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OPERATIONAL CHARACTERISTICS OF THE EVAPORATIVE LIGHT SCATTERING DETECTOR USED IN ANALYSIS OF ETHOXYLATED ALCOHOLS

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ABSTRACT

Influence of the temperature of a drift tube and flow rate of nitrogen as nebulizing gas with 100% hexane and 40% hexane plus 60% (5% H_2O in 2-propanol) as mobile phases on the respones of ELS detector for selected standards of ethoxylated alcohols was examined. For optimal conditions (1.5 dm^3 N_2/min, 50 °C) calibration curves for both mobile phases were created. The response of ELSD for standards not undergoing evaporation is mainly affected by the composition of the mobile phase.

INTRODUCTION

UV detector, most frequently used in liquid chromatography, is excellent in analysis of ethoxylated alkylphenols, mainly thanks to its compatibility with gradient mode, high sensitivity and a straight-line relationship between the response and the mass of the substance under determination. On the other hand, ethoxylated alcohols and acids which do not possess chromophore groups have to be derivatized.

Literature offers two derivatization methods. The first one is the reaction with phenyl isocyanate[1-5] and the other is the esterification of ethoxylates with 3,5-dinitrobenzoil chloride in the presence of pyridine.[6] The derivatized ethoxylates, absorbing in the UV range, are not observed to essentially change their affinity to normal-phase packings. Some elongation of retention times is observed on reversed phases due to the higher hydrophobicity of the ethoxylated alcohol molecules following their reaction with phenyl isocyanate or dinitrobenzoyl chloride. Such additional step contributes both to elongation of the analysis time and to the error of the method. Which is more, the peaks of the components determined overlap the peaks of the derivatising reagent. Unfortunately, it is also necessary for quantitative calculations to find correction coefficients for the respective derivatized oligomers, as their UV absorption depends on the length of the polyoxyethylene chain.

Traces of ethoxylated alcohols and alkylphenols are also determined using a fluorescent detector.[7,8] Alkylphenol ethoxylates can be determined without derivatization but ethoxylated alcohols with no fluorescent groups in a molecule need conversion into their appropriate derivatives in reaction with 1-anthroylnitryl. The other derivatising agents include 1-naphtyl isocyanate[9,10] and naphtoyl chlorides.[11] Because of its very low detection limit (high sensitivity) fluorescent detector is very often used in analysis of waste water samples (environmental analysis).

RI detector is not suitable for analysis of such complex mixtures (finding the distribution range), as it is only applicable under isocratic conditions[12] and have quite low sensitivity, which may be decisive in trace determination. However, it is useful in determination of the total content of PEG in ethoxylation products under isocratic conditions with either CH_3OH or CH_3CN as an organic modifier of a mobile phase.[13,14] Flame-ionisation detectors (FID) are not widely used in HPLC either, mainly because of problems with repeatability.

The most promising for HPLC is the ELS detector. Although recent versions of the ELS detector have sensitivities comparable with the RI detector, yet its applicability in the analysis of high-boiling substances using a gradient and solvents with absorptions in the UV range (tetrahydrofurane, acetone, toluene, dimethylformamide, etc.) gives it a significant advantage over all other types of detectors. Its compatibility with gradient mode may be useful in direct analysis of ethoxylation products distribution both on normal and reverse phases. The use of light scattering on aerosol particles for non-volatiles detection was first reported by Ford and Kennard who described their 'Evaporative Analyser' in 1966.[15] In 1978, Charlesworth published an excellent work[16] on the operational characteristics of the ELS detector. While the signal formation mechanism has not been studied thoroughly yet,[17-24] more and more papers are published year by year on the analysis of glycerides,[25-28] carbohydrates[29] phospholipides,[30-32] saccharides,[33] steroids,[34] surfactants,[35-37] amino acids[38] and other ones[39-42] using this detector.

The ELS detector is best applicable with columns of diameters equal to or even less than 2.1 mm (narrow bore) for which the consumption of a mobile phase is drastically lower (for ϕ = 2.1 mm it is five times as low as for the widely used columns with ϕ = 4.6 mm). Beside purely economic benefits, there is also the advantage of evaporation temperature. As it is obviously easier to evaporate 0.3 cm^3/min (which is an optimum flowrate for columns with ϕ = 2.1 mm) than 1.5 cm^3/min (optimum flowrate for columns with ϕ = 4.6 mm) of a mobile phase, lower temperatures may be used in the former case without a risk of evaporation of the components of the mixture subjected to analysis.

The aim of the work is to show the correlations between the response of the ELS detector and the temperature of the drift tube and a flow rate of the nebulizing gas (N_2); Additionaly the calibration curves and the dependence of the response of the ELSD on the composition of the mobile phase for standards of ethoxylated alcohols are presented.

EXPERIMENTAL

Reagents

Hexane and 2-propanol of HPLC grade were obtained from BDH Laboratory Supplies (U.K.). High purity water was obtained through HP 661A Water Purifier (Hewlett Packard, U.S.A.). Nitrogen of technical grade (99.8%)

used as nebulizing gas was supplied by Linde Gas Poland. 1-dodecanol (C12OH) was purchased from Merck (Germany). Standards of ethoxylated alcohols were obtained either from Fluka Chemie AG (Switzerland) (Pentaethylene glycol monodecyl ether - C10EO5, Pentaethylene glycol monotetradecyl ether - C14EO5, Pentaethylene glycol monoheksadecyl ether - C16EO5, Nonaethylene glycol monododecyl ether - C12EO9) or from Nikko Chemicals Co., Ltd. (Japan) (Monoethylene glycol monododecyl ether - C12EO1, Triethylene glycol monododecyl ether - C12EO3, Pentaethylene glycol monododecyl ether - C12EO5, Heptaethylene glycol monododecyl ether - C12EO7, Pentaethylene glycol monooctadecyl ether - C18EO5); digit after EO indicate number of oxyethylene groups. All with purity at least 97 %.

Apparatus

The HPLC system consisted of a Hewlett Packard HP 1050 Liquid Chromatograph connected with ELS detector [Varex II - (U.S.A.)] by a 90 cm capillary with 0.25 mm I.D. The samples were introduced directly to detector without a chromatographic column by Waters 717 plus autosampler (Waters, U.S.A.) with 25 μL loop. The flow of a mobile phase was 0.3 cm^3/min. Channel A was hexane and B was 5% H_2O in 2-propanol. Each experimental point was an average of 5 injections. After changing a detector's working parameters (N_2 flow, drift tube temperature) the system was stabilized for at least 20 min. A personal computer with Grams/386 for Chromatography software (Galactic, U.S.A.) were used for data collection and quantification of peak areas. Sampling rate was set on 25 Hz. Results of integration were exported to Excel spreadsheet (Microsoft Corporation, U.S.A.) and after calculation of averages and standard deviations were sent to Grapher (Golden Software, U.S.A.) which was used for drawing curves and for determination of correlation parameters.

RESULTS AND DISCUSSION

Response Of ELSD For Ethoxylated Alcohols Standards

Formation of the ELS detector signal comprises three complex processes:

1. Nebulization of an effluent from the chromatographic column.

2. Evaporation of the lower boiling solvent and concentrating the higher boiling component to be determined.

3. Scattering of incident light on such aerosol particles.

For a given mobile phase, the value of the response of the ELS detector depends on three parameters, namely the following:

a) drift tube temperature,
b) linear rate of the nebulizing gas (N_2), and
c) flowrate of a mobile phase.

This paper refers only to analysis of the effect of a) and b), assuming the flowrate of a mobile phase of 0.3 cm^3/min as an optimum for chromatographic columns with ϕ = 2.1 mm (as results from the height of the theoretical plate), and frequently recommended by manufacturers of LC equipment (Hewlett Packard, Macherey Nagel, Waters etc.).

Later in this paper, the effect of the kind of the solvent used in normal-phase separation on the detector response is investigated for the selected standards of ethoxylated alcohols. Shown below are the relationships between the ELS detector response, drift tube temperature, flowrate of the nebulizing gas (N_2) and the mass of the component determined, for limiting values of the optimal gradient used in a system of normal phases by Bear,[43] that is for 100% hexane (beginning of the gradient), and 40% hexane + 60% (5% H_2O in 2-propanol) (end of the gradient).

Effect Of Drift Tube Temperature On ELSD Signal

Hexane as a mobile phase

The temperature effect was investigated in the range 30-120 °C and found to be significant for the response of C12OH and lower oligomers (Fig. 1). The alcohol stops being 'visible' for the ELS detector as early as above 40 °C, whereas the first homologue, C12EO1, is not detectable just above 50 °C. The value of the signal of the latter is twice as small with temperature growth from 30 to 40 °C. Up to 50 °C, the response of homologues containing not less than three oxyethylene groups is the same. For C12EO3, the value of the signal drops by half at 90°C; for C12EO5 it does at 120 °C. For C12EO9, it is not

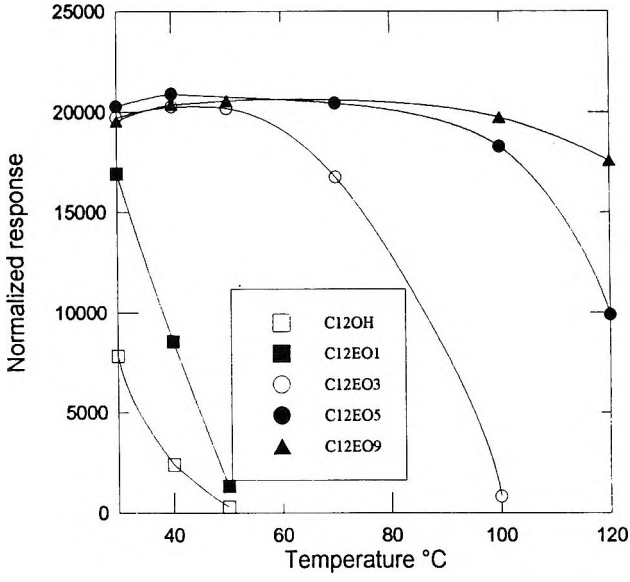

Figure 1. Relationships between the ELS detector response and the detection temperature for polyethylene glycol monododecyl ether homologue standards (mobile phase = 100 % hexane, V=1.5 N_2 dm^3/min).

temperature dependent between 30 and 100 °C. Experimental results show that for relatively volatile components, the detector signal depends on their vapor pressure.

40%Hexane + 60%(5%H_2O in 2-propanol) as a mobile phase

Unlike in the case of hexane, for a mixture 40% hexane + 60% (5% H_2O in 2-propanol) as a solvent the curves illustrating the relationship between the response of the ELS detector and detection temperature (except for the most volatile C12OH and C12EO1), have distinct maximums, namely 60 °C for C12EO3, 80 °C for C12EO5 and 110°C for C12EO9. Contrary to the highly volatile hexane, to evaporate 2-propanol which has lower volatility the drift tube is heated to a higher temperature, obviously producing an effect on the course of evaporation of the ethoxylates. The process is earliest commenced (even before complete evaporation of 2-propanol) for ethoxylated alcohols with the shortest polyoxyethylene chains (Fig. 2). C12EO1 stops being visible to the ELS detector above 50 °C, C12EO3 does above 100 °C and C12EO5 does above 130 °C. All the curves are convex parabolas ($y=ax^2+bx+c$, with $a<0$).

