore the performance, the capillary was washed with 1N HCl (2 min) and 0.2N NaOH (5 min), and rinsing with water in between. However, as a result of such acid and alkaline washes, the electroosmotic flow varied considerably, and the day-to-day RSD for the migration times were greater than 3%. Under such circumstances, reliability of peak identification based on migration time was poor. Besides variations in the electroosmotic flow, other factors such as temperature fluctuations due to Joule heating and inadequate temperature control would also contribute to migration irreproducibility. To improve analyte identification and migration reproducibilty in capillary electrophoresis, the uses of migration indexes[18] and normalization of the migration time of the analytes[18,19] have been reported. In this study, the approach of normalizing migration time by dividing the migration time of the porphyrins by the migration time of a reference standard

was utilized. MP was used as the reference standard, and the RSD values for the reproducibility of the normalized migration times are presented in Table 1. It is clearly evident that the migration time reproducibility was greatly improved with the use of normalized migration times. Day-to-day RSD values ranged from 0.2 - 0.64%, corresponding to a minimum of 4-fold improvement.

Table 1

Reproducibilities of the Migration Times and Peak Areas of ZnPP and PP, With and Without Normalization.

	ZnPP	PP
(I)	**Migration Times in Min (% RSD)**	
Run-to-run (n = 8)	7.81 (1.1)	9.15(1.1)
Day-to-day (n = 8)	7.82 (3.1)	9.26 (3.4)
(II)	**Normalized Migration Times (% RSD)[a]**	
Run-to-run (n = 8)	0.89 (0.26)	1.05 (0.12)
Day-to-day (n = 8)	0.89 (0.64)	1.05 (0.20)
(III)	**% RSD for Absolute Peak Areas**	
n = 5	12.6	12.5
(IV)	**% RSD for Normalized Peak Areas[a]**	
n = 5	4.3	2.1

a Migration times and peak areas were normalized by dividing by those of MP.

In addition to improvement in migration reproducibility, MP could also serve as an internal standard in quantitation. Run-to-run variations and quantitation errors due to diffusion during injection[20,21], accidental hydrodynamic flow (caused by unequal sample and buffer elevations), surging of solutions in the capillary due to changing of the electrolyte reservoirs, and the like could be minimized by the use of an internal standard[22]. MP was chosen as the internal standard as it is not naturally occurring in whole blood, and was well separated from the porphyrins of interest, such that it does not interfere with porphyrin quantitation. The structural resemblance between MP, PP and ZnPP (all contain 2 carboxylic acid groups) implies that their migration characteristics would possibly be similar. The large RSD values observed for the peak areas of ZnPP and PP (Table 1) could be partly attributable to the adsorption of the porphyrins. When the ratios of the peak areas of the analytes to that of the internal standard were taken as responses, RSD values dropped considerably, in agreement with literature RSD values for analyses with internal standard (e.g., comparison with Table 1 in ref. 22).

Extraction of Blood

Different solvents have been employed for the extraction of erythrocyte porphyrins. Solvents such as DMSO-acetone mixture[9], aqueous acetone[10] and aqueous ethanol[12] have been used. Attempts to adopt these extraction methods with analysis by MEKC were however, not successful. In the case of DMSO-acetone mixture, whole blood was first prediluted with aqueous acetic acid before extracting with DMSO-acetone mixture[9]. When the supernatant was injected into the MEKC system, broad peaks were observed, probably due to the pH gradient existing across the boundaries between the acidic sample zone and the alkaline electrophoresis buffer. It was also noted that broader peaks were obtained for porphyrins dissolved in aqueous acetone and aqueous ethanol, compared to porphyrins dissolved in DMF. It appeared that the choice of sample solvent to be injected has an effect on the peak efficiency. DMF is a strong solvent for the porphyrins, is water miscible, and is suitable for our MEKC analysis. To date, there has been no report on the use of DMF as the extracting solvent. The feasibility of extracting erythrocyte porphyrins with DMF was thus evaluated in this investigation.

Unspiked blood and blood samples spiked with ZnPP and PP were extracted with different volumes of DMF containing MP as the internal standard. After centrifugation, the yellowish extracts were directly injected into

the capillary column. Peak areas of ZnPP and PP relative to that of MP were calculated and compared with those of the standards, taking into account the amounts of ZnPP and PP extracted from unspiked blood. The analytical recoveries of the porphyrins obtained with different volumes of DMF are summarized in Table 2. As can be seen, larger volumes of DMF resulted in higher analytical recoveries of the porphyrins. Recoveries greater than 94% were obtained when extracted with 1.5 mL of DMF. Besides acting as an extraction solvent, DMF also precipitates proteinaceous materials from the blood. With smaller volumes of DMF, the blood proteins were probably not precipitated sufficiently and thus interfered with the porphyrins' recovery. Although filtration is not required, attempts were made to remove fine particles possibly present in the supernatant solutions, to prevent clotting of the capillary. Poor recoveries of the porphyrins were obtained when polytetrafluoroethylene (PTFE) filters were used. About 50% of ZnPP and PP were retained on the filter, while only about 38% was recovered for MP. Similar observations were also reported when porphyrin solutions were filtered through Nylon membranes[23,24]. Other filter which has lower affinity for porphyrins was recommended, if the filtration step was deemed necessary[24].

Table 2

Effect of Volume of DMF on the Analytical Recovery of Porphyrins Added to Normal Blood.

	% Recovery		
Volume of DMF (mL)	**ZnPP (220 pmol added)**		**PP (50 pmol added)**
0.3		62	78
0.7		74	84
1.5	94	98	

Despite the quantitative analytical recovery achievable with DMF extraction, there are drawbacks of direct injection of the supernatant solution. The capillary suffered from short column life as the performance of the capillary deteriorated beyond restoration after about 60 injections. This was not observed with injections of porphyrin standards. Although no other fluorescent compounds or fluorescence quenching compounds which interfered with the electropherograms were observed, it appeared that compounds capable of interacting with the capillary wall and shortening the life of the capillary were

simultaneously being extracted. The yellowish colour of the extract also suggested the presence of other UV-absorbing substances. In addition, the use of large volumes of DMF inevitably results in the dilution of the porphyrin concentration, hampering detection especially for samples containing low concentrations of porphyrins. A solution to both problems is to perform sample cleanup and preconcentration on a solid phase extraction (SPE) cartridge. Solid particulates could also be removed from the sample solutions after passing through the cartridges.

Extraction with Sample Cleanup

Sample preparation on SPE cartridges have been reported for porphyrins extracted from urine, faeces and tissues[24-26]. However, no such treatment on erythrocyte porphyrins has been performed. The conditions for quantitative extraction of erythrocyte porphyrins and subsequent sample cleanup on commercially available C-18 cartridges were investigated in this study. The performance of two different sorbent capacities, 330 mg (Sep-Pak Plus) and 120 mg (Sep-Pak Light) were compared. Supernatants of spiked blood extracted with 1.5 mL of DMF was first diluted with 2 mL of 0.5N acetic acid before loading into the C-18 cartridge. The purpose of the dilution step is to modify the polarity of the solvent so that porphyrins would be retained on the C-18 cartridge. Upon loading of the sample solution, a dark red band was formed near the inflow of the cartridge. No movement of the band was observed with subsequent washing with water and 20% aqueous DMF solution. When eluted with DMF, the red band diffused into broader bands, indicating differential elution. No signals were obtained from the first 120 mL and 350 mL of eluates from the Sep-Pak Light cartridge and Sep-Pak Plus cartridge, respectively. These volumes were needed to displace the residual water from the cartridges and to move the porphyrin band further down the cartridges. To elute the retained porphyrins, 600 mL of DMF was needed for the Sep-Pak Plus cartridge, while most of the porphyrins were detected in eluates of 300 mL for the Sep-Pak Light cartridge. The eluates were slightly yellowish in color, with most of the pigments still retained on the cartridges, demonstrating the effectiveness of the SPE cartridges in removing most of the matrix components. The Sep-Pak Light cartridge was judged to be superior as the analytes could be retained and collected with half the volume of eluant needed for the Sep-Pak Plus cartridge.

In most of the previous studies, blood samples were prediluted with an appropriate aqueous solution to achieve a more complete extraction[9,12]. Rossi et al.[9] reported a better extraction efficiency when the blood was prediluted with 0.5N acetic acid, instead of water. In our study, DMF extraction of unspiked blood, with and without predilution with acetic acid, followed by sample cleanup were compared. With predilution, extraction efficiencies of ZnPP and PP were both increased, with a considerable increase in the case of ZnPP. The increase is presumably attributable to the lysing of the erythrocytes, releasing the more strongly bound ZnPP[27]. The supernatants from DMF extraction with predilution with acetic acid, however, gave rise to broad peaks when injected directly into the MEKC system, probably due to the pH differences existing at the boundaries between the sample zone and the electrophoresis buffer. Hence with sample cleanup, exchange of the sample solution to one that is more appropriate for injection could be performed. The column life of the capillary was also prolonged as a consequence of sample cleanup. More than 120 injections could be carried out with reasonable performance of the capillary.

Analytical recoveries of ZnPP and PP from the Sep-Pak Light cartridge were first evaluated with standard porphyrin solutions in DMF, following the washing and elution steps described under Experimental. Near quantitative recoveries (> 95%) of the porphyrins were obtained, indicating minimum loss of analytes from the washing step and the effectiveness of DMF elution. Next, analytical recoveries of porphyrin standards spiked into normal blood, together with the concentrations of ZnPP and PP originally present in the blood sample were determined by the method of standard additions. A series of normal blood samples spiked with porphyrin standards were prepared for the calibration plot. These blood samples were then subjected to the extraction procedures and analysed by MEKC. The ratios of the peak areas of ZnPP or PP to that of MP were plotted against the amount of porphyrins added. Correlation between the relative peak areas and the amount of added porphyrins were generally good in the concentration range of 0.035 - 0.67 μmol/L for PP, and 0.16 - 2.9 μmol/L for ZnPP. Due to the higher fluorescence of PP than ZnPP, considerably lower concentration range was studied for the extraction recoveries of PP. Correlation coefficient values of at least 0.993 were obtained for both the ZnPP and PP lines. Extrapolation of the lines to the horizontal axis gave a measure of the porphyrins content in normal blood. The ZnPP value was found to be 0.27 μmol per liter of whole blood, and PP was found to be 0.03 μmol per liter of whole blood. These values fall into the range reported in literature[9]. To calculate the analytical recoveries of the porphyrins, relative peak areas of extracted ZnPP and PP were compared to those for the

standard solutions, corrected for the amount of porphyrins already present in the blood. The analytical recoveries are shown in Table 3. The recovery varied from 88 - 100%. The reproducibilities for the extraction recovery of ZnPP and PP were evaluated by repeating 5 analyses on blood samples from one normal human subject. RSD values were about 6% for both porphyrins.

Table 3

Analytical Recovery of ZnPP and PP Added to Normal Blood.

Compound	Added Amount (pmol)	% Recovery [a]
ZP	70	88
	240	99
	590	92
PP	15	96
	501	100
	120	98

[a] Spiked blood samples were prediluted with 0.5N acetic acid, extracted with DMF, cleaned up on Sep-Pak Light cartridges, and analysed by MEKC, as described under Experimental. The results represent the average of triplicate extractions. The concentrations of endogeous ZnPP and PP were subtracted prior to calculating recoveries.

Fig. 2 shows an electropherogram of a blood extract from a normal adult female human subject. The slight difference in the migration times of the porphyrins in Figs. 1 and 2 was probably due to matrix effects. Coproporphyrin I, which was extracted in some blood samples[8,11], was not detected in this study. Its presence , however, would not cause an interference problem, as it migrates between ZnPP and MP in our electrophoretic conditions. The limits of detection (LOD) determined from the actual injections of low concentrations of the porphyrins were found to be 50 nmol/L and 8 nmol/L for ZnPP and PP, respectively, at a signal-to-noise ratio of 3. With an injection volume of approximately 10 nL, the minimum detectable amounts for ZnPP and PP were about 500 amol and 80 amol, respectively. Further improvement in the LOD for ZnPP could be achieved with the proper choice of excitation wavelength. An excitation filter centered at 410 nm and wider bandwidth (e.g., 20 nm) should sufficiently cover the range of excitation

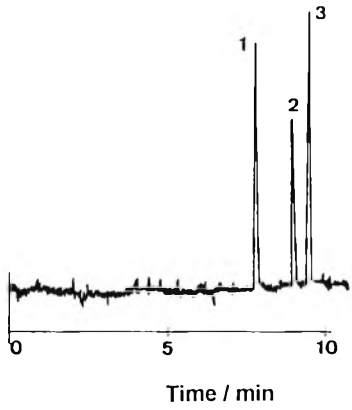

Figure 1. Optimized MEKC separation of ZnPP, PP and MP. Conditions: buffer, 20 mmol/L CAPS (pH 10.8), 20 mmol/L SDS, 8% DMF; capillary, 65 mm i.d. x 67.2 cm (45.2 cm to the detector); applied voltage, 21 kV. Peaks: 1 = ZnPP (0.2 mmol/L), 2 = MP (0.05 mmol/L), and 3 = PP (0.07 mmol/L).

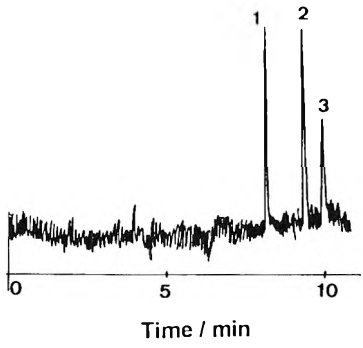

Figure 2. Electropherogram of a normal adult female blood extract. Electrophoretic conditions and peak identities same as in Fig. 1.

wavelengths required for maximum fluorescence of ZnPP and PP. Nevertheless, the sensitivity achieved with epi-illumination fluorescence microscopy detection was comparable to those reported using capillary electrophoresis / laser-induced fluorescence system[16], and approaching those reported for HPLC / fluorescence system[17].

CONCLUSIONS

Th:s paper demonstrated that simultaneous extraction of ZnPP and PP from blood samples was possible with DMF. With sample cleanup on a C-18 SPE cartridge, quantitative analytical recoveries of ZnPP and PP of greater than 88% were achieved. Although the extracts were slightly yellowish in color, effective separation coupled with specific fluorescence detection permitted analysis of the porphyrins with very little interference by endogenous compounds. The porphyrins were identified by their normalized migration times, with respect to that of an internal standard, MP. The C-18 cartridge acted as a guard column to protect the capillary from undesirable adsorption of contaminants, thus aiding in prolonging the column life. It also improved the sensitivity of the method by preconcentrating the analytes. The LODs obtained for ZnPP and PP were 50 nmol/L and 8 nmol/L, respectively.

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QUANTITATIVE HPTLC DETERMINATION OF SALICYCLIC ACID IN TOPICAL ACNE MEDICATIONS

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ABSTRACT

A method was developed for determination of salicylic acid in anti-acne pharmaceutical preparations involving separation on a preadsorbent high performance thin layer chromatography (HPTLC) silica gel plate with fluorescent phosphor, detection by fluorescence quenching, and quantification by densitometric scanning. Salicylic acid was directly detected and quantified on the plate at levels as low as 1 μg, and no interference was encountered from other ingredients in the medication formulations.

The method was applied to the analysis of a commercial gel preparation with a label value of 2.0% salicylic acid, and an average value of 99.2% of theoretical was obtained (n=16). Accuracy was validated by analysis of a 10% benzoyl peroxide anti-acne gel spiked with 2% salicylic acid, and recovery averaged 100.0% (n=10). The coefficients of variation for the analyses were 3.9 and 5.6%, respectively. Advantages of the new HPTLC method are discussed.

INTRODUCTION

In earlier studies, quantitative high performance thin layer chromatography (HPTLC) methods were developed for the active ingredients sulfur[1] and benzoyl peroxide[2] in a variety of topical ointment, cream, and liquid pharmaceutical preparations for control of acne. Other acne medications contain salicylic acid as the active ingredient, and in this paper we report an HPTLC method with scanning densitometry for their analysis. The official methods for determination of salicylic acid raw material and formulated pharmaceutical products involve titration with sodium hydroxide, ultraviolet spectrophotometry, or high performance column liquid chromatography.[3,4] The quantitative HPTLC method, which is based on separation of salicylic acid on preadsorbent silica gel plates containing fluorescent indicator, detection by fluorescence quenching, and *in situ* densitometric scanning at 244 nm, has advantages over the earlier methods and is shown to be sufficiently rapid, accurate, and precise for use in routine pharmaceutical analysis.

EXPERIMENTAL

Standard Solution

A standard solution (1.00 μg/μL) was prepared by dissolving 25.0 mg of reagent grade salicylic acid (Aldrich Chemical Co.) in reagent grade methanol in a 25 volumetric flask.

Thin Layer Chromatography

HPTLC was carried out on 10 x 20 cm Whatman LHPKDF high performance silica gel plates with 19 lanes, preadsorbent spotting area, and fluorescent phosphor (catalog no. 4806711). Standard and sample solutions were applied to the preadsorbent using a 10 μL Drummond digital microdispenser. Plates were developed with n-pentyl formate-chloroform-formic acid (2:7:1) for a distance of 7 cm beyond the silica gel preadsorbent interface in a paper-lined, solvent-saturated Camag twin trough chamber and then dried in a fume hood. Separated salicylic acid zones were detected by inspection under 254 nm UV light in a viewing cabinet and scanned using a Shimadzu CS-930 densitometer in the single-beam reflectance mode at 244 nm. This wavelength of maximum absorption was obtained by an *in situ*

spectral scan of a 6.00 μg standard zone between 200 and 370 nm.

Analysis of Samples

The medication analyzed to test the new method was a gel with a label declaration of 2% salicylic acid purchased in a local pharmacy without prescription. Approximately 500 mg of the sample was accurately weighed into a 15 ml vial, and 12.5 ml of methanol was added by pipet. The sample was dissolved by vigorous shaking.

A spiked acne gel medication having 10% benzoyl peroxide as the active ingredient and many other ingredients in common with the salicylic acid medication was used to test the accuracy of the new method. The spiked sample was prepared by weighing 500 mg of the gel and 10.0 mg of salicylic acid into a 15 ml vial and dissolving in 12.5 ml of methanol. A calibration curve was determined from the scan areas and spotted weights of 2.00, 4.00, and 6.00 μL aliquots of the 1.00 μg/μL standard using a Quattro curve-fitting program on an IBM-PC.

The percent salicyclic acid in the unknown was determined by spotting duplicate 4.00 μL aliquots of the standard and duplicate 5.00 μL aliquots of the sample, which would contain a theoretical value of 4.00 μg of salicylic acid according to the label value. The weight of salicylic acid in the sample was calculated by multiplying the weight of salicylic acid in the standard zones (4.00 μg) by the ratio of the average sample peak area to the average standard peak area. Percent salicylic acid was calculated by dividing the weight of salicylic acid by the weight of sample in the 5.00 μL aliquot and multiplying the quotient by 100.

The method was validated by analyzing the spiked sample as described above and calculating recovery by comparing the amount determined experimentally with the theoretical 2.00% fortification level. A blank containing 500 mg of sample without spike was also analyzed.

RESULTS AND DISCUSSION

Methanol completely dissolved the salicylic acid in the standard and

samples, but other insoluble sample ingredients settled to the bottom of the volumetric flasks and did not interfere with spotting. Salicylic acid produced a tight band with R_f 0.71 across the lane of the high performance preadsorbent silica gel plate when viewed under 254 nm UV light. Despite the presence of many ingredients in the medications analyzed, including Carbomer 940, dioctyl sodium sulfosuccinate, disodium EDTA, phenoxyethanol, triethanolamine, and polyglycerylmethacrylate, only one additional fluorescence-quenched zone, with an R_f of 0.61, appeared in sample chromatograms, and it did not interfere with scanning the salicylic acid zone. The benzoyl peroxide in the spiked sample migrated near the solvent front because of the high strength (polarity) of the mobile phase compared to methylene chloride-methanol (1:1), which gave an R_f value of 0.50 for benzoyl peroxide on the same layer.[2]

The calibration curve was repeated many times and found to have a typical linearity correlation coefficient (r value) of 0.93, with a range of 0.83-0.97, for 2-6 μg/spot of salicylic acid. In order to simplify and optimize the reliability of the analysis, quantification was performed by comparing the sample with a single standard having a similar zone area within the linear range, rather than constructing a new calibration curve on each plate. Standards and samples were always chromatographed together on each plate to correct for inevitable slight layer variations. The least intense zone that could be reproducibly scanned was 1.00 μL of standard (1.00 μg), which produced a scan area of about 1000 counts. The 244 nm maximum absorption determined from an *in situ* spectral measurement was quite different from the 270-280 nm detector setting used in HPLC.[4]

Four different samples of the salicylic acid medication with a 2.0% label value were analyzed four times each, for a total of 16 analyses. The overall average obtained was 1.98% salicylic acid, 99.2% of the label value. Standard deviation values for the replicate analyses averaged 3.9%. As a further measure of precision, the percent difference between the scan areas of the duplicate samples and standards spotted in each analysis was typically about 3%, with a range of 0.4 to 9%.

Four different portions of the benzoyl peroxide gel, spiked with 2.00% salicylic acid, were each was analyzed two or three times (total of 10 analyses). Recovery of the spike averaged 100.0% with a 5.6% standard deviation, which verifies the accuracy of the method and the value obtained for the analysis of the unknown. Blanks analyzed in the fortified sample assays did not show the presence of any interferences at the R_f of the analyte.

The quantitative HPTLC method described has high sample throughput because up to eight duplicate samples can be analyzed on a single plate along with the required duplicate standards, and it has adequate sensitivity, selectivity, accuracy, and precision for routine use in a pharmaceutical analytical laboratory. It is more selective than the earlier spectroscopic and titration methods because of the separation step. In contrast to HPLC, insoluble, inactive ingredients do not have to be filtered prior to TLC because the layer is not reused, and an internal standard is not required because samples and standards are separated under essentially identical conditions on the same plate. Other types of samples can be analyzed using the same basic procedure unless an interfering fluorescence-quenched zone migrates with the salicylic acid-zone. It is only required to dissolve the salicylic acid completely in methanol at a concentration that allows an aliquot of sample between 1 and 10 μL to produce a separated zone with a scan area similar to the 4 μL standard.

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DISPLACEMENT THIN LAYER CHROMATOGRAPHY OF MORPHINE AND ITS SEMI-SYNTHETIC DERIVATIVES

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ABSTRACT

Morphine and its sixteen semisynthetic derivatives of pharmacological interest were subjected to planar chromatography. Adequate stationary and mobile phases for both elution and displacement chromatography were found. All these components can be separated by elution mode of development. At the same time, several morphine derivatives can be the member of displacement train. Experiments and results using either straight-phase or reverse phase chromatography with both elution and displacement types of developments are detailed.

INTRODUCTION

Displacement chromatography is based on the well known phenomenon that the more strongly retarded compounds can displace the less strongly retarded ones, as it has been known for a long time[1]. In 1943, Tiselius classified chromatographic separation according to the mode of development, such as elution, frontal and displacement chromatography[2], which was widely used in the forties and early fifties for separation of various compounds, such as proteins, peptides, amino acids, etc.[3-5].

The overwhelming majority of these separations was done using column arrangement of the stationary phase, and this column displacement chromatography was adopted to the high performance liquid chromatographic conditions by Horváth et al. in 1981[6] when they separated numerous types of organic compounds using high performance displacement chromatography. At the same time, their experiments and publications have started an essential boom of displacement separations for preparative purposes. In 1982, displacement chromatography on planar arrangement of the stationary phase was used to scout the optimum conditions for high performance displacement separations[7], and this effort was continued through the '80s[8-12].

Spacer displacement thin layer chromatography (SD-TLC) employs spots or bands of component(s) which are inserted between the two displaced components to improve their separations. Thereby resolution of displacement thin layer chromatography of some drugs and metabolites was increased, and the detection of the tiny bands of the displaced components was made easier without the use of the spacers.

Detailed accounts on the displacement chromatography of ecdysteroids (insect moulting hormones that can be found in insects and also in plants) were published to demonstrate the separating power of planar displacement chromatography, as well as the application of forced flow development at planar displacement chromatography.[13-15]

Our recent research[16] has been focused on the chromatographic behaviour of morphine and their derivatives with pharmacological interest[17]. To determine hydrophobicity of morphine derivatives, a series of reverse phase and straight phase TLC plates were checked, some of them were originated from commercial sources, others were home-made[16]. Our investigations were expanded to find conditions for displacement chromatography.

Table 1

The Structures of Compounds that Were Investigated

		- - - - - - Substituents, such as - - - - - -				
Compound		**R_1 N**	**7-8**	**R_2 6**	**R_3 3**	**R_4 14**
Morphine	(B)	$-CH_3$	=	-OH	-OH	-H
Azidomorphine	(C)	$-CH_3$	DH	$-NH_3$	-OH	-H
ECAM	(D)	$-CH_2$-cPr	DH	$-NH_3$	-O-Et	-H
14-Hydroxy-dihydro-morphine	(E)	-Met	DH	-OH-	-OH	-OH
Dihydromorphine	(F)	-Met	DH	-OH-	-OH	-H
Norazido-ethyl-morphine	(G)	-H	DH	$-N_3$	-O-Et	-H
CAM	(H)	$-CH_2$-cPr	DH	$-N_3$	-OH	-H
14-Hydroxy-azido-codeine	(I)	-Met	DH	$-N_3$	-O-Met	-OH
14-Hydroxy-azido-morphine	(J)	-Met	DH	$-N_3$	-OH	-OH
Ethylmorphine	(K)	-Met	=	-OH	-O-Et	-H
Norazidomorphine	(L)	-H	DH	$-N_3$	-OH	-H
6-Amino-dihydro-morphine	(M)	-Met	DH	$-NH_2$	-OH	-H
Normorphine	(N)	-H	=	-OH	-OH	-H
Nalorphine	(O)	-allyl	=	-OH	-OH	-H
Codeine	(P)	-Met	=	-OH	-O-Met	-H
Azidocodeine	(Q)	-Met	DH	$-N_3$	-O-Met	-H
14 Hydroxy-dihydro-codeine	(R)	-Met	-DH	-OH	-O-Met	-OH

Abbr.: DH: dihydro -cPr: -cyclopropyl
-Met: -methyl -Et: -ethyl
=: double bond

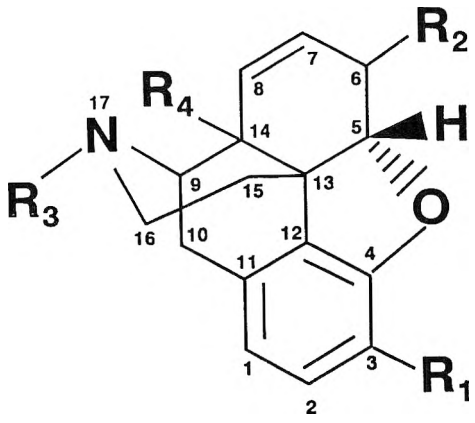

Figure 1. The structure of morphine and its derivatives. The substituents are listed in Table 1.

EXPERIMENTAL

Stationary phases were purchased from E. Merck (Darmstadt, Germany). TLC plates silica gel 60 F_{254} pre-coated on glass, 20x20 cm, layer thickness 0.25 mm (Art. No. 5715), and TLC plates RP-18 $F_{254}S$, pre-coated on glass, 20x20 cm, layer thickness 0.25 mm (Art. No. 15389) were used.

Mobile phases for displacement development contained triethanolamine (alt., Reanal, Budapest, Hungary) or tetrabutylammonium chloride (pract., Kat. No. 86870, Fluka, Buchs, Switzerland) for straight phase or reverse phase displacement chromatography, respectively. Solvents were purchased from commercial sources in the highest available quality.

Morphine and its semisynthetic derivatives were kind gifts of Alkaloida Pharmaceutical Works (Tiszavasvári, Hungary). The structures of morphine derivatives used for separations are given in Fig. 1 and Table 1.

All TLC developments were performed in glass chambers (Desaga, Heidelberg, Germany) at ambient temperature. After developments, the TLC plates were dried and spots were visually detected at daylight as well as under UV light of 254 nm (using UV lamp of Desaga).

RESULTS

Fig. 2 gives the separation of morphine derivatives using elution type development on silica stationary (straight) phase and with several different running systems.

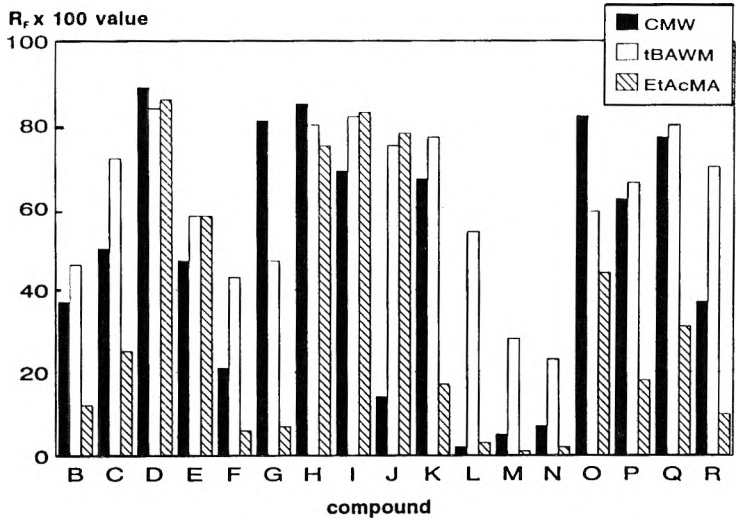

Figure 2. RF values that characterize thin layer chromatography of morphine and its derivatives on silica stationary phase using chloroform-methanol-water (7:5:1, v/v), t-butanol-ammonia-water-methanol (20:1:4:2, v/v) and ethyl acetate-methanol-ammonia (18:2:1, v/v) mobile phases. B. C, D, ..., R refer the compounds given in Table 1.

Fig. 3 gives TLC of morphine derivatives using n-butanol-0.2 M hydrochloric acid-water-methanol (20:1:4:2, v/v); some spots were displaced, others were eluted by the mobile phase. The eluted spots were both under and over the displacer front.

Figs. 4-6 give displacement chromatography of morphine derivatives on silica using chloroform, dichloromethane, dichloroethane carrier and triethanolamine displacer.

Fig. 7 gives displacement development on reverse phase stationary phase. Not all the morphine derivatives were displaced by the displacer front,

as several components remained behind the displacer front.