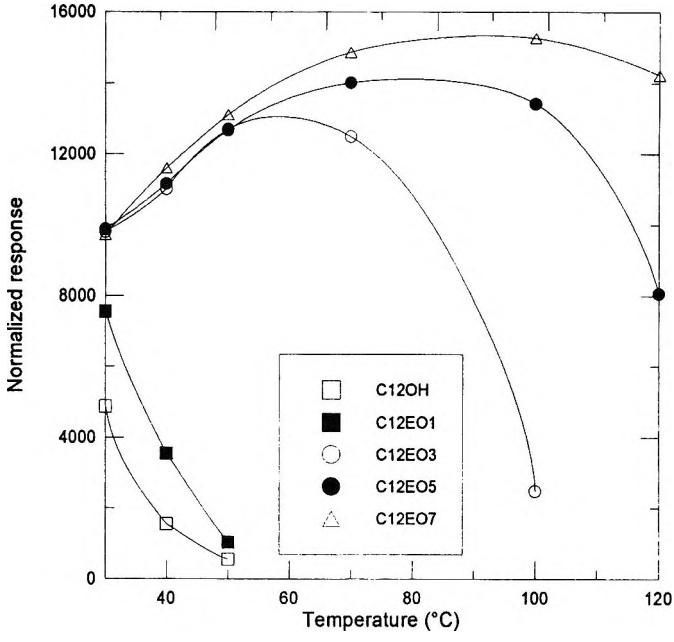

Figure 2. Relationship between the ELS detector response and the detection temperature for polyethylene glycol monododecyl ether homologue standards (mobile phase = 40 % hexane + 60 % (5 % H_2O in 2-propanol, $\forall$=1.5 dm^3 N_2 /min).

Standards containing shorter polyoxyethylene chains give 'narrower', while those with longer polyoxyethylene chains give 'wider' parabolas. 'Narrow' parabolas for the 'lower' standards show that evaporation of 2-propanol is accompanied by simultaneous evaporation of an ethoxylate. The left branches of the curves may indicate incomplete evaporation of 2-propanol which 'dilutes' the surfactant causing weaker light scattering which results in poor response. It may well be that the parabolic shape of the curves, other than for hexane, is under the influence of hydrogen bonds formed between the surfactants and 2-propanol.

For both extreme values of the gradient, 50 °C was selected as an optimum temperature. Such temperature is high enough to effect complete hexane evaporation while preventing evaporation of the homologues being determined (except C12OH and C12EO1). At such temperature, the

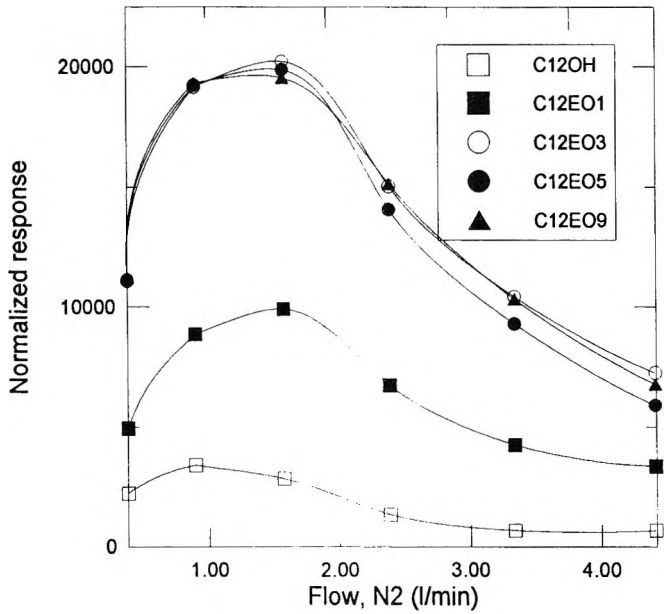

Figure 3. Relationship between the ELS detector response and the flowrate of N_2 for polyethylene glycol monododecyl ether homologue standards (mobile phase = 100 % hexane, T_{det}=50°C).

evaporation of 40% hexane + 60% (5% H_2O in 2-propanol) is probably incomplete and the resulting signal is lower than for hexane but, the higher homologues (C12EO3 and C12EO5) are not evaporated either.

Effect Of Volume Flow (Linear Velocity) Of Carrier Gas (N_2) On ELSD Signal

Hexane as a mobile phase

The same standards of ethoxylated alcohols (C12OH, C12EO1, C12EO3, C12EO5, C12EO7, C12EO9) were used to analyze the effect of linear velocity of N_2 on the value of the ELS detector response. The course of all the curves is similar to Gaussian curve with a maximum around 1.5 dm^3/min (35 mm N_2) and a distinct upward slope on the left side of the curves up to the maximum, which is followed by a drop of the value of the response with increasing linear

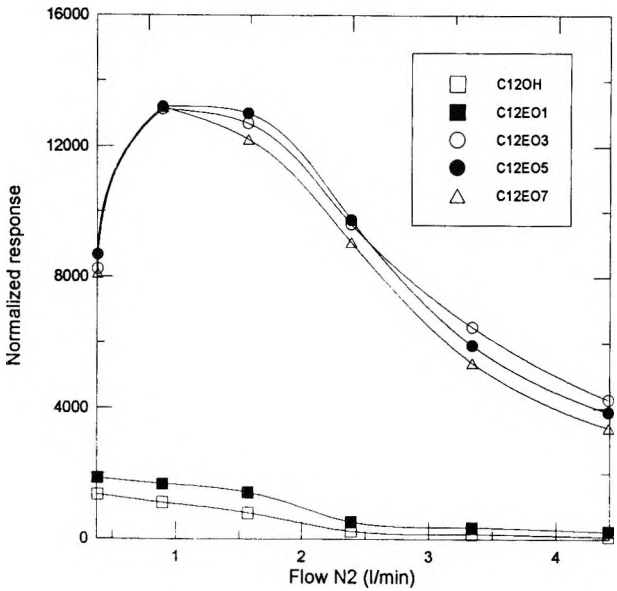

Figure 4. Relationship between the ELS detector response and the flowrate of N_2 for polyethylene glycol monododecyl ether homologue standards (mobile phase = 40 % hexane + 60 % (5% H_2 O in 2-propanol, T_{det} =50°C).

velocity of the nebulizing gas. (Fig. 3). The curves seem to indicate that the detector signal is dependent on the size of the aerosol particles of the ethoxylates rather than on their quantity, since with a growth in the linear velocity of the nebulizing gas, the quantity of the aerosol particles is inevitably higher while their size is smaller. Because the detector's signal also declines with an increase of linear velocity, the detector response is proportional to the particle size (right branches of the curves). The course of the left side of the curves at such low detection temperature (50 °C) and poor eluate nebulization may indicate incomplete evaporation of hexane which 'dilutes' the substances determined and cause poorer light scattering. Moreover, for C12EO3 and higher oligomers the course of the curves is almost identical. On the other hand, the value of the response at a maximum for C12EO1 is twice as small as for the oligomers referred to above, and almost ten times as small for C12OH (Fig. 3).

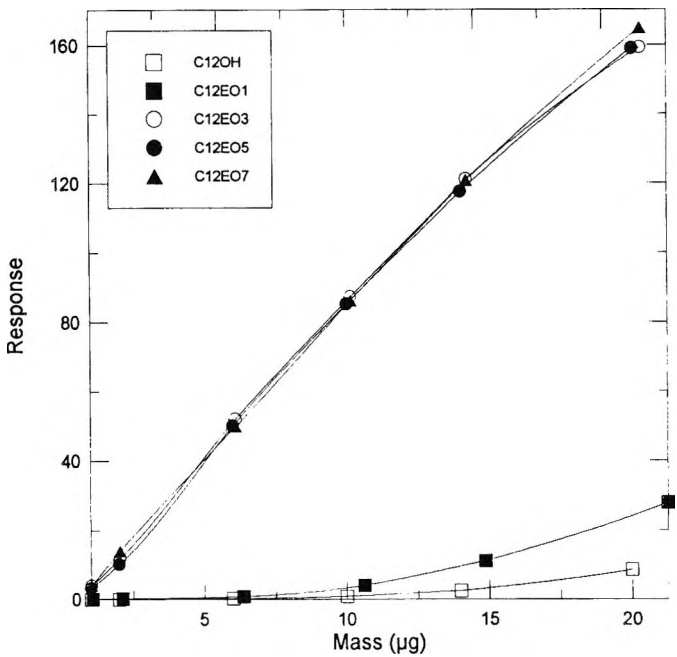

Figure 5. Calibration curves of polyethylene glycol monododecyl ether homologue standards with the same lengths of hydrophobic and different lengths of hydrophilic chains (mobile phase = 100 % hexane, V =1.5 dm^3 N_2 /min, T_{det} =50°C).

40 % Hexane + 60% (5% H_2O in 2-propanol) as a mobile phase

At 50 °C, the signal of all the ethoxylated alcohols (except C12OH and C12EO1) dissolved in 40% hexane + 60% (5% H_2O in 2-propanol) is the same; so the effect of the flowrate of the nebulizing agent was analyzed at such temperature. The resulting relationships are shown in Fig. 4.

No differences are observed in the course of the curves, though the maximum signal is about 13000, much lower than the maximum for hexane (20000).

For both extreme values of the gradient, an optimum flowrate of the nebulizing gas (in other words, the highest detector response) is 1.5 dm^3 N_2/min (35 mm), so the study was continued for that flowrate of N_2.

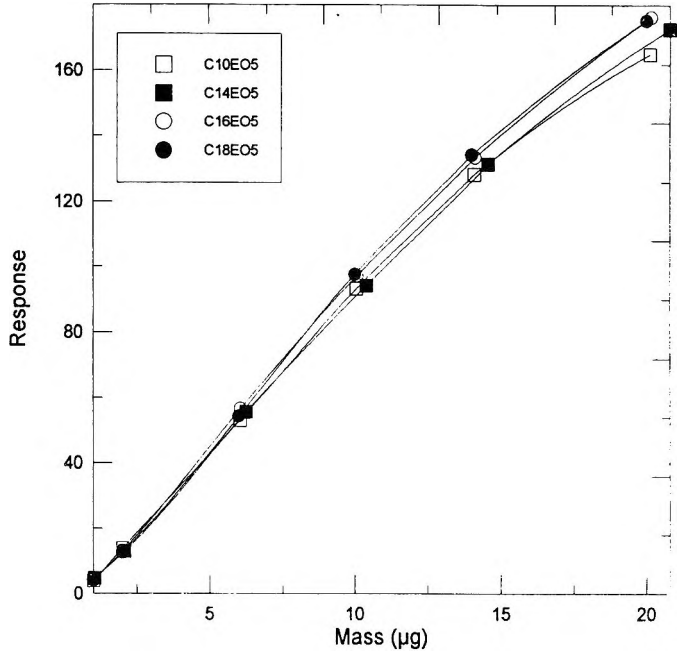

Figure 6. Calibration curves of pentaethylene glycol alkyl ether homologue standards with the same lengths of hydrophobic and different lengths of hydrophilic chains; (mobile phase = 100 % hexane, V=1.5 dm^3 N_2/min, T_{det}=50°C).

Dependence Of ELSD Response On The Mass Of Ethoxylated Alcohol

Hexane as a mobile phase

The relationships between the ELS detector response and the mass of ethoxylated alcohols and C12OH are shown in Figures 5 and 6 (at an optimum drift tube temperature and optimum flowrate of the nebulizing gas: 50 °C, 1.5 dm^3/min). The curves were ploted in a range 1-20 µg. Both for standards of ethoxylated alcohols having the same lengths of their hydrophobic and different lengths of their hydrophilic chains (C12EO3, C12EO5, C12EO7, C12EO9), as well as the same hydrophilic and different hydrophobic chain lengths (C10EO5, C12EO5, C14EO5, C16EO5, C18EO5), the detector response is the same within the margin of error and is not linear in the studied range 1-20 µg.

Table 1

Exponential Correlation Parameters ($Y=b*X^a$) for Ethyxylanted Alcohols for Hexane as a Mobile Phase

	Correlation Parameter		
Ethoxylate	**a**	**b**	**R^2**
C12EO3	1.2451±0.13407	4.52994±2.103E-05	0.98986
C12EO5	1.3107±0.14793	3.79955±3.0457E-05	0.98889
C12EO7	1.22077±0.14434	4.81896±2.8247E-05	0.98785
C10EO5	1.2396±0.16074	4.87505±4.2735E-05	0.98628
C14EO5	1.21462±0.11137	5.09298±1.0409E-05	0.99266
C16EO5	1.26393±0.1493	4.80086±3.195E-05	0.98786
C18EO5	1.25772±12728	4.87743±66.34E-05	0.99107

The average values of a and b for all standards are 1.2500 (σ-0.0298) and 4.6850 (σ=0.3926) respectively.

The only exceptions are C12OH and C12EO1 standards, having the highest volatility and undergoing evaporation in the drift tube under detector working parameters (Fig. 5).

Even the earliest measurements with ELS detectors showed that the relationship between the detector response and the mass of various components is non-linear,[43] though the results shown in a double-logarithmic scale tended to form straight lines with slopes of about 1.82.[44] So the function of the detector response should be of the exponential type:

$$Y=b*X^a$$

where: a - response coefficient.

The parameters of those correlations are shown in Table 1.

40% Hexane + 60%(5%H_2O in 2-propanol) as a mobile phase

Calibration curves of the detector response were found for the mixture of 40 % hexane + 60% (5% H_2O in 2-propanol) as a solvent in the same optimum conditions (1.5 dm^3/min, 50°C) as for hexane. The curves were also ploted in a

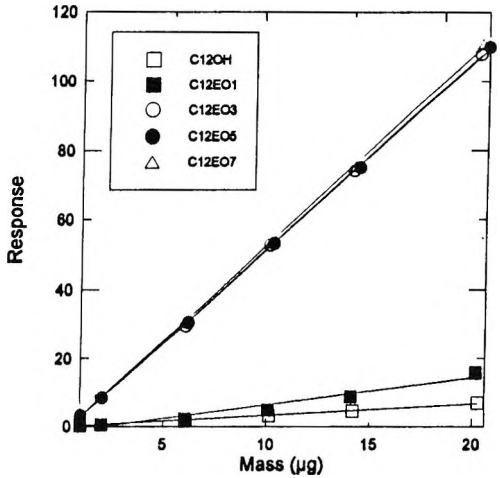

Figure 7. Calibration curves of polyethylene glycol monododecyl ether homologue standards with the same lengths of hydrophobic and different lengths of hydrophilic chains (mobile phase = 40% hexane + 60 % (5% H_2O in 2-propanol, V=1.5 N_2 dm^3/min N_2, T_{det} = 50°C).

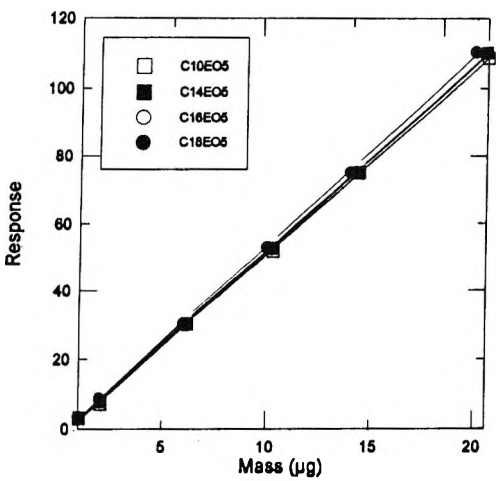

Figure 8. Calibration curves of pentaethylene glycol alkyl ether homologue standards with the same lengths of hydrophilic and different lengths of hydrophobic chains (mobile phase = 40% hexane + 60 % (5 % H_2O in 2-propanol, V=1.5 N_2 dm^3/min, T_{det} = 50°C).

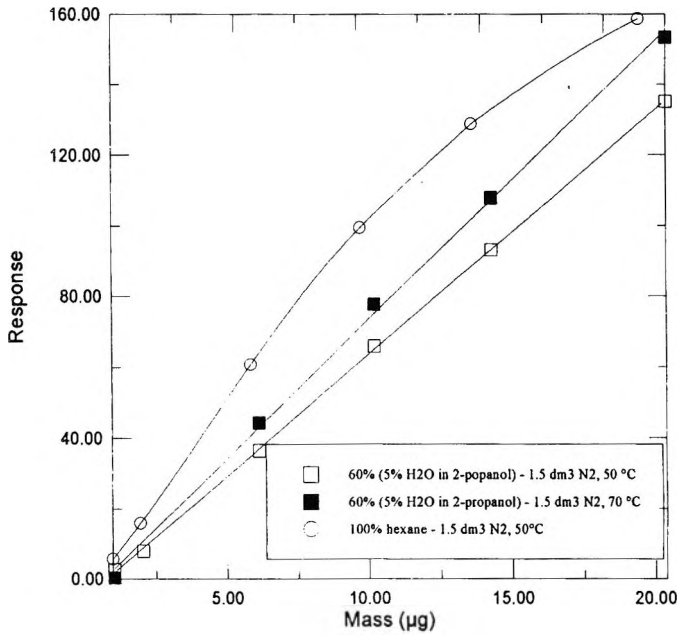

Figure 9. Comparison of the ELS detector response for C12E07 under various operating conditions.

range 1-20 µg. Contrary to hexane in the range under examination, all the curves are straight lines (Fig. 7 and 8). The most volatile standards - C12OH and C12EO1, which are evaporated at 50°C show lines with much lower slopes (0.3329 and 0.8022 respectively). At the same time however, as previously observed for the curves showing the dependence of the detector response on the drift tube temperature and nebulizing gas flowrate, the value of the signal was about 30% smaller than it was for hexane. Linear correlation parameters of the standards above are shown in Table 2.

Dependence Of ELSD Response On The Composition Of The Mobile Phase

Fig. 9 illustrates the differences in the run of the calibration curves for C12EO7 for hexane and 40% hexane + 60% (5% H_2O in 2-propanol) as a mobile phase. For the latter system, the calibration curve was plotted at 50 and 70 °C. As the standard is not evaporated at 70 °C while the mixture 40% hexane + 60% (5% H_2O in 2-propanol) is undoubtedly better evaporated than

Table 2

Linear Correlation Parameters (Y=a*X+b) forEthoxylated Alcohols for 60 % (5 % H_2O in 2-propanol) as a Mobile Phase

	Correlation Parameter		
Ethoxylate	**a**	**b**	**R^2**
C12EO3	5.4691±0.0813	-2.81351±0,07083	0.99985
C12EO5	5.45463±0.0800	-2.76133±0.07194	0.99981
C12EO7	5.56501±0.0740	12.66079±0.04936	0.99973
C10EO5	5.43627±0.087553	-3.35764±0.10297	0.99976
C14EO5	5.50475±0.0947	-3.31288±0.13873	0.99982
C16EO5	5.49277±0.1178	-2.76655±0.32474	0.99960
C18EO5	5.62852±0.1056	-2.83525±0.19375	0.99969

The average values of a and b for all standards are: 5.5073 (σ=0.0627 and -2.9297 (σ=0.2618) resepectively.

in 50°C, the run of the this curve is closer to the one plotted for hexane. The straight-line shape of the curve obtained for 40% hexane + 60% (5% H_2O in 2-propanol) as a mobile phase is quite far (about 20% in the middle section of the range) from the curve plotted for hexane which is 'positively deflected' from it while both at low and high concentrations, the responses are very close for the two mobile phases.

Since the ELSD responses for ethoxylates non-evaporating under optimal detection conditions ($\dot{V}$ = 1.5 dm^3 N_2/min, T_{det} = 50 °C) are statistically the same and only dependent on the composition of a mobile phase, the arbitrarily selected C12EO7 was used to create calibration curves for 10, 20, 30, 40, 50, 60% (5% H_2O in 2-propanol) as a mobile phase (with hexane complement). As expected, an increase in the content of 2-propanol in a mobile phase was accompanied by a decrease of the slopes of the plotted lines with simultaneous 'intensification' of the linear shape of the curves, indicated by greater linear correlation coefficients (Fig. 10).

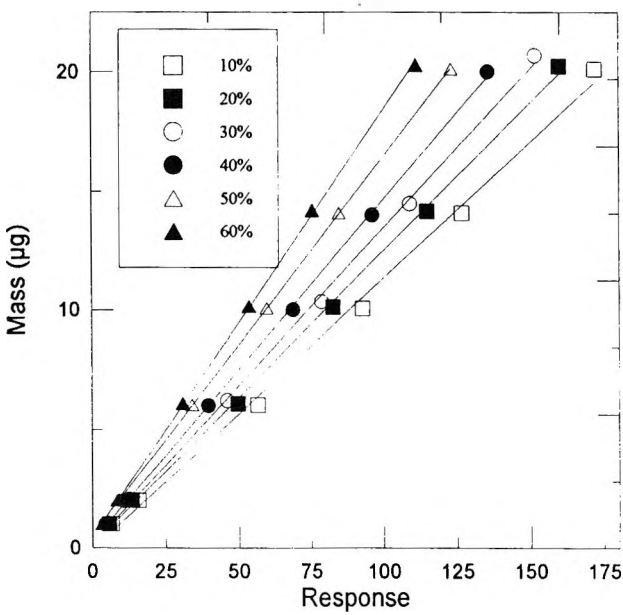

Figure 10. Mass=f(response) for various compositions of a mobile phase for heptaethylene glycol monododecyl ether C12EO7(V=1.5 dm^3 N_2/min, T_{det}=50°C).

Fig. 11 illustrates the dependence of the slopes of the curves shown in Fig. 10 on the composition of a mobile phase (% B) and retention time. In the range under examination, the relationship is illustrated by a parabola and may be described by the equation:

$Y = 1.4143*10^{-5} * X^2 + 2.78094*10^{-4} * X + 1.11003*10^{-1}$ (R^2_a=0.997097, R^2_b= 0.971322) for the composition of a mobile phase (where X= %B), and

$Y = 1.68331*10^{-5} * X^{-2} + 1.92203*10^{-4} * X + 1.110186*10^{-1}$ (where $X=t_r$) for retention times.

The latter shows a direct relationship between the composition of a mobile phase and the correction coefficient by which the peak area of a given homologue with a specific retention time, t_r, is to be multiplied to obtain its quantity expressed in µg.

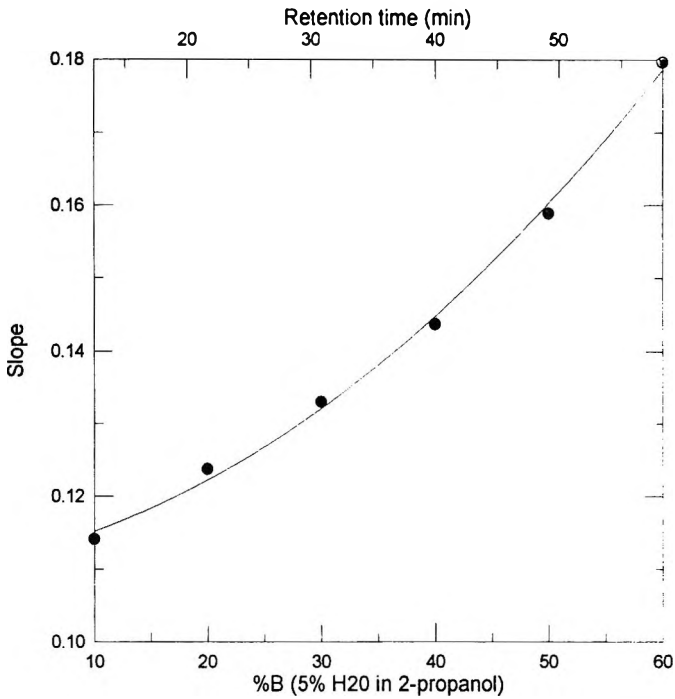

Figure 11. Relationships between the slope of the straight lines shown in Figure 13 and the concentration of (5 % H_2O in 2-propanol) in hexane and retention times.

SUMMARY

A study carried out with the use of an ELS detector indicates that the ELSD response, in the range under examination (valuable from the practical point of view) is the same for all studied standards of ethoxylated alcohols (except for C12OH with C12EO1, and C12EO2 which have the lowest boiling points), whereas the UV-VIS detector gives a response dependent on the length of the hydrophilic chain. However, the detector signal for these standards depends to a significant extent on the composition of a mobile phase and is the greatest for 100% hexane under optimum conditions (N_2 flowrate and drift tube temperature). The run of the calibration curves for a mobile phase containing 2-propanol is linear, while for hexane is linear in log-log scale with a response

coefficient equal to 1.25. The difference between the response for 100% hexane and a mobile phase containing 2-propanol, probably caused by different evaporation of these solvents, is much smaller for higher detection temperatures (>70 °C), at which on the other hand, standards of the lower ethoxylated alcohols undergo evaporation.