DISCUSSION

Thin-layer chromatography of morphine and its derivatives has been widely used to differentiate these compounds (for reviews, see[19-20]). Running systems with three or more solvents can be preferentially used, and the proper choice of the mobile phase can be made in knowledge of the main component(s) to be best separated from the others. Moreover, the use of several analyses in various running solvents can improve both the qualitative and quantitative evaluation of the results. The R_F values indicated in Fig. 2. show that practically any morphine derivatives can be well separated, and the identification of the morphine derivatives can be adequately done using all three different mobile phases given here.

Another possibility is to use an adequate ratio of the mixtures of n-butanol-acetic acid-water-methanol and n-butanol-ammonia-water-methanol[21]. Thereby changing the ratio of the running solvents containing acidic (acetic acid) and basic (ammonia) constituents, the different compounds show diverging dependence, which also serves for the optimization of separations. It is important to avoid the use of nonvolatile components, such as sodium- or phosphate ion containing buffers, because of their very limited migration with the mobile phase through the silica stationary phase. In this case, the various morphine derivatives migrate with very different R_F values, and the displacer can be easily removed from the zones of components which are eluted by the displacer (that is that components migrate slower then the displacer front, thereby their zones are contaminated with the displacer).

Fig. 3 shows TLC of morphine derivatives on silica using n-butanol-0.2M hydrochloric acid-water-methanol (20:1:4:2, v/v) mobile phase. While some morphine derivatives were eluted with the mobile phases, several components become part of the displacement train, that is they were displaced by the displacer train. This system has two basic advantages. First of all, the eluted components were either behind or ahead of the displacer front. Thereby, the whole plate was well utilized by the components showing various mobility. The second advantage is related to the composition of the displacing system, i.e. all components of the mobile phase can be easily removed if any one of the separated components is to be prepared. At the same time, this system

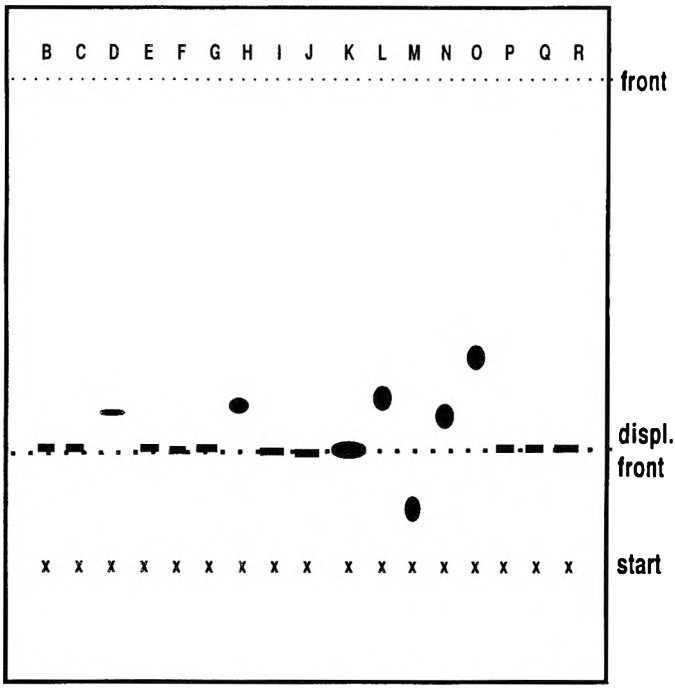

Figure 3. Thin layer chromatography of morphine and its derivatives on silica stationary phase using n-butanol-hydrochloric acid-water-methanol (20:1:4:2, v/v) mobile phase.

(n.butanol-0.2M hydrochloric acid-water-methanol (20:1:4:2, v/v)) is rather sensitive to the changes of temperature, which should be kept at 22 $\pm$ 2 °C, for reproducible results.

Figs. 4, 5, and 6 give displacement chromatogram of the morphine derivatives (see Table 1.) on silica stationary phase using chloroform, dichloromethane and dichloroethane carriers, respectively. Neither morphine, nor any of the morphine derivatives showed any migration when the plain carrier was used as mobile phase (not shown), however, all of these components migrated when the adequate amount of triethanolamine displacer was added to the mobile phase. Neither carrier was selective with any of the morphine derivatives, as norazidomorphine, normorphine and 6-amino-dihydromorphine were not displaced by the triethanolamine using either chloroform, or

dichloromethane or dichloroethane, while all other compounds were displaced by triethanolamine.

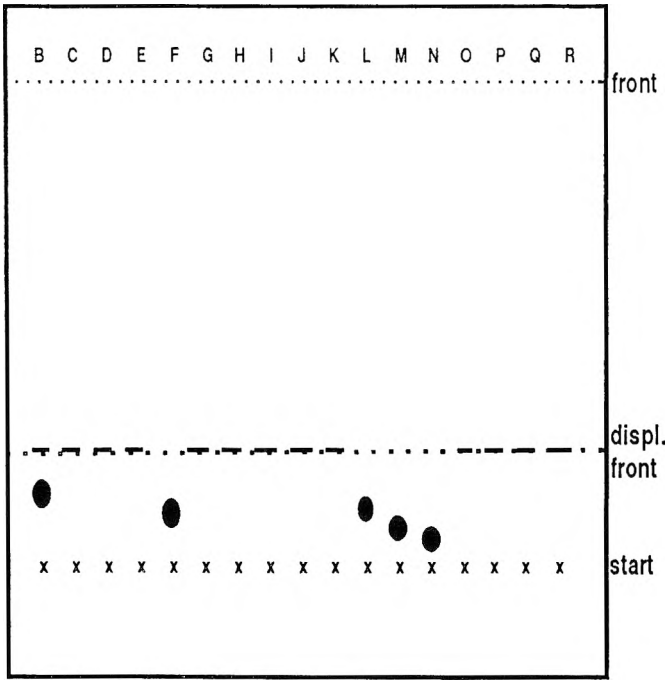

Figure 4. Thin layer displacement chromatography of morphine derivatives on silica using chloroform carrier and triethanolamine displacer.

The ratio of displacer to carrier was 5:95, 3:97 and 10:90 (v/v), in the experiments presented in Figs. 4, 5 and 6, respectively. However, displacers with lower (1:99 v/v) and higher (20:80) ratio could also generate fully developed displacer train after a relatively short development of the displacer front (not shown).

Planar displacement chromatography has hitherto been restricted to the use of straight stationary phases (silica[9-14] and alumina layers[12]). Fig. 7 presents displacement thin layer chromatography of some morphine derivatives on reverse phase stationary phase, that is on TLC plates RP-18 $F_{254}S$ (E. Merck). Our efforts to generate displaced bands using non-polar stationary

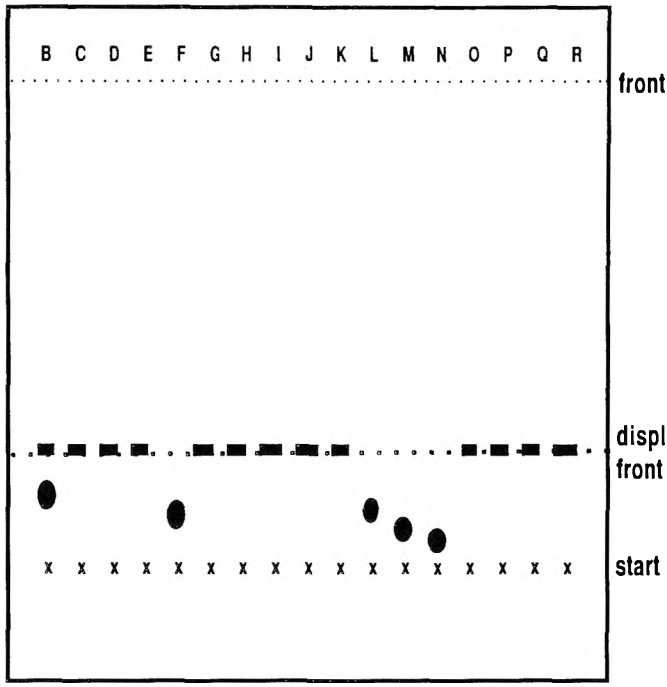

Figure 5. Thin layer displacement chromatography of morphine derivatives on silica using dichloromethane carrier and triethanolamine displacer.

phases have earlier been unsuccessful, although several other reverse phase plates were tried, such as other commercially available reverse phase TLC plates as well as home made, paraffin-coated silica plates. Although the paraffin impregnated silica plates were successfully used at the determination of hydrophobicity of morphine derivatives, displacement chromatography on these plates has never given adequate results. The basic problem was that morphine and its derivatives did not migrate, but remained in the place of their origin.

We suppose that non-migration of these spots is related to the limited wettability of these plates with aqueous-organic mobile phases, and the special surface characteristics of the non-polar stationary phases on the TLC plates RP-18 $F_{254}S$ (Merck) somehow circumvented the problem of too strong adsorption

of morphine derivatives to the surface of the stationary phase, like with the wettability of the RP-18 stationary phase with aqueous eluent (where the percentage of water is even higher than 80%).

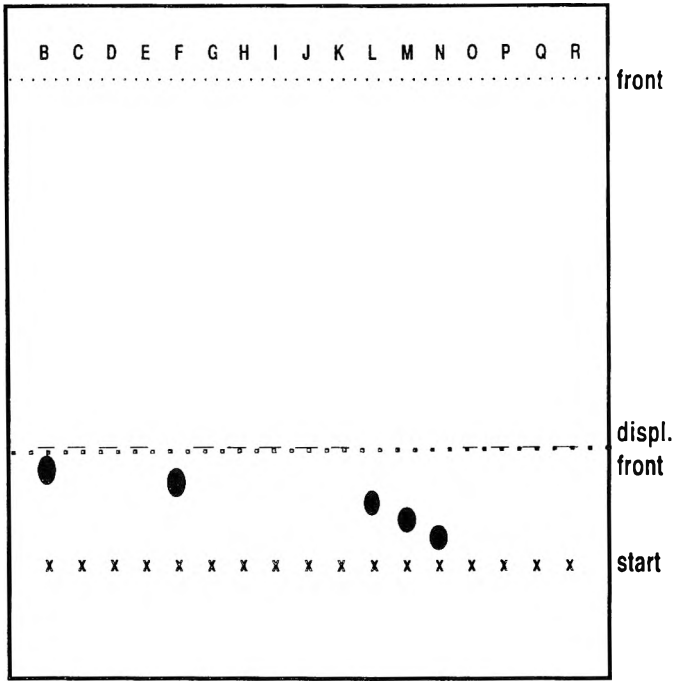

Figure 6. Thin layer displacement chromatography of morphine derivatives on silica using dichloroethane carrier and triethanolamine displacer.

The use of reverse phase stationary phases for displacement chromatography has recently found new important applications for the separation of macromolecules, such as peptides, proteins and nucleic acids on a preparative scale[24-29]. Preparative (e.g. industrial) scale separations require optimization of the conditions of displacement chromatography which can be conveniently done using planar stationary phases. Recently, reverse phase planar displacement chromatography has been performed with forced-flow of the mobile phase[30]. Thereby the development of the displacement train is faster, more effective and more reproducible, than propagation of the mobile phase by capillary forces.

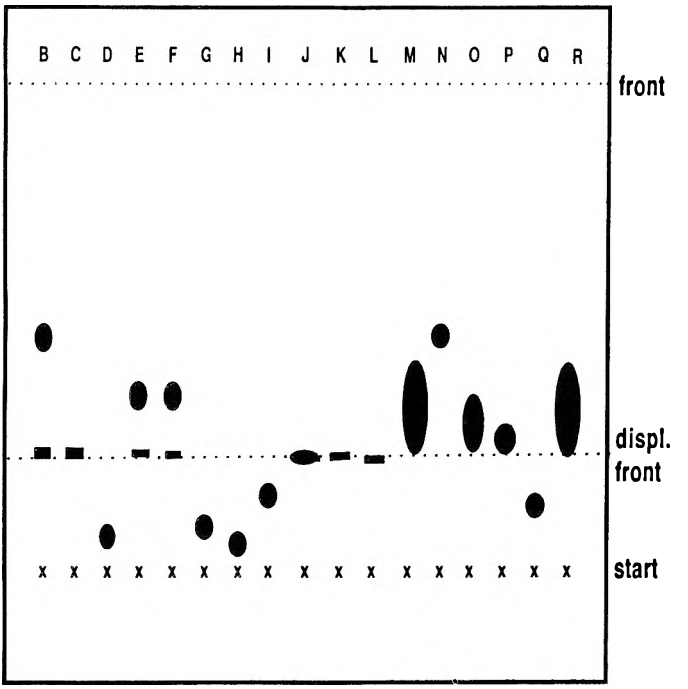

Figure 7. Reverse phase thin layer displacement chromatography of morphine derivatives on RP-18 F_{254} S TLC plates using 10% (w/v) tetrabuthylammonium chloride displacer in acetonitrile-water (30:70 v/v) carrier.

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OPTIMIZATION OF HPLC SEPARATION OF CARBAMATE INSECTICIDES (CARBOFURAN, HYDROXYCARBOFURAN AND ALDICARB) BY EXPERIMENTAL DESIGN METHODOLOGY

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ABSTRACT

The experimental design methodology has allowed to optimize the chromatographic separation of polar pesticide molecules (carbofuran, hydroxycarbofuran and aldicarb) by HPLC, owing to the determination of mathematical equations. Second degree equations have been elaborated, dependent upon injected volume, eluent flow and eluent composition. These equations have allowed us to plot Isoresponse Curves which are used to easily visualize and optimize separations. Interactions between eluent flow and polarity are emphasized.

INTRODUCTION

Generally, the analyst has to perform many experiments in order to find optimal practical conditions for the development of pesticide residue analytical methods.

The chromatographic analysis depends on many factors; their influences are not easily controlled. The practical conditions for optimal separation of similar molecules are usually determined by independant or step-by-step variations of factors. Owing to the purposed methodology, a cheaper and more direct strategy can be followed, which is an interesting alternative to a Simplex methodology. Until now, experiments planning methodology was not often applied to the optimization of residue analysis.[1]

An *a priori* experimental design methodology has been planned in order to determine mathematical equations applicable to the separations of organic residues by classic chromatographic techniques (High Performance Liquid Chromatography); separations of carbamate insecticide residues were then performed using the previous equations.

THE DESIGN OF EXPERIMENTAL METHODOLOGY

Table 1

Experimental Matrix of 2^3 Plan

	A	B	C	Y
Test 1	-1	-1	-1	y1
Test 2	+1	-1	-1	y2
Test 3	-1	+1	-1	y3
Test 4	+1	+1	-1	y4
Test 5	-1	-1	+1	y5
Test 6	+1	-1	+1	y6
Test 7	-1	+1	+1	y7
Test 8	+1	+1	+1	y8
Level -1	a	a'	a"	
Level +1	b	b'	b"	

For the A factor, the value a is set at level -1 and b at level +1; for B, the value a' is set at level -1 and b' is set at level +1; for C, the value a" is set at level -1 and b" at level +1.

The effects of factors which could modify the separation are measured, their interactions are displayed, and those which affect the results are pointed out. In a second time, analytical expressions of influences are set up and used to determine the best combination of experimental conditions.

We briefly describe the method, refering to cited literature for general justification.[2,3,4,5] The difference **y** between retention times of 2 molecules, distinguished by HPLC, is governed by **k** factors (A, B, C...), for instance eluent mixture polarity, injected volume, etc. Each factor has been brought to 2 values (levels a (-1) and b (+1)), corresponding to the limits of a reasonable variation interval. So, 2 experiments had to be performed (Table 1). The influences of the factors, and their interactions have been evaluated as the $\mathbf{h_n}$ coefficients. For instance, with 3 factors, the $\mathbf{h_n}$ coefficients are listed as follows:

$h_A = 1/8(- y1 + y2 - y3 + y4 - y5 + y6 - y7 + y8)$
$h_B = 1/8(- y1 - y2 + y3 + y4 - y5 - y6 + y7 + y8)$
$h_C = 1/8(- y1 - y2 - y3 - y4 + y5 + y6 + y7 + y8)$
$h_{AB} = 1/8(+ y1 - y2 - y3 + y4 + y5 - y6 - y7 + y8)$
$h_{AC} = 1/8(+ y1 - y2 + y3 - y4 - y5 + y6 - y7 + y8)$
$h_{BC} = 1/8(+ y1 + y2 - y3 - y4 - yS - y6 + y7 + y8)$
$h_{ABC} = 1/8 (- y1 + y2 + y3 - y4 + y5 - y6 - y7 + y8)$
$H = 1/8(+ y1 + y2 + y3 + y4 + y5 + y6 + y7 + y8)$

h_A, h_B, h_C display effects of the A, B, C factors, respectively.
h_{AB}, h_{AC}, h_{BC} characterize first degree interactions between A and B, A and C, B and C, respectively.
h_{ABC} characterizes the second degree interaction between A, B and C.
y1, y2, ..., y8 are results for test 1, test 2, ..., test 8, respectively.
H is the average of the results.

There is an interaction between 2 factors, A and B, for instance, if the effect of A depends on the level of B. and *vice-versa*. The calculation of the global effect of A (h_A) results from 8 measured responses y_i. The variance analysis of the results allows us to determine if the observed effect can be explained by measurement uncertainties or if it is significant. The variance analysis is described in detail in the literature.[6,7] Then, the response is expressed as a linear mathematical equation, for instance, in the event of 3 significantly influential factors, without significant interactions:

$$Y = H + h_A * A + h_B * B + h_C * C$$

or, when interactions are significant:

$$Y = H + h_A * A + h_B * B + h_C * C + h_{AB} * AB + h_{AC} * AC + h_{BC} * BC + h_{ABC} * ABC$$

Table 2

Validation Assay

	A	B	C	Y
Level -1	a	a'	a"	y1
Level +1	b	b'	b"	y4
Level 0	d=(a+b)/2	d'=(a'+b')/2	d"=(a"+b")/2	y5
Level x	c	c'	c"	y6
-1<x<+1	a<c<b	a'<c'<b'	a"<c"<b"	

Four experiments were performed, setting a, b, c and d values to A, a', b' c' and d' to B, a", b", c" and d" to D. (a, b, c, d, a', b', c', d', a", b", c", d")$\in$[-1; +1].

To validate this mathematical equation, an additional experiment set has to be carried out, in which new values have been affected to factors, inside of the previous intervals, for example 0 and c (Table 2). If the value between the experimental response and the calculated one does not exceed 5%, the linear equation is confirmed, and can be used to choose the best values of the factors. In the opposite case, a complementary experiment set, also called *centered composite plan*, has to be performed. In Table 3, an experimental matrix for the study of 3 factors has been planned. It contains main tests (factors are fixed at levels of - 1 and + 1), six centered points (level 0), repaired for statistical results treatment, and α points (factors are fixed at levels - α and + α): the α value and the number of centered points depend on **k** (factors number). In a three factors plan, α = 1.6. From the effects matrix of the centered composite plan, a more complex mathematical equation (quadratic one) can be built up:

$$Y = a_o + a_1A + a_2B + a_3C + a_4AB + a_5AC + a_6BC + a_7A^2 + a_8B^2 + a_9C^2$$

Table 3

Centered Composite Plan: Experimental Matrix

	A	**B**	**C**	**Y**
Test 1	-1	-1	-1	y1
Test 2	+1	-1	-1	y2
Test 3	-1	+1	-1	y3
Test 4	+1	+1	-1	y4
Test 5	-1	-1	+1	y5
Test 6	+1	-1	+1	y6
Test 7	-1	+1	+1	y7
Test 8	+1	+1	+1	y8
Test 9	0	0	0	y9
Test 10	0	0	0	y10
Test 11	0	0	0	y11
Test 12	0	0	0	y12
Test 13	0	0	0	y13
Test 14	0	0	0	y14
Test 15	$-\alpha$	0	0	y15
Test 16	$+\alpha$	0	0	y16
Test 17	0	$-\alpha$	0	y17
Test 18	0	$+\alpha$	0	y18
Test 19	0	0	$-\alpha$	y19
Test 20	0	0	$+\alpha$	y20
Level $-\alpha$	d	d'	d"	
Level -1	a	a'	a"	
Level 0	(a+b)/2	(a'+b')/2	(a"+b")/2	
Level +1	b	b'	b"	
Level $+\alpha$	e	e'	e"	

A routine scientific calculator is enough to run matrix calculations,[8] as described below. a_i coefficients are calculated from the matrix with real values, as reported in Table 4.

Table 4

Effects Matrix of the Centered Composite Plan (X)
(Y): Matrix of Measured Responses.
$y = (y9 + y10 + y11 + y12 + y13 + y14)/6$

	-----(X)-----									(Y)
	A	**B**	**C**	**AB**	**AC**	**BC**	**A^2**	**B^2**	**C^2**	
T1	a	a'	a"	aa'	aa"	a'a"	aa	a'a'	a"a"	y1
T2	b	a'	a"	ba'	ba"	a'a"	bb	a'a'	a"a"	y2
T3	a	b'	a"	ab'	aa"	b'a"	aa	b'b'	a"a"	y3
T4	b	b'	a"	bb'	ba"	b'a"	bb	b'b'	a"a"	y4
T5	a	a'	b"	aa'	ab"	a'b"	aa	a'a'	b"b"	y5
T6	b	a'	b"	ba'	bb"	a'b"	bb	a'a'	b"b"	y6
T7	a	b'	b"	ab'	ab"	b'b"	aa	b'b'	b"b"	y7
T8	b	b'	b"	bb'	bb"	b'b"	bb	b'b'	b"b"	y8
T9	(a+b)/2	(a'+b')/2	(a"+b")/2	(a+b)/2 • (a'+b')/2	(a+b)/2 • (a"+b")/2	(a'+b')/2 • (a"+b")/2	(a+b)/2 • (a+b)/2	(a'+b')/2 • (a'+b')/2	(a"+b")/2 • (a"+b")/2	y9
T15	d	(a'+b')/2	(a"+b")/2	d • (a'+b')/2	d • (a"+b")/2	(a'+b')/2 • (a"+b")/2	dd	(a'+b')/2 • (a'+b')/2	(a"+b")/2 • (a"+b")/2	y15
T16	e	(a'+b')/2	(a"+b")/2	e • (a'+b')/2	e • (a"+b")/2	(a'+b')/2 • (a"+b")/2	ee	(a'+b')/2 • (a'+b')/2	(a"+b")/2 • (a"+b")/2	y16
T17	(a+b)/2	d'	(a"+b")/2	(a+b)/2 • d'	(a+b)/2 • (a"+b")/2	d' • (a"+b")/2	(a+b)/2 • (a+b)/2	d'd'	(a"+b")/2 • (a"+b")/2	y17
T18	(a+b)/2	e'	(a"+b")/2	(a+b)/2 • e'	(a+b)/2 • (a"+b")/2	e' • (a"+b")/2	(a+b)/2 • (a+b)/2	e'e'	(a"+b")/2 • (a"+b")/2	y18
T19	(a+b)/2	(a'+b')/2	d"	(a+b)/2 • (a'+b')/2	(a+b)/2 • d"	(a'+b')/2 • d"	(a+b)/2 • (a+b)/2	(a'+b')/2 • (a'+b')/2	d"d"	y19
T20	(a+b)/2	(a'+b')/2	e"	(a+b)/2 • (a'+b')/2	(a+b)/2 • e"	(a'+b')/2 • e"	(a+b)/2 • (a+b)/2	(a'+b')/2 • (a'+b')/2	e"e"	y20

$$[A] = \begin{bmatrix} a_0 \\ a_1 \\ a_2 \\ a_3 \\ a_n \end{bmatrix} \qquad A = (X'X)^{-1}X'Y$$

A = a_i coefficients matrix X = tests matrix
X' = transposed tests matrix X^{-1} = inverted tests matrix
Y = responses matrix

The quadratic equation has to be validated before using it to choose the best values of the factors.

The process can require variable changes, new study intervals, or the split of the **k** factors study into 2 studies of **k'** and **k"** factors (k' + k" = k).

dt being the difference of the retention times, and **t** the average analysis time, the optimal chromatographic separation of 2 molecules M and M' is reached fitting with the highest value of the $\frac{dt}{t}$ ratio (the highest separation associated with the lowest analysis time).

Applications to carbamate residue analysis are reported.

EXPERIMENTAL

Carbofuran (CAR), hydroxycarbofuran (OHC) and aldicarb (ALD) are polar pesticides belonging to the N-methylcarbamate chemical family (Figure 1).[9] Residue analysis was usually designed by HPLC.[10,11,12]

Products and Materials

- acetonitrile, methanol, HPLC grade, S.D.S. (Peypin, France),
- deionized water, Seral System (Grosseron, St Herblain, France),
- sodium hydroxide, analytical grade, Osi (Paris, France),

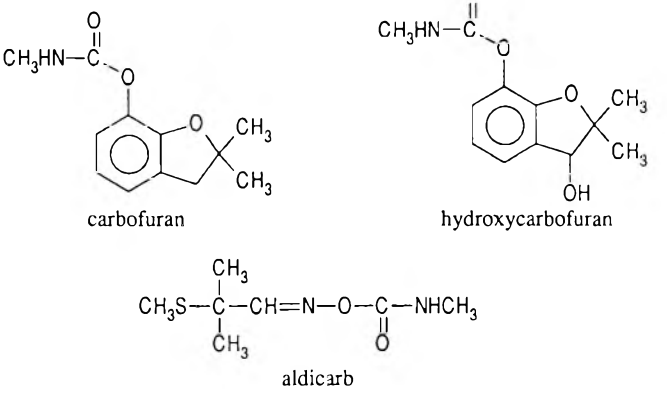

Figure 1. Carbofuran (CAR), Hydroxycarbofuran (OHC) and Aldicarb (ALD).

- hydrochloric acid, analytical grade, Osi,
- o-phthaldialdehyde (OPA), analytical grade, Fluka (Buchs, Switzerland),
- sodium tetraborate, decahydrate, analytical grade, Osi,
- helium N55,1'Air Liquide (Paris, France),
- standards: carbofuran, aldicarb, 99.8%, Cluzeau Info Labo (Ste Foy, France), hydroxycarbofuran, pure, Bayer (FRG).

Syringes, 25 and 1000 μL, Hamilton (Reno, NE, US).

HPLC

- injector, U6K, Waters (Milford, MA, US),
- chromatograph 600E Multisolvent Delivery System, Waters,
- detector, 470 Scanning Fluorescence Detector, Waters,
- integrator, 740 Data module, Waters,
- pumps, PCRS, Waters,
- temperature controller, TCM, Waters,
- stainless steel column, Lichrosorb RP-18 (5 μm), 250 mm x 4.6 mm I. D., Merck (Darmstadt, FRG),
- stainless steel pre-column, Lichrosorb RP- 18 (5 μm), 2.5 mm x 4.6 mm I. D., Merck.

Post-column derivatization was used to detect CAR, OHC and ALD. The eluate was continuously mixed with sodium hydroxide 0.1 N at 80°C, and

then OPA reagent (100 mg/L, in sodium tetraborate aqueous solution (20 g/L)) was added at 25°C (ref. 12, 13). The N-methylcarbamates derivatization reaction sequence is presented Figure 2. Fluorescent products resulted, which were detected

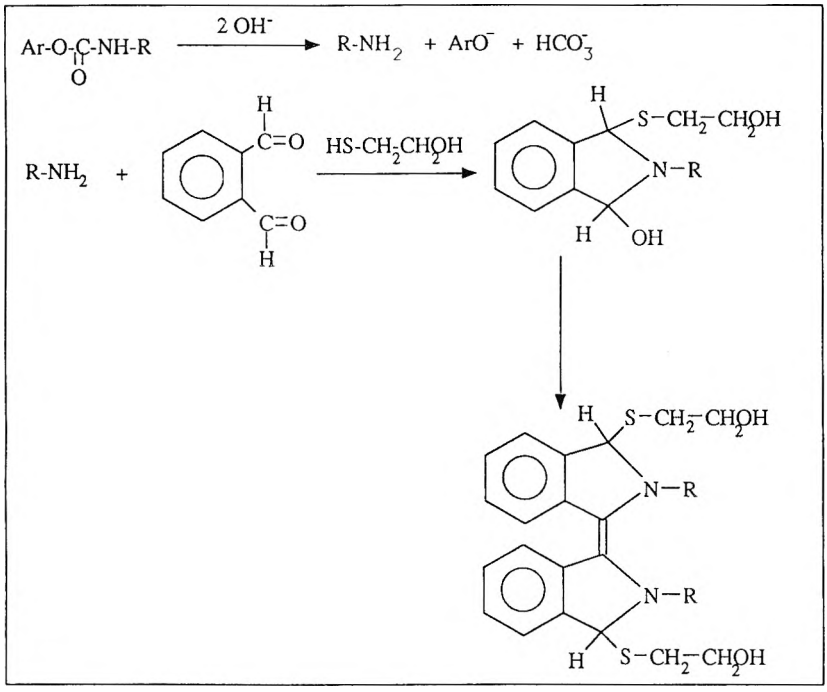

Figure 2. Post-column derivatization of N-methylcarbamates. The reaction yields a highly fluorescent compound which is used to detect low carbamate concentrations (1 μg/L).

The chromatographic parameters were:

- column temperature: 30°C,
- eluent sparge: He, 50 mL/min,
- sodium hydroxyde pump flow: 0.5 rnL/min,
- OPA reagent pump flow: 0.5 mL/min,
- excitation wavelength: 339 nm,
- emission wavelength: 445 nm,
- hydrolysis temperature: 80°C.

Mixture solutions of CAR, OHC and ALD [3 x 25 µg/L (injected volume: 400 µL), and 3 x 500 µg/L (injected volume: 20 RL)] were prepared in acidified water (pH 4) / methanol / acetonitrile mixture (45/10/45). Injections were spaced 1 hour apart, in order to reach the stationary rate; injections were repeated three times.

RESULTS

The studied factors were:

- injected volume (A)
- eluent flow (B)
- composition (polarity) of ternary eluent mixture (C)

The composition of ternary eluent mixtures (water / acetonitrile / methanol) has been expressed as a polarity coefficient, due to the following arbitrary **p** coefficients: $p_{water} = 1.0$, $p_{methanol} = 0\ 9$ and $p_{acetonitrile} = 0\ 5$. So, in this scale, the polarity of the mixture of water, methanol and acetonitrile (45/10/45 v/v/v) is 0.765.

The measured responses were:

$$\frac{dt}{t}(1) = \frac{\text{aldicarb retention time - hydroxycarbofuran retention time}}{0.5\ (\text{aldicarb retention time + hydroxycarbofuran retention time})}$$

$$\frac{dt}{t}(2) = \frac{\text{carbofuran retention time - aldicarb retention time}}{0.5\ (\text{aldicarb retention time + carbofuran retention time})}$$

A chromatogram is displayed Figure 3.