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THE COMPARATIVE INVESTIGATION OF SEVERAL STATIONARY PHASES CONTAINING IMINODIACETIC FUNCTIONAL GROUPS FOR THE HIGH PERFORMANCE CHELATING EXCHANGE CHROMATOGRAPHY

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ABSTRACT

Three chelating ion-exchangers having iminodiacetic acid functional groups immobilised at the surface of different substrates (silica gel, hydrophilic and hydrophobic polymer matrices) were compared for the separation of various alkaline-earth and transition metal ions. The retention of metal ions on two commercially available Diasorb IDA silica (250 mm x 4 mm id.) and Tosoh TSK Gel Chelate 5 PW (75 x 7 mm id.) columns and column packed with poly(styrene-divinylbenzene) substrate coated with Phthalein Purple dye was investigated in maleate, tartrate and oxalate mobile phases. Metal-ion retention increased

with pH and with a decrease in eluent concentration. The complexing ability of ion-exchangers decreased in the order IDA-silica > TSK Gel Chelate 5 PW > polystyrene-divinylbenzene coated with Phthalein Purple dye. The selectivity of separation was similar for IDA-silica and TSK Gel Chelate 5 PW and was slightly different for the dye coated column. The chromatographic separation of metal ions is demonstrated in oxalate and tartrate mobile phases.

INTRODUCTION

Chelating ion exchangers containing iminodiacetic acid (IDA) functional groups have been widely used for the preconcentration of traces of transition metal ions from waters with complex matrices. Their main advantages are good selectivity and good kinetics of complexation. The latter property allows the application of iminodiacetic acid containing sorbents for the on-line preconcentration and also for the direct chromatographic separation of metal ions.

In the last decade a great interest in the application of different iminodiacetic acid containing ion-exchangers for the ion-chromatographic separation of metals has appeared.[1-10] The application of octadecylsilica dynamically modified with dodecyliminodiacetic acid for the isocratic separation of alkaline-earth metal ions was described.[1] The tartrate eluent was used for the separation of four metals. The covalent attachment of iminodiacetic acid to the polymer or silica surface produced chelating phases of increased working stability. Thus, Toei[2] used the commercially available polymer based column TSK-Gel Chelate 5-PW bearing IDA groups. The separation of magnesium, calcium and strontium was achieved with o-cresolphtalein complexone as a component of the mobile phase and magnesium and calcium were determined in sea water. It should be noted this column packing was originally designed for the metal-chelate affinity chromatography of proteins and peptides in neutral media so its stability in acid eluent was suspect.

This problem can be overcome by using silica based sorbents. The higher stability with good separation efficiency and selectivity for alkaline, alkaline-earth and transition metals has been demonstrated for iminodiacetic acid bonded silica.[3-5] It was found that both complexation reactions and ion-exchange interactions occurred on the stationary phase. As a result, the ion-chromatographic separation of alkaline, alkaline earth and transition metal ions was possible with different mineral and organic acids used as the eluent.

chromatographic separation of alkaline, alkaline earth and transition metal ions was possible with different mineral and organic acids used as the eluent.

Another type of chelating ion-exchanger with IDA groups was investigated by Jones and co-workers.[6-10] He studied a number of HPLC resins impregnated with dyes containing iminodiacetic acid functional groups such as Xylenol Orange,[6-9] Methyl thymol Blue[6-10] and Phthalein Purple.[10] These Methylthymol chelating ion-exchangers proved to be extremely successful for the separation of a number of alkaline earth and transition metal ions using step gradient elution. The application of impregnated resins to the chromatographic determination of different metal ions in concentrated brines, sea water was described. To decrease the influence of complex matrices on the concentration and the separation of metal ions the eluents of increased ionic strength (0.5 - 1.0 M solutions of KNO_3 or KCl) were used. It allowed the supression of the ion-exchange equilibria in chromatographic systems and therefore to use mainly the chelating ability of resins coated with dyes for the separation.

So, it can be seen that the several chromatographic approaches including chelating ion-exchangers with IDA groups have been studied. However, in order to establish the general properties and the optimal substrates for the high-performance separation of transition metal ions, more detailed investigations are required. In this study three different types of iminodiacetic acid containing ion-exchangers and their metal-ion chromatographic behaviour were evaluated.

MATERIALS

Apparatus

An isocratic ion-chromatographic system consisted of LKB 2150 HPLC titanium pump (Bromma, Sweden), a Rheodyne 7010 polyether-ether-ketone (PEEK) liquid six port injection valve (Rheodyne, Cotati, CA, USA) fitted with 100 μL PEEK sample loop and post-column detection system. The latter included a Constametric III HPLC pump (LDC, Riviera Beach, Fl, USA) used for delivery of 4-(2-pyridylazo)-resorcinol (PAR) reagent, a zero dead volume PTFE tee followed by a 1.4 m, 0.3 mm ID PTFE reaction coil and Dionex Spectral Array detector (Dionex, Sunnyvale, CA, USA) set at 490 nm.

Columns

Tosoh (Tokyo, Japan) and JV BioChemmack (Moscow, Russian Federation),

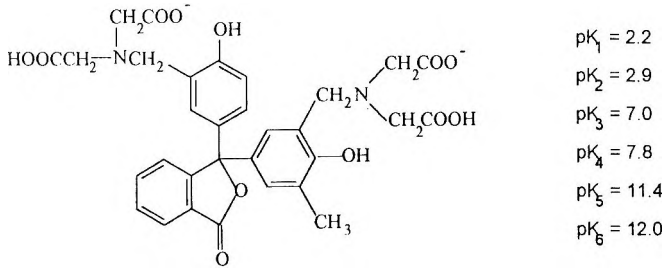

Figure 1. The structure and pK values of Phthalein Purple.

correspondingly.

Phthalein Purple (Sigma Chemicals, Poole, UK) was used to impregnate 8.8 μm particle size polystyrene divinylbenzene neutral hydrophobic resin (Dionex, Sunnyvale, CA, USA). The modified resin was packed in a 100 x 4.6mm ID PEEK column.

Reagents

Phthalein Purple (Sigma Chemicals, Poole, UK) or *o*-Cresolphthalein-3',3"-bis-methyleniminodiacetic acid (Fig. 1) was used for impregnation of polystyrene divinylbenzene neutral hydrophobic resin. Common reagents were supplied by BDH (Poole, UK) except PAR and Zn-EDTA which were obtained from Fluka (Glossop, UK). Metal stock solutions were Spectrosol standard solutions obtained from BDH. Other reagents were of analytical grade. All solutions were prepared using deionised water from Milli-Q system (Millipore, Bedford, MA, USA).

RESULTS AND DISCUSSION

Comparative Characteristics of Chelating Ion-exchangers

As described earlier the nature of bonded chelating agent is a key factor responsible for the selectivity of the stationary phase. IDA exhibits both ion-exchange properties for metal ions attributed to free carboxylate groups and a

Table 1

Characteristics of Chelating Sorbents Containing Iminodiacetic Acid Functionality

N	Name	Particle Size, μm	Diameter Pore, nm	Matrice	pH Range Stability	Concentration of Ligand μMole/g	Capacity for Transition Metals
1	Tosoh TSK Gel Chelate 5-PW	10	100	hydrophilc polymer*	2-12		24 μmole/ml Cu(II)[12]
2	IDA-silica	6	15	silica	1-7	130	
3	Phtalein Purple	8.8	12	neutral PST-DVB	0.5-11	157	22.1 μmole/g Zn(II)

strong complexing property especially for transition metal ions due to the formation of a stable 6-member ring complex including nitrogen and carboxylic groups. According to literature data the complexing ability of Phthalein Purple molecules is lower than N-alkylsubstituted iminodiacetic acid.[11] The type of immobilisation of molecules at the surface of substrates determines the availability of IDA groups for binding of metal ions. So, one can conclude from Table 1, only 14 mole% of the dye was "active" assuming 1:1 ratio of metal to ligand binding.

Besides the type of immobilisation of IDA groups at the surface of the matrix, the concentration of immobilised ligands, their distribution at the surface and the nature of matrix can also impact on the selectivity of separation. Some of above properties of studied IDA chelating ion-exchangers are presented in Table 1. As can be seen from Table 1 the studied sorbents have hydrophilic and hydrophobic polymers and silica as a backbone. Nevertheless, both the concentration of immobilised ligand and the capacity of studied chelating exchangers have similar magnitudes. It should mean equal retention for columns of equal volume assuming similar complexing ability. Practically equal values of column volume were calculated for TSK-Gel Chelate 5 PW column and IDA-silica column namely 3.3 mL and 3.14 mL, respectively. The volume of the Phthalein Purple column was about 2.5 times smaller.

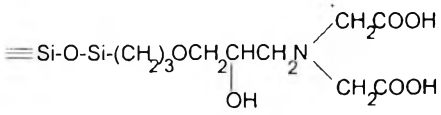

Figure 2. The structure of silica based chelating ion-exchanger

The main drawbacks of polymeric substrates are swelling and shrinking with change in pH and ionic strength of eluent. However, both the hydrophilic polymer in the case of TSK Gel Chelate 5PW and polystyrene- divinylbenzene neutral hydrophobic resin coated with Phthalein Purple had no tendency for volume change in various eluents.

It would be expected a litttle difference in acid-base properties for Phthalein Purple (Fig. 1) in solution and in immobilised state at the polymer surface. In case of iminodiacetic acid covalently bonded to silica (Fig. 2) the change in acid-base properties of chelating ion-exchangers is possible due to secondary interactions with residual silanol groups.

Dependence of Retention of Transition Metal Ions on Concentration of Eluent

The selectivity of chelating exchangers and the retention of transition metal ions can be controlled by altering the conventional stability constants of the chelates, through the pH of the eluent or through the concentration of complexing acid in the eluent. The solutions of dicarboxylic acids of different complexing and acidic ability (maleic, tartaric and oxalic) were used as eluents for some transition metal ions. Fig. 3 shows the dependence of the logarithm of capacity ratio (log k') of transition metal ions on the logarithm of the concentration of maleic acid. Maleic acid has a relatively weak complexing ability and buffering property in the studied pH range. As shown in Fig. 3 the retention of metal decreased in order IDA-silica > TSK Gel Chelate 5PW > Phthalein Purple column. The elution order of transition metal ions

$$Mn < Co < Cd < Zn < Ni < Pb < UO_2 < Cu$$

was the same for IDA-silica and TSK Gel Chelate 5PW columns and was in a good agreement with data published for other IDA-chelating cation- exchange resins such as Chelex 20 (BioRad) and MetPac CC-1 (Dionex).[13,14] A worse separation selectivity of the Zn/Cd pair, but better separation selectivity for the

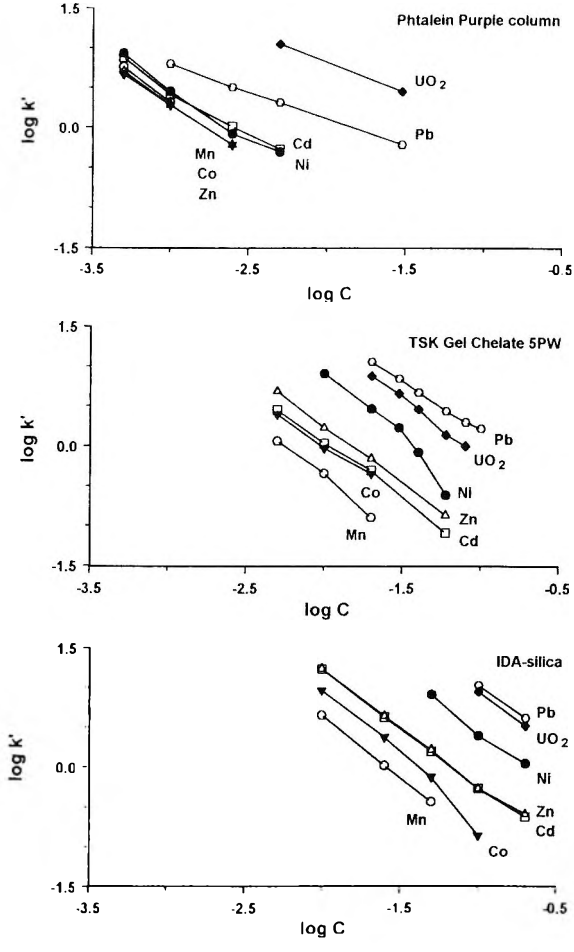

Figure 3. Dependence of the retention of transition metal ions on the concentration of maleic acid.