The effects matrix is recorded Table 5, chosen values of - 1 and + 1 levels are indicated. They agree with usual chromatographic parameter ranges. The effects values reported in Table 6 result from the calculation process of $\mathbf{h}_n$ coefficients discussed above; the following linear equations have been deduced:

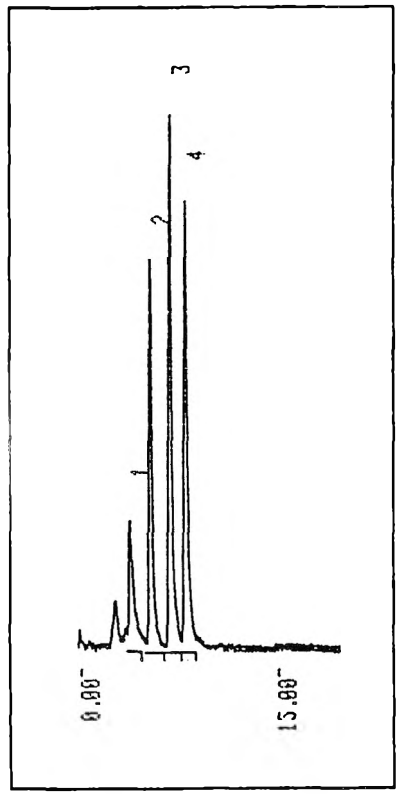

Figure 3. HPLC chromatogram; hydroxycarbofuran (2), aldicarb (3) and carbofuran (4). The factors A, B and C are, respectively, set at the following values: 210 μL, 0.75 mL/min and 0.765.

$$\frac{dt}{t}(1) = 0.324 - 0.012A + 0.015B + 0.181C - 0.018AC + 0.019BC \qquad (1)$$

$$\frac{dt}{t}(2) = 0.231 - 0.008A + 0.017B + 0.171C - 0.008AC + 0.018BC \qquad (2)$$

(A, B. and C in interval [- 1; + 1])

Table 5

Effects Matrix of the HPLC Separation Study

	A	B	C	AB	AC	BC	ABC	M	dt/t(1) ($x10^3$)	dt/t(2) ($x10^3$)
Test 1	-	-	-	+	+	+	-	+	141±0	63±2
Test 2	+	-	-	-	-	+	+	+	152±14	58±6
Test 3	-	+	-	-	+	-	+	+	133±1	57±3
Test 4	+	+	-	+	-	-	-	+	147±1	61±1
Test 5	-	-	+	+	-	-	+	+	504±5	380±6
Test 6	+	-	+	-	+	-	-	+	437±6	355±0
Test 7	-	+	+	-	-	+	-	+	566±7	456±11
Test 8	+	+	+	+	+	+	+	+	511±4	418±8

Level -1 20 μL 0.5mL/ min 0.665

Level +1 400 μL 1.0mL/ min 0.865

Table 6

Observed Effects

	dt/t(1)	dt/t(2)
h_A	**-0.012**	**-0.008**
h_B	**0.015**	**0.017**
h_C	**0.181**	**0.171**
h_{AB}	0.002	-0.001
h_{AC}	**-0.018**	**-0.008**
h_{BC}	**0.019**	**0.018**
h_{ABC}	0.001	0.002
H	0.324	0.231

The effects (h_n) which are influential at 5% risk are bold values; other effects are not significant at 40% risk.

Table 7

Validation Tests of the Linear Equation 1 (dt/t(1)): It is not Validated

	A	0	-0.5	0	-1
Experimental Conditions	**B**	0	1	0.2	-0.6
	C	0	0	0.5	-0.5
Calculated dt/t(1)		0.324	0.345	0.356	0.197
Experimental dt/t(1)		0.236	0.253	0.419	0.233
Deviation (%)		27	26	15	17

Table 8

Validation Tests of the Linear Equation 2 (dt/t(2)): It is not Validated

	A	0	-0.5	0	-1
Experimental Conditions	**B**	0	1	0.2	-0.6
	C	0	0	0.5	-0.5
Calculated dt/t(1)		0.231	0.252	0.322	0.144
Experimental dt/t(1)		0.153	0.171	0.270	0.122
Deviation (%)		34	32	16	22

Validation tests are reported Tables 7 and 8. They did not support the previous equations. So, a centered composite plan of experiments had to be performed in order to determine the quadratic equations.

The effects matrix of the centered composite plan is reported Table 9. From the matrix calculation, the following quadratic equations have been extracted:

$$10^3 \frac{dt}{t}(1) = 3.52 + 0.5A + 0.70B - 11.36C - 0.16AB - 0.61AC - 0.81BC + 0.05A^2 - 0.02B^2 + 9.17C^2 \quad (1')$$

$$10^3 \frac{dt}{t}(2) = 3.34 + 0.24A + 0.62B - 10.85C - 0.16AB - 0.24AC - 0.63BC + 0.08A^2 - 0.05B^2 + 8.64C^2 \quad (2')$$

A, B and C factors range, respectively, from 0.020 to 0.400 mL/min, from 0.5 to 1.0 mL/min and from 0.665 to 0.865 (arbitrary unit).

Results of the validation tests are recorded in Tables 10 and 11, in agreement with the above two equations.

DISCUSSION

The relationship between $\frac{dt}{t}(1)$ or $\frac{dt}{t}(2)$ and the three factors (injected volume (A), eluent flow (B) and eluent polarity (C)) yields quadratic equations. The eluent composition (C) is the main factor acting on the separation of the 3 molecules; the coefficients linked to this factor, its square value and interactions are higher than other coefficients. So, the separations of those molecules are thought to be mainly under control of the difference of solvation of the molecules. In a previous study,[14] concerning the HPLC separation of simazine and atrazine, the role of eluent polarity and eluent flow have already been emphasized.

According to these equations, the HPLC separations have been plotted, in the way of Isoresponse Curves: Figure 4 shows isoresponse curves illustrating Equation 1' and Figure 5 shows isoresponse curves illustrating Equation 2'. On those plots, the injected volume (A) is set at its central value (210 μL). Isoresponse curves, in every case, are parabolic portions or straight line segments, as would be generally expected.[2] They can be used to determine parameter combinations that lead to a desired separation. For instance, when the injected volume is 210 μL, the eluent flow is 0.75 mL/min and the eluent mixture composition is water / methanol / acetonitrile (42/10/48) (polarity 0.75), $\frac{dt}{t}(1)$ and $\frac{dt}{t}(2)$ are expected to be 0.205 and 0.160, respectively. The experimental results are 0.202 and 0.161, respectively, in good agreement with the expected ones.

Table 9

Effects Matrix of Centered Composite Plan

K	A	B	C	AB	AC	BC	A^2	B^2	C^2	dt/t (1)	dt/t (2)
1	0.097	0.60	0.705	0.06305	0.06839	0.45825	0.00941	0.4225	0.4970 3	0.155	0.079
1	0.323	0.60	0.705	0.20995	0.22772	0.45825	0.10433	0.4225	0.4970 3	0.157	0.086
1	0.097	0.90	0.705	0.0873	0.06839	0.6345	0.00941	0.81	0.4970 3	0.174	0.097
1	0.323	0.90	0.705	0.2907	0.22772	0.6345	0.10433	0.81	0.4970 3	0.168	0.091
1	0.097	0.60	0.825	0.0631	0.08003	0.53625	0.00941	0.4225	0.6806 3	0.399	0.305
1	0.323	0.60	0.825	0.2100	0.26648	0.53625	0.10433	0.4225	0.6806 3	0.387	0.304
1	0.097	0.90	0.825	0.0873	0.08003	0.7425	0.00941	0.81	0.6806 3	0.398	0.305
1	0.323	0.90	0.825	0.2907	0.26648	0.7425	0.10433	0.81	0.6806 3	0.373	0.294
1	0.210	0.75	0.765	0.1575	0.16065	0.57375	0.0441	0.5625	0.5852 3	0.243	0.163
1	0.020	0.75	0.765	0.015	0.0153	0.57375	0.0004	0.5625	0.5852 3	0.263	0.181
1	0.400	0.75	0.765	0.30	0.306	0.57375	0.16	0.5625	0.5852 3	0.230	0.155
1	0.210	0.5	0.765	0.105	0.16065	0.3825	0.0441	0.25	0.5852 3	0.236	0.152
1	0.210	1.0	0.765	0.210	0.16065	0.765	0.0441	1.00	0.5852 3	0.251	0.172
1	0.210	0.75	0.665	0.1575	0.13965	0.49875	0.0441	0.5625	0.4422 3	0.137	0.060
1	0.210	0.75	0.865	0.1575	0.18165	0.64875	0.0441	0.5625	0.7482 3	0.536	0.443

To run the matrix calculation, a constant value (K) is needed. A: injected volume (μL). B: eluent flow (mL/min). C: eluent mixture polarity.

According to the plots $\frac{dt}{t}(2) = 0.100$ when:

1 - (A = 210 μL; B = 0.6 cm^3/min; C = 0.72) or
2 - (A = 210 μL; B = 0.8 cm^3 /min; C = 0.65).

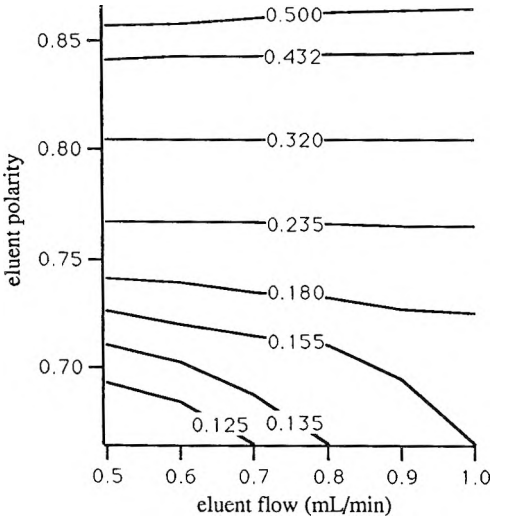

Figure 4. Isoresponse curves resulting from the quadratic Equation 1' ($\frac{dt}{t}(1)$). Injected volume is 210 μL. $\frac{dt}{t}(1)$ values are reported on the curves.

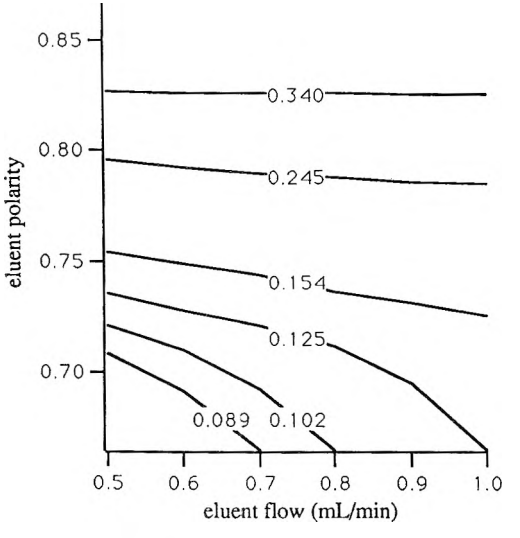

Figure 5. Isoresponse curves resulting from the quadratic Equation 2' ($\frac{dt}{t}(2)$). Injected volume is 210 μL. $\frac{dt}{t}(2)$ values are reported on the curves.

This example shows that a very polar eluent is not required to separate CAR and ALD well.

Table 10

Validation Tests of the Quadratic Equation 1' (dt/t(1)): The Equation is Validated

	A (μL)	210	115	210
Experimental Conditions	**B (mL/min)**	0.75	1.0	0.8
	C	0.765	0.765	0.815
Calculated dt/t(1)		0.243	0.258	0.362
Experimental dt/t(1)		0.243	0.253	0.356
Deviation (%)		0.0	2.0	1.7

Table 11

Validation Tests of the Quadratic Equation 2' (dt/t(2)): The Equation is Validated

	A (μL)	210	115	210
Experimental Conditions	**B (mL/min)**	0.75	1.0	0.8
	C	0.765	0.765	0.815
Calculated dt/t(2)		0.164	0.175	0.278
Experimental dt/t(2)		0.163	0.171	0.270
Deviation (%)		0.0	2.3	3.0

CONCLUSION

According to our study, mathematical equations which characterize separation of molecules could be established quickly; of course, other factors

(nature of the stationary phase, temperature of the column,...)[14] could be studied. Elaboration of mathematical equations and plot of isoresponse curves are easy to derive, with simple computers. A direct strategy for detecting significant chromatographic parameters can be defined, using experimental design.

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CHARACTERIZATION OF PROTEINTYPE PROTEINASE INHIBITORS BY HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS

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ABSTRACT

A method based on high performance capillary electrophoresis (HPCE) has been developed for identification and quantitative determination of inhibitors from leguminous seeds. The method allows specific characterization of Kunitz soybean trypsin inhibitor (KSTI) and Bowman Birk inhibitor (BBI) from soybean (*Glycine max.*, L.). Standard curves for the inhibitors showed a satisfactory linearity between normalized peak area and sample concentration, and a good repeatability of the method was found. Identification of protein peaks as proteinase inhibitors was based on their binding to trypsin. The system also proved useful for determination of the number and specificity of inhibitor sites on

the individual inhibitors and for binding studies with monoclonal antibodies against the inhibitors.

INTRODUCTION

Proteintype proteinase inhibitors for both trypsin and chymotrypsin are widespread in plants.[1] The inhibition of these digestive enzymes are expected to account for some of the antinutritional problems observed when inhibitors are present in food and feed and especially soybean constituents have been the subject for various investigations in this connection.[2,3]

The inhibitors present in soybeans are divided into two groups of isoinhibitors, the Bowman Birk inhibitors (BBI) and the Kunitz soybean trypsin inhibitors (KSTI).[1] BBI isoinhibitors form a group of approximately eight doubleheaded inhibitors with molecular weights of 8 kD and high cystine contents.[4] The KSTI represents a group of three isoinhibitors with molecular weights of 20.1 kD and two disulfide bridges.[5]

As a wide variety of isoinhibitors with different properties often occurs in soybeans as well as in other plant varieties[1], measurements of the inhibitor activity by enzymatic assays does not always provide sufficient informations.[3] The evaluation of the significance of a certain level of inhibitor activity[6] may also require information on the number and relative amounts of the individual inhibitors and their specificities, number of inhibitor sites and affinities for enzymes.[7]

For rapid evaluation of the inhibitor contents in large numbers of samples, techniques based on monoclonal antibodies in enzyme linked immunosorbent assays (ELISA) have proven useful.[8-10] As basis for these types of assays and for various other reasons it is also important to obtain knowledge on the specificity of the mAb and if possible the epitope to which the mAb binds on the inhibitor. The ELISA offers the possibilities for such analyses.[11,12] However, the ELISA involves hydrophobic interaction of the first layer with the plastic surface of the microtiter wells used for ELISA. This hydrophobic interaction has been suspected to cause partly denaturation of the protein[13] and in addition the molecular interaction does not necessarily occur at the same rate in solution as when one of the components has been subjected to immobilization. There is, therefore, a need for supplementary methods for efficient characterization of the inhibitors. The use of HPCE offers a technique with the possibility of studying complex formation without immobilization or

labelling of the reactant and still employing buffer conditions suitable for the binding reactions to occur.[14]

Previously, we have developed an HPCE method for separation of proteintype inhibitors of proteinases and their complexes with monoclonal antibodies and enzymes.[7] This HPCE method used MECC with cholate micelles and taurine in the buffer or mobile phase. The cholate micelles give both hydrophobic and ionic interactions with the proteins whereas the applied zwitterion, taurine, protects against adsorption of proteins to the capillary wall.[7,14]

The present paper describes uses of MECC with a buffer system and pH suitable for detailed characterization of proteintype proteinase inhibitors. The binding between these inhibitors and the enzymes, trypsin and chymotrypsin, as well as the monoclonal antibodies against the inhibitors are studied. The technique allows specific determination of proteins which have binding sites for the enzymes and epitopes corresponding to paratopes on the antibodies.

MATERIALS

Apparatus

The apparatus used was an $HP^{3D}CE$ (Hewlett-Packard, Waldbronn, Germany) with a 614 mm x 0.05 mm I.D. fused-silica capillary. Detection was performed by on-column measurements at a position 530 mm from the injection end of the capillary.

Samples and Reagents

Bowman Birk inhibitor (BBI) from soybean, Kunitz soybean trypsin inhibitor (Type I-S) (KSTI), porcine pancreas trypsin and bovine pancreas chymotrypsin were obtained from Sigma (St. Louis, MO, USA). Monoclonal antibodies raised against the inhibitors from soybean were from the collection in this laboratory.[12] Trigonellinamide, disodium hydrogenphosphate, cholic acid and taurine (2-amino-ethanesulphonic acid) were obtained from Sigma.

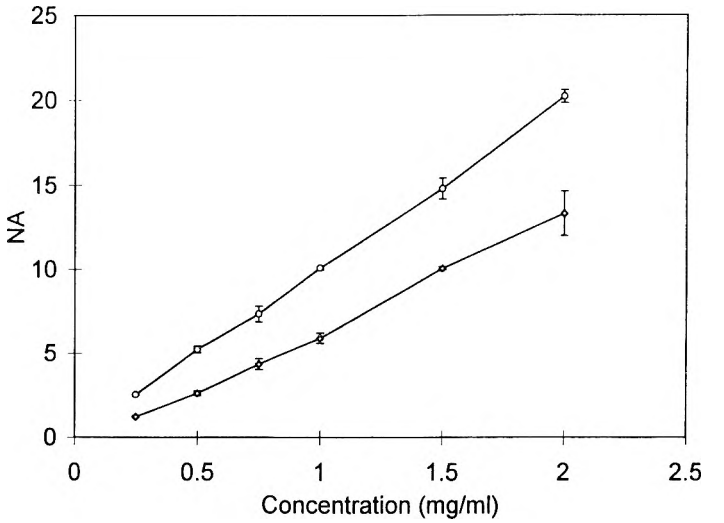

Figure 1. Normalized areas (NA) measured in triplicate from electropherograms of varying amounts of BBI ( ) and KSTI (O).

METHODS

All analyses with enzymes were made with solutions of enzymes in running buffer freshly prepared from standard solutions of enzymes in 1 mM HCl. The samples were introduced from the positive end of the capillary by vacuum injection (2 sec., 25 mbar). On-column detection at 200 nm was applied. Buffer solutions were filtered through a 0.2 μm membrane filter before use. Washing with 0.1 M NaOH for 2 min and buffer for 5 min were performed between each analysis.

RESULTS

The applicability of HPCE for quantitative determination of inhibitors was evaluated by the linearity obtained when normalized peak areas were plotted against sample concentration. Triplicate runs were performed at 6 different concentrations of the inhibitors, BBI and KSTI (Fig. 1). Different slopes were found due to different response factors of the inhibitors at 200 nm.

The linearity studies also included trypsin, which is a proteinase inhibited by BBI and KSTI, α-lactalbumin as a reference protein/inert protein and a marker molecule; trigonellin amide. The data obtained are displayed in Table 1 and the presented relative standard deviations for the NA determinations are given as the highest of the six relative standard deviations obtained. For all components tested, good correlation was found between normalized peak areas and sample concentration (Table 1). Similarly, the variation in migration time was examined. Peak identification by migration time was seen to be somewhat enhanced by use of migration times relative to the internal markers. In addition, the use of an internal marker provides a reference for alignment of electropherograms of different compounds.

Table 1

Correlation Between Normalized Peak Area (NA) and Sample Concentration as well as Relative Standard Deviation (Rel Std Dev, Calculated from n Runs) for Migration Time (MT) and Relative Migration Time (RMT)

Sample	Conc. range (mg/ml)	NA=f(C)* Corr. coeff.	NA rel std dev/(n) (%)	MT rel std dev/(n) (%)	RMT** rel std dev/(n) (%)
BBI	0.25-2.0	0.9979	<9.9 /(4)	0.58/(24)	0.53/(24)
α-lactalbumin	0.25-2.0	0.9936	<11.8/(3)	0.99/(18)	0.63/(18)
KSTI	0.25-2.0	1.0000	<6.5 /(4)	1.02/(24)	1.07/(20)
Trypsin	0.25-2.0	0.9960	<5.5 /(3)	2.84/(18)	2.06/(18)
TGA	0.06-0.5	1.0000	<7.5 /(4)	0.66/(20)	

* C; Concentration of sample

**RMT; MT relative to MT of TGA (trigonellin amide) used as internal standard

The designation of particular protein peaks to inhibitors was investigated by the ability of the protein to form complexes with the proteinase enzymes, hence resulting in appearance of a complex peak with migration time different from the individual components. All mixed samples were allowed to form complexes in 5 mM phosphate buffer at pH 7.5, and MECC of the

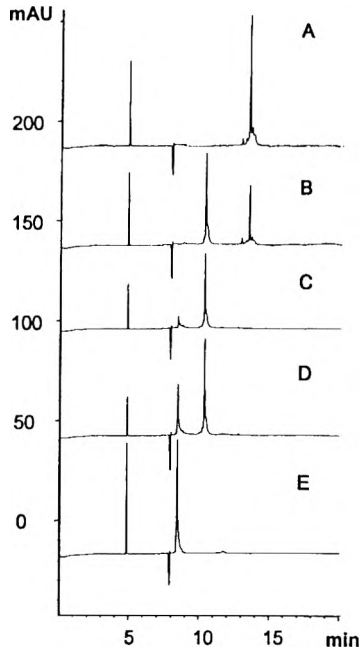

Figure 2. Complex formation of KSTI and trypsin at different mixture ratios of A: KSTI (2.4 mg/ml) + TGA (0.6 mg/ml). Electropherogram E shows porcine trypsin (2.73 mg/ml) + TGA (0.27 mg/ml). For B, C and D the molar ratios of KSTI to trypsin was 1.6, 0.8 and 0.4 respectively.

mixtures were performed immediately. Fig. 2 shows results from the mixing of KSTI and trypsin at different molar ratios followed by electrophoresis. KSTI is seen to have one inhibitor site for trypsin as the NA of the complex peak did not increase regardless of whether the enzyme or the inhibitor is present in a molar excess. Self digestion of trypsin was investigated by repeated runs of a KSTI-trypsin mixture at pH 8.2, which did not show degradation of the complex peak (Fig. 2, C) during more than 3 hours.

In Fig. 3, the binding between KSTI and a monoclonal antibody against KSTI is shown at various molar mixing ratios, with the mAb-KSTI complex having a migration time different from those of the individual molecules. As expected, a molar excess above 2 of KSTI to mAb was necessary to observe a peak for unbound KSTI, implying that the mAb bound two KSTI molecules per mAb molecule (Fig. 3, C).

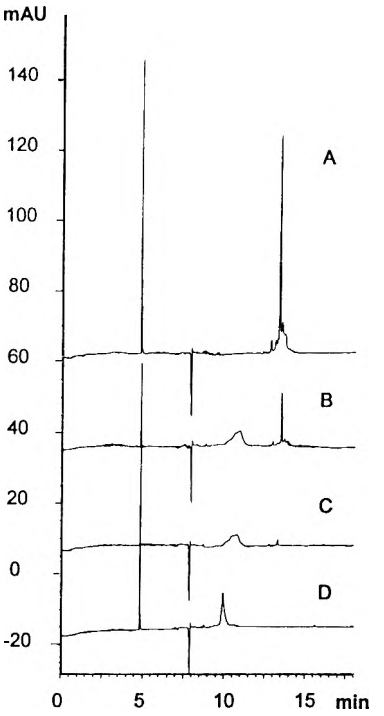

Figure 3. Complex formation of KSTI and a monoclonal antibody with specificity for KSTI. Electropherogram A (KSTI (2 mg/ml) + TGA (1 mg/ml)) shows the mixture used for incubation with the mAb. B: KSTI:mAb in molar ratio of 3.6:1; C: KSTI:mAb in molar ratio 1.8:1; D: mAb (1.07 mg/ml) + TGA (0.6 mg/ml).

The ability of KSTI to simultaneously bind trypsin and the mAb was investigated by mixing the mAb-KSTI complex from Fig. 3 with trypsin at different molar ratios (Fig. 4). The inhibitor site for KSTI was seen to be different from the mAb binding site as simultaneous binding was observed.

The chymotrypsin-trypsin inhibitor, BBI, was subjected to similar studies, with incubation of BBI with chymotrypsin at different molar ratios (Fig. 5). Similarly to the KSTI-complexes, the formation of complex peaks resulted in changed MT's.

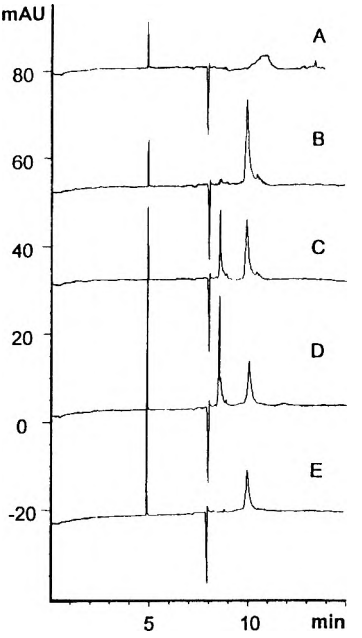

Figure 4. Simultaneous binding of mAb and trypsin to KSTI. A: Mixture of mAb and KSTI in a molar ratio of 1:1.8. The KSTI + TGA mixture employed in A, B and C are identical to Fig. 3, A. B: KSTI:mAb:trypsin in a molar ratio of 1:0.56:1.5; C: KSTI:mAb:trypsin in a molar ratio of 1:0.56:3; D: mAb (0.84 mg/ml) + trypsin (0.5 mg/ml) + TGA (0.5 mg/ml); E: mAb (1.07 mg/ml) + TGA (0.6 mg/ml).

DISCUSSION

HPCE was investigated for its applicability as a method of characterization of proteintype proteinase inhibitors. The linear correlation shown between NA and the concentrations of the analytes (Table 1) enables the use of NA as a measure of the amount of individual proteins. As the response factors differ for the individual proteins (Fig. 1), an evaluation of the amounts of proteins in the complex peaks from NA requires knowledge of the response factor of the complexes. Instead, an internal marker, TGA, has been included in the inhibitor samples used for mixing with other proteins. The NA of TGA

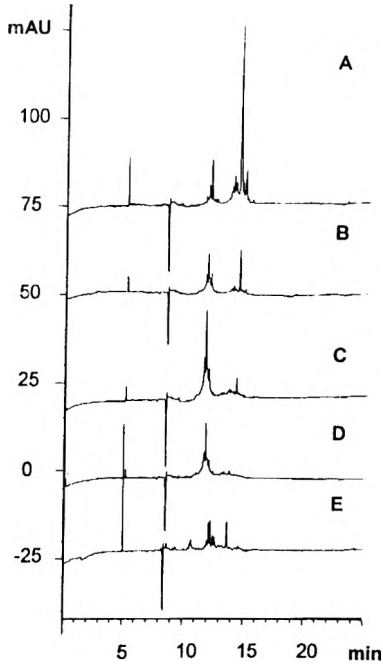

Figure 5. Complex formation of BBI with chymotrypsin. A: Shows the mixture used for incubation with chymotrypsin; BBI (2.73 mg/ml) + TGA (0.1 mg/ml); B: BBI:chymotrypsin in a molar ratio of 2.7:1; C: BBI:chymotrypsin in molar ratio 1.4:1; D: BBI:chymotrypsin in a molar ratio 0.8:1; E: chymotrypsin (2.4 mg/ml) + TGA (0.2 mg/ml).

was also shown to be linearly correlated with the concentration (Table 1), and the amount of TGA observed in an electropherogram with inhibitor present can therefore be used as a measure for the total amount of the inhibitor present in the sample. The use of an internal marker may provide a better determination of the mixing ratios than when calculated from the microlitres actually mixed due to the errors correlated to pipetting of volumes of a few microlitres.

In Fig. 2, the complex formation between KSTI and porcine trypsin is illustrated. High affinities are usually observed for the binding of inhibitors to trypsin and chymotrypsin and the reaction rate is high resulting in fast complex formation following mixing of the compounds.[6,7] With mixing of bovine chymotrypsin and KSTI no complex formation was observed, confirming the findings that KSTI does not have an inhibitor site for chymotrypsin[15] although

this has been indicated in some studies.[16] These results also indicate that the system now developed does not favour unspecific complexation.

When mixing KSTI with mAb, a broad peak was observed (Fig. 3). This peak broadening may be due to a mixture of mAb complexed with one or two KSTI molecules. With addition of trypsin to the mAb-KSTI mixture, a new complex peak was formed with markedly changed MT and peak shape (Fig. 4, B and C). In a sample with trypsin and mAb, no interaction of the trypsin and mAb was observed (Fig. 4, D and E), confirming that trypsin binds to KSTI in the KSTI-mAb complex.

With binding of BBI to chymotrypsin, a range of inhibitor-enzyme complex peaks appeared, which possibly is due to the presence of various isoforms of BBI as well as of chymotrypsin.

In conclusion, MECC offers a method requiring limited sample amounts compared to traditional methods and with complex formation in solution which complements traditional methods as ELISA and enzymatic assays. With the ability of HPCE to be used for quantitative analyses of the proteins, the method presented enables knowledge of the number and specificity of inhibitor sites on the individual isoinhibitors present in a given sample as well as characterization of mAb binding to the inhibitors.

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APPLICATION OF SUPERCRITICAL FLUID EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY FOR THE DETERMINATION OF SOME ANABOLIC AGENTS DIRECTLY FROM BOVINE TISSUE SAMPLES

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ABSTRACT

The aim of the present work was to evaluate the feasibility of supercritical fluid extraction (SFE) of some growth-promoting anabolic steroids directly from bovine tissue samples with carbon dioxide as a supercritical fluid. The study was divided into two phases. The first phase involved the optimization of SFE parameters versus recovery and repeatability. Results were obtained by high performance liquid chromatography (HPLC) or supercritical fluid chromatography (SFC). The second set of studies consisted of SFE from the real samples monitored by the combination of high performance liquid chromatography

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atmospheric pressure chemical ionization mass spectrometry (HPLC/APCIMS). The limit of detection was about 10 ppb, but 100 ppb is the practical detection limit for SFE-HPLC/MS analysis under the experimental conditions used in this study. The results from this work suggested that SFE may be useful for isolation of target compounds from complex biological samples.