pair Cd/Co was obtained for the silica based column. A relatively low separation selectivity and different elution order were demonstrated for Phthalein Purple column. This difference in elution order can also be connected with the weaker complexing ability of the immobilised dye in comparison with maleic acid used as the eluent. It should be noted that the Tosoh TSK-Gel Chelate 5PW column retained the ions of nickel (II), copper (II), lead (II) and uranyl in maleic acid based eluents using a pH lower than the

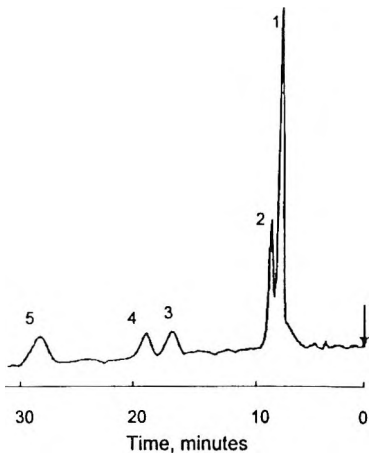

Figure 4. Separation of transition metals. Stationary phase, TSK Gel Chelate 5PW; mobile phase, 0.01 M tartaric acid; flowrate, 0.8 mL/min; photometric detection (490 nm, 0.3 aufs), derivatization with PAR-Zn-EDTA reagent at 1 mL/min flowrate. Sample: 10 μL of standard solution containing (1) 12 ppm Mg^{2+}; (2) 5 ppm Mn^{2+}; (3) 5 ppm Co^{2+}; (4) 5 ppm Cd^{2+}; (5) 5 ppm Zn^{2+}.

Table 2

Capacity Factors (k') of Some Transition Metals on Different Columns in Tartaric Acid Eluents

Metals	Phtalein Purple 0.001M	TSK Gel Chelate 5PW 0.01M	IDA-Silica 0.1M
Mn(II)	2.21	1.35	2.27
Co(II)	2.29	3.85	3.50
Zn(II)	2.68	7.12	5.41
Cd(II)	3.03	4.44	7.15
Pb(II)	6.76	no elution	no elution
Cu(II)	no elution	no elution	no elution

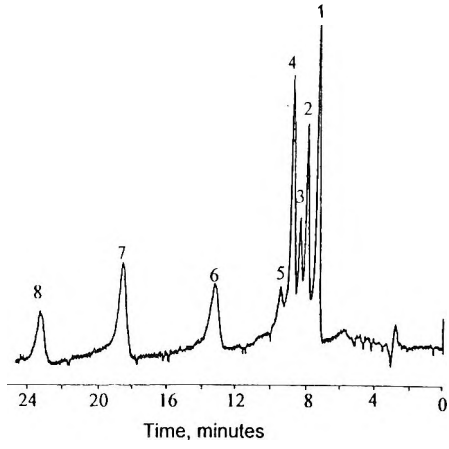

Figure 5. Separation of transition metals. Stationary phase, IDA-silica; mobile phase, 0.09 M tartaric acid; flowrate, 0.8 mL/min; photometric detection (490 nm, 0.2 aufs), derivatization with PAR-Zn-EDTA reagent at 1 mL/min flowrate. Sample: 100 μL of standard solution containing (1) 0.7 ppm Mg^{2+}; (2) 0.7 ppm Ca^{2+}; (3) 0.7 ppm Sr^{2+}; (4) 1.4 ppm Ba^{2+}; (5) 1.4 ppm Mn^{2+}; (6) 1.4 ppm Co^{2+}; (7) 1.4 ppm Cd^{2+}; (8) 1.4 ppm Zn^{2+}.

stability range of the corresponding matrix. This leads to dissolution of the resin and strongly restricts the applicability of this column for the determination of the above mentioned metal ions.

One possible solution of this problem is the use of eluents containing a stronger complexant such as tartaric and oxalic acid. However, as shown in Table 2 the use of 0.01 M tartaric acid as eluent does not provide the elution of copper (II) and lead (II) from TSK Gel Chelate 5 PW column. Also, 0.1 M tartaric acid does not provide elution of these metals from the IDA silica column. It is interesting that the observed elution order for Zn(II) and Cd(II) is different for TSK Gel Chelate 5 PW and IDA-silica columns. The typical separations of alkaline earth and transition metal ions are presented in Fig. 4,5.

Fig. 6 shows the retention times of transition metal ions as function of pH in 0.02M oxalic acid eluent on TSK Gel Chelate 5 PW and IDA-silica columns. Using this eluent it was possible also to elute the strongly retained Cu(II) and In (III) from TSK Gel Chelate 5 PW over the whole of the studied pH range. At the same time little or no retention was observed for any of the transition metal ions except copper on the Phtalein Purple column. The observed retention order of the metal ions in oxalate eluent changed in comparison with maleic acid based eluent (Fig. 3). First of all, the weaker retention of UO_2^{2+} is

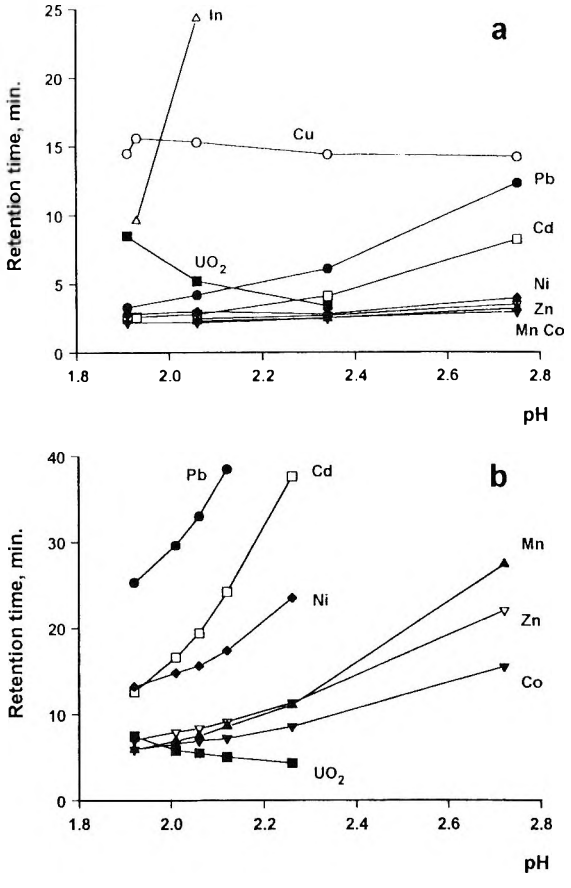

Figure 6. Influence of pH on retention times of transition metal ions in 0.02M oxalyc acid eluent. Stationary phases are (a) TSK Gel Chelate 5PW and (b) IDA-silica. Flow-rate, (a) 1 mL/min and (b) 0.8 mL/min.

connected with the higher stability of its oxalate complex. Secondly, the retention of manganese(II) and cadmium(II) was markedly increased relative to the other transition metal ions. Cadmium eluted after nickel and manganese eluted after zinc. Oxalate therefore improves the selectivity of separation for the pair Zn(II)/Cd(II) in the case of the IDA-silica and for the pair Cd(II)/Co(II) in the case of the TSK Gel Chelate 5 PW. More extensive mobile-phase complexation of separated metals occurred in oxalate eluents. Thus using 0.1 M oxalic acid the elution of strongly retained In(III) and Th^{4+} was obtained (Fig. 7).

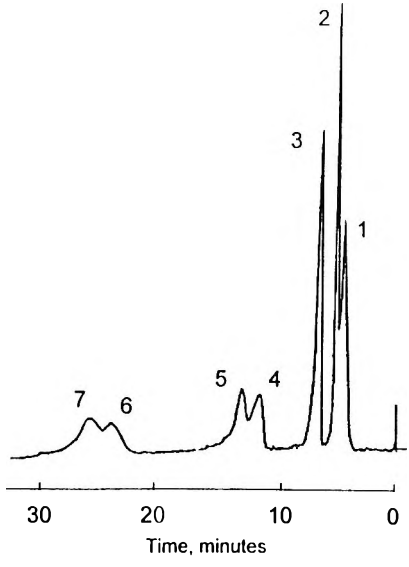

Figure 7. Separation of transition metals. Stationary phase, IDA-silica; mobile phase, 0.1 M oxalic acid; flowrate, 0.7 mL/min; photometric detection (490 nm, 0.2 aufs), derivatization with PAR-Zn-EDTA reagent at 1 mL/min flowrate. Sample: 100 μL of standard solution containing (1) impurities of alkaline-earth metal ions; (2) 0.8 ppm Co^{2+}; (3) 1.3 ppm Cd^{2+}; (4) 2.1 ppm Pb^{2+}; (5) 4.3 ppm UO^{2+};(6) 1.6 ppm In^{3+}; (7) 2.7 ppm Th^{4+}.

It should be mentioned that the feasibility of using tartaric and oxalic acid eluents is strongly restricted by the very low solubility of tartrates and especially of oxalates of alkaline-earth and transition metals. So, the injection onto chromatographic columns equilibrated with 0.01 M tartaric/oxalic acid of the sample solution containing at least 20 ppm one of each of magnesium, calcium, strontium and barium produced the splitting and distortion of chromatographic peaks and led to the marked decrease in efficiency of separation. Evidently, it takes place due to the formation of a precipitate of insoluble salts for a short time at the top of the column.

CONCLUSION

The comparison of different chelating ion-exchangers bearing iminodiacetic functionality confirmed the complexation in the stationary phase was the most important factor for retention of metal ions. Iminodiacetic acid bonded to silica

showed the strongest retention of metal ions, and the Phthalein Purple coated column the weakest retention. Under the conditions studied the use of IDA silica and TSK Gel Chelate 5PW columns are of preference for the isocratic separation of transition metal ions. However, the applicability of the latter column is restricted by dissolution in acid media. So strong complexing eluents must be used for complete regeneration and elution of strongly retained copper(II) and iron (III). The solubility of the complexes of alkaline earth and transition metals in some dicarboxylic acid solution may restrict the application of the studied chromatographic conditions to the analysis of samples containing elevated concentration of these metals.

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A SENSITIVE ASSAY FOR CYCLOPHOSPHAMIDE IN HUMAN PLASMA UTILIZING MASS SPECTROSCOPY

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ABSTRACT

This study describes a highly sensitive, solid-phase extraction-HPLC/MS/MS assay for cyclophosphamide in human plasma, with a minimum quantifiable level of 0.025 μg mL^{-1}. Plasma samples (0.5 mL) were mixed with 0.01 M ammonium acetate buffer (1.5 mL, pH 4.9) and internal standard (D4 deuterated compound, 50 μL, 5 μg mL^{-1}), and then extracted using Isolute CH(EC) cartridges (100 mg). Contaminants were washed from the cartridges with 0.01 M ammonium acetate:methanol (90:10, 1 mL), prior to the elution of the compounds of interest with 0.1 M ammonium acetate:methanol (50:50, 300 μL). An aliquot (200 μL) of the eluent was injected onto the chromatography system, which consisted of a LiChroCART 4-4 RP-select B, 5 μm cartridge precolumn, protecting a LiChrospher 60 RP-select B, 5 μm (250 x 4 mm id) cartridge analytical column.