INTRODUCTION

Many samples encountered by the analytical chemist consist of trace components in a complex and interfering matrix. For the identification and quantitation of these components, isolation from the matrix is usually necessary. Sample preparation methods are generally both time and solvent consuming. According to a recent survey, two thirds of the analysis time is devoted to sample preparation and this step accounts for at least one-third of the errors generated during the performance of an analytical method.[1] Extraction with supercritical fluids is one of the most rapidly developing methods for separation substances from various matrices. The term dense gas is also used, but it covers both the liquid and supercritical states of a fluid and the term supercritical is applied only when the critical parameters have been exceeded. Comprehensive reviews on principles, theory, techniques and applications of supercritical fluid extraction (SFE) have been published.[2-6]

Supercritical fluid extraction is a relatively new analytical isolation technique which offers advantages of low temperature processing, recovery of a solvent-free extract, rapid extraction resulting from high mass transfer due to higher diffusivity and lower viscosity in comparison to organic solvents.[7] The selectivity and solvent power depend on the density, which can be varied during the extraction procedure by controlling pressure and temperature. By changing the extraction conditions class-selective extractions and fractionation of the extract can be achieved. By far the most widely used extraction fluid has been supercritical carbon dioxide (CO_2)[2]. Its preferential use is due to the fact that it is an chemically inert, inexpensive, non-toxic and non-flammable extraction gas with mild critical parameters (31.1^{o}C critical temperature and 7.38 MPa critical pressure).[8] This enables the application of low extraction temperatures thereby reducing the risk of analyte degradation during the extraction. Due to the gaseous nature of CO_2 at low pressure SFE can be combined with chromatographic techniques reviewed e.g., by Chester et al.,[6] Hawthorne et

al.,[9] Jinno and Saito.[10] By adding a modifier (*e.g.*, MeOH, H_2O) it is possible to adjust the solvating power of CO_2. Many other supercritical fluids have been also used *e.g.*, N_2O, SF_6, CH_3OH, H_2O and $CHClF_2$.[11-13]

Supercritical fluid extraction has been used for industrial-scale separations and the isolation of a variety of compounds.[2,4] On the analytical scale SFE has also attracted considerable attention and a variety of techniques have been used for on-line analyses combined with other chromatographic methods.[6] An increasing number of quantitative applications of SFE especially for the extraction of environmental pollutants has been reported in recent years.[14-22] SFE is utilized also in the field of food science to isolate not only natural food components such as fat,[23] cholesterol[24] and volatiles[25-26] but also natural but not desirable components like mycotoxins[27] and organic contaminants such as pesticides and PAHs.[28-32]

Only limited studies have been published on the use of supercritical fluids for the extraction of polar drug residues in the bovine tissues. Ramsey *et al.*[33] have used the combination of SFE/SFC/MS/MS for the determination of residues of a small group of veterinary drugs in freeze-dried pig's kidney. The results showed the detection of 1 mg/kg of spiked samples for the four drugs diethylstilbestrol, dienestrol, hexestrol and trimethoprim.

The combination of SFE-HPLC has been used for the determination of polar drugs, steroidal compounds and sulfonamides from animal food, blood plasma and chicken tissue.[34-37] Also, solubility's of some steroidal compounds have been conducted by SFE-HPLC.[38] The concentration of drugs investigated in most previous studies were, however, so high that true residue analysis is out of question.

The aim of the present study was to develop a unified multiresidue extraction procedure for the isolation of estrogenic and anabolic agents from bovine tissues using supercritical fluid extraction techniques. The goal of this work was also to evaluate the feasibility of extracting these growth-promoting target compounds at low parts per billion level directly from bovine tissue samples with carbon dioxide as a supercritical fluid. The method is compared with a traditional three-phase liquid-liquid extraction procedure used previously for related studies. The analytes were monitored by HPLC and identified by UV detection and mass spectrometry.

MATERIALS AND METHODS

Chemicals

All solvents, buffers and common reagents were purchased from Fisher Scientific (Rochester, NY, USA) unless otherwise noted. A standard stock solution of the seven drugs (500 μg/ mL dichlromethane) was prepared from pure standards purchased from Sigma Chemical Co (St. Louis, MO, USA). The drugs were dexamethasone (DEX), diethylstilbestrol (DES), medroxy progesterone (MDP), melengestrol acetate (MGA), trenbolone (TBOH), triamcinolone acetate (TACA) and zeranol (ZER). Figure 1 shows structures of these seven growth promoting drugs investigated in this study.

Tissue samples

About 200 g of beef steak, purchased from a local supplier, was homogenized to a smooth paste in a Waring blender (2 min/full speed). The paste was spread onto Petri trays and freeze-dried using a VirTis Unitrap II (VirTis, Gardiner, NY, USA). The lyophilized tissue sample was then ground using a mortar & pestle. Portions of the powdered muscle or liver (1 g) were mixed with methanol (10 mL), spiked with the appropriate quantity of each anabolic compound by using the standard stock solution, allowed to equilibrate for one hour whilst being sonicated and then evaporated to dryness under a stream of dry nitrogen. All samples were thoroughly mixed prior to being loaded into the SFE extraction cell.

Three-phase liquid-liquid extraction

The liquid-liquid extraction was carried out according to the procedure reported by Hsu et al.[39] and Covey et al.[40] Muscle or liver tissue were homogenized in the same way as described above. Five-gram aliquots of bovine liver or muscle homogenate were then vortex-mixed with 10 mL of 0.04 M NaOAc. The pH of the solution was brought to 4.2 - 4.7 with glacial acetic acid. The enzymatic hydrolysis was initiated by adding 100 μL of β-glucuronidase (Sigma Chemical Co., St. Louis, MO, USA). After 8 hour of enzymatic hydrolysis at 37°C, 20 mL of acetonitrile was added followed by mixing on a vortex mixer for 30 seconds. The homogenate was then centrifuged (5000 rpm for 20 min) and the supernatant (30 mL) was then

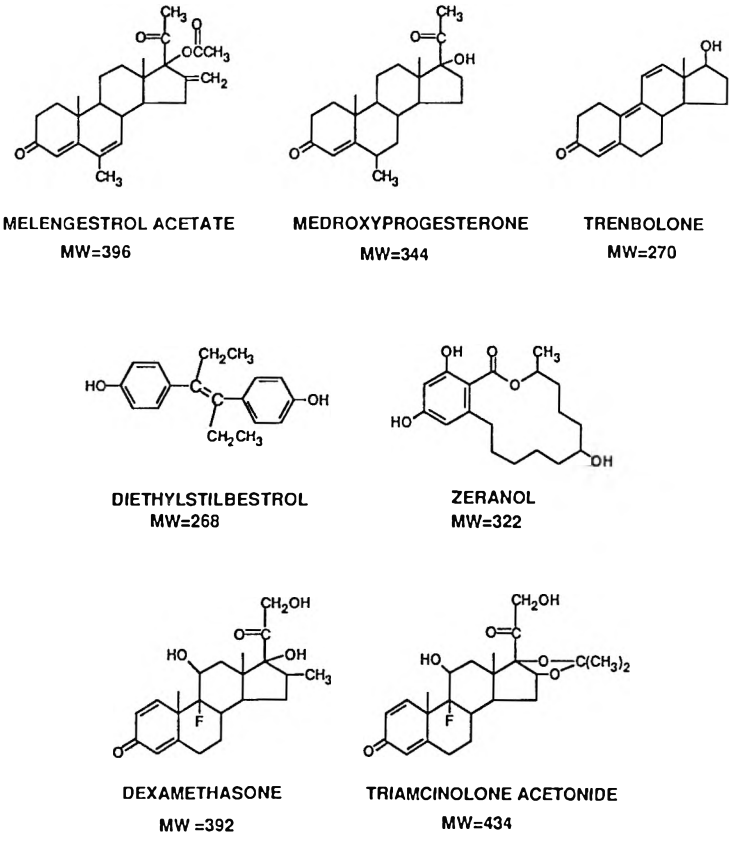

Figure 1. Structures of growth promoting drugs investigated in this study.

transferred to a clean test tube. Hexane (8 mL) and dichloromethane (2 mL) were added and mixed by rotation for 3 minutes. Samples were then centrifuged (2000 rpm for 2 min). A three-phase liquid system was obtained. The middle layer (15 mL in acetonitrile) was transferred to a 20 mL scintillation vial and evaporated to dryness under nitrogen. The liver and muscle extracts were each dissolved in 1 mL of dichloromethane prior to analysis.

Supercritical fluid extraction (SFE)

A Lee Scientific Series 600 micro-scale Extraction System (Lee Scientific, Inc., Salt Lake City, UT, USA) was used for sample preparation while the analytes were analyzed by high performance liquid chromatography (HPLC) combined with UV-detection or mass-spectrometry (MS).

Samples were injected (standards in the solvent) or weighed (spiked tissue samples) into a 2 mL extraction cell (Valco guard column, Valco Instruments Co. Inc. TX, USA) located external to the instrument. A restrictor was directed through the oven into the collection system external to the capillary SFC instrument (Lee Scientific, Inc., Salt Lake City, UT, USA). During the SFE procedure the temperature of the oven was kept constant at 60°C. Restrictors used in this study were cut to the desired length from fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA). The Guthrie-type restrictor was homemade. A schematic of the supercritical fluid extraction system is shown in the owner's manual of the instrument. SFC-grade CO_2 in an aluminum cylinder with helium head pressure from Scott Specialty Gases (Plumbsteadville, PA, USA) was used for all SFE experiments.

Analytes were collected by placing the restrictor into a 2 mL auto sampler vial containing 1 mL of precooled (+ 5°C) methanol (MeOH). The cooling effect of expanding CO_2 was sufficient to keep the vial cool to prevent the loss of analytes. After the extraction samples were evaporated to dryness under nitrogen and prior to HPLC analysis they were dissolved in 100 μL of eluent containing 30% of methanol/acetonitrile (50/50) and 70% mM ammonium formate.

High performance liquid chromatography (HPLC)

Dissolved tissue extract residue as well as standard mixtures were injected into a Waters HPLC system, Model # 510 (Waters, Inc. Milford, MA, USA) using a Rheodyne injector, Model # 7125 (Rheodyne Inc., Cotati, CA USA) with a 20 mL sample loop. A fixed-wavelength (245 nm) UV-detector, Model #440 (Waters, Inc. Milford, MA, USA) was used for monitoring the seven drugs. The data were recorded on a Hewlett-Packard integrator, Model # 3390A (Hewlett-Packard, Palo Alto, CA, USA).

The HPLC flow rate used was 1.0 mL/min and the column utilized in this

system was 5 cm x 4.6 mm ID, 5 μm, Supelcosil (Supelco, Inc. Bellefonte, PA, USA). The mobile phase was composed of methanol, acetonitrile and 20 mM ammonium formate controlled by a Waters automated gradient controller, Model # 680 (Waters, Inc. Milford, MA, USA). The linear gradient program of 19 minutes was initiated with the low organic solvent composition (5%) while the final mobile phase composition was mainly the mixture of methanol and acetonitrile (95%).

Quantitation of the seven drugs recoveries by SFE from tissue samples was accomplished by comparison of HPLC peak areas between the analytical standards of the drugs and fortified tissue samples (100 ppb of the each drug). Triplicate samples were used for recovery and repeatability studies.

Supercritical Fluid Extraction-Supercritical Fluid Chromatography (SFE/SFC)

The micro-scale Extraction System Series 600 based on cryogenic solute focusing combined to capillary Supercritical Fluid Chromatography Series 600 (Lee Scientific, Inc., Salt Lake City, UT, USA) was used to determine the optimum extraction time. Samples were placed into the 2 mL extraction cell (Valco Instruments Co. Inc., TX, USA). During the SFE procedure the temperature of the SFC oven was maintained at 60°C, and the cryofocusing T-piece located inside the SFC oven was cooled by the external CO_2 tank. The temperature of extraction cell was 60°C. Three successive 20 minute extractions were accomplished from the same sample at 400 atm (1 atm = 0.10132 MPa). After the extraction, the 10-port valve was switched to the column position and the normal SFC run was commenced. A SB-cyanopropyl-50, 50 mm x 10 m fused-silica capillary column coated with a 25 μm film thickness (Lee Scientific, Inc., Salt Lake City, UT, USA), was used for the separation. SFC-grade carbon dioxide (Scott Specialty Gases, Plumsteadville, PA, USA) was used as the extraction fluid as the mobile phase. The oven temperature was kept constant at 120°C. The frit restrictor was used for the mobile phase restriction providing an average linear velocity of 1.2 cm/sec (at 100 atm). The temperature of the flame ionization detector (FID) was 375°C. The separation was accomplished by applying a pressure program as follows: 100 atm initial pressure held constant for 10 min, then ramped to 415 atm at a constant ramp rate of 10 atm/min.

Mass Spectrometry (MS)

A Sciex TAGA 6000E tandem triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with a standard atmospheric pressure ionization (API) source was used in these experiments. The heated pneumatic nebulizer interface was used for combined high performance liquid chromatography/mass spectrometry (LC/MS) experiments. To produce atmospheric pressure chemical ionization (APCI) mass spectra the mass ranged scanned was from m/z 250-450 while the $[M+H]^+$ ion was monitored for each individual solute in the selected ion monitoring (SIM) mode.

RESULTS AND DISCUSSION

The goal of this work was to investigate the use of supercritical fluid extraction (CO_2) for the isolation of growth promoting anabolic steroids directly from fortified bovine tissue samples and to determine them by high performance liquid chromatography coupled with UV or mass spectrometry detection.

The present study was divided into two phases. The first phase of investigation involved studies to optimize the extraction parameters (temperature, pressure, time, restrictor) versus recovery and repeatability. The matrix was an inert filter paper or fortified freeze-dried bovine muscle tissue sample. Results were obtained by HPLC-UV or SFC-FID. The second set of studies consisted of SFE from three-phase liquid-liquid liver extracts and from freeze-dried muscle tissue sample monitored by HPLC/MS. In order to get satisfactory recovery and repeatability results, a good reference material was required. Spiking directly onto the sample can cause too high recoveries.[35] Therefore, spiked samples were prepared according to Ramsey et al.[33] The drugs were first diluted in dichloromethane and the solution was then thoroughly mixed with freeze-dried matrix

SFE conditions were optimized for the seven drugs by segnentially varying the following parameters. Temperature: 50, 60, 80 and 100°C; extraction pressure: 250, 300, 350 and 400 atm (1 atm = 0.10132 MPa); extraction time: 10, 20, 30, 40, 50, 60 and 120 min; restrictor: Guthrie-type (homemade), 20 cm long x 15 or 21 mm ID linear. The optimization was done by keeping three of the variables constant while varying the other. The collected analytes were

then measured by HPLC with UV or MS detection. SFC-FID was also used to determine the optimum extraction time.

Table 1

Effect of Temperature and Restrictor on the Recovery of the Drugs Investigated

Compound	60°C 21 mm a) %	80°C 21 mm a) %	80°C 15 mm a) %	100°C 15 mm b) %	100°C 21 mm b) %
DEX	59	93	98	37	65
TBOH	69	109	102	110	68
TACA	70	80	68	58	69
ZER	77	85	80	69	72
DES	68	84	100	67	64
MDP	74	76	68	55	55
MGA	71	77	80	63	61

a) extraction time: 60 min; pressure: 400 atm.
b) extraction time: 30 min; pressure: 400 atm.

In the optimization studies the stock solution (500 ng of each compound) was deposited onto a piece of filter paper located in the extraction vessel. Table 1 and Figure 2 show the results from optimizing SFE conditions for recovery of the seven drugs. SFE/SFC-FID data shown in Figure 2 show that the 20 min extraction is not sufficient to extract all the analytes and at least 40 min is required. A 60 min extraction produced about 80% recoveries. The three extracts were carried out successively from the same sample. Table 1 shows that, by increasing the extraction temperature to 80°C, the recoveries (e.g. DEX) were also increased compared to the results obtained at 60°C. At the same time, however, the fat contained in the samples caused difficulties with UV-detection. In this study alumina or other absorbents were not used for retaining fat in the extraction vessel. Because of this it might be that the recoveries at 100°C were even lower than at 80°C even through the extraction time at 100°C was only 30 min. The effect of pressure on the SFE recoveries of seven drugs was also conducted. Although the data are not presented, it was

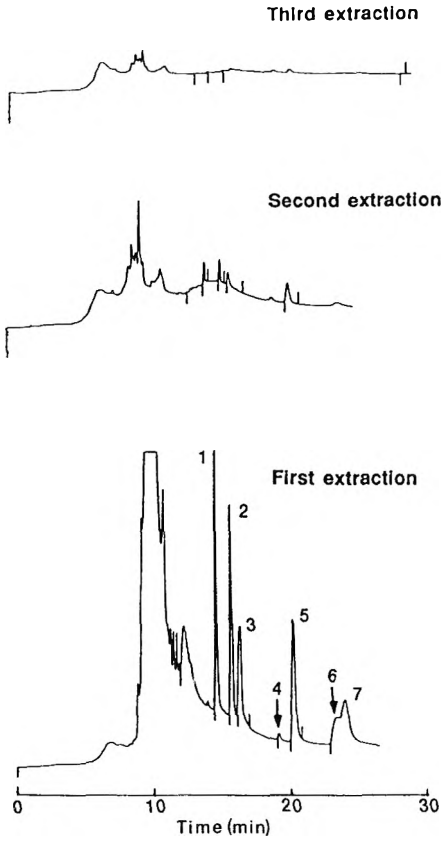

Figure 2. SFE/SFC-FID chromatograms of spiked (500 ng of each compound) three-phase liquid-liquid extract residue of bovine muscle after three successive 20 min extractions at 400 atm and 60°C.
1 = melengestrol acetate, 2 = medroxyprogesterone, 3 = trenbolone, 4 = diethylstilbestrol, 5 = dexamethasone, 6 = zeranol, 7 = triamcinolone acetonide.

found out that 400 atm gave the best recoveries for all the drugs. The pressure limit of the system was 415 atm.

To maintain the pressure in a supercritical fluid extraction system, restrictors have to be used at the outlet. This restrictor is often a piece of fused-

silica capillary tubing of 5-25 μm i.d.. These might, however, cause plugging when the sample matrices contain high concentrations of water or fat. To avoid this problem heated variable restrictors have been designed which "sense" the plugging before it happens.[41] Despite our use of linear restrictors in this work plugging rarely occurred. This may be due to our use of freeze-dried samples prior to SFE. The restrictor type also has an effect on recoveries. The linear restrictor, 20 cm length x 21 μm i.d. produced the best recoveries ranging from 57 % (DEX) to 112 % (TBOH).

Improved results were obtained if the restrictor was prerinsed with a small amount of methanol (100 mL). This was especially true with TACA, ZER, DES and MDP, where recoveries improved approximately 10% by prerinsing the restrictor. In practice this procedure, however, became impractical and was discontinued. The Guthrie-type restrictor plugged easily, especially when tissue samples were used even though it gave quite good results with pure standards.

To determine the optimum conditions for isolating all seven compounds, a compromise must be met. The optimized conditions were as follows: extraction time 60 min, extraction temperature 60°C, extraction pressure 400 atm with a linear restrictor 20 cm x 21 μm i.d. Using these optimum conditions, the recoveries and repeatabilites shown in Table 2 were obtained. SFE was done from spiked (100 ppb of each drug) freeze-dried bovine muscle tissue sample. It is shown that repeatability and recovery were poor with polar drugs such as dexamethasone (DEX), triamcinolone acetonide (TACA) and zeranol (ZER) with recovery varying from 44% to 58%.

The more non polar drugs including melengestrol acetate (MGA), medroxyprogesterone (MDP) and diethylstilbestrol (DES) recoveries were quite good varying from 83 to 91%. The overall recovery was 69% with an R.S.D. of 19.7%. Trenbolene (TBOH) could not be measured in these experiments, due to interference from coeluting compounds. One reason for the variable recoveries may be due to the position of the extraction cell. In this work it was positioned horizontally rather than the preferred vertical position to achieve better recoveries and especially more repeatable results. At the 100 ppb concentration level repeatability was not acceptable, but at 1 ppm concentration level the R.S.D. was 5%. Recovery of DES was only 34.0 % which is quite consistent with the results of Karlsson et al.[36]

Table 2

Recovery and Repeatability of SFE from Freeze-Dried Muscle Tissue Sample. Fortified with 100 ppb of the Drugs.

Compound	Recovery %	R.S.D. %	n
DEX	57	13.6	3
TACA	44	17.7	3
ZER	58	12.1	3
DES	83	34.0	3
MDP	91	11.2	3
MGA	83	29.5	3
$\bar{x}$	69	19.7	

$\bar{x}$ = mean of recoveries

Extraction of pure steroids from the filter paper using 5% of MeOH as a modifier gave a recovery of *ca.* 50% for DEX. It should be noted that individual sample matrices affect the extraction parameters.[38] In this work, the matrix was freeze-dried bovine muscle. The matrix effect is clearly shown in Figure 3, where A) shows the HPLC-UV chromatogram after SFE of pure drugs from filter paper, B) shows the same trace after SFE of spiked freeze-dried muscle tissue sample and C) shows the HPLC-UV chromatogram after SFE of spiked three-phase liver extract. Even if MeOH is not used as a modifier fat and fat-related compounds coelute and interfere with the interpretation of the HPLC-UV chromatogram especially in the case of the liver sample.

Quite often analytes are collected directly into organic solvent,[42] but they can be trapped first on sorbents and after that diluted with a small amount of an organic solvent.[43] Analytes could be collected also directly into a SPE mini-column for further sample preparation.[44] In this study the compounds were collected directly into MeOH to simplify the collection system. No loss of the target compounds appeared to occur using this collection procedure.

Figure 4 A), B) and C) show the HPLC/API/MS analysis of the SFE extract from freeze-dried muscle tissue sample and three-phase liquid-liquid extract

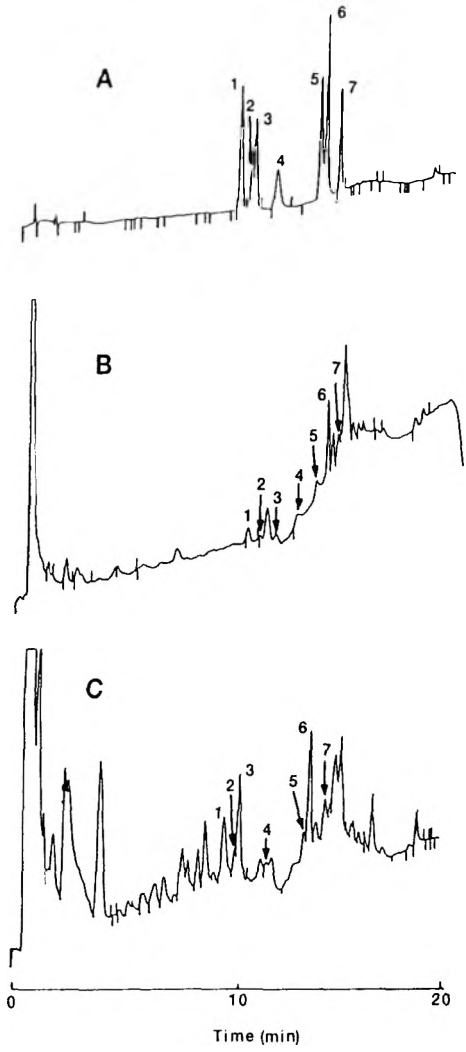

Figure 3. HPLC-UV chromatograms of A) SFE of the stock solution (20 ng of each compound) in filter paper, B) SFE from spiked (500 ppb) freeze-dried muscle tissue sample, C) SFE from three-phase liquid-liquid extract from liver residue spiked to 100 ppb. SFE conditions: 60 min/ 60°C/400 atm.
1 = dexamethasone, 2 = trenbolone, 3 = triamcinolone acetonide, 4 = zeranol, 5 = diethylstilbestrol, 6 = medroxyprogesterone, 7 = melengestrol acetate.

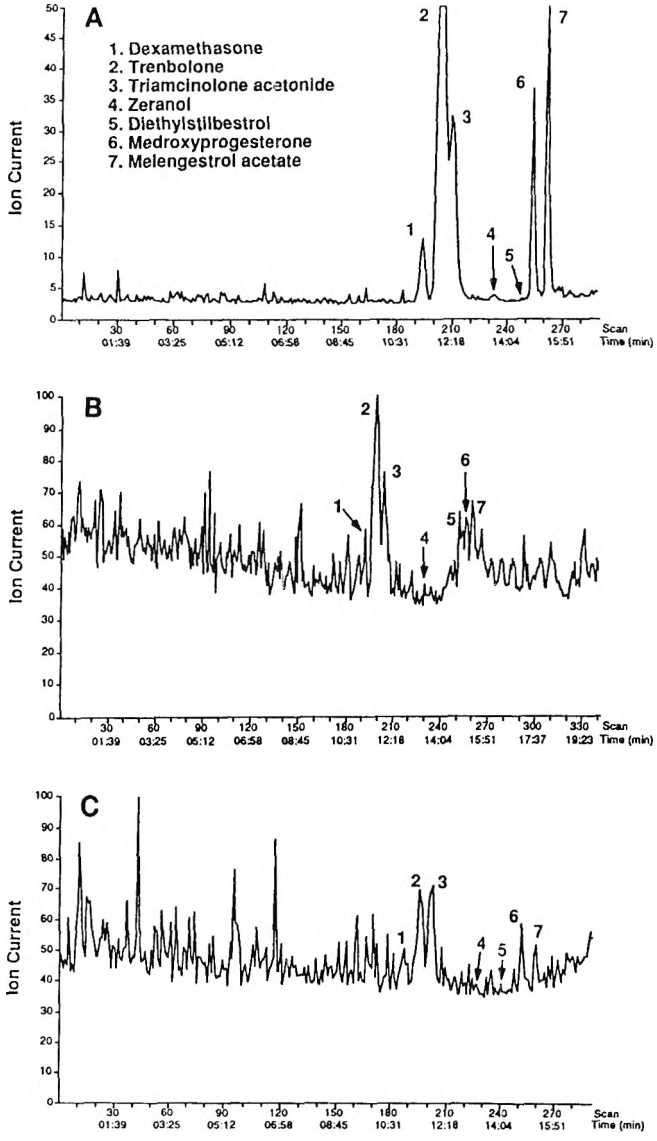

Figure 4. (SIM)HPLC-APIMS analysis of A) freeze-dried muscle tissue sample spiked to 100 ppb, B) three-phase liquid-liquid liver extract spiked to 100 ppb, and C) three-phase liquid-liquid liver extract spiked to 10 pbb of the seven drugs.

under selected ion monitoring (SIM) conditions. In Figure 4A) the muscle sample was spiked with the seven drugs at the level of 100 ppb and in Figure 4B) the three-phase liver extract residue was spiked at the same level. The liver sample (three-phase extract) in the study of Figure 4C) was fortified with 10 ppb of the seven drugs. As expected the liver sample was more complex than the muscle sample. It appears that the 100 ppb level is the practical detection limit for HPLC/MS analysis under these experimental conditions for both samples. The 10 ppb level (Figure 4C) was reached, but the S/N ratio was unsatisfactory. The identification of zeranol and diethylstilbestrol was less certain, but for trenbolene, triamcinolone acetate, medroxy progesterone and melengestrol acetate the 10 ppb level could be detected. In previous literature reports the detection limit has been reported only to 1 ppm for diethylstilbestrol by SFE/SFC/MS/MS with no data from recovery and repeatability.[33]

CONCLUSIONS

The goal of this work was to study the feasibility of supercritical fluid extraction using CO_2 as an extraction fluid to isolate some growth promoting compounds directly from bovine tissue sample and to analyze them by (SIM)HPLC/API/MS. Conventional methods for the determination of these analytes exist, but they are time-consuming with excessive use of organic solvents and low recoveries.

The results of this work show that (SIM)HPLC/MS provides an alternative method for monitoring selected anabolic compounds isolated from tissue samples by SFE. The 100 ppb level was the practical detection limit for all the drugs investigated while the 10 ppb level could be reached for some. The disadvantage of the single quadrupole mass spectrometer operation is that there is little structural information available. However, for target analyses single quadrupole instruments operated under selected ion monitoring (SIM) conditions can offer a fast and reliable method. For quantitative identification tandem mass spectrometry offers additional analytical capabilities.

Supercritical fluid extraction has some desirable analytical advantages so that this approach still deserves more investigation. One should not expect SFE to replace all other forms of sample preparation steps. Some sample cleanup may still have to be done after or before supercritical fluid extraction. It seems, however, that modern SFE instrumentation with multiple extraction cells, higher pressure limits and modifier addition systems will provide more

versatility for the isolation of target compounds from complex biological samples.

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DETERMINATION OF KETOROLAC IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AFTER AUTOMATED ON-LINE SOLID PHASE EXTRACTION

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ABSTRACT

An automated solid phase extraction-based procedure for the determination of ketorolac in human plasma was developed and validated. Solid phase extraction of ketorolac tromethamine and ketoprofen (internal standard) was performed on disposable C-18 cartridges directly from plasma samples spiked with the corresponding standards after dilution with saline. All operations were performed automatically by means of a switching-valve assembly in the sample preparation module (Prospekt system), and sample analysis were carried out by on line connection with the HPLC system. Linearity of the analytical methodology was assessed at the concentration range 25-2500 ng/mL ketorolac tromethamine. However, accurate and precise determinations at 5 and 10 ng/mL were obtained by the use of weighing in calibration curve fitting. The mean absolute recoveries for ketorolac tromethamine and the internal standard were 87.7% and 72.9% respectively. Respective intra- and inter-assay precision values were below 7.2 and 18.0%, the later at the

5 ng/mL concentration level of ketorolac tromethamine which was considered as the lower limit of quantitation (3.4 ng/mL in terms of free acid form). Intra- and inter-assay accuracy, expressed as relative error in percentage, were less than 9.5 and 8.8% respectively. The minimal sample handling and the obtained precision and accuracy at the wide range of concentration levels tested make this method suitable for routine quantitations of ketorolac in human plasma.

INTRODUCTION

Ketorolac tromethamine (KT) is an orally and parenterally active agent with potent analgesic and antiinflammatory activity associated with the inhibition of cicloоxygenase.[1,2] KT has been shown to be effective in the management of acute pain[3,4] as well as in the improvement of occular inflammation.[5]

Analytical methods for the determination of ketorolac in plasma and other biological fluids have been developed mainly for pharmacokinetic studies. Except for a method in which zinc sulphate-deproteinized serum is used,[6] the recently reported analytical techniques combine liquid-liquid extraction of the drug into organic solvents such as diethyl ether or ethyl acetate, with HPLC-based quantitations.[7-9] The most common disadvantages of liquid extraction are the large sample handling and the long time consuming although effective sample clean-up and relative high detection levels are often achieved using such techniques.

In this paper, a new automated solid phase extraction (SPE) based procedure for the determination of ketorolac in human plasma is described. The method uses ketoprofen (KP) as internal standard and combines minimal sample preparation with the lowest reported limit of quantitation.

MATERIALS

Chemicals and Reagents

KT was purchased from Chemo Ibérica (Barcelona, Spain), KP was obtained from Sigma (St. Louis, MO, USA) and was used as internal standard.