The mobile phase was methanol:0.1M ammonium acetate buffer (pH 4.9, 60:40), run at a flow rate of 1 mL min^{-1}. Detection was by mass spectroscopy using a Finnigan MAT TSQ 700 triple stage quadrupole machine, fitted with an APCI interface. For quantification, the parent ions at $^{m}/_{z}$ 261 and 265 (cyclophosphamide and D4 internal standard respectively) were selected by quadrupole 1, collisonally dissociated in the octapole, and daughters ions at $^{m}/_{z}$ 120 and 124 then monitored via quadrupole 3. The calibration lines were linear over the range 0.025 to 1.0 μg mL^{-1}, with no evidence of any systematic deviation. The within- and between-day precision and accuracy were examined at five levels (0.025, 0.05, 0.5, 0.8 and 1 μg mL^{-1}, n=6), with values of <14% and within ±5% respectively. Loss of compound was not observed (0.05 and 0.8 μg mL^{-1}, n=6) after 3 freeze/thaw cycles, storage of the extracted samples for 24 h at room temperature and at 0 to 5°C, and after 4 weeks storage at -15 to -25°C. Diluting samples from 80 and 10 μg mL^{-1} to within the range of the standard curve was shown not to affect the assay. Although the recovery of cyclophosphamide and the internal standard was only 20%, the use of the deuterated compound ensured an accuracy and precision within the validation criteria. The utility of the assay was demonstrated using plasma from a patient who had received an IV infusion of cyclophosphamide at 1000 mg m^{-2}, given over 1 h.

INTRODUCTION

Cyclophosphamide is an alkylating agent which is used in the treatment of a variety of tumour types. The compound is activated in the liver by cytochrome P450 to phosphoramide mustard[1] and, as a result, its pharmacokinetics can be effected by agents which induce or inhibit P450 metabolism.[2] It is also now fairly well established that cyclophosphamide induces its own metabolism on repeated dosing.[3]

Several analytical methods have been developed for the measurement of cyclophosphamide in human plasma, although many of the early radiolabelled techniques[4] probably lacked specificity. The most commonly used methods have been gas chromatography with[5] or without[6] derivatization of the parent molecule.

HPLC has not been widely used to quantify cyclophosphamide in biological fluids because of its poor sensitivity. However, an HPLC/UV technique has been reported by Rustum and Hoffman[7] which had a detection limit of 0.3 $\mu g\ mL^{-1}$

We report a highly sensitive solid-phase extraction-HPLC assay for cyclophosphamide in human plasma using atmospheric pressure chemical ionization (APCI) mass spectrometry as the method of detection. The extraction stage is rapid because it utilizes solid-phase technology, and the method highly specific as a result of the detection method.

MATERIALS AND METHODS

Materials

Cyclophosphamide ([2-(bis-2-chloro-ethyl) aminotetrahydro-2H-1,3,2-oxaphosphorine-2-oxide]), was obtained from Sigma Chemicals Ltd, (Poole, UK) (Figure 1). The internal standard was a D4-deuteration of cyclophosphamide, (4,4, 6,6 tetradeuterated [2-(bis-2-chloro-ethyl) amino-tetrahydro-2H-1,3,2-oxaphosphorine-2-oxide]), generously supplied by the Cancer Research Unit, University of Newcastle-Upon-Tyne, UK (Figure 1). Cyclophosphamide and the internal standard were supplied as greater than 99% pure. Methanol was of HPLC grade and obtained from Rathburn Chemicals Ltd, (Walkerburn, UK). Ammonium acetate and acetic acid were of analytical grade and obtained from Fisons PLC, (Loughborough, UK) and BDH Ltd, (Poole, UK) respectively. Isolute extraction cartridges were obtained from International Sorbent Technology Ltd (Hengoed, Wales, UK). Human plasma was collected using sodium fluoride and potassium oxalate as the anticoagulant and stored frozen at -15°C to -25°C until required for analysis.

Preparation of Standards

Calibration samples were prepared by spiking control human plasma (0.5 mL) with cyclophosphamide (50 μL of the appropriate standard prepared in water) to give nominal concentrations of 0.025 to 1.0 $\mu g\ mL^{-1}$. Triplicate samples were run at the top (1.0 $\mu g\ mL^{-1}$) and bottom (0.025 $\mu g\ mL^{-1}$) levels.

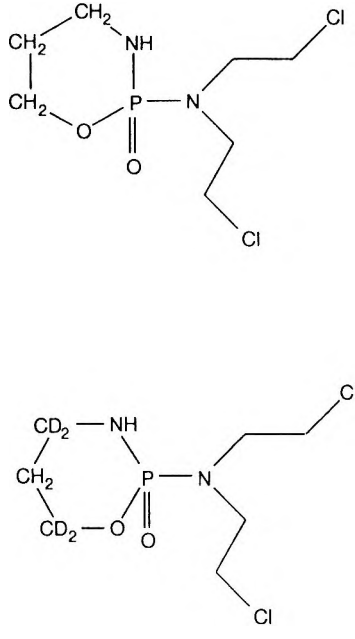

Figure 1. Chemical structure of cyclophosphamide and deuterated cyclophosphamide.

Validation samples were prepared at 5 levels by spiking human plasma (0.5 mL) with aliquots of cyclophosphamide (50 μL) to give nominal concentrations of 0.025, 0.050, 0.50, 0.80 and 1.0 μg mL^{-1}.

Instrumentation and Operating Conditions

The HPLC system consisted of a LiChroCART 4-4 RP-select B, 5 μm cartridge precolumn (Merck, Germany), protecting a LiChrospher 60 RP-select B, 5 μm (250 x 4 mm id) cartridge analytical column (Merck, Germany). A mobile phase of methanol:0.1 M ammonium acetate buffer (pH 4.9, 60:40) was pumped at 1.0 mL min^{-1} using a Hewlett Packard 1050 quaternary pump. The mass spectrometry system consisted of a Finnigan MAT TSQ 700 triple stage quadrupole mass spectrometer with an APCI/ESI interface. Data capture was achieved using the Finnigan MAT Instrument Control software (ICL, Version 7.2) and ICIS II (Version 7.0) application software running on a DEC 3100

workstation (ULTRIX Version 4.2a operating system). Samples were injected on to the system using a Gilson ASPEC XL autosampler.

The HPLC eluent was introduced into the APCI source of the mass spectrometer via an electronically actuated valve (Jones Chromatography, Clywd, UK), which diverted the first 3 minutes of each run to waste. Other conditions were as follows: vaporizer and capillary temperatures of 450 and 175°C respectively; a corona discharge of 5 μA; mass analyzers (quadrupoles 1 and 3) tuned to unit resolution (10% valley definition); argon collision gas pressure of 0.5 mtorr and a collision energy of -45 eV.

For quantification, the parent ions at $^{m}/_{z}$ 261 and 265 (cyclophosphamide and D4 internal standard respectively) were selected by quadrupole 1, collisonally dissociated in the octapole collision cell, and daughter ions at $^{m}/_{z}$ 120 and 124 then monitored via quadrupole 3. The scan time per dissociation was set to 0.5 s giving a cycle time of 1 s.

Assay Procedure

Plasma samples (0.5 mL) were mixed with 0.01 M ammonium acetate buffer (1.5 mL, pH 4.9) and internal standard solution (50 μL, 5 μg mL^{-1}), except for blank samples where 50 μL of water was substituted for the internal standard solution. Each sample was then extracted using Isolute CH(EC) cartridges (100 mg) preconditioned with methanol (2 mL) and 0.01 M ammonium acetate buffer (1 mL). Contaminants were washed from the cartridge with 0.01 M ammonium acetate:methanol (90:10, 1 mL), prior to the elution of the compounds of interest with 0.1 M ammonium acetate:methanol (50:50, 300 μL). An aliquot (200 μL) of the eluent was injected onto the LC/MS/MS system.

Validation Studies

The assay was validated by assessing accuracy and precision, both within and between runs. For the former, the accuracy (Eq 1) and precision (Eq 2) of the assay was examined at the 5 validation levels (n=6) in one run. In the case of the between-run assessment, one sample from each of the 5 validation levels was assessed in 6 separate runs. The stability of samples under various conditions (0.05 and 0.8 μg mL^{-1}, n=6) was also investigated, together with the effect of dilution from levels of 80 (n=4) and 10 μg mL^{-1} (n=6).

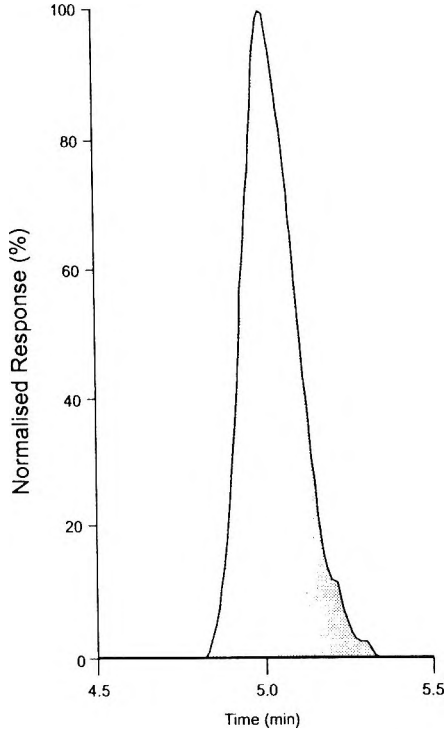

Figure 2. Typical mass chromatogram of a cyclophosphamide peak (1.05 μg mL^{-1}).

The acceptance criteria of the assay was based on Shah et al.[8] Acceptable mean accuracy and precision was defined as being within ±20% at the lowest validation level and ±15% at the remaining levels. In addition, no more than 2 samples at any one level could exceed the above limits, with overall a total of 75% of the individual samples being within specification. With the exception of the recovery experiment, these criteria were applied to the satellite studies using the ±15% limits.

$$\text{Accuracy (M\%D)} = \frac{\text{Observed conc.-expected conc. X 100}}{\text{Expected conc.}} \quad (1)$$

$$\text{Precision (CoV)} = \frac{\text{Standard deviation X 100}}{\text{Mean}} \quad (2)$$

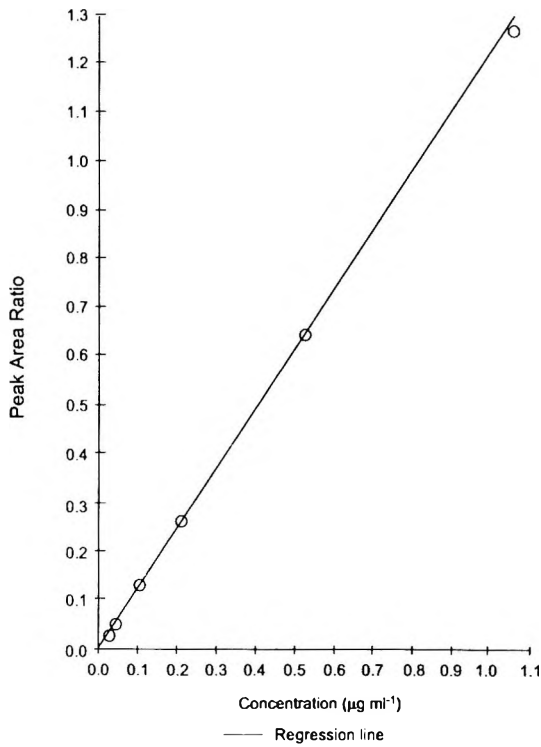

Figure 3. Typical calibration curve.

Utility of Method

To demonstrate the utility of the assay, the plasma concentrations of cyclophosphamide were measured in a cancer patient who was involved in a phase II drug interaction study. Cyclophosphamide was given as a 1 h intravenous infusion (1000 mg m^{-2}) 3 h after the administration of tirapazamine, a novel anticancer agent currently undergoing clinical assessment. For cyclo- phosphamide, plasma samples were obtained for up to 4.5 h following the start of the infusion and analyzed for parent compound as described above.