Sodium chloride, sodium acetate and acetic acid (reagent grade) were supplied by Merck (Darmstadt, Germany). Methanol and acetonitrile (for HPLC) were obtained from Scharlau (Barcelona, Spain). All HPLC and SPE solvents were filtered through 0.5 μm Millipore filters and thoroughly degassed in an ultrasonic bath before use. Sodium heparinate (161.4 USP heparin units/mg) was obtained from Kraeber (Hamburg, Germany). Water was purified through a Milli-Q system (18 MΩ cm resistivity).

Instrumentation

Sample injections and SPE were made with a Prospekt (Programmable On-line Solid-Phase Extraction Technique) system (Spark Holland, Emmen, Netherlands). The complete system consisted of a solvent delivery unit (SDU) with a purge pump and a six-port solvent selection valve, an autosampler (Marathon) which was cooled at 4°C, and a programmable on-line sample preparation module (the main Prospekt SPE controller unit). Chromatographic separations were performed using Waters HPLC equipment (Waters, Mildford, MA, USA) consisting of a M-600 pump, a M-486 U.V. detector, a RCM 8 x 10 radial compression module and a M-845 data and chromatography control station using Waters ExpertEase Chromatography software (V.3.0).

METHODS

Standard Preparation

Stock solutions of KT and the internal standard KP were prepared weekly in methanol at a concentration of 1 mg/mL. Working standard solutions (0.1 to 200 μg/mL for KT and 10 μg/mL for KP) were prepared daily by dilution of stock solutions with methanol:water (1:9) mixture.

Plasma Collection and Sample Preparation

Human blood was obtained from healthy donors into heparinized tubes (0.75 mg sodium heparinate/mL blood). Plasma was obtained by blood

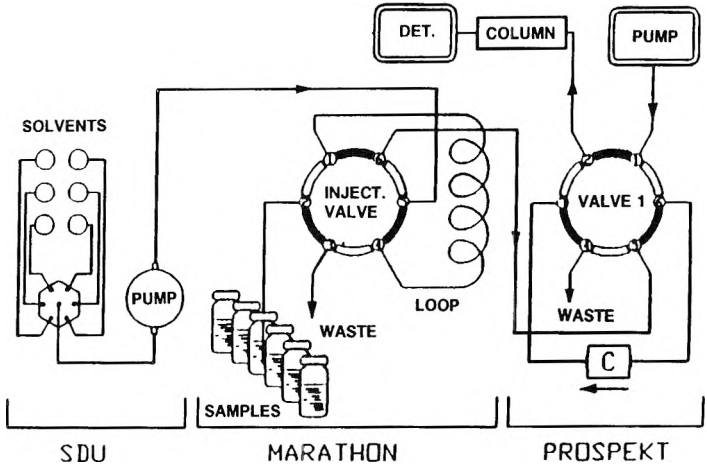

Figure 1. Schematic diagram of the SPE-HPLC system.

centrifugation at 2000 x g (4°C) for 15 minutes. After centrifugation, plasma was pooled and stored frozen at -80°C in polypropylene tubes until use.

Triplicate sets of standard calibration curves of KT in human plasma were prepared on three different days for inter-assay method validation. Intra-assay validation data was obtained using six calibration curves, all prepared on the same day. Sample preparation was performed as follows: 500 µl aliquots of pooled blank plasma were placed in 1.2 mL autosampler glass vials. 550 µl of NaCl 0.9% were further added and the mixture was vigorously vortexed. Finally, 25 µl aliquots of KT and KP working standard solutions were added to obtain the desired final plasma concentrations. Samples were analyzed on the same day of preparation.

Solid Phase Extraction and Chromatography

Automated SPE from spiked plasma samples was performed on the Prospekt system which was switched on-line to the liquid chromatograph. Samples were automatically loaded on disposable cartridges, purged with the appropriate solvents for clean-up and were subsequently eluted to the HPLC system. The configuration of the whole SPE-HPLC system is illustrated in Figure 1.

Extraction procedure was conducted as follows: Solid phase C-18 disposable cartridges (10 x 3 mm I.D., Analytichem) were first conditioned with 2 mL of methanol (solvent 1) followed by 2 mL of Milli-Q water (solvent 2) and 2 mL of 50 mM sodium acetate pH 3.5 (solvent 3). All solvents were flushed at a flow rate of 2 mL/min. Sample loading was achieved by switching the sample loop valve and allowing the on line conection of the SDU with the C-18 cartridge through the sample loop. Sample (1 mL) was then directed to the cartridge at a flow rate of 1 mL/min solvent 3 during 2 minutes. For sample clean-up, 1.5 mL of methanol:0.1% acetic acid (20:80) mixture were flushed through the cartridge at a flow rate of 1.5 mL/min. Elution was performed by on-line connection of C-18 cartridge with the HPLC system by means of the switching valve (valve 1, Figure 1).

Chromatographic separations were carried out using a Nova-pak C-18 radial pak cartridge (10 x 0.8 cm, 4 μm, Waters) coupled to a Newguard RP-18 cartridge guard column (15 x 3.2 mm, 7 μm, Applied Biosystems, San Jose, CA, USA). The mobile phase consisted of acetonitrile (solvent A) and 0.1% acetic acid (solvent B). A linear gradient program was used from 30% to 60% solvent A in 10 min, 60% solvent A was maintained for 2 minutes and increased to 100% in 3 minutes. Flow rate was set at 2 mL/min. UV detection was performed at 313 nm from 0 to 7.2 min and at 258 nm from 7.2 min to the end of the analysis.

Quantitation

Quantitation was performed by the internal standard method. For calibration graphs the peak area ratio of KT/KP were plotted versus known concentrations of KT. Data were fitted by weighed least-squares linear regression using the reciprocal of the square concentration values as the weighing factor. A weighed least-squares regression was used because of the clear improvement in the precision and accuracy for the back calculated values, mainly at low concentration levels. Data were automatically processed with ExpertEase Chromatography Software (V.3.0).

Linearity was assessed by comparing the obtained values after peak area ratio normalization by the corresponding KT effective concentration.

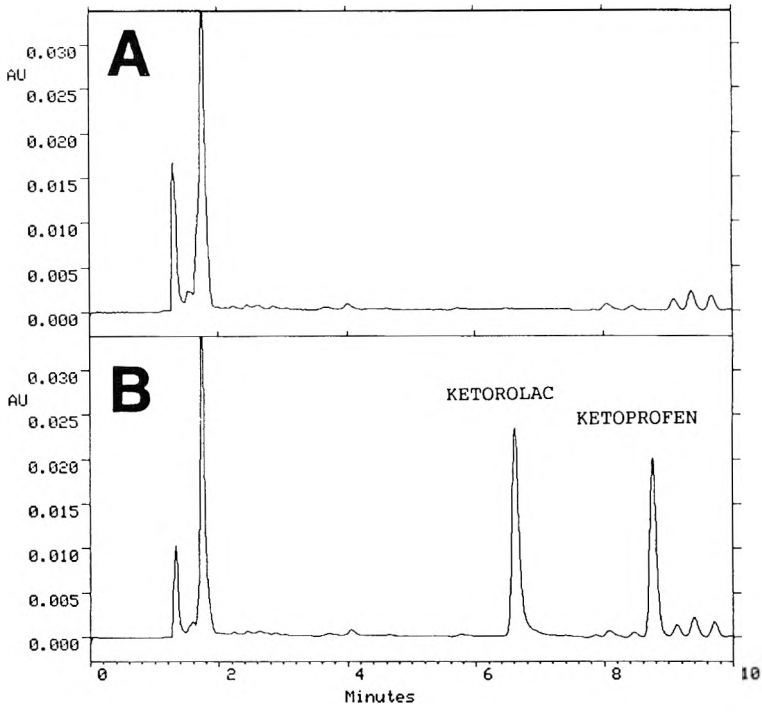

Figure 2. Representative chromatographic profiles of: (A), human blank plasma sample and (B), plasma sample spiked with 500 ng/mL KT and 250 ng/mL KP (internal standard).

Recovery, Accuracy and Precision

To estimate the recovery of the analytical procedure, stock standard solutions of KT and KP were diluted with methanol:water (1:9) mixture and were directly injected into the HPLC system (reference solutions, injection volume of 100 µl). Dilutions were properly corrected to ensure that the same total standard amounts were injected into the HPLC system when compared with those introduced to the C-18 cartridges from spiked plasma samples. The absolute recoveries were established by comparing the absolute peak areas for spiked plasma samples after the extraction procedure with those of the reference solutions. Mean recovery values were obtained from duplicate sets of calibration standard samples prepared each day of the validation process.

Intra- and inter-assay precision and accuracy were determined at seven concentration levels of KT (5, 10, 25, 50, 250, 500 and 2500 ng/mL plasma). For this purpose, the first analyzed set of standard spiked plasma samples was used to construct the calibration graph. The remaining samples were analyzed as unknowns and KT concentrations determined from these samples against the initially obtained calibration curve. Precision was expressed as coefficient of variation (C.V.) and accuracy as relative error (R.E.), both in percentage.

Statistics

One way analysis of variance was used to perform comparisons among the different calculated parameters at the concentration levels tested.

RESULTS AND DISCUSSION

HPLC chromatographic profiles for human blank plasma and plasma spiked with KT and KP (internal standard) are depicted in Figures 2-A and B respectively. As it is shown in Figure 2-B, both peaks were well resolved. No endogenous interfering peaks were found in blank plasma samples at their retention times (Figure 2-A) indicating a good selectivity for the chromatographic method. UV detection for KT was performed at 313 nm whereas wavelength was changed to 258 nm for KP detection due to the low molar absorptivity of this compound at 313 nm. This mechanism prevented the use of higher concentrations of internal standard in plasma samples which could presumibly influence the solid phase extraction process.

The linearity of the analytical procedure was assessed in the concentration range 5-2500 ng/mL KT. For this purpose KT/KP peak area ratios were normalized by KT effective concentration (Table 1). Comparison by means of analysis of variance showed significant differences ($p < 0.05$) among the normalized ratios. These differences were further attributed (Newman-Keul's multiple comparison test) to the higher responses obtained at the lower KT concentrations (5 and 10 ng/mL). However, the use of weighed linear regression in calibration curve fitting allowed accurate and precise measurements at these concentration levels (see Tables 2 and 3). For twelve

calibration graphs the obtained mean regression coefficient and slope values were r = 0.9986 (0.08% C.V.) and b = 0.003470 (2.2% C.V.) respectively.

Table 1

Normalized Response and Recovery for Ketorolac and Ketoprofen from Spiked Plasma (n=12)

Effective Ketorolac Tromethamine (ng/mL)	Normalized Response Ratio ($x10^3$)	Recovery of Ketorolac (%)	Recovery of Ketoprofen (%)
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
5	4.16 ± 0.52	92.2 ± 26.46	75.0 ± 4.14
10	4.03 ± 0.24	90.9 ± 7.52	72.3 ± 2.45
25	3.53 ± 0.22	85.3 ± 4.60	73.1 ± 3.95
50	3.50 ± 0.09	84.4 ± 3.18	72.6 ± 3.75
250	3.52 ± 0.06	87.1 ± 6.49	71.2 ± 2.19
500	3.47 ± 0.09	85.5 ± 5.31	72.4 ± 3.64
2500	3.49 ± 0.10	88.4 ± 5.33	73.8 ± 2.45
Overall		87.7 ± 8.41	72.9 ± 3.22

The recovery of the analytical procedure for KT was calculated at the entire concentration range tested, whereas recovery for KP was estimated at the working concentration of 250 ng/mL plasma. Results are summarized in Table 1. No statistically significant differences ($p < 0.05$) were found among recovery values obtained at the different KT concentration levels, which ranged from 84.4% at 50 ng/mL to 92.2% at 5 ng/mL, the mean overall value being 87.7 ± 8.41. Likewise, no statistically significant differences ($p > 0.05$) concerning the recovery of the internal standard were found among the concentration levels of KT indicating that KP recovery was unaffected by the presence of increasing amounts of KT in the plasma samples. Recovery for KP ranged from 71.2% to 75.0%. The mean overall recovery for KP was 72.9 ± 3.22.

Precision and accuracy for the determination of KT were calculated from the measured concentration data obtained in replicate calibration curves as described in material and methods. Results are shown in Table 2. Intra-assay precision values ranged from 1.0% at 250 ng/mL to 7.2% at 5 ng/mL

Table 2

Intra- and Inter-Assay Precision and Accuracy for Ketorolac

Effective KT Concentration (ng/mL)	Concentration Found (ng/mL)	C.V. (%)	R.E. (%)
Intra-assay (n= 5)			
5	5.5	7.2	9.5
10	10.8	2.1	7.7
25	23.8	3.6	-4.8
50	49.6	2.8	-0.7
250	254.6	1.0	1.8
500	512.5	1.2	2.5
2500	2582.9	2.7	3.3
Inter-assay (n = 6)			
5	5.3	18.0	5.8
10	10.9	10.6	8.8
25	25.2	7.7	0.7
50	49.8	3.6	-0.4
250	253.7	2.6	1.5
500	503.1	2.6	0.6
2500	2529.4	5.1	1.2

whereas intra-assay accuracy ranged from -4.8% at 25 ng/mL to 9.5% at 5 ng/mL. Overall precision and accuracy were also estimated for the three days of analysis (inter-assay), in this case, the coefficients of variation ranged from 2.6% at 250 and 500 ng/mL to 18.0% at 5 ng/mL, and the relative errors ranged from -0.4% at 50 ng/mL to 8.8% at 10 ng/mL (Table 2).

To evaluate the goodness of the calibration curve fitting, back-calculated concentration values for KT were obtained from each calibration graph used during method validation. The obtained mean back-calculated values, as well as the precision and accuracy estimations for each KT concentration level, are summarized in Table 3. Precision and accuracy were always below 5.3% at the entire range of concentrations tested.

Table 3

Precision and Accuracy of Back-Calculated Values from Calibration Curves for Ketorolac (n = 12)

Effective KT Concentration (ng/mL)	Concentration Found (ng/mL)	C.V. (%)	R.E. (%)
5	4.9	2.9	1.9
10	10.5	4.1	5.2
25	24.2	5.3	2.9
50	49.1	2.2	1.6
250	252.1	1.1	1.0
500	499.1	2.9	0.4
2500	2515.6	3.5	0.6

In conclusion, the method described here allows rapid and fully automated extraction and chromatography of ketorolac from human plasma samples, with minimal sample preparation (virtually only plasma dilution and internal standard addition is required). Minimal sample handling as well as the fully automation of the extraction process made this technique specially useful for routine analysis and, probably, would justify the good accuracy and reproducibility obtained. The experimental limit of quantitation for KT was set at 5 ng/mL (3.4 ng/mL in terms of ketorolac free acid form) taking into account the acceptable precision obtained at this concentration (7.2% and 18.0% for intra- and inter-assay C.V. respectively). This limit of quantitation provides enough sensitivity to follow thoroughly the plasma levels of ketorolac for pharmacokinetic and biopharmaceutical studies. The high sensitivity of the proposed methodology could be useful to measure the low plasma levels obtained after the screening of some transdermal formulations where plasma levels below 92 ng/mL (C_{max} value) have been recently reported.[10]

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BAND BROADENING IN MICELLAR LIQUID CHROMATOGRAPHY

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ABSTRACT

The effect of temperature on efficiency in micellar liquid chromatography (MLC) has been investigated using an SDS micellar mobile phase and a C_{18} stationary phase. Application of the Knox equation to plate count data yielded crucial information about band broadening in MLC. The improvement in chromatographic efficiency with temperature is due to a decrease in both the A (flow anisotropy) and C (stationary phase mass transfer) terms of the Knox equation. The decrease in the A term can be attributed to a shift in the position of the equilibrium of the solute away from the micelle and towards the bulk solvent; the decrease in the C term with temperature can be explained in terms of surfactant adsorption which tends to increase both the thickness and viscosity of the stationary phase. By increasing the operating temperature of the column, less surfactant is adsorbed on the stationary phase.

Due to concerns about the validity of the Knox equation to describe band broadening in MLC, it was necessary to consider other studies of micellar mobile phases in relation to their role in MLC, in order to obtain physically meaningful values for the A,

B, and C terms of the Knox equation. When the different equilibria involving the micelle and surfactant were taken into account, the low rate of surfactant desorption could not be ignored as the underlying cause of band broadening in MLC. If the desorption rate of surfactant molecules on an alkyl bonded phase is too low, the result is a disturbance in the various equilibria involving the micelle and surfactant monomer. This disturbance would explain the continued adsorption of SDS on the stationary phase at concentrations in excess of the critical micelle concentration of the surfactant. The low rate of surfactant desorption would also affect the dynamics of micellization which play a crucial role in MLC mobile phase mass transfer.

INTRODUCTION

In 1980, Armstrong and Henry[1] first demonstrated that aqueous micellar solutions can be used as mobile phases in reverse phase liquid chromatography (RPLC). They called this technique pseudophase or micellar liquid chromatography (MLC). Since the first report by Armstrong and Henry, the potential applications and unique capabilities of MLC have been investigated. More than one hundred papers to date including several review articles and a symposium series volume[2-7] have been published on the unique advantages of MLC.

Clearly, micellar mobile phases have certain advantages over traditional hydro-organic mobile phases in RPLC, e.g, direct injection of biologicals, resolution of optical isomers via chiral micelles, and unusual selectivity to name a few. However, there is a problem with MLC - it tends to be less efficient than conventional RPLC.

Dorsey et al[8] were the first to address this problem. They believed the reduction in column efficiency was due to slow mass transfer, which arises principally from poor wetting of the stationary phase. Dorsey demonstrated that chromatographic efficiency in MLC can be improved by adding a small amount of propanol, 3% by volume, to the mobile phase. Yarmchuk and Cline-Love,[9] on the other hand, attributed the reduced efficiency associated with ionic micellar mobile phases to poor mass transfer between the micelle and the stationary phase, with the micelle exit rate constant being the limiting factor for

hydrophobic solutes. Borgerding and Hinze[10] concluded that poor mass transfer within the stationary phase itself, resulting from adsorption of surfactant onto the alkyl bonded phase, is responsible for the low efficiencies observed in MLC. They demonstrated that addition of an alcohol, such as isopropanol, to a nonionic micellar solution reduces the amount of surfactant adsorbed on the stationary phase, resulting in a more efficient separation. In contrast to what has been reported by other workers, Cassidy[11] in a recent study on band broadening in MLC concluded that improvement in solute mass transfer which can occur upon addition of propanol to an SDS micellar solution is due to changes in the structure of the micelles, not mass transfer effects related to the loading of surfactant on the bonded phase.

Clearly, there is disagreement among workers concerning the reason for the low efficiencies evidenced in MLC. While the addition of a medium chain length alcohol such as propanol to a micellar mobile phase has been shown to improve column efficiency significantly, the presence of an alcohol in the mobile phase can affect the retention mechanism by shifting the equilibrium of the solute away from the stationary phase and the micelle and toward the bulk aqueous phase.[12] In addition, the added organic modifier can greatly complicate the interpretation of plate count data because the properties of the micelles in these so-called hybrid mobile phases are also influenced by the presence of the alcohol,[13-14] but it is not clear to what extent. Hence, there is a limit to the information that can be garnered about the underlying cause of the reduced efficiencies in MLC from experiments involving alcohol containing micellar solutions.

Therefore, a different approach, varying the operating temperature of the column, was employed in this study to better understand the causes of band broadening in MLC. Yarmchuk and Cline-Love in 1984[9] showed that increasing the operating temperature of the column in micellar RPLC enhances the efficiency of the separation process in MLC. However, Yarmchuk's study was limited to the C_1 alkyl bonded phase. Unlike a C_{18} column, the efficiency of a C_1 column does not improve significantly with the addition of an alcohol such as propanol to the micellar mobile phase. Furthermore, the unusual selectivity exhibited by C_1 bonded phase columns towards ionogenic solutes in MLC is well known to many workers in the field of micellar RPLC.[15-16] For example, the retention time of some ionogenic compounds on C_1 bonded phase columns actually increases with increasing micelle concentration, which is opposite of what is considered normal retention behavior in MLC. This effect will occur with compounds that possess the same charge as the surfactant and is

considered to be an excluded volume effect, because the compound is excluded not only from the micelle, but from the double layer that surrounds the micelle. Since this effect is not observed with C_{18} and C_8 alkyl bonded phases, the so-called antibinding behavior probably occurs as the result of a different form of surfactant monomer association with the methyl bonded phase, which implies that C_{18} and C_1 bonded phase columns interact very differently with ionic surfactants. Although some workers[15-16] have argued that antibinding behavior occurs on C_1 columns as a result of the methyl stationary phase not adsorbing appreciable amounts of surfactant, Berthod[17] has in fact shown that ionic surfactants, such as SDS and CTAB, exhibit maximum adsorption on C_1, not C_{18} or C_8 alkyl bonded phases.

Because the effect of temperature on surfactant adsorption and micellar structure[18-20] is reasonably well understood, we believe that re-examining the relationship between column temperature and efficiency in MLC with a C_{18} bonded phase column can yield additional information and insight into the causes of band broadening in MLC for stationary phases with serious wetting problems. In this paper, we present the results of a comprehensive study on the effects of temperature, and flow rate on efficiency in MLC using an SDS micellar mobile phase and a C_{18} alkyl bonded stationary phase.

EXPERIMENTAL

1. HPLC System

All high performance liquid chromatographic (HPLC) measurements were made with a Rainin 81-20 M analytical HPLC system which incorporated two Rainin Rabbit HP pumps (Rainin Instruments, Woburn, MA), an Apple MacIntosh computer as the controller and data station, a Model 7125 Rheodyne injection valve with a 30 μL loop (Cotati, CA), and a Rainin Dynamax Mixer. The detector was a variable wavelength Knauer UV/Visible spectrometer (West Berlin, Germany). The extra column volume of the system was less than 60 μL.

The analytical column was an Apex I 5-μm octyldecyldimethyl silane ODS (100 mm x 4.6 mm, Jones Chromatography, Lakewood, CO). A silica guard column placed between the injector and the pump saturated the mobile phase with silicates, minimizing dissolution of the column packing. Both the

analytical column and mobile phase reservoir were water-jacketed and temperature controlled with a Haake (Berlin, Germany) circulator. The dead volume of the column was determined by injecting different solutions such as methanol, methanol-water, or water onto the columns. Dead volume measurements obtained for micellar mobile phases were comparable to the values obtained for methanol-water mobile phases. This volume, approximately 1.00 mL, was used in all k' calculations. The k' values reported in this study were averages of at least triplicate determinations.

2. Materials

Sodium dodecyl sulfate (SDS) was obtained from BDH Chemicals and was purified prior to use by first dissolving it in ethanol followed by addition of charcoal to the solution. The charcoal was then separated from the mother liquor via filtration, and the SDS was recrystallized from the mother liquor. The test solutes which were obtained from Aldrich and Sigma were used as received. Stock solutions of the test solutes were prepared in methanol and diluted to the appropriate working concentration with 0.05 M SDS for the micellar system or 30% methanol in water for the hydro-organic system. Working concentrations of injected solutes were; acetophenone (57 μg/mL), benzene (40 μg/mL), nitrobenzene (55 μg/mL), methylbenzoate (65 μg/mL), toluene (45 μg/mL), p-nitrophenol (60 μg/mL), phenol (45 μg/mL), and coumarin (30 μg/mL).

The SDS micellar mobile phase solutions were prepared by dissolving the appropriate amount of surfactant in HPLC grade water and filtering the solution twice with a 0.45 μm Nylon membrane filter. All mobile phase solutions were prepared using HPLC grade solvents which were degassed prior to use. pH measurements were made on these solutions using a Chem Trix pH meter. The pH of each solution was approximately 7.3. For the test solute p-nitrophenol (pKa = 7.1), the pH of the mobile phase was adjusted to 3.0 prior to use to prevent deprotonation of the phenol.

3. Procedure

The analytical column was not considered to be equilibrated with the mobile phase unless the retention times were constant. Chromatograms were obtained with mobile phase flow rates varying from 0.1 mL to 3.5 mL/minute.

Flow rates were measured by collecting the effluent in a 10 mL graduated cylinder for a sufficient length of time to ensure collection of at least 7 mL. The effect of temperature on efficiency in MLC and conventional RPLC was investigated using the following mobile phases: 0.02M SDS, 0.05M SDS, and a solution of 30% methanol in water. The Foley-Dorsey method[21] was used to compute the number of theoretical plates . Although there are many methods available for the calculation of chromatographic efficiency, Bildingmeyer and Warren[22] have shown that it is the most accurate manual method for plate count calculation. Berthod[23] also found this to be true. Because the Foley-Dorsey equation for plate count corrects for the asymmetry in skewed peaks, reliable chromatographic figures of merit can be obtained from tailing peaks using this method.

The Knox equation[24] was used in this study to assess the contributions of flow anisotropy, longitudinal diffusion, and other band broadening processes to the final peak bandwidth. This equation is the most widely accepted plate height equation in chromatography and can be expressed in the following form

$$h = Av^{1/3} + B/v + Cv \qquad (1)$$

where A, B, and C are the constants of the Knox equation, h is the reduced plate height ($h = H/d_p$, where d_p is the stationary phase particle diameter, and H is the column plate height), and v is the reduced mobile phase velocity ($v = ud_p/D_m$, where u is the mobile phase linear velocity (cm/sec) and D_m is the solute mobile phase diffusion coefficient). H was computed from the plate count data as an average of triplicate determinations. Values for the A, B, and C terms of the Knox equation were obtained by a nonlinear least squares fitting of the data using the Levenburg-Marquardt algorithm. Each regression analysis was examined by influence statistics to verify the fidelity of the results which is a legitimate concern when analyzing noisy collinear data.

RESULTS AND DISCUSSION

A series of chromatograms were run to illustrate the advantages of higher operating temperatures for micellar mobile phases. Figure 1 shows the separation of a five-component test mixture on a C_{18} column using a 30% methanol/70% water mobile phase. Figure 2 shows the separation of the same test mixture using a 0.05M SDS mobile phase at 25^0 and 45^0 C. For the 0.05M SDS mobile phase, increasing the operating temperature of the system

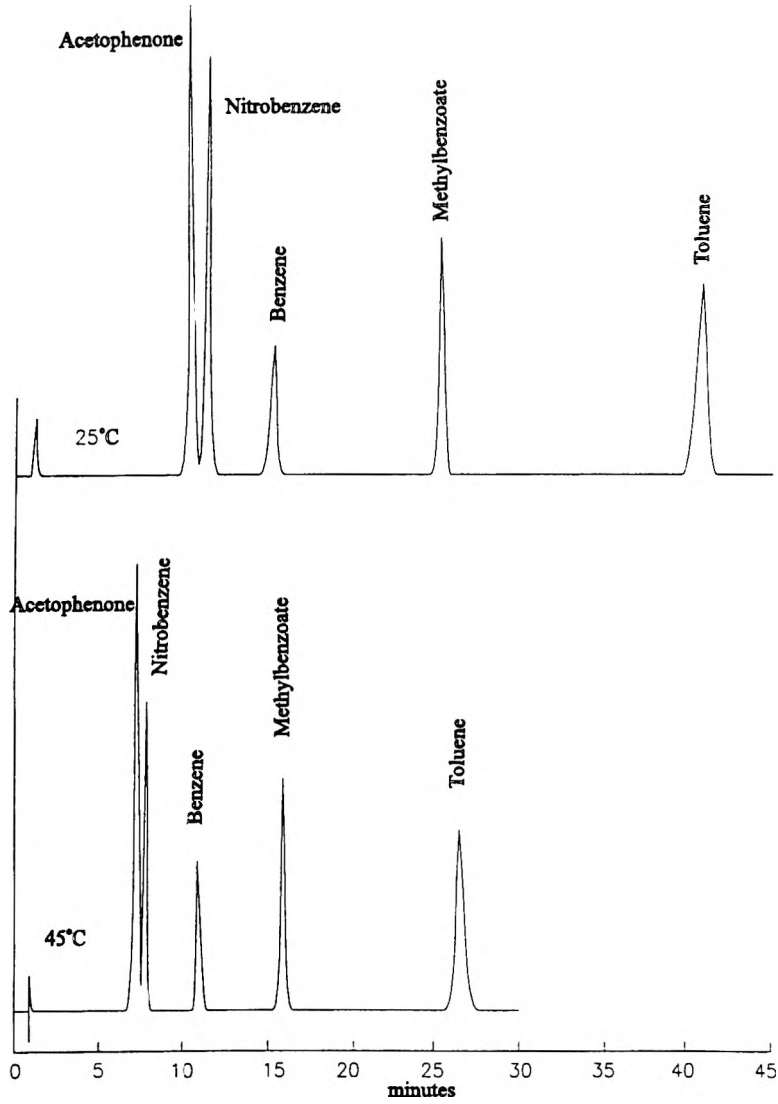

Figure 1. Chromatograms of the test mixture at 25^0 and 45^0 C for the 30% methanol in water mobile phase. An Apex I C-18 column (10 cm, 5micron particle size) was used; the flow rate of the mobile phase was 1.0 mL/min. A = nitrobenzene, B = acetophenone, C = Benzene, D = methylbenzoate, and E = toluene.

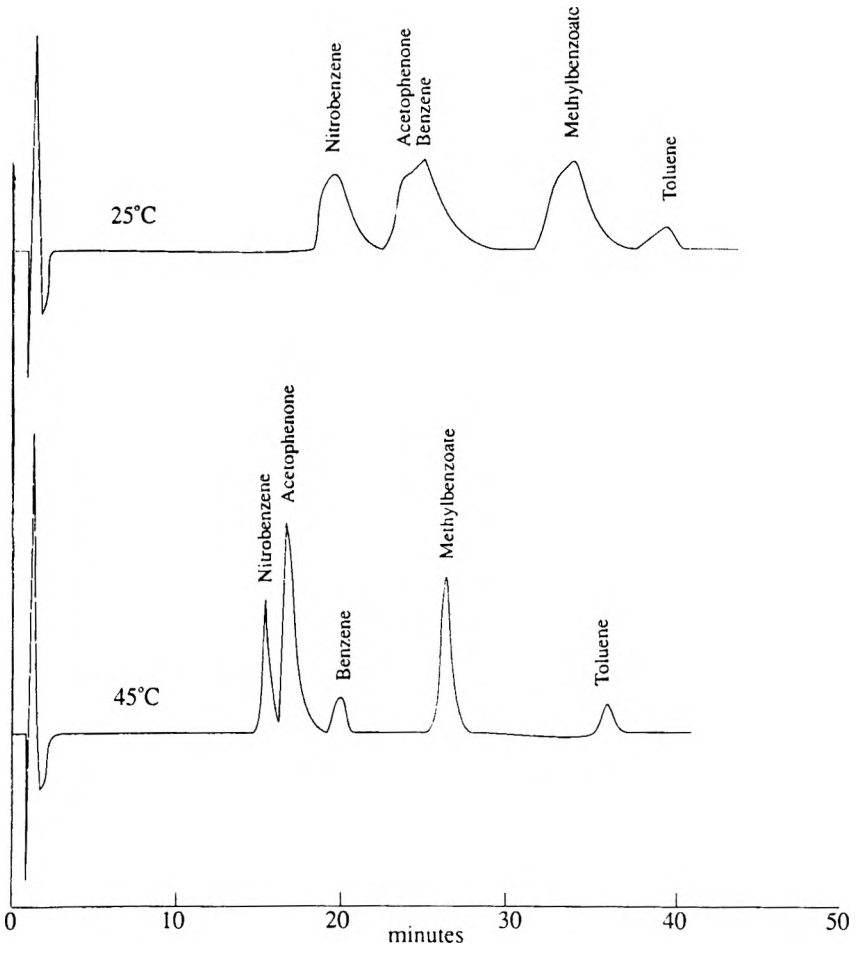

Figure 2. Chromatograms of the test mixture at 25^0 and 45^0C for a 0.05M SDS mobile phase. A = nitrobenzene, B = acetophenone, C = Benzene, D = methyl benzoate, and E = toluene.

improved both the resolution and efficiency of the separation. For the methanol in water mobile phase, there was little improvement in either resolution or efficiency.