Table 1

Precision and Accuracy Data for the Assay of Cyclophosphamide in Human Plasma

Study	Parameter (n=6)	Observed Conc. ($\mu g\ mL^{-1}$) 0.025*	0.050*	0.500*	0.800*	1.00*
Within-Day	Mean ($\mu g\ mL^{-1}$)	0.0247	0.0520	0.523	0.843	1.07
	S.D. ($\mu g\ mL^{-1}$)	0.0014	0.0070	0.016	0.028	0.04
	CoV (%)	5.86	13.5	3.00	3.33	3.14
	M%D (%)	-4.26	0.87	1.49	2.25	3.98
Between-Day	Mean ($\mu g\ mL^{-1}$)	0.0258	0.0518	0.525	0.826	1.05
	S.D. ($\mu g\ mL^{-1}$)	0.0031	0.0031	0.023	0.029	0.04
	CoV (%)	8.14	6.04	4.41	3.46	3.76
	M%D (%)	0.00	0.55	2.01	0.22	1.59

*Nominal concentration; Individual accuracies were within the acceptance criteria.

RESULTS

The chromatograms for cyclophosphamide and the internal standard were free of interfering endogenous peaks, characteristic of this highly specific detection method (Figure 2). Retention times for both cyclophosphamide and the internal standard were in the region of 5 mins. The calibration lines were linear over the range 0.025 to 1.0 $\mu g\ mL^{-1}$, with no evidence of any systematic deviation (Figure 3).

The accuracy and precision of the assay was within the acceptance criteria. For the within-day data the mean precision was 5.86% at 0.025 $\mu g\ mL^{-1}$, 13.5% at 0.05 $\mu g\ mL^{-1}$, 3.00% at 0.5 $\mu g\ mL^{-1}$, 3.33% at 0.8 $\mu g\ mL^{-1}$ and 3.14% at 1.0 $\mu g\ mL^{-1}$ with mean accuracies of -4.26%, 0.87%, 1.49%, 2.25% and 3.98% respectively (Table 1).

Similar results were observed for the between-day data with a mean precision of less than 9% and a mean accuracy of within ±2% (Table 1).

Table 2

Freeze/Thaw and Dilution Data for the Assay of Cyclophosphamide in Human Plasma

Parameter	Observed Conc. ($\mu g\ mL^{-1}$) 2 Freeze/Thaw		3 Freeze/Thaw		Dilution	
	0.05*	0.80*	0.05*	0.80*	80*	10*
Mean ($\mu g\ mL^{-1}$)	0.0514	0.852	0.0509	0.861	84.4	10.8
S.D. ($\mu g\ mL^{-1}$)	0.0030	0.027	0.0035	0.026	5.66	0.2
CoV (%)	5.82	3.19	6.94	3.00	6.70	1.57
M%D (%)	-0.13	3.34	-1.13	4.47	6.63	5.28

n=6 except for dilution study at 80μ mL^{-1} where n=4

*Nominal concentration; Individual accuracies were within the acceptance criteria.

Table 3

Stability of Samples After Extraction and Storage at 0 to 50°C for 24 h and Ambient Temperature for 24 h

Condition	Parameter (n=6)	Observed Conc. ($\mu g\ mL^{-1}$) 0.050*	0.80*
Fridge (0-5°C)	Mean ($\mu g\ mL^{-1}$)	0.0568	0.856
	S.D. ($\mu g\ mL^{-1}$)	0.0019	0.024
	CoV (%)	3.32	2.83
	M%D (%)	10.3	3,84
Ambient	Mean ($\mu g\ mL^{-1}$)	0.0574	0.874
	S.D. ($\mu g\ mL^{-1}$)	0.0032	0.030
	CoV (%)	5.51	3.45
	M%D (%)	11.4	6.05

*Nominal concentration; Individual accuracies were within the acceptance criteria.

Table 4

Stability of Samples Stored at -15 to -25°C

Parameter (n=6)	Observed Conc. (μg mL^{-1}) 1 Week 0.050*	1 Week 0.80*	2 Week 0.050*	2 Week 0.80*	4 Week 0.050	4 Week 0.80*
Mean (μg mL^{-1})	0.0589	0.858	0.0545	0.833	0.0572	0.861
S.D. (μg mL^{-1})	0.0029	0.018	0.0020	0.031	0.0022	0.031
CoV (%)	4.93	2.11	3.60	3.72	3.81	3.55
M%D (%)	14.6	4.15	5.79	1.05	11.2	4.45

*Nominal concentration; Individual accuracies were within the acceptance criteria.

Table 5

Mean Recovery (± S.D.) of Internal Standard and Cyclophosphamide from Human Plasma

Component (n=6)	Recovery (%) 0.025*	0.50*	1.0*
I.S	23.9 ± 5.8	23.2 ± 6.2	25.4 ± 9.9
Cyclo	20.2 ± 5.1	21.6 ± 5.9	24.7 ± 10.0

*Nominal concentration

The stability of plasma samples at 0.05 and 0.8 μg mL^{-1} was examined under a variety of conditions. There was no evidence for any loss of cyclophosphamide, or any affect upon the quantification process, after 2 and 3 freeze/thaw cycles (Table 2), after storage of the extracted samples for 24 h at ambient temperature, after storage of the extracted samples at 0 to 5°C for 24 h and when kept in the freezer at -15°C to -25°C for up to 4 weeks (Tables 3 and 4).

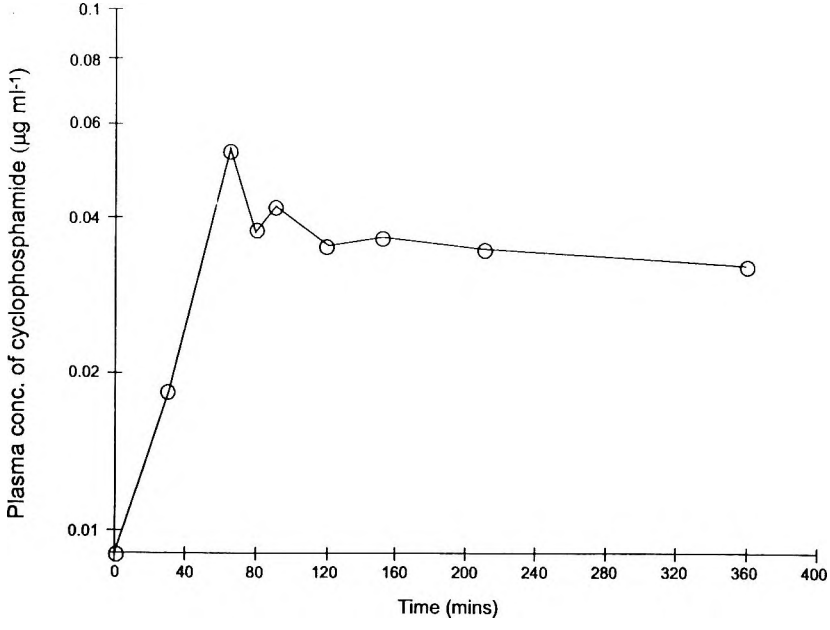

Figure 4. Plasma concentrations of cyclophosphamide following a 1 h intravenous infusion of 1000 mg m^2.

The recovery of cyclophosphamide and the internal standard was examined at the low, middle and high validation levels as part of the within-day validation. As would be expected, the recovery of both compounds was identical within the limits of the assay, being in the region of 20% (Table 5). Although the recovery was low, it did not have a significant effect upon the precision and accuracy of the assay.

The upper calibration range of the assay was low in relation to peak plasma concentrations that can be typically expected in adult patients (40-100 μg mL^{-1}) receiving a therapeutic dose of cyclophosphamide (1000-1500 mg m^2). In order to allow the analysis of such samples, the effect of dilution with control plasma was examined at cyclophosphamide concentrations of 80 and 10 μg mL^{-1}. Dilution of these samples was shown not to affect the quantification of cyclophosphamide with an accuracy and precision well within the acceptance criteria (Table 2).

Plasma samples from a patient treated IV with cyclophosphamide have been successfully analyzed using this assay, demonstrating the utility of the method (Figure 4).

DISCUSSION

This study reports the validation of a highly sensitive assay for cyclophosphamide in human plasma utilizing mass spectrometry as the method of detection. The assay has been developed to study the interaction of cyclophosphamide with other novel anticancer agents which may share the same metabolic pathway. The technique also has the potential for studying the pharmacokinetics of cyclophosphamide in situations where plasma volume is limited, such as paediatrics and small rodents. For the latter, however, further validation work would be required to ensure the performance of the assay.

The recovery of cyclophosphamide and the deuterated internal standard was low, being in the region of 20%. Such a low recovery is not always ideal and can introduce unacceptable assay variability. However, in the present study this was clearly not the case, most likely reflecting the use of a deuterated internal standard.

We did not attempt to identify the reason for the low recovery in terms of a poor extraction or retention by the solid-phase extraction cartridge. However, it is likely that the sensitivity of the assay could be improved even further by a modification of the solid-phase extraction procedure.

The short run time and simple extraction procedure for the current assay gives the potential for a high throughput of samples. Furthermore, detection by mass spectrometry affords this assay a high degree of specificity. This can be particularly important when dealing with cancer patients who are often treated with a variety of concomitant medications.

In conclusion, we have developed a highly sensitive LC/MS/MS assay for cyclophosphamide in human plasma. Over the range 0.025 to 80 $\mu g\ mL^{-1}$, the assay has been demonstrated to have an accuracy and precision within ±15%. The compound was stable in plasma after up to 3 freeze/thaw cycles and for storage for up to 4 weeks in the freezer. No compound loss was detected after the extracted samples were stored at room temperature and in the fridge for up to 24 h.

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DETERMINATION OF INTRACELLULAR AND EXTRACELLULAR NITRITE AND NITRATE BY ANION CHROMATOGRAPHY

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ABSTRACT

A highly sensitive method for the simultaneous direct detection as well as the ratio of intracellular and extracellular nitrite (NO_2^-) and nitrate (NO_3^-) in human macrophage (Mϕ), plasma and urine has been developed. Samples were deproteinized with the organic solvent acetonirile, lyophilized and reconstituted in buffer, determination and quantification of nitric oxide (NO) stable end products NO_2^- and NO_3^- was performed utilizing an isocratic high performance liquid chromatography (HPLC) with an anion exchange column. A mobile phase of 20 mM NaCl with 1mM mono sodium phosphate-NaH_2PO_4 at pH = 7.0 was used. Analyte anions were detected by direct UV-wavelength at 210 nm. Sensitivity in intracellular and extracellular fluids were 0.01 µmol/L for both anions with recovery rates of 99.6-99.4% for NO_2^- and NO_3^-. This method has successfully been applied to the determination of nitrite and nitrate in plasma and urine of normal human

volunteers as well as in cell line U-937 macrophage cytosol/supernatant. The mean concentration of NO_2^- in normal plasma (n=22) was 3.1 ± 0.4 μmol/L and NO_3^- 10.3 ± 0.3 μmol/Lwith ratio of NO_2^-:NO_3^- = 0.3; in normal urine (n=22) NO_2^- was 380 ± 61 μmol/L and NO_3^- 1345 ± 270 μmol/L with ratio of NO_2^-:NO_3^- = 0.3; in intracellular human macrophage fluid (n=21) NO_2^- was 0.66 ± 0.08 mol/L/10^6 Mϕ, and NO_3^- 0.68 ± 0.09 mol/L/10^6 Mϕ with ratio NO_2^-:NO_3 = 1; and in extracellular human macrophage fluid (n=21) NO_2^- was 0.03 ± 0.01 mol/L/10^6 Mϕ and NO_3^- 3.03 ± 0.33 mol/L/10^6 Mϕ with ratio NO_2^-:NO_3^- = 0.01.