In a simple reversed phase separation, there is initially a modest increase in efficiency due to a lowering of the mobile phase viscosity as the

operating temperature is increased. However, heat dissipation[25] is a problem in conventional reversed phase columns (e.g., particle size 5 microns, 4.6 mm id, pressure drop of a few hundred bar, flow rates of 2mL/min or greater). The inner core of the packed bed can be warmer than the wall region by 0.5 to several degrees Centigrade, with the overall result that a radial temperature profile is generated within the column which can cause non-uniformity in the migration velocity of the solute. Hence, at very high column temperatures (ca. 55 degrees Centigrade), N levels off and then decreases because of thermal gradients within the column.[26]

Table 1

Variation of Efficiency and Asymmetry with Temperature for the Test Mixture *Solutes. The Mobile Phase was 30% Methanol in Water.

	25°C		35°C		45°C		55°C		65°C		**73°C	
Compounds	**N**	**B/A**	**N**	**B/A**	**N**	**B/A**	**N**	**B/A**	**N**	**B/A**	**N**	**B/A**
Nitrobenzene	3900 (k'=10.4)	1.2	4300 (k'=7.9)	1.3	4200 (k'=6.5)	1.3	4700 (k'=5.4)	1.2	3900 (k'=4.6)	1.3	3200 (k'=3.9)	1.4
Acetophenone	3500 (k'=9.2)	1.3	4200 (k'=7.2)	1.1	4100 (k'=5.9)	1.2	4800 (k'=5.0)	1.2	3200 (k'=4.2)	1.2	2800 (k'=3.6)	1.3
Benzene	4100 (k'=14.5)	1.3	4500 (k'=11.7)	1.0	4800 (k'=9.8)	1.0	5100 (k'=8.3)	1.0	5000 (k'=7.0)	1.1	----------	
Methylbenzoate	4800 (k'=25.2)	1.1	4700 (k'=18.5)	1.3	4100 (k'=14.6)	1.2	5300 (k'=11.7)	1.3	4600 (k'=9.5)	1.2	----------	
Toluene	5100 (k'=40.9)	1.1	4900 (k'=31.8)	1.0	5100 (k'=25.5)	1.0	5400 (k'=20.8)	1.1	4900 (k'=17.0)	1.0	4700 (k'=14.5)	1.0
***p-Nitrophenol	4300 (k'=11.4)	1.3	4000 (k'=8.0)	1.3	4200 (k'=6.1)	1.2	4000 (k'=4.7)	1.3	3000 (k'=3.6)	1.4	----------	

*ODS column

**Only a few experiments were performed at 73°C because of our concern about damage to hydrolysis of the bonded phase.

***For p-nitrophenol a 20% methanol in water mobile phase with the pH adjusted to 3.0 was used because the capacity factor value of the arene was less than 5 in 30% methanol in water at 25°C.

The relationship between temperature and efficiency for the hydro-organic mobile phase (see Table 1) adheres to the simple model described above. However, the large increase in efficiency with temperature for the micellar mobile phase (see Table 2), which is greater than 100% for most of the solutes chromatographed, cannot be explained by this model, which suggests that different processes are responsible for band broadening in MLC and are also responsible for the dramatic improvement in efficiency at elevated temperatures in SDS micelle mediated liquid chromatography.

Table 2

Variation of Efficiency and Asymmetry with Temperature for the Test Mixture *Solutes. The Mobile Phase was 0.05M SDS.

	25°C		35°C		45°C		55°C		65°C		**73°C	
Compounds	**N**	**B/A**	**N**	**B/A**	**N**	**B/A**	**N**	**B/A**	**N**	**B/A**	**N**	**B/A**
Nitrobenzene	310	3.5	900	2.4	1600	2.8	2300	2.4	2700	2.0	4200	1.1
	(k'=15.8)		(k'=13.6)		(k'=12.2)		(k'=10.7)		(k'=9.3)		(k'=8.9)	
Acetophenone	280	2.5	400	3.4	980	2.4	1800	1.0	2100	1.0	3300	1.5
	(k'=19.4)		(k'=16.0)		(k'=13.6)		(k'=11.2)		(k'=9.8)		(k'=9.5)	
Benzene	1300	2.0	1800	1.3	3300	1.6	4500	1.4	4500	1.2	----------	
	(k'=17.8)		(k'=17.0)		(k'=16.3)		(k'=15.2)		(k'=13.7)			
Methylbenzoate	570	2.1	900	2.4	2000	2.6	2800	2.7	4700	1.2	----------	
	(k'=25.9)		(k'=24.1)		(k'=21.4)		(k'=19.1)		(k'=16.8)			
Toluene	2000	1.8	2500	1.7	3100	1.0	3300	1.0	3900	1.1	5900	1.1
	(k'=33.0)		(k'=31.6)		(k'=30.6)		(k'=28.4)		(k'=26.1)		(k'=25.5)	
***p-Nitrophenol	2600	2.1	3300	2.2	4900	1.2	4500	1.4	5300	1.2	----------	
	(k'=9.2)		(k'=8.7)		(k'=7.6)		(k'=5.8)		(k'=4.9)			

*ODS column

**Only a few experiments were performed at 73°C because of our concern about damage to the column due to hydrolysis of the bonded phase.

***For p-nitrophenol, a 0.02M SDS solution with the pH adjusted to 3.0 was used as the mobile phase because the k' value of the arene was less than 5 with 0.05M SDS at 25°C. On some C_{18} columns, p-nitrohphenol exhibited a split peak with the 0.02M SDS mobile phase at column temperatures in excess of 35°C. Evidently, p-nitrophenol is sensitive to changes in the structure of the C_{18} which occur as a consequence of hydrolysis of the bonded phase, a process catalyzed by elevated temperature and low pH.

1. Knox Plots

To better understand the nature of the relationship between temperature and efficiency in MLC, the Knox equation was used to study band broadening at different temperatures for two micellar mobile phase, a 0.02M SDS and a 0.05M SDS. Because of concern for the effect of viscous heat dissipation which can obscure the interpretation of HETP curves at temperatures above 45 degrees Centigrade,[26] only solutes which exhibited large increases in N with temperature, at or below 45 degrees Centigrade, were used in the Knox plot studies. The diffusion coefficient (D) of each test solute (see Table 3) was obtained from the literature[27,28] and represented a weighted average of free and micelle bound solubilizate, since the Taylor dispersion technique[29] was used in these referenced studies to determine D.

Table 3

Diffusion Coefficients of Selected Arenes.

Solute	Mobile Phase	Diffusion Coefficient (x 10^6 cm^2/sec)		
		25^0C	35^0C*	45^0C*
p-nitrophenol	0.02M SDS	4.35**	5.56	6.93
Benzene	0.05M SDS	6.80***	8.70	10.8

*The diffusion coefficient (D) at 35 and 45 degrees Centigrade was computed using the value of D at 25^0C and Waldens rule: $D_x = D_{298}$ $[T_x/T_{298}]$ $[e_{298}/e_x]$, where x is equal to 308^0K (35^0C) or 318^0K (45^0C) and e is the viscosity of the bulk solvent, water, obtained from the CRC Handbook.

**from reference 22. The Taylor dispersion method was used to measure the diffusion coefficient of p-nitrophenol in 0.02M SDS.

***from reference 23. The Taylor dispersion method was used to measure the diffusion coefficient of benzene in 0.05M SDS.

Figure 3 is a plot of reduced plate height versus reduced mobile phase velocity for p-nitrophenol at 25^0, 35^0, and 45^0 C on a C_{18} column with a 0.02M SDS mobile phase at pH=3. Table 4 lists the A, B, and C parameters of the Knox equation at these three temperatures. The noticeable improvement in

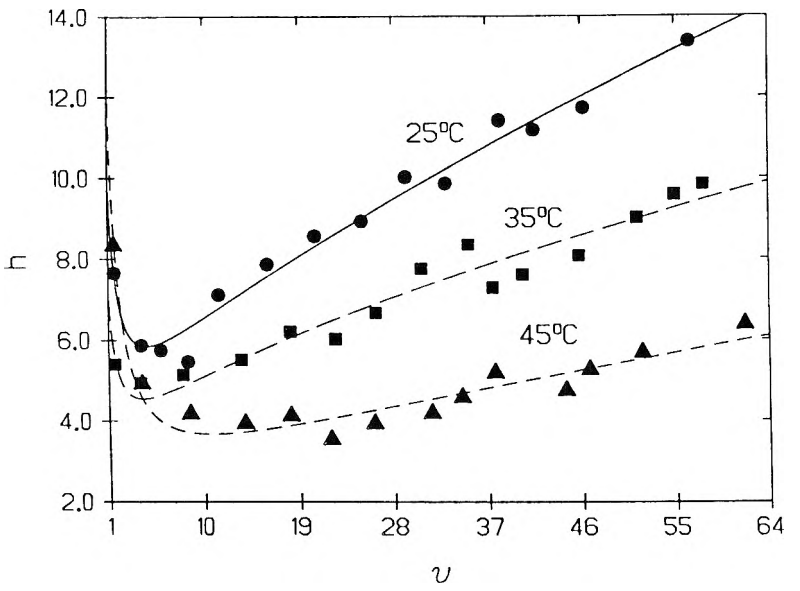

Figure 3. Reduced plate height versus reduced mobile phase velocity for p-nitrophenol on C-18 with a 0.02M SDS mobile phase at 25°, 35°, and 45°C. Each data point in the plot is an average of triplicate determinations.

chromatographic efficiency with temperature is due to a decrease in both the A (flow anisotropy) and C (stationary phase mass transfer) terms of the Knox equation. By comparison, in conventional RPLC there is generally an increase in the C term with temperature,[30] which further reinforces the conclusion that different processes are responsible for band broadening in MLC.

The decrease in the A term with temperature for p-nitrophenol can be explained by the change in the equilibrium constant for the solute between the micellar aggregate and the bulk solvent, i.e., K_2.[12] As temperature is increased, K_2 decreases, and the distribution of solute between the micelle and bulk solvent is altered. The result is that less solute is bound to the SDS micelle in the mobile phase as the operating temperature of the column is increased. Since it is easier for an arene to transfer between different solvent flow streams within the column when it exists as free solute in water instead of a bound species, the net result is that a decrease in eddy diffusion occurs which in all likelihood is responsible for the decrease in the A term of p-nitrophenol.

Table 4

Knox Equation Parameters* for p-Nitrophenol and Benzene at Various Temperatures for a 0.02M and 0.05M SDS Mobile Phase.

p-Nitrophenol (0.02M SDS)

Temperature	A	B	C	R^2
25°C	2.34(±0.14)	7.43(±0.69)	0.074(±.013)	0.99
35°C	1.96(±0.14)	5.16(±0.66)	0.030(±.011)	0.99
45°C	1.06(±0.13)	11.4(±0.65)	0.026(±.010)	0.99

Benzene (0.05M SDS)

Temperature	A	B	C	R^2
25°C	3.4(±1.1)	5.57(±2.2)	0.16(±0.22)	0.93
45°C	2.7(±0.23)	11.4(±0.54)	0.05(±.04)	0.99

*The uncertainty in A, B, and C was determined from the statistical parameters of the least squares fitting.

The decrease in the C term with temperature for p-nitrophenol can be explained in terms of surfactant adsorption which tends to increase both the thickness and viscosity of the stationary phase.[31-33] By increasing the operating temperature of the column and mobile phase, less surfactant is adsorbed on the stationary phase.[34] The rate of solute mass transfer between the mobile and stationary phase is also increased. In addition, the fluidity of the tethered alkyl chains and imbibed surfactant should increase with temperature which will also lessen stationary phase mass transfer resistance. The overall result is an improvement in column efficiency.

The large increase in the B term of the Knox equation with temperature (from 25°C vs 45°C) is due to a decrease in the viscosity of the bulk solvent. There is also a shift in the position of the equilibrium of the solute away from the micelle and towards the bulk solvent[9] as temperature is increased, also resulting in an increase in the diffusion rate of the solute because the rate of molecular diffusion is greater for free solubilizate than bound solubilizate. However, the decrease in the value of the B term of p-

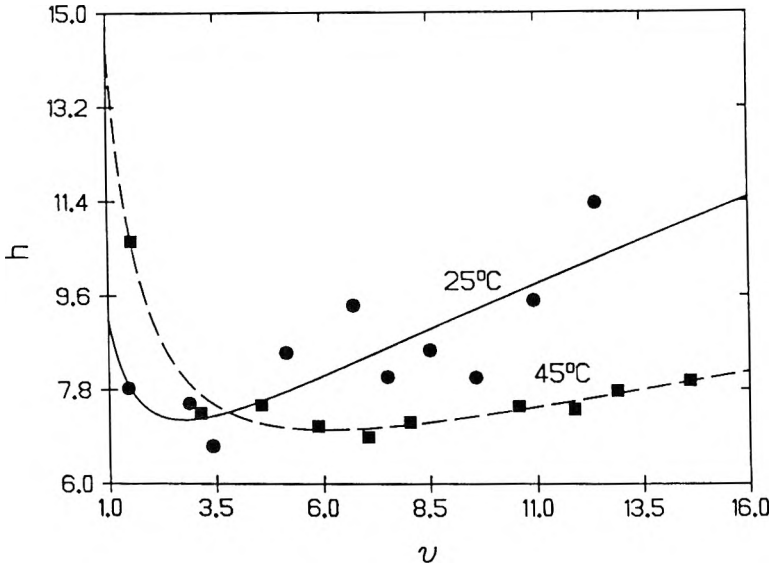

Figure 4. Reduced plate height versus reduced mobile phase velocity for benzene on C-18 with a 0.05M SDS mobile phase at 25^0 and 45^0C. Each data point in the plot is an average of triplicate determinations.

nitrophenol as column temperature is increased from 25^0 to 35^0 C cannot be explained in terms of either a change in viscosity or a shift in the position of the micelle solute equilibrium and is probably an anomaly unique to p-nitrophenol. Because the influence statistics show the regression equation is not overwhelmed by instability, we do not believe this result is a statistical artifact arising from poorly fitted data.

In Figure 4, a plot of reduced plate height versus reduced mobile phase velocity is shown for benzene at 25^0 and 45^0 C on a C_{18} column with an unbuffered 0.05M SDS mobile phase. Table 4 lists the A, B, and C parameters of the Knox equation at 25^0 and 45^0 C for benzene. Again, the improvement in chromatographic efficiency with temperature is due to a decrease in both the A and C terms of the Knox equation. Interestingly enough, Berthod and Hinze[28] also reported that reduced chromatographic efficiencies in MLC can be attributed to large increases in both the A and C terms of the Knox equation.

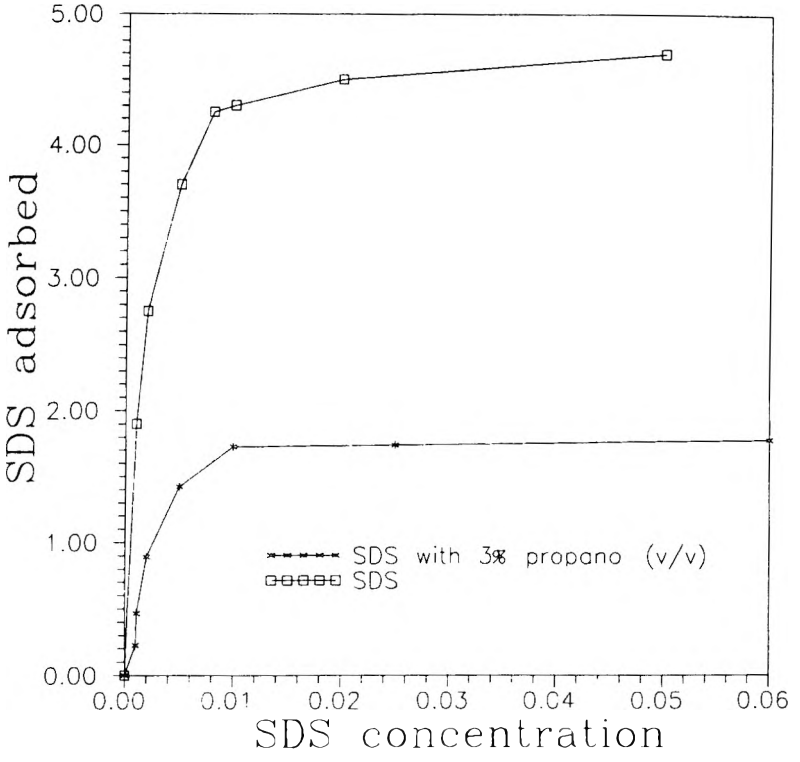

Figure 5. Adsorption isotherms of SDS on C-18 with and without propanol in the mobile phase. (micromoles/meter-squared vs. molarity of SDS). Adsorption data were obtained by pumping the appropriate concentration of surfactant in the mobile phase through the column (data from references 17 and 44).

2. Kinetics of Surfactant Desorption

Although the fit of the flow rate data to the Knox equation was good, there are concerns about the validity of this model to describe band broadening in MLC,[34] which is not surprising because the Knox equation is based on experimental data and correlations found to hold true in both packed column gas chromatography and conventional RPLC. However, MLC differs from gas chromatography or conventional RPLC due to the secondary chemical equilibria, so it is possible that one cannot model explicitly all of the salient features of band broadening in micellar RPLC using the Knox equation. Clearly, studies of micellar mobile phases in relation to their role in MLC are crucial for obtaining physically meaningful values for the A, B, and C terms of

the Knox equation. Therefore, we examined the adsorption isotherm of SDS on C_{18} to better understand the meaning of the changes in the A, B, and C terms of the Knox equation with temperature. In Figure 5, the adsorption isotherm of SDS on C_{18} at 25°C is shown. The data used in this figure was obtained by Berthod and coworkers.[17] An examination of the figure reveals a very interesting result: SDS continues to adsorb on the stationary phase at concentrations in excess of the cmc of the surfactant. This behavior is surprising in view of the fact that once the concentration of surfactant in the mobile phase exceeds the cmc, all of the added surfactant monomer should be micellized. In other words, this plot should show a cessation in the adsorption of SDS at concentrations in excess of the cmc. However, if the system is not at equilibrium, we will not see a cessation in SDS adsorption.

In micellar RPLC, the surfactant is involved in three distinct equilibria. First, there is an exchange of surfactant monomer between the micelle and the bulk aqueous phase.[35-37] This process is very fast and is characterized by a relaxation time, t_1, which is in the microsecond range. The second equilibrium involves the break-up and reformation of the micelle.[38-40] The kinetics of micellar dissociation are very complex and are characterized by a relaxation time, t_2, which is in the millisecond range. Finally, the surfactant monomer in the mobile phase is in equilibrium with molecules of surfactant adsorbed on the stationary phase. Although there is no published data on the rate of SDS desorption on hydrophobic silica, SDS desorption rates on solid surfaces such as carbon black or nylon[41-42] are found to be quite low.

In all likelihood, neither t_1 nor t_2 are responsible for band broadening in MLC. Because the exit rate constant of an SDS molecule from an SDS micelle is about 10^7 s^{-1} and the micelle surfactant association rate is nearly diffusion controlled, it is reasonable for one to assume that t_1 processes are instantaneous on a RPLC time scale. If t_2 (the stability or average lifetime of a micelle) influenced efficiency in MLC, then t_2 and chromatographic efficiency should share a similar relationship with SDS concentration. Clearly, this is not the case (see Figure 6). The average life-time of an SDS micelle increases with increasing SDS concentration up to 0.20 M SDS and then decreases.[43] However, column efficiency in MLC is observed to decrease with increasing SDS concentration, which is probably due to an increase in the viscosity of these mobile phases. Therefore, the reduction in efficiency associated with micellar mobile phases cannot be directly attributed to the dissociation kinetics of the micelle.

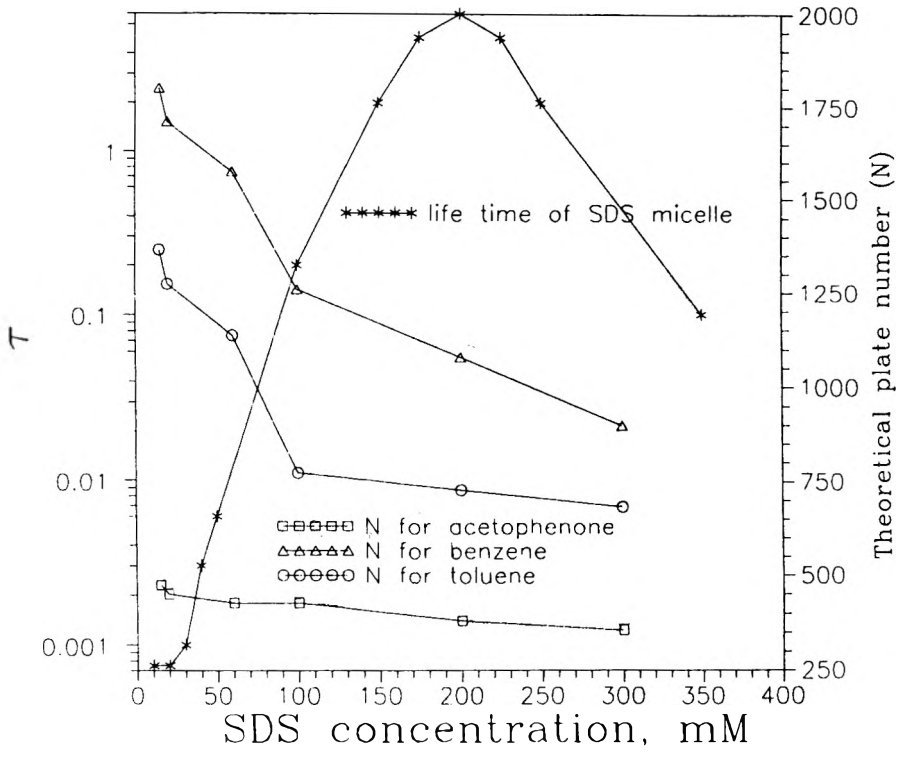

Figure 6. N and τ_2 versus concentration of SDS. Plate count data were obtained on an Apex I C-18 column; the flow rate of the mobile phase was 1.0 mL/min. τ_2 data were obtained from reference 43.

If the desorption rate of surfactant molecules on the stationary phase is too low, the result is a disturbance in the various equilibria involving the micelle and the surfactant monomer (see Figure 7). This disturbance would explain the continued adsorption of SDS on the stationary phase at concentrations in excess of the cmc. The low rate of surfactant desorption would also affect the dynamics of micellization which play a very important role in mobile phase mass transfer within the column. Assuming rapidly established equilibria between the micelle and surfactant monomer in the mobile phase and between the solute in the bulk solvent (water) and the micelle, the rate of establishment of the equilibrium between surfactant monomer in the mobile and stationary phase is then rate determining in MLC. If the rate is too slow, solute mass transfer between the mobile and stationary

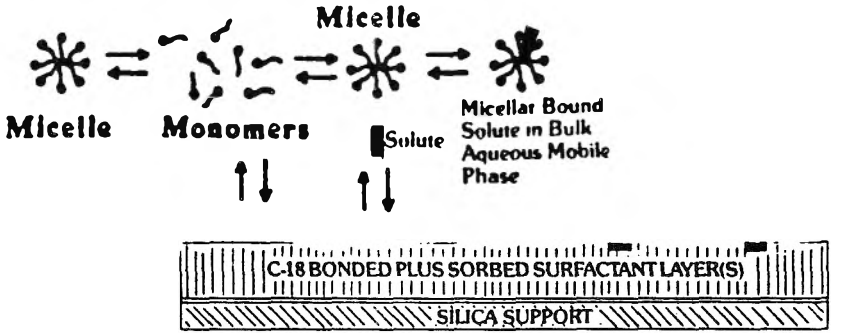

Figure 7. Equilibria in micellar reversed phase liquid chromatography. (Figure adapted from references 10 & 43.)

phases is sluggish resulting in significant broadening of the chromatographic peaks. Hence, one cannot ignore the possibility that in MLC the underlying cause of the inefficiency is the low rate of surfactant desorption which would explain the disagreement among workers concerning the reason for the low efficiencies evidenced in MLC.

3. Organic Modifiers vs Temperature

When propanol is added to an SDS micellar mobile phase (3% by volume), the adsorption of SDS on C_{18}[44] ceases after 10 millimolar SDS (see Figure 5) which is close to the cmc of the surfactant. The break observed in the SDS adsorption isotherm suggests that adsorbed surfactant monomer and the SDS micelles are at equilibrium within the column. Probably, surfactant desorption is occurring at a faster rate when a so-called hybrid mobile phase is used, which could explain the difference in shape between the adsorption isotherm of SDS and SDS hybrid micellar mobile phases. The increase in the surfactant desorption rate could also explain why efficiencies approaching those of conventional RPLC are obtained with these mobile phases, e.g., 0.05 M SDS with 3% propanol v/v.

Scott and Simpson[45] have shown that propanol preconcentrates at the C_{18} bonded phase/mobile phase interface. In other words, over 90% of the C_{18} stationary phase is covered by propanol at a concentration of 3% w/v. The preconcentration of propanol at the stationary phase probably causes the C_{18}

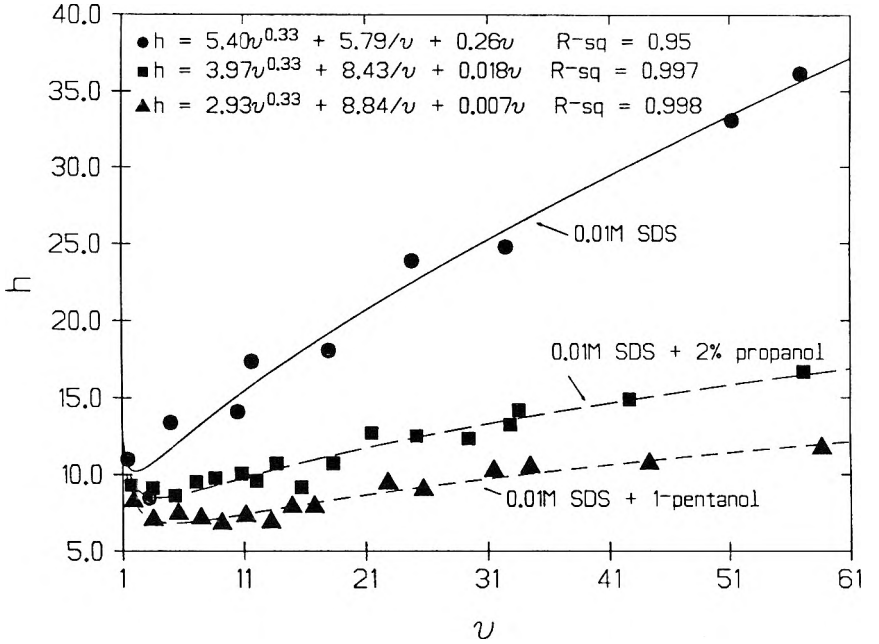

Figure 8. Reduced plate height versus reduced mobile phase velocity at 25^0 for coumarin on Apex I C-18 with a 0.01M SDS mobile phase with and without organic modifier. Each data point shown in the plot is an average of triplicate determinations.

chains to tilt toward the surface normal of the bonded phase,[46] which could explain why desorption kinetics of SDS would be more favorable on propanol modified C_{18}. In the absence of propanol, the C_{18} chains are probably tilted away from the surface normal. The net result is a less liquid-like stationary phase.[46]

The models hypothesized by researchers in microemulsion formation, in which low levels of moderate chain length alcohols are believed to enhance the flexibility of the surfactant film separating the aqueous hydrocarbon domains[47] could also be applicable to this system since the tethered C_{18} chains are analogous to an interfacial surfactant film.

Preliminary studies carried out in our laboratory on the effects of alcohol mobile phase modifiers on efficiency in MLC have shown that addition of a small amount of propanol or pentanol to a micellar mobile phase yields

improved efficiencies in MLC because of a large decrease in both the A and C terms of the Knox equation (see Figure 8), which suggests that both temperature and alcohol additives such as propanol or pentanol enhance the efficiency of the MLC separation process in much the same manner. Interestingly enough, the increase in the B term of coumarin as a result of the addition of propanol or pentanol to the micellar mobile phase can also be attributed to a decrease in the viscosity of the mobile phase and a shift in the position of the equilibrium of the solute away from the micelle and towards the bulk solvent.[12]

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF HALOPERIDOL IN PLASMA

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ABSTRACT

A sensitive, reproducible and accurate high performance liquid chromatographic (HPLC) method for the quantitative determination of haloperidol in plasma has been developed and validated. Sample preparation involves extraction of haloperidol and diazepam (internal standard) from 0.5 mL plasma. The separation was carried out in a stainless steel, resolve C_{18} column with a mobile phase composed of a mixture of 55% methanol and 45% HPLC water containing 0.2 M ammonium acetate and adjusted to an apparent pH 7.2. The mobile phase was pumped at a flow rate of 1.5 mL/min. The column oven temperature was adjusted at 38°C and the effluent was monitored at 249 nm. The retention times for the internal standard and haloperidol were found to be 5.1 and 6.3 minutes, respectively. Peak-height ratios of the drug to the internal standard were used for the quantification of haloperidol in the plasma samples. The average (±SD) absolute and relative recovery of haloperidol were

97±3.6% and 100 6±1.52%, respectively. The intraday coefficients of variation (CVs) ranged from 1.74 to 4.68%, while the interday CVs varied from 2.31 to 5.23%. The detection limit for haloperidol in plasma was found to be 5 ng/mL.

INTRODUCTION

Haloperidol is a potent neuroleptic drug of the butyrophenone series, widely prescribed for the treatment of acute and chronic psychotic syndromes, abnormal movements and confused states in elderly and hospitalized patients[1]. The drug is readily absorbed from the gastrointestinal tract. A serum half-life ranging from 10 to 19 hours and an oral bioavailability of about 60% were reported.[2] This low bioavailability is attributed to the first-pass effect. Large intersubject variation in the pharmacokinetics of haloperidol in both healthy volunteers and psychiatric patients was reported.[1-3] Because of the overall low plasma levels following single dose of haloperidol and due to the need for close plasma drug monitoring, specially in psychiatric patients, a highly sensitive, simplified, and accurate assay for the drug is essential.

Several analytical methods have been reported for the assay of haloperidol in biological fluids. These include: gas chromatography (GC),[4,5] gas chromatography combined with mass spectroscopy (GCMS),[6] radioreceptor assay,[7,8] radioimmunoassay (RIA),[9,10] and high performance liquid chromatographic assay.[11-15] The GC and GCMS methods required highly sophisticated equipment and are not amenable to rapid and routine clinical assay. The radioreceptor assay is not specific. The RIA methods are sensitive, but need expensive materials and impractical for routine analysis. The HPLC methods proved to be sensitive and convenient techniques for the determination of haloperidol in biological fluids. Some of the previous methods needed a relatively large plasma volume (2 mL) which may not be always available.[15] In others an electrochemical detector was necessary for conducting the assay.[14]

In this report, a simple, sensitive and reproducible HPLC assay for the determination of haloperidol in plasma is described.

MATERIALS AND METHODS

Materials

Haloperidol and the internal standard diazepam (Sigma Chem. Co., St. Louis, MO, U.S.A.), glacial acetic acid, ammonium acetate and hydrochloric acid (BDH Chemicals Ltd., Poole, U.K.), sodium hydroxide (E. Merck AG, Darmstadt, Germany) were used without further purification. Methanol, chloroform and ether (BDH Chemicals Ltd., Poole, U.K.) were HPLC grade.

Instruments

The following instruments were utilized: A Model LC-10 AD solvent delivery pump, a Model SPD-10 AV UV-Vis detector, a Model CTO-10 A column oven, and a Model C-R4A Chromatopac computing integrator (Shimadzu Corporation, Koyato, Japan), a Model 7010 Rheodyne injector (Rheodyne Inc., Catati, CA, U.S.A.), stainless steel column (Resolve C_{18}, 150 mm length x 3.9 mm i.d., 5 μm particles, Waters Associates, Milford, MA, U.S.A.), and a Model CFC-301 Gallenkamp centrifuge (Gallenkamp, Loughborough, England).

Standard Stock Solutions

An accurately weighed sample of 10 mg haloperidol and 10 mg diazepam were dissolved in methanol in two separate 100 mL volumetric flasks to give standard stock solution of 100 μg/mL.

Chromatographic Conditions

The mobile phase was a mixture of 55% methanol and 45% HPLC water containing 0.2M ammonium acetate and adjusted to an apparent pH 7.1-7.3 with acetic acid. It was degassed daily by passing it through 0.45 μm membrane filter (Millipore, Bedford, MA, U.S.A.).

The mobile phase was pumped at a flow rate of 1.5 mL/min, which produced back-up pressure of about 210 kg/cm^2. The column oven temperature

was adjusted at 38°C. The effluent was monitored at 249 nm and attenuation at 0.0005 AUFS. The chart speed was 2.5 mm/min.

Analytical Procedure

To a screw-capped (silicone coated) 15 mL glass test tube, 0.5 mL plasma and 25 µl of the internal standard (1 µg/mL) were added. The mixture was shaken on a vortex mixer for 30 sec. Five millilitres ether and 3 mL of 0.1N HCl were added for extraction and the mixture was shaken on a vortex mixer for one min and centrifuged for 3 min. at 3000 rpm. The aqueous phase containing the drug was transferred to 15 mL glass stoppered centrifuge tube containing 7 mL chloroform and 0.5 mL 1N NaOH. The mixture was shaken gently for 2 min and the chloroform layer was transferred to another 10 mL glass centrifuge tube and evaporated to dryness under vacuum at 45°C. The residue was reconstituted with 0.5 mL mobile phase, vortexed for 1 min and an aliquot of 20 µl was directly injected into the loop injector.

Application

The in vivo percutaneous absorption of haloperidol from experimental methylcellulose gel formulation developed in our laboratory were studied in rabbits. Ten white New Zealand male rabbits weighing 4.2-4.5 kg were utilized. The hairs were removed from the back of the rabbits with an animal clipper. The gel formulation containing a dose of 10.0 mg haloperidol was spread uniformLy to the back of each rabbit over an area equal to 3.14 cm^2. Blood samples (1.5 mL) were collected from the marginal vein of the left ear via a cannula into heparinized tubes before drug administration and at 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 hrs post-dosing. The plasma was harvested and stored at -20°C pending analysis.

RESULTS AND DISCUSSION

The solvent system used provided good separation of the drug and its internal standard with no interference from plasma substances. Figure 1 represents a typical chromatogram of: blank plasma (A), plasma containing the drug (B), and plasma containing haloperidol and the internal standard (C). Using the assay method the retention times for the internal standard and

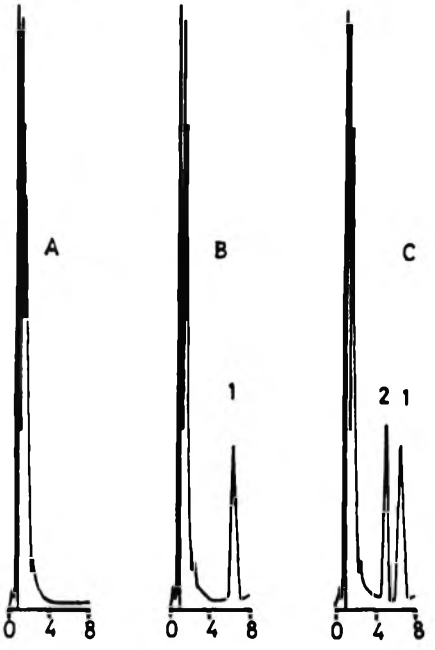

Figure 1. Chromatograms from (a) blank human plasma, (b) plasma spiked with haloperidol and (c) human plasma spiked with haloperidol and the internal standard.

1 = Haloperidol
2 = Diazepam (Internal Standard)

haloperidol were 5.1 and 6.3 minutes, respectively. The presence of ammonium acetate (0.2 M) in the mobile phase and heating the column to 38°C improved separation and yielded sharp peaks.

Quantification

Peak-height ratios of the drug to the internal standard were used in preparing four different standard curves in plasma and mobile phase by spiking 0.5 mL drug-free plasma samples and 0.5 mL mobile phase samples with the drug standard to produce a final concentration of 10, 20, 40, 60 and 100 ng/mL haloperidol. The standard plots were constructed over a period of three weeks. Least squares linear regression analysis of the mean standard calibration lines

for mobile phase and plasma samples resulted in the following equations:

$Y = 0.0097\ X - 0.0077$, $r = 0.999$ (Plasma)
and $Y = 0.0102\ X - 0.0048$, $r = 0.999$ (Mobile phase)

Analysis of variance of the slope, intercept and correlation coefficient of the four standard curves from plasma indicated non-significant difference ($F=5.11$, $p>0.05$). These results confirm the linearity of the standard curves and the excellent reproducibility of the assay method.

Recovery

The absolute recovery of haloperidol and the internal standard (diazepam) were determined by comparing the peak-height of the drug obtained from spiked plasma with the peak-heights obtained by the direct injection of pure aqueous drug standard at three different concentrations (30, 50 and 80 ng/mL). The relative recovery of the drug was calculated by comparing the concentrations obtained from the drug-supplemented plasma to the actual added concentrations. The results of the recovery studies are shown in Table 1. The average absolute and relative recovery of haloperidol were found to be 97±3.6% and 100.6±1.52%, respectively.

Table 1

Absolute and Relative Recovery of Haloperidol from Human Plasma*

Conc. (ng/mL)	Mean Peak Heights (cm)		Absolute Recovery %	Relative Recovery % Mean±SD
	Aqueous	Plasma		
30	0.86±0.05	0.80±0.2	93.02	100.94±5.25
50	1.30±0.08	1.28±0.04	98.46	98.73±2.95
80	1.89±0.11	1.90±0.14	100.53	106.3±2.4
Diazepam (I.S.)				
50	1.82±0.08	1.77±0.07	97.25	

*Twelve replicate analyses of each concentration.

Precision

The intraday precision was evaluated by replicate analysis of plasma samples containing haloperidol at three different concentrations (30, 50 and 80 ng/mL). The intraday precision showed a coefficient of variation (CV) of 1.74 to 4.68% (Table 2). The interday precision was similarly evaluated over 3-weeks period. The interday CVs ranged from 2.31 to 5.23% (Table 2).

Table 2

Intraday and Interday Precision of Haloperidol in Human Plasma

Intraday*			Interday**		
Added Conc. (ng/mL)	Measured Conc. (ng/mL)	Bias***	Added Conc. (ng/mL)	Measured Conc. (ng/mL)	Bias***
30			30		
Mean	30.7	2..3	Mean	30.3	1.0
S.D.	1.2		S.D.	1.59	
CV%	3.93		CV%	5.23	
50			50		
Mean	48.74	-2.52	Mean	49.36	-1.28
S.D.	2.28		S.D.	1.48	
CV%	4.68		CV%	3.0	
80			80		
Mean	83.18	3.97	Mean	85.1	6.38
S.D.	1.45		S.D.	1.96	
CV%	1.74		CV%	2.31	

*Mean values represent five different plasma samples for each concentration.
**Interday precision was determined from 12 different runs over 3-weeks period at the three concentrations.
***Bias=100 X (measured concentration-added concentraton) / added concentration.

Sensitivity

The minimum detectable amount which is defined as the amount in nanograms that give peak height equals to twice the background noise was found to be 5 ng of haloperidol per 1 mL of plasma samples.

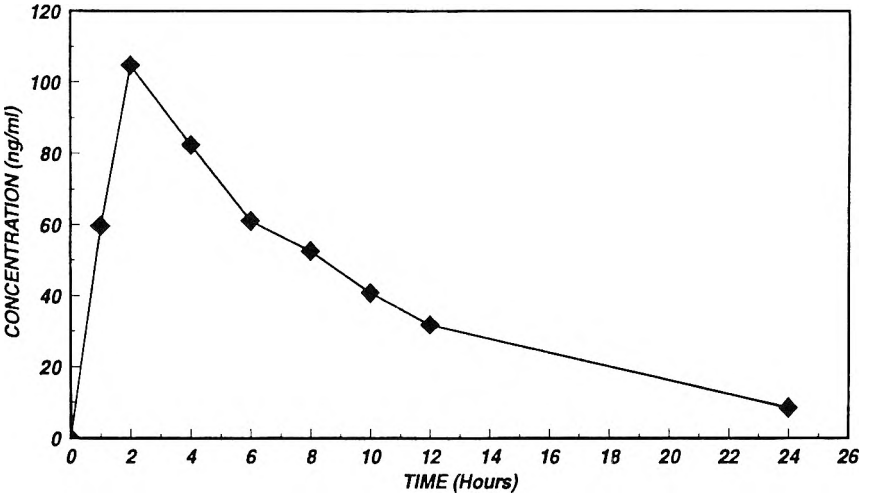

Figure 2. Mean plasma concentration-time profile of haloperidol after transdermal administration of 10 mg dose to ten rabbits.

Specificity

The specificity of the method was evaluated by analysing drug-free blank plasma from several healthy male volunteers. No interference from endogenous plasma constituent was observed at the retention times of haloperidol and the internal standard.

Application

Figure 2 shows the mean plasma concentration-time profile of haloperidol after transdermal administration of haloperidol (10 mg) from the developed gel formulation to each rabbit. The mean pharmacokinetic parameters calculated from the individual rabbit data (AUC, C_{max}, T_{max}, K_{el}, $t_{1/4}$ and MRT) are presented in Table 3.

Table 3

Mean (±SD) Pharmacokinetic Parameters of Haloperidol after Transdermal Administration of 10 mg Dose to 10 Rabbits

Parameters	Value
AUC (ng.hr/mL)	1027.14±254.84
C_{max} (ng/mL)	108.60± 36.20
T_{max} (hr)	2.20±0.42
K_{el} (hr^{-1})	0.121±0.013
$t_{1/2}$ (hr)	5.77±0.64
MRT (hr)	8.94±1.59

AUC = Area under the plasma concentration-time curve.
C_{max} = Peak plasma concentration. T_{max} = Peak time.
K_{el} = Elimination rate constant.
$t_{1/2}$ = Elimination half-life.
MRT= Mean residence time.

CONCLUSION

The HPLC method developed in this study has the sensitivity, accuracy and reproducibility which makes it valuable in many applications, specifically in pharmacokinetic studies and blood level monitoring of haloperidol.

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A RAPID HPLC METHOD FOR THE DETERMINATION OF RAFFINOSE FAMILY OF OLIGOSACCHARIDES IN PEA SEEDS

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SUMMARY

Two HPLC techniques for the determination of raffinose family oligosaccharides (RFO - raffinose, stachyose and verbascose) in pea are described.. A reverse phase HPLC (RP-HPLC) on Silica C_{18} column using demineralized water as mobile phase enabled determination of the total content of RFO within less than 10 minutes. Ion Moderated Partition HPLC (IMP-HPLC) using a strong cation exchanger, in calcium form, with demineralized water as mobile phase allowed the determination of all the individual saccharides within 25 minutes. In both RP-HPLC and IMP-HPLC refractometric detection was used. The methods

were compared on a representative pea sample series, and in both techniques the lowest detectable concentration in sample solution was calculated to be 5 mg saccharide/mL, i.e., the smallest detectable amount of each saccharide was 100 ng.

INTRODUCTION

Legumes are an important input in many countries for animal and human consumption. They are an excellent source of proteins (20-40 %) and carbohydrates (50-60 %) and fairly good sources of thiamine, niacin, calcium, and iron.[1] However, per capita consumption of legumes in the US and other industrialised countries has fallen considerably in recent years.[2] This could be due to the fact that legumes have certain undesirable flavours, flatus factors, and anti-nutrients, and even toxic substances.

Flatulence is the most common symptom associated with pulse consumption, but its social implications are overshadowed by more serious accompanying consequences. Abdominal pain and diarrhoea are often experienced by susceptible individuals, especially children, causing pulses to be less readily accepted. Unfortunately those affected are often most at risk from malnutrition and pulses may be the only affordable source of good quality protein available to them. The production of flatus by monogastric animals is due to colonic fermentation of carbohydrates which escape breakdown in the stomach and small intestine. The oligosaccharides, raffinose, stachyose and verbascose, which are common in legume seeds, are thought to be the major producers of flatulence when those foods are consumed. These saccharides contain either one, two or three galactose units joined to sucrose by α - D - 1-6 linkages. Owing to the absence of an α-galactosidase enzyme capable of hydrolysing the α-1,6-galactosidic linkage, these oligosaccharides accumulate in the lower intestine and undergo anaerobic fermentation by bacteria, which may result in the production of diarrhoea, flatus gas and attendant discomfort.[3] Fibre polysaccharides and indigestible starch have also been associated with flatulence,[4] whilst the volatile fatty acids, that are produced when carbohydrates are fermented in the colon, have been suggested to have positive effects on cholesterol and carbohydrate metabolism. The decrease in faecal pH, due to production of acids, has also been suggested to be a preventive factor against the occurrence of cancer in the large bowel.[5] The benefits of oligosaccharide ingestion arise from an increased population of indigenous bifidobacteria in the colon which, by its antagonistic effect, suppresses the activity of putrefactive

bacteria and thereby reduces the formation of toxic fermentation products.[6]

Instrumental techniques for the RFO determination such as GLC, HPLC have been used.[7,8,9,10] As an alternative technique for the determinaton of such non-reducing oligosaccharides, high performance capillary electrophoresis (HPCE) has been reported[11] which is based on separation of borate complexes of the saccharides in an electric field, and has been shown to be rapid, cheap and efficient. Both chromatographic (HPAC - PAD) and electrophoretic (HPCE) techniques were compared for the RFO determination in pea[12] and the results obtained were very close. However, both techniques are expensive for those laboratories with low resources which would like to analyse these legume components in a large number of samples, for instance, in genetic studies.

The aim of this work was to develop and validate suitable analytical methods for the RFO screening in a large pea collection, i.e., hundreds of samples. The present study had two main purposes: firstly to find a rapid and relatively cheap HPLC method for RFO determination, and secondly to replace the toxic and relatively expensive mobile phase (MeCN:H_2O) which is commonly used in conventional IR-HPLC carbohydrate determination. The method developed will be used for the screening of pea varieties for flatulence-causing saccharides and will be a factor in:

- finding pea varieties with low RFO levels
- finding parental lines suitable for the breeding of new varieties
- searching for genetic dependence

MATERIAL AND METHODS

Samples, Chemicals and Instrumentation

Dry pea seeds were obtained from the Research Institute of Technical Crops and Legumes (Šumperk, Czech Republic). Four pea samples of different genotypes (iso-line 1 to 4) were provided from John Innes Centre (Norwich, UK). Sugar standards (stachyose, raffinose, sucrose, maltose, glucose, galactose and fructose) were obtained from SIGMA-ALDRICH, Ltd. (Prague, Czech Republic). The stainless steel (250x8 mm ID) chromatographic column with a strong cation exchanger OSTION LG KS 0803, 4.2 % DVB (Spolek pro chemickou a hutní výrobu, Czech Republic) in calcium form was filled in our laboratory. Reverse phase chromatography columns (stainless steel 250x4 mm

ID) Separon SGX RPS (high carbon loaded ODS), 7μm and Separon SGX C_{18}, 5 μm were purchased from Tessek (Prague, Czech Republic). A desalting guard column served strong cation exchanger HEMA BIO SB, 10 μm (30x3 mm ID) and strong anion exchanger HEMA BIO Q, 10 μm (30x3 mm ID) from Tessek. Other chemical products were of analytical grade.

The HPLC analysis was performed on a modular chromatograph from Thermo Separation Products (Watrex Praha, Czech Republic) consisting of isocratic pump ConstaMetric 3200, sample injector Rheodyne 7725i (20 μl loop) and differential refractometer RefractoMonitor IV. The chromatograms were evaluated with a PC system using CSW software (DataApex, Prague, Czech Republic).

HPLC Conditions

These chromatographic methods were employed for RFO determination in pea extracts:

1) RP-HPLC: An analytical column Separon SGX RPS 7 μm or Separon SGX C_{18} 5 μm with guard columns (Separon RPS or Separon C_{18}) at ambient temperature were eluted with demineralized water at a flow rate of 1 mL/min or 0.7 mL/min, respectively. The injection volume was 20 μl.

2) IMP-HPLC: Analytical column filled with strong cation exchanger OSTION LG KS 0403, 17 - 20 μm in Ca^{++} form heated to 80°C was fitted with desalting guard columns and eluted by degassed demineralized water at a flow rate of 0.4 mL/min. The injection volume was 20 μl. Refractometric (sensitivity $1x10^{-6}$ RIU/10 mV) detection was employed in both RP-HPLC and IMP-HPLC methods.

3) HPAC-PAD analysis of oligosaccharides in representative pea samples was performed on a DIONEX system as described by Frias et al.[10]

Calibration

An external standard calibration method was used. In the case of IMP-HPLC six concentration levels of sucrose, raffinose and stachyose were measured (30 - 1000 mg/l) and four levels in the case of RP-HPLC.

Coefficients of calibration equations describing the relationship between concentration of sugar and peak area were found by linear regression.

Sample Treatment

An extract of RFO from ground pea sample was obtained, using a 80% ethanol-water mixture, by two ways, i.e., boiling under reflux and by sonication.

Boiling under reflux: Two g of ground pea sample was weighed into a 50mL Erlenmeyer flask, 20 mL of 80% ethanol added and boiled under reflux for 60 minutes. After cooling the mixture was transferred into a 200mL volumetric flask and made up to volume with demineralized water. The extract was then filtrered through a membrane (0.45 μm) and analyzed by HPLC.

Sonication: One hundred mg of ground pea sample was placed in a 5 mL vial, 2 mL of 80% ethanol added, vial stoppered and placed in a sonic bath for 20 minutes. The suspension was transferred into a 25mL volumetric flask and made up to volume with demineralized water. After filtration through a membrane filter (0.45 μm) the extract was analyzed by HPLC.

RESULTS AND DISCUSSION

Column characteristics are summarised in Tables 1 and 2. Chromatograms of the model mixture of saccharides and a pea sample extract are shown in Figures 1 and 2, respectively. It is clear that using RP-HPLC the RFO were not sufficiently resolved for the saccharides, i.e., raffinose, verbascose and stachyose could not be quantified individually but only as their sum. On the other hand the IMP chromatography allows good separation of the individual α-galactosides (see Figures 1 and 2).

In spite of the higher efficiency of the RP-HPLC column Separon C_{18} compared with that of the Separon RPS column, the latter column was used for routine analyses due to a larger retention time gap between raffinose and ethanol resulting in better quantitative results of RFO. The column life of the Separon RPS was more than 100 analyses without regeneration (column rinsing with 80% ethanol overnight) and more than 500 analyses on the same column could be carried out without significant loss of column efficiency. The column life of the OSTION column was more than 200 analyses without regeneration

Table 1

Reverse Phase Column Performance

Saccharide	Retention Time (min)		Column Efficiency (N/m)		Resolution	
	Separon SGX RPS 7μm	Separon SGX C_{18}, 5μm	Separon SGX RPS, 7μm	Separon SGX C_{18}, 5μm	Separon SGX RPS, 7μm	Separon SGX C_{18}, 5μm
Glucose	2.11	3.15	3050	7600	-	-
Galactose	2.10	3.11	3080	9500	-	-
Fructose	2.17	3.23	3100	9400	-	-
Maltose	2.45	3.63	3300	11400	1.03	1.70
Sucrose	2.62	4.24	3800	13800	0.52	2.18
Stachyose	3.18	5.40	4800	14700	1..51	3.59
Raffinose	3.39	5.85	4600	15700	0.55	1.23

Table 2

Ion Moderated Partition Column Performance

Saccharide	Retention Time (min)	Column Efficiency (N/m)	Resolution
Stachyose	11.97	13600	-
Raffinose	13.25	15600	1.54
Sucrose	14.82	17100	1.79
Maltose	14.65	17300	-
Glucose	17.47	11500	2.40
Galactose	19.42	20800	1.64
Fructose	21.18	21300	1.38

(column rinsing with 0.1 M - $Ca(NO_3)_2$ overnight). Desalting guard columns withstood approximately 50 analyses, whilst more than 800 analyses are possible using the same analytical column.

The external standard calibration method was used for quantitative analysis. The calibration analyses were carried out using stachyose, raffinose and sucrose. Since the standard of verbascose was not available the qualitative analysis of verbascose was based on the published data of elution order of saccharides on cation exchanger (IMP-HPLC);[14] the higher degree of polymerization of saccharide the shorter retention time. Hence, verbascose as a pentasaccharide was assumed to elute before a tetrasaccharide, i.e., stachyose. The identity of the verbascose peak was also confirmed from previous studies.[10,12] The calibration results obtained are shown in Tables 3 and 4.

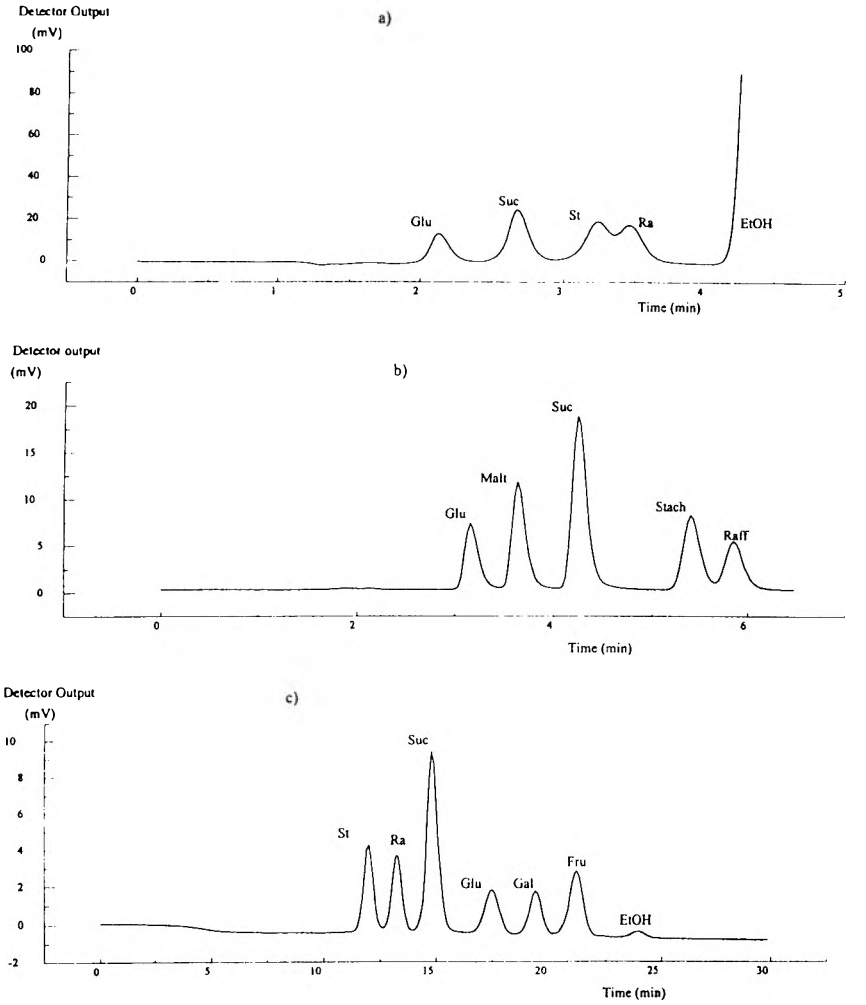

Figure 1. Chromatograms of standard mixture of saccharides; (a) RP-HPLC on Separon SGX RPS of 100 mg/mL glucose (Glu), 200 mg/mL of sucrose (Suc), 200 mg/mL of Stachyose (St) and 200 mg/mL of raffinose (Ra); (b) RP-HPLC on Separon SGX C_{18} of 104 mg/mL glucose (Glu), 93 mg/mL of maltose (Malt), 194 mg/mL of sucrose (Suc), 118 mg/mL of Stachyose (St) and 99 mg/mL of raffinose (Ra); (c) IMP-HPLC of 120 mg/mL of stachyose (St), 100 mg/mL of raffinose (Ra), 250 mg/mL of sucrose (Suc), 100 mg/mL of glucose (Glu), 100 mg/mL of galactose (Gal) and 120 mg/mL of fructose (Fru).

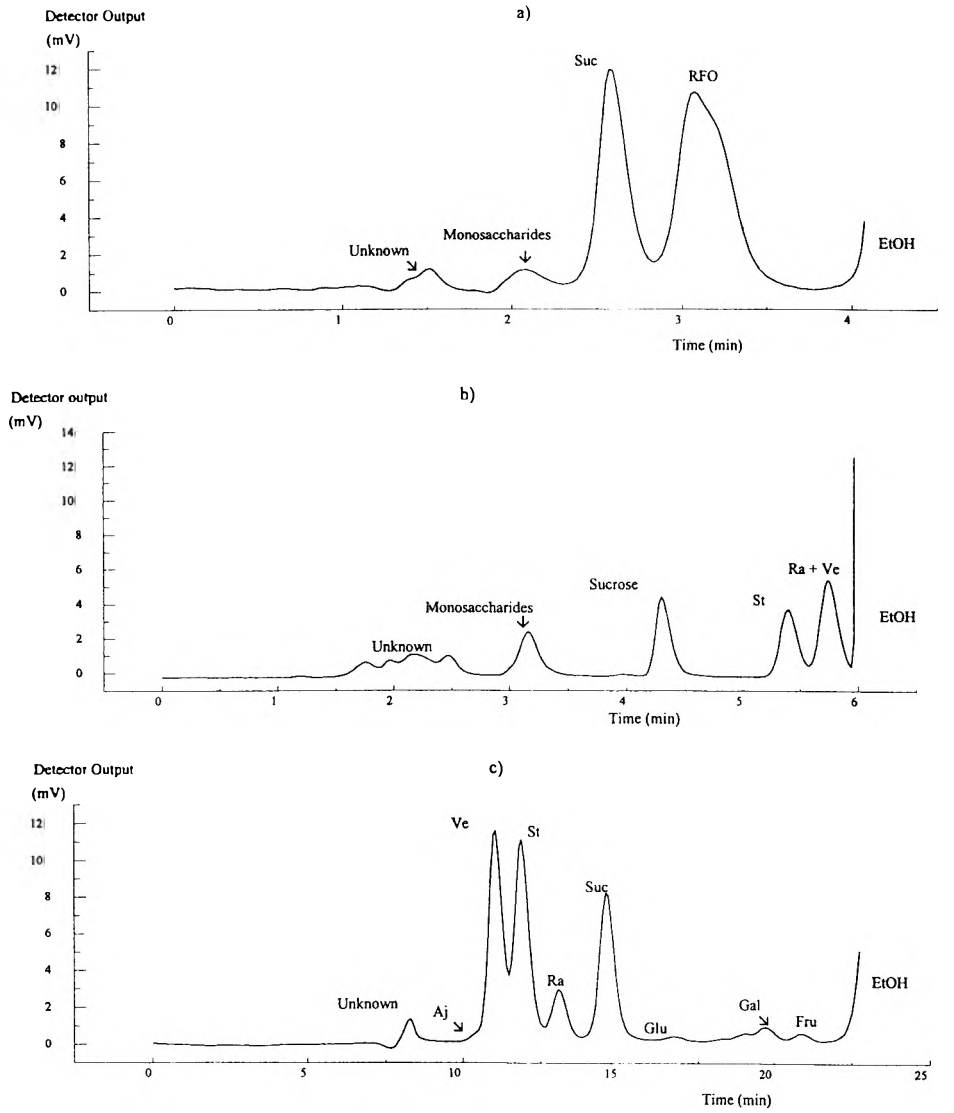

Figure 2. Chromatograms of pea sample extracts (pea variety Uladovskij 387); (a) RP HPLC on Separon RPS; (b) RP HPLC on Separon C_{18}; (c) IMP HPLC on OSTION LG KS 0403 in Ca form.

Table 3

Results of Calibration Analysis (RP-HPLC); Coefficients of Calibration Equations Y = a x X + b; Y = Peak Area (mV.s) and X = Concentration (μg/mL); Four Calibration Levels

Saccharide	a	b	Correlation Coefficient	Concentration range (μg/mL)
Stachyose	1.59	2.3	0.9987	50 - 500
Raffinose	1.68	5.8	0.9997	25 - 250
Sucrose	1.62	3.8	0.9995	50 - 500

Table 4

Results of Calibration Analysis (IMP-HPLC); Coefficients of Calibration Equations Y = a x X + b; Y = Peak Area (mV.s) and X = Concentration (μg/mL); Six Calibration Levels

Saccharide	a	b	Correlation Coefficient	Concentration range (μg/mL)
Stachyose	0.756	30.8	0.9990	100 - 1000
Raffinose	0.604	12.1	0.9982	20 - 200
Sucrose	0.744	38.0	0.9997	100 - 1000

From these data it is clear that both chromatographic methods showed good linearity between peak area and concentration for each saccharide. An average response factor (ratio of peak area and saccharide concentration) of stachyose and raffinose was used for the quantitative analysis of RFO determined by RP-HPLC. In the case of IMP-HPLC the quantitative analysis of individual saccharides was based on their own calibration equation (stachyose, raffinose and sucrose) and quantitative analysis of verbascose was based on calibration results of stachyose. For both HPLC techniques the lowest detectable concentration in sample solution analysed was found to be 5 μg of saccharide/mL, i.e., the smallest detectable amount of saccharide was 100 ng.

A comparison between the extraction efficiency of a sonication technique and boiling under reflux was made. It was found that the latter

technique was twice as efficient as the sonication technique (Figure 3) and the optimum extraction time for boiling was found to be 60 minutes (Figure 4).

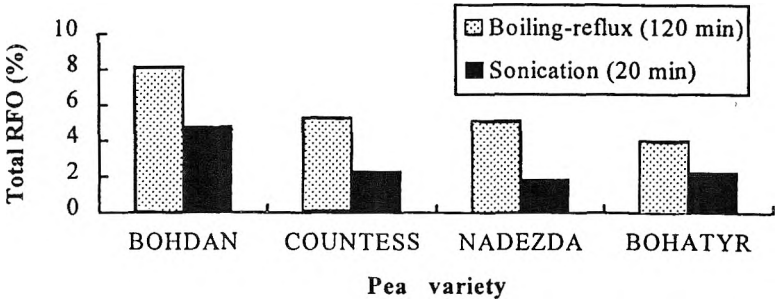

Figure 3 Extraction efficiency - comparison of two techniques

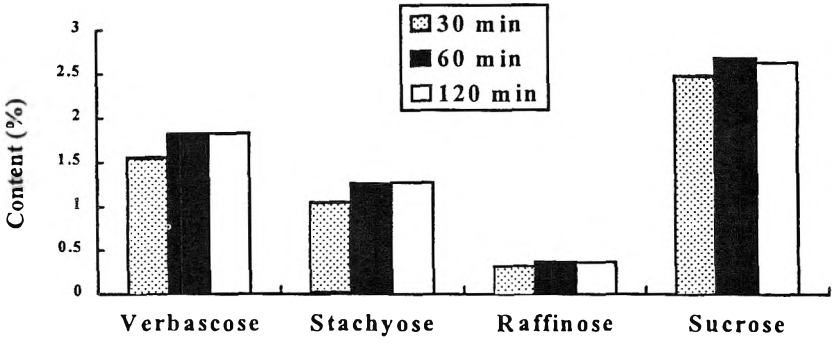

Figure 4 - Time course of extraction efficiency of boiling under reflux technique (pea sample Uladovskij 387)

The relative standard deviation and repeatability of the HPLC analyses and recoveries of RFO are summarised in Table 5. The RSD of HPLC analyses were calculated on the basis of results of 10 repetitive injections of the same sample extract. The repeatability of the HPLC techniques was obtained on the basis of 10 repetitive analyses of the same pea sample (Uladovskij 387). Recovery figures are an average of two different standard additions at levels corresponding to 50 % and 100 % of the actual saccharide content.

The IMP HPLC was compared with HPAC-PAD method (High

Performance Anion-exchange Chromatography with Pulsed Amperometric Detection) developed at the John Innes Centre and the Institute of Food Research in Norwich (UK).[10] On the basis of the figures shown in Table 6 it can be stated that the IMP HPLC results are in good agreement with those obtained with HPAC-PAD.

Table 5

Statistics of HPLC Analyses

	RP HPLC			IMP HPLC
	Verbascose	Stachyose	Raffinose	Sum of RFO
RSD of injection (%)	1.2	1.3	2.8	2.8
Repeatability (%)	2.6	3.8	5.8	4.2
Recovery (%)	-	93	94.5	95.2

Table 6

Comparison of IMP HPLC (I)* with HPAC-PAD (II)

Sample	Verbascose		Stachyose		Raffinose		Total RFO	
	I	II	I	II	I	II	I	II
iso-line 1	3.12	2.16[b]	2.03	1.45[b]	0.69	0.56[b]	5.84	4.17[b]
iso-line 2	5.47	3.74[b]	3.48	2.62[b]	1.15	1.05[b]	10.10	7.41[b]
iso-line 3	4.71	3.63[b]	4.86	2.48[b]	1.26	0.92[b]	10.83	7.03[b]
iso-line 4	3.99	4.21[b]	4.32	3.54[b]	1.28	1.51[b]	9.59	9.26[b]
BOHDAN	4.03	3.37	3.11	2.87	0.98	0.88	8.12	7.12
COUNTESS	1.25	1.34	2.22	2.38	0.55	0.64	5.31	4.97
NADEZDA	1.49	1.63	2.84	2.93	0.81	0.78	5.14	5.34
BOHATYR	1.97	1.73	2.35	2.21	0.99	1.03	4.02	4.36

*average of three replicates (for RSD see Table 5)

b) data from Frias et al., 1995[10] (with permission).

CONCLUSION

Both RP and IMP chromatography methods give acceptable results for quantitative analyses of the RFO in pea. The RP HPLC is a rapid method suitable for the determination of total RFO, especially in cases of large sample sets. The IMP HPLC enables the determination of the individual RFO, i.e., verbascose, stachyose and raffinose in pea samples within 30 minutes. Similar repeatability (< 5%) and recovery of RFO (93 - 95 %) was found for both methods.

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SEPARATION OF TRIAZINE HERBICIDES BY ION-INTERACTION HPLC AND APPLICATION TO SURFACE WATERS

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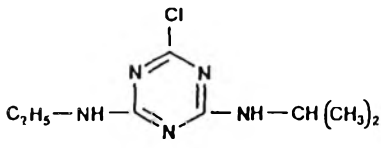
ABSTRACT

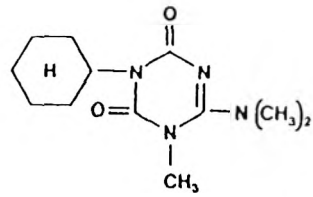
An ion-interaction reverse phase chromatographic method is presented for the separation and determination of atrazine, hexazinone, prometryn, propazine, simazine, terbuthylazine. The separation is carried out in isocratic conditions on a octadecyl silica stationary phase, using an hydroorganic solution (acetonitrile/water 45/55) of 5.0 mmol/L octylamine o-phosphate at pH 6.4 as mobile phase, with spectrophotometric detection at 230 nm. The detection limit (around 1.0 μg/L) reached without derivatization makes the method suitable for the analysis of surface waters without preconcentration. Application to river and lagoon water samples are reported.

INTRODUCTION

Triazine herbicides, widely used as pre-emergence weed-killers, are characterized by a very high chemical stability, a low water solubility, a low volatility and a high trend to be absorbed in organic and clayey colloids. All these properties lead to a consequent long persistence in the environment.



atrazine



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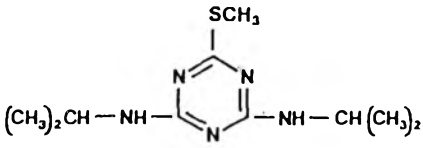
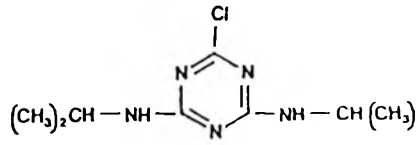
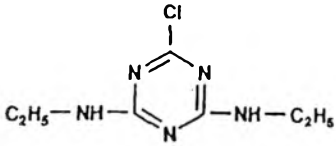


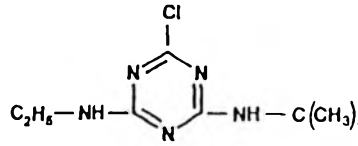
prometryn



propazine



simazine



terbuthylazine

Figure 1. Chemical structures of the analytes.

Their toxicity, expressed as oral LD50 for rats, ranges from 182 (cyanazine) to more than 5000 mg/Kg (simazine, propazine, anilazine)[1, 2].

As concerns structure, the triazine derivatives generally used as weed-killers are symmetric triazines in which two carbon atoms of the aromatic ring are substituted with two alkylamine- groups and the third by a chloro-, a methoxy- or an azido- group. About twenty five triazine herbicides are used, being atrazine and simazine the most widely employed[2].

Preconcentration methods are often necessary before the analytical determination. They make use of supercritical fluid extraction[3-5]; off-line[6-10] and on-line[11-14] solid-phase extraction with various sorbents; liquid-liquid extraction[15-16]; microwave techniques[17], size-exclusion chromatography[18].

The chromatographic techniques are the most widely used for triazine herbicide analysis: gas chromatography with thermoionic[19], atomic emission[20], selective N and P[5, 6, 18, 21-23], mass spectrometry[6, 13, 22, 24] detection systems and HPLC analysis with UV detection[5, 7-10, 2, 16, 18, 25]. Examples are also reported[26] of gas-liquid chromatography, of HPLC thermospray MS[11] and MS-MS[27] methods.

In this paper an ion-interaction chromatographic method which permits detection limit of around 1.0 μg/L without preconcentration is presented. The method is applied in the separation of terbuthylazine, prometryn, atrazine, hexazinone, simazine and propazine (chemical structures reported in Figure 1).

EXPERIMENTAL

Chemicals and Reagents

Ultrapure water from Millipore MilliQ system was used for the preparation of the solutions. Sodium nitrate was analytical grade Merck reagent. Prometryn, propazine, simazine, terbuthylazine, hexazinone, atrazine were analytical grade LabService Analytica reagents. Octylamine, ortho-phosphoric acid and HPLC grade acetonitrile were Fluka chemicals.

Apparatus

The chromatographic analyses were performed with a Merck-Hitachi Lichrograph chromatograph Model L- 6200 equipped with a two channel D-2500 chromato-integrator interfaced with a UV-Vis detector L-4200 and with a conductivity detector with temperature control L-3720 from the same firm.

Absorbance measurements were performed with a Hitachi model 150-20 spectrophotometer.

For pH measurement a Metrohom 654 pH-meter provided with a

combined glass-calomel electrode was employed.

Chromatographic Conditions

A Spherisorb Phase Separation S5 ODS-2 (5 μm, 250 x 4.6 mm) cartridge column was used as the stationary phases together with a guard pre-column Merck Lichrospher RP-18 (5 μm).

In the optimization study the mobile phases were prepared at different concentrations of octylammonium o-phosphate and acetonitrile and brought to pH 6.4 with o-phosphoric acid. The pH value so obtained in the hydroorganic solution is known as operational pH. The optimal conditions are: 5.0 mmol/L octylammonium phosphate in acetonitrile/water 45/55.

The chromatographic system was conditioned by eluting the mobile phase until a stable baseline signal was obtained; a minimum of 1 hour was necessary at flow rate of 1.0 mL/min. After the use, the column was washed by flowing a 50/50 v/v water/acetonitrile mixture (1.0 mL/min for one hour), and then acetonitrile (0.7 mL/min for 20 min).

Our results fit the model[28, 29] according to which the interaction reagent contained in the mobile phase is bound onto the surface of the stationary phase through adsorption and electrostatic forces, giving rise to an electrical double layer. The interaction properties of the original reverse phase packing material are therefore modified. The new surface is able to simultaneously retain anions and cations[30-32].

Preparation of Herbicide Standard Solutions

Standard solutions, 10.00 mg/L were prepared in acetonitrile for successive dilutions of 400.00 mg/L acetonitrile solutions and preserved in brown bottles at 4°C for a maximum time of a month; the work solutions were prepared in acetonitrile/water (30/70) just before the injection in the HPLC system.

Sampling and Storage of Surface Waters

Surface water samples were collected in 1 L pyrex glass with wide neck and PP-screw-capwal bottles, previously washed with 0.2 mol/L

hydrochloric acid and repeatedly rinsed with ultrapure water. During the sampling, bottles were rinsed twice with the sample water, then filled and tightly capped. The entire sample was immediately filtered in the laboratory through Millipore 0.45 μm filters and stored at 4°C. All the analyses were performed within three days.

RESULTS

Table 1 shows for the triazine derivatives here investigated, the absorptivity values evaluated at 230 nm (which wavelenght offers the best average sensitivity in the separation) and the absorptivities at the wavelenght of the maximum absorbance. In order to obtain the best resolution and sensitivity in the lowest total analysis time, the experimental conditions of organic modifier, ion-interaction reagent concentration and flow-rate were optimized.

Table 1

Absorptivity Values at the Wavelenghts of Maximum Absorbance and at 230 nm.

	λ_{max}	ε_{max} (L mol^{-1} cm^{-1})	ε_{230nm} (L mol^{-1} cm^{-1})
atrazine	220	$(3.72\pm0.05)10^4$	$(2.26\pm0.04)10^4$
hexazinone	244	$(1.40\pm0.03)10^4$	$(8.98\pm0.11)10^3$
propazine	220	$(3.11\pm0.03)10^4$	$(1.72\pm0.04)10^4$
prometryn	220	$(4.26\pm0.05)10^4$	$(3.60\pm0.04)10^4$
simazine	220	$(3.24\pm0.05)10^4$	$(1.71\pm0.02)10^4$
terbuthylazin	222	$(3.93\pm0.06)10^4$	$(2.51\pm0.04)10^4$

Figure 2 shows, as an example, the separation of a mixture containing hexazinone, simazine, atrazine, propazine, terbuthylazine, prometryn, at concentration of 6.0 μg/L each, obtained in the optimized conditions, namely: octylammonium o-phosphate 5.0 mmol/L in acetonitrile/water (45/55), flow rate: 0.7 mL/min.

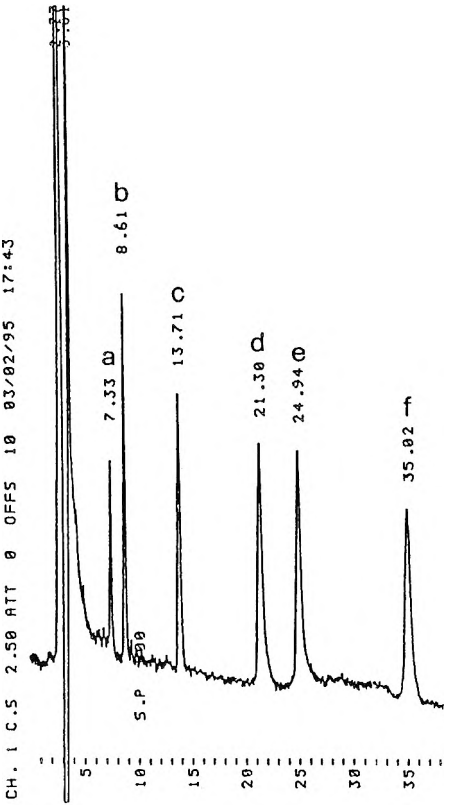


Figure 2. Chromatogram of the standard mixture. a: hexazinone, b: simazine, c: atrazine, d: propazine, e: terbuthylazine, f: prometryn, 6.0 μg/L each. Stationary phase: Phase Separation Spherisorb 5S ODS-2, 250 x 4.6 mm (5 μm) cartridge-type column, mobile phase: 5.0 mmol/L octylammonium o-phosphate in acetonitrile/water (45/55), pH 6.4; flow rate 0.7 mL/min. Spectrophotometric detection at 230 nm.

Calibration Curves

Under the optimized conditions the calibration curves reporting the peak areas (as evaluated by the integrator) vs standard concentrations were built. Standard concentrations range between 5.0 and 40 μg/L for all the pesticides.

Table 2

Slopes (m) of the Regression Equation y= mx and Correlation Coefficients (r^2) of the Plot Analyte Peak Area (y) vs Analyte Standard Concentration (x).

Analyte	m	r^2
atrazine	$(8.15\pm0.05)\ 10^2$	0.9996
hexazinone	$(2.50\pm0.04)\ 10^2$	0.9979
propazine	$(9.72\pm0.13)\ 10^2$	0.9992
prometryn	$(1.47\pm0.01)\ 10^3$	0.9997
simazine	$(8.97\pm0.06)\ 10^2$	0.9996
terbuthylazine	$(1.41\pm0.01)\ 10^3$	0.9998

In all cases linear plots were obtained: Table 2 reports the regression equation and the correlation factors and Table 3 reports the detection limits (*d.l.*), calculated through the sensitivity *S* (expressed as the peak area given by the integrator for 1 μg/L solution) and the evaluation in the chromatogram of a peak area (*a*) corresponding to an average signal to noise ratio = 3 ($d.l. = a/S$ (μg/L)). Detection limits resulted of 3.0 μg/L for hexazinone and equal or lower than 1.0 μg/L for the other herbicides.

Application of the Method to Surface Water Samples

Figures 3-5 (A) show the chromatograms recorded under the optimized conditions for some samples of surface waters and namely samples of Po and Dora Riparia rivers, which flow near Turin and collect effluents from agricultural zones, and a sample of Venice lagoon water, collected near the outlet of the Dese River. No herbicide, at least at the detectable level, seems to be present in all the samples studied.

Figure 3-5 (B) show the chromatograms of the same samples added with a mixture of hexazinone, simazine, atrazine, propazine, terbuthylazine, prometryn, at concentration of 8.0 (Figure 4) and 10.0 μg/L (Figures 3 and 5) each. The recovery yields, evaluatedc through standard addition method are

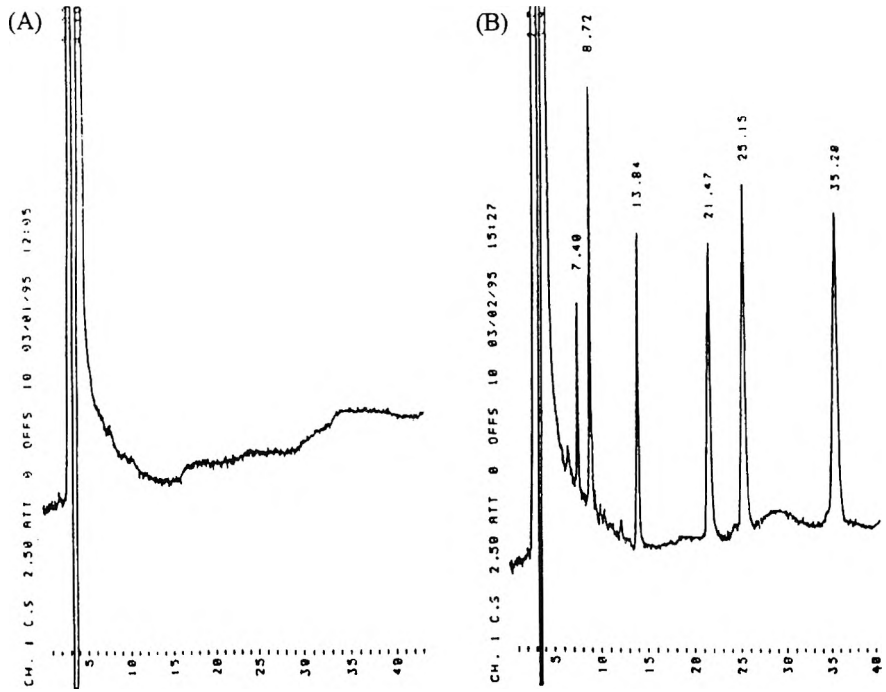


Figure 3. Chromatogram of a native (A) and spiked (B) sample of Po river water with 10.0 μg/L of hexazinone, simazine, atrazine, propazine, terbuthylazine and prometryn. Experimental conditions as in Figure 2.

always greater than 96% for all the herbicides, so thus showing a negligible matrix interference.

The results indicate that the method proposed can be successfully applied without preconcentration treatment in the analysis of surface waters, where the generally reported maximum concentration of herbicides is 30 μg/L[33]. The method proposed here does not require any pretreatment or derivatization process, except for filtration through a 0.22 μm membrane.

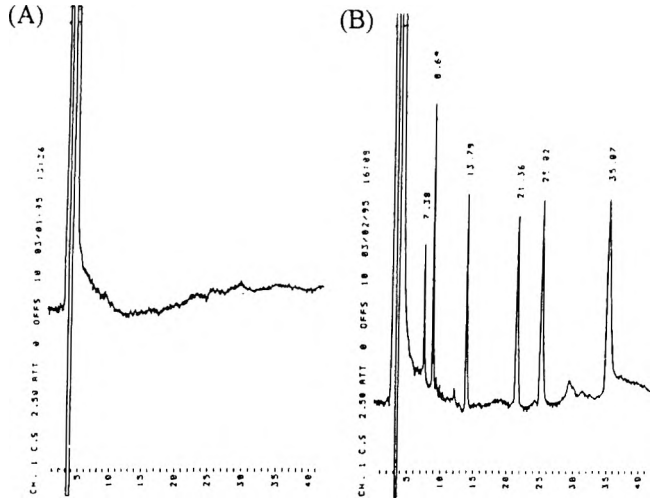


Figure 4. Chromatogram of a native (A) and spiked (B) sample of Dora Riparia river water with 8.0 μg/L of hexazinone, simazine, atrazine, propazine, terbuthylazine and prometryn. Experimental conditions as in Figure 2.

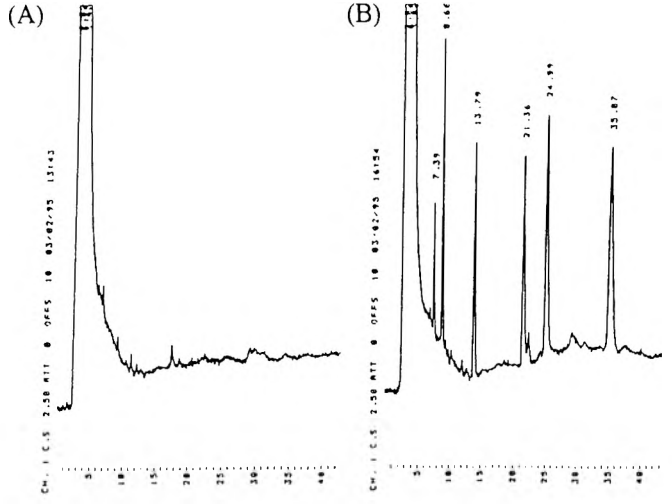


Figure 5. Chromatogram of a native (A) and spiked (B) sample of Venice lagoon water with 10.0 μg/L of hexazinone, simazine, atrazine, propazine, terbuthylazine and prometryn. Experimental conditions as in Figure 2.

Table 3

Detection Limits (μg/L) of the Analytes Investigated in the Optimized Chromatographic Conditions*

Analyte	Detection Limit (μg/L)
atrazine	1.0
hexazinone	3.0
propazine	0.8
prometryn	0.8
simazine	1.0
terbuthylazine	0.9

* Stationary phase: Phase Separation Spherisorb 5S 250 x 4.6 mm (5μm) cartridge column; mobile phase: octylamine 5.0 mmol/L in water/acetonitrile solution (55/45) brought to pH 6.4 with o-phosphoric acid, flow-rate 0.7 mL/min; spectrophotometric detection at 230 nm.

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SOFTWARE REVIEW

PAPYRUS, Version 7.0, Research Software Design Corp., 2718 SW Kelly Street, Portland, OR 97201, Price: $99.00.

Every scientist knows the difficulties involved in building and maintaining a reference file. Papyrus is a bibliographic database specifically designed to help in this task. The purpose of Papyrus is to allow the user to build a database of literature references easily and to manipulate that database, including the possibility of outputting selected citations in accordance with the requirements of any journal.

The program is a DOS-based program, but it can also be installed and operated within Windows. The installation process is fairly simple, straightforward and fast. The manuals (a reference and a tutorial) are very user-friendly and easy to follow. As a result, learning to use the database is quite simple, although some of the data manipulation can become complicated; e.g., the creation of an output format to a required layout. I found Papyrus to be intuitive and I was able to set up a reference database effortlessly and quickly.

For entering references, the software provides a large number of input templates: for journal articles, books, chapters, patents, maps, these, etc. Each input template has two levels of fields. The most common fields, such as year of publication, title, etc., are always visible. The less-used fields (at least according to Papyrus' way of thinking), such as author affiliation or address, are hidden, but can be made visible at will. The user can develop a keyword dictionary which will allow him to do intelligent searches later on. He canm enter an abstract and attach unlimited "note cards" to each reference. Each field can be up to 8K characters long, which should be sifficient for virtually all situations. However, the verbose user can use the unlimited note cards feature to enter further comments.

The references in the database can be manipulated in the standard way. The user can search the database either according to specific fields or globally. Logical operations AND, OR and NOT are allowed in the searches. Lists can be built (either alphabetical or numerical) and outputted to the screen, printer or to a file. References can be grouped into sub-libraries according to several criteria. Housekeeping chores, such as maintaining the journal list, or the keyword list, is relatively straightforward.

Papyrus has some nice features which make it easier to build and maintain the library. For example, once a particular keyword has been defined, all the user needs to do is type any reasonable abbreviation of the keyword, and Papyrus will fill in the rest. The same holds true for journal names. Another nice feature is the possibility of seeing all of the prvious items in a field and choosing an item from that list to be inserted into the field.

The program has some limitations. The fact that it is DOS-based makes it very difficult to interact with other programs. For example, putting a citation directly into a word processor (especially a Windows-based program) manuscript is awkward. Also, since thw working screens in Papyrus are text screens, it is impossible to add figures to references or to use unusual characters such as sub- and superscripts (although Papyrus does allow you to print sub- and superscripts using special control characters).

Another limitation is that there is no Back Screen option. If you make a mistake and wish to go back to correct the error, you are "out of luck." Your only recourse is to hit the ESC key, which takes you back all the way to relevant main sub-menu.

These shortcomings notwithstanding, I found Papyrus to be a very useful package. Its low cost, ease of use, friendliness and range of features make it very attractive. If you are looking for a references manager, Papyrus is highly recommended.

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J. LIQ. CHROM. & REL. TECHNOL., 19(1), 163–164 (1996)

THE BOOK CORNER

GAS CHROMATOGRAPHY: ANALYTICAL CHEMISTRY BY OPEN LEARNING, Ian A. Fowlis, John Wiley and Sons, New York, 1995; 258 pages; ISBN 0-471-95467-5 and in paperback as ISBN 0-471- 95468-3.

The text provides a working knowledge of gas chromatography and gas chromatographs. It is recommended for those who are just assuming duties for gas chromatographic determinations and for those who may have been taught how to perform a specific chromatographic determination and desire a fundamental understanding of the technique. Chemistry students who are ready for courses in analysis will find this text an excellent introduction to chromatographic practice.

The chapters cover the basic instrumentation, packed and capillary systems, injectors, detectors, data handling, quantitative and qualitative analyses. Additional sections provide introductory information on combined techniques, analysis of less-volatile substances, and environmental analysis. Specific topics generally are easy to locate from consulting the table of contents or index. Thus the student has a good reference text as well as a good learning guide.

The relationships among fundamental chromatographic parameters are dealt with concisely and in an easy-to-follow manner. Although the text disclaims coverage of chromatographic theory (the student is referred to another text in the *Analytical Chemistry by Open Learning* series for this information.), it does provide sufficient information for a basic understanding of the chromatographic process. In this respect it is weak only the the order in which a couple of topics are presented: The importance of carrier gas selection is mentioned a couple of times before an explanation is presented; and focusing or refocusing is discussed before it is defined (except perhaps in a 60-word sentence on temperature programming). These are exceptions to the the generally clear and logical way concepts and practice are presented.

The repeated emphasis on the importance of the time spent in the mobile phase is especially welcomed for it has been my experience that students should learn that concept first.

The text stresses to the point of redundancy that no text can provide the understanding of practical experience in the laboratory. Nevertheless the reasons behind standard chromatographic technique are clearly explained. I found only one exception: the importance of the position of the needle tip during injection leaves the student without any guide or reference for knowing what should be done.

The "Fowlis Rule" for determining which compounds may be successfully handled by gas chromatography comes from the author's years of experience. If a solute does not dissolve in ethyl acetate, one had best look for another method for separation, according to the rule.

The figure on the split/splitless injector is confusing. Although the pathway for carrier to septum purge is clearly open, the pathways to the column and split outlet appear blocked. I found only one incidence of lack of clarity in the text: "... the programmed temperature vaporising injector sometimes called the split/splitless injector."

Two omissions are puzzling. The author gives considerable (and deserved) credit to Walter Jennings for work in capillary chromatography and uses the "DB" series in cataloguing chromatographic phases. Nevertheless, Jennings' 1987 text, *Analytical Gas Chromatography*, is not listed in the Bibliography. Finally, I am not sure how the author kept from drawing a lesson from the statement that "on-column injection ... is the method of choice for the *discriminating* chromatographer."

Reviewed by
Leonard H. Ponder, FAIC
Shimadzu Scientific Instruments, Inc.
Columbia, Maryland

J. LIQ. CHROM. & REL. TECHNOL., 19(1), 165–169 (1996)

LIQUID CHROMATOGRAPHY CALENDAR

1996

FEBRUARY 5 - 7: PrepTech'96, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. R. Stevenson, 3338 Carlyle Terrace, Lafayette, CA 94549, USA, or Ms. Joan Lantowski, ISC Technical Conferences, Inc., 30 Controls Drive, P. O. Box 559, Shelton, CT 06484, USA.

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

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

MARCH 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.

APRIL 17 - 19: VIIth International Symposium on Luminescence

Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Université de Nice, Fracnce. Contact: Prof. W. R. G. Baeyens, University of Ghent Pharmaceutical Institute, Lab or Drug Quality Control, Harelbekestraat 72, Ghent, Belgium. Email: willy.baeyens@rug.ac.be.

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

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

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

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

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

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

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

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities,

Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

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

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

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

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

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

1997

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

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

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

1998

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

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

1999

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

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

2000

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

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

2001

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

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

2002

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

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

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Following are acceptable reference formats:

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

Book:

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

Chapter in a Book:

1. C. T. Mant, R. S. Hodges. "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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