INTRODUCTION

NO_2^- and NO_3^- are the stable metabolic products of NO, an intermediate in the metabolism of the amino acid L-arginine.[1-4] NO is synthesized by mammalian cells from the guanidino nitrogen atom of L-arginine and molecular oxygen (O_2) by the enzyme NO-synthase.[5,6] NO as an intermediate is rapidly oxidized in vivo to the nitrogen oxides, NO_2^- and NO_3^- by molecular O_2 or superoxide anion (O_2^-).[7]

Previously the nitrates present in biological fluids have been determined by colorimetric procedures and nitrite by diazotization and coupling reactions.[8] The reduction of nitrate to nitrite by a cadmium column and determination of NO_2^- by diazotization,[9] has been the more commonly employed method,[10-12] where NO_2^- reacts with the Griess reagent to form a purple azo dye and NO_2^- determined spectrophotometrically.[13-15] Determination of inorganic NO_2^- and NO_3^- by the kinetic cadmium-reduction methods is a refinement of this approach.[16] Other authors have detected NO_2^- and NO_3^- simultaneously, but with low sensitivity in plasma.[17-20]

We have developed a method for the simultaneous separation, determination and quantification of intracellular and extracellular NO_2^- and NO_3^- anions by an anion-exchange high performance liquid chromatography. This technique has proven to be a rapid and highly sensitive simple approach for the evaluation of the nitric oxide stable end products (NO_2^-, NO_3^-).

MATERIALS AND METHODS

Apparatus

An isocratic high performance liquid chromatography (HPLC) (Perkin Elmer, Norwalk, CT, USA) was used for simultaneous separation of NO_2^- and NO_3^- anions in intracellular and extracellular fluids such as human macrophage, plasma, and urine. The HPLC system included: a UV-785A programed absorbance detector, Perkin Elmer; a Diode Array Detector (DAD)-235C Perkin Elmer; an advanced LC sample processor ISS 200, Perkin Elmer; an isocratic-gradient IC-pump Perkin Elmer; an interface 600 series LINK PE-NELSON; a computer Digital DECpc LPv 466d2, linked to a printer (Hewlett-Packard Laser Jet 4 Plus); a pre-column (PRP-×100, guard 250×2.3mm) with an exchange capacity of 200 μeq/gr to provide an effective filter and coupled to a anion exchange post column (PRP-×100, 150×4.1 mm ID) containing a strong basic poly-styrene-divenylbenzene-trimethyleammonium exchanger (Hamilton, Reno, NE, USA).

Reagents

Inorganic salts used for standard analytes and mobile phase electrolyte were analytical reagent grade. NaCl and monosodium phosphate NaH_2PO_4 (Fluka Chem Co, Ronkonkoma, NY, USA) were used for the buffer solutions. The nitrogen standard used were NO_3^- (1mL =1mg) and NO_2^- (1mL =0.25 mg) (Ricca Chem. Co. Arlington, TX, USA).

The standards were prepared in Electrochemical-Millipore-Q-pure-water as a stock solution containing NO_2^-=125 μg/mL and NO_3^-=500μg/mL 10mL total volume, aliquotted in 10 vials (1 mL) and stored at -70 C. The Millipore-Q-water (18.2 megohm-cm) was double filtered by vacuum through Nylon 66-membrane filter, 47 mm diameter (Alltech Assoc. Co. Deerfield, IL, USA) and a Sep-Pak-Cartridges-C-18 from Millipore System, (Millipore, Milford, MA, USA) to yield Electrochemical-Millipore-Q-water.

The mobile phase was prepared with Electrochemical pure-water to which was added 20 mM NaCl and 1mM NaH_2PO_4. The final pH of the mobile phase was pH = 7.0. The resultant mobile phase buffer solution was vortexed and then filtered through a 0.2 μ Nylon 66-membrane filter and degassed under strong vacuum for 20 minutes using a Pierce filtration system (Pierce Chemical Co., Rockford, IL, USA).

Chromatography

The mobile phase (20 mM NaCl with 1mM NaH_2PO_4) was pumped through the isocratic HPLC-anion chromatography system with a flow rate of 1 mL/min, producing a background pressure of between 500-700 psi. Injection volumes that we used were between 20-100 µl. The isocratic HPLC-system was run at ambient temperature (20-24 C). Samples for analysis were thermostated at 4 C.

Detection of NO_2^- and NO_3^- anions was performed by direct UV-detection using a 210 nm wavelength. Detection by the DAD-system was performed with a spectrum of 205-300 nm as inorganic anions absorb less in the mid UV-wavelength than mobile phase anions. Anion retention times and run times were alternated by changing eluent strength and flow rate without overlapping other anions.

Cell Culture

Human monocyte/macrophage cell line U-937 (American Type Culture Collection-ATCC, Rockville, MD, USA) was grown in suspension culture at 37°C in a humidified atmosphere containing 5% CO_2 in a tissue culture flasks (75 cm^2/250 mL) in 20 mL in a culture medium of RPMI 1640 containing 10% FBS (fetal bovine serum), 30% bicarbonate, 5% penicillin/streptomycin, and 2% fungizone (Biologos, Naperville, IL, USA). Cell density was maintained between approximately 5 x 10^5 and 1 x 10^6/mL. Culture medium was changed on days 3 and 5 and every day there after by adding 10 mL of fresh medium and removing 10 mL of medium. When culture media was changed macrophages were centrifuged at 500 x g for 5 minutes.

Sample Preparation

Human blood, urine and the human macrophage cell line U-937 were used for study. Blood and urine was obtained from healthy adults. Samples were centrifuged at 3000 × g for 10 minutes at 4°C and the supernatant aliquotted in microtubes, 1.5 mL and stored at -70°C. Human macrophage cultures (cell line U-937) (1 x 10^6 cells/mL) were centrifuged at 500 × g for 5 minutes at 4°C and supernatants were aliquotted and stored at -70°C. The macrophage pellet was dissolved in Dulbeco's phosphate buffered saline,

(Sigma Chem. Co., St. Louis, MO, USA) and the cell membranes disrupted by sonicator (Sonifer 250 at cycle 3 for 60 sec.) (Fisher, Itasca, IL USA) and centrifuged at 3000 × g for 10 minutes at 4°C then the cytosol was aliquotted and stored at -70°C.

Sample Deproteinization

A) Assay for plasma deproteinization was performed by adding 250 µl of acetonitrile to 500 µl plasma in a 1.5 mL microcentrifuge tube and then vortexed for 10 sec. The protein sedimentation was performed by centrifugation for 15 minutes at 3000 × g. The supernatant was treated with an additional 250 µl acetonitrile [v:v], vortexed and centrifuged. Plasma supernatants were lyophilized in a vacuum system (vacuum = 839 u, heater = 70°C, time = 2 h) (AS 160 automatic speedvac, Savant, Inst. Inc. New York, NY, USA) and reconstituted in a volume or 100-500 µl of buffer pH = 7 (mobile phase) which yielded supernatants of consistent quality. The supernatant was transferred to insert assembled amber target vials with teflon/silicone/teflon septums (National Scientific Co., Lawrenceville, GA, USA) and placed in the autosampler processor and the simultaneous detection of nitrite and nitrate performed.

B) Assay for urine and macrophage cytosol and supernatant was performed by adding 250 µl of acetonitrile to 1mL of macrophage cytosol and supernatant or urine in 1.5 mL centrifuge tubes and vortexed and centrifuged as for plasma. The resultant supernatant was filtered through a disposable Anopop-IC syringe filter (0.2 µ, 10 mm) (Alltech Inc., Deerfield, IL, USA) and the purified supernatant was lyophilized and reconstituted in 100-1000 µl of buffer pH = 7 and transferred to insert vials of the autosampler to be injected in the HPLC column.

Validation of HPLC Methodology

Comparison of HPLC methodology with the Griess-Saltzman assay

Validation of our anion liquid chromatography methodology for the detection of NO_2^- and NO_3^- beyond that obtained from comparison of sample values to calibration curves of known standards was performed by a comparison of the HPLC methodology to the Griess-Saltzman reaction assay.

NO_2^- and total NO_3^-/NO_2^- were determined by Griess-Saltzman reaction.[13-15] NO_2^- was determined before NO_3^--reduction, and the total NO_3^-/NO_2^- was determined after reduction of NO_3^- to NO_2^- by nitrate reductase,[21] using nitrate/nitrite Cayman's Kit (Alexis Corporation, San Diego, CA, USA). The first step is measurement of NO_2^- by the Griess-Saltzman reaction before reduction in prepared samples. Standard $NaNO_2$ was made in concentrations of between 5 and 80 μM. Human plasma were deproteinized at a v:v of 1:1, lyophilized and reconstituted (concentrated 5 times) in Buffer pH = 7. The Griess reagent consisted of 1 part 0.1% naphthylethylenediamine dihydrochloride in distilled water and 1 part 1% sulfanilamide (or sulfanilic acid) in 5% concentrated H_3PO_4, the 2 parts being mixed together within 12 h of use and kept chilled. Addition of the Griess-Reagent to the samples converted NO_2^- yielding a deep purple azo compound. The second step was the stoichiometric reduction i.e. conversion of NO_3^- to NO_2^- utilizing 0.1 u/mL nitrate reductase, and addition of the Griess-Reagent which converted NO_2^- into a azo compound. Spectrophotometric measurement of absorbancy at 540 nm (spectrophotometer model MK-II Titertek Multy Skan-Plus, ICN Biomedical, Irvine, CA, USA) of the azo chromophore allowed determination of the nitrite concentration in the measured specimens.

Additionally, we obtained and analyzed plasma samples from 22-healthy adult volunteers. These samples were used for paired determination of nitrite and nitrate by the Griess-Saltzman reaction and compared to our method of anion-chromatography (HPLC) detection using the techniques as outlined above.

Recovery

The accuracy of our HPLC methodology was established by the "spiked" recovery approach. The recoveries for both anions were performed by spiking standards of the NO_2^- and NO_3^- into human control plasma. Intra-assay variation was estimated by calculating the mean and standard deviation of the concentration of six samples of two different spiked standards of NO_2^- and NO_3^-. Inter-assay variation was estimated from the concentration of spiked samples determined in six consecutive chromatographic assays. The precision of the assay was demonstrated by the coefficient of variation of the measured concentrations of the spiked NO_2^- and NO_3^- standards in human control plasma.

Statistical Analysis

Data are expressed as mean ± standard error of the mean. NO_2^- and NO_3^- values are expressed as μmol/L. The ratio of NO_2^-:NO_3^- is presented as an index of relative partitioning of nitric oxide metabolism. Test statistics used include one factor and two factor analyses of variance (ANOVA). Results were considered significant at the .05 level.

RESULTS

Initial calibrations were performed by analyzing data from standard samples. The chromatography software program determined calibration levels for unknown component amounts in individual samples by comparing response peak areas with those responses obtained from known standard samples. In an attempt to improve the reliability of this approach, more than one standard was used. A standard calibration curve with five levels of concentration was used for NO_2^- (0.015-1.95 μg/mL) and NO_3^- (0.98-125 μg/mL) with a sample size of 20 μl. The area of the external standard curves generated linear correlation coefficients between 0.9986-1.0000 for NO_2^- and NO_3^-. The peak purity for NO_2^- and NO_3^- was 1.000. Detection limits were based on the ratio of peak analyte area to that of the external standard area.

NO_2^- and NO_3^- values were determined in 86 samples of control plasma (n=22) and urine (n=22) of healthy adults as well as in human macrophage cytosol (n=21) and supernatant (n=21). The peak for NO_2^- and NO_3^- anions appeared with different retention times and their recognition was by standard peak run times for both anions by UV-detection (Figures 1, 2 and 3). Each sample was calculated by software data acquisition and expressed as μmol/L.

Using direct UV-detection we found the plasma NO_2^- of 22 healthy controls to be 3.1 ± 0.4 μmol/L and NO_3^- 10.3 ± 0.7 μmol/L with a ratio of NO_2^-:NO_3^- = 0.3, and that urine NO_2^- was 380 ± 61 μmol/L and NO_3^- 1345 ± 270 μmol/L with a ratio of NO_2^-:NO_3^- = 0.3 (Table 1). Human macrophage cytosolic NO_2^- after washing and 2 h of incubation in media at 37°C was 0.66 ± 0.08 mol/L/10^6 Mϕ, and NO_3^- 0.68 ± 0.09 μmol/L/10^6 Mϕ with a ratio of NO_2^-: NO_3^- = 1, and extracellular (supernatant) NO_2^- was 0.03 ± 0.01 μmol/L/10^6 Mϕ and NO_3^- 3.03 ± 0.33 mol/L/10^6 M¢ with a ratio of NO_2^-:NO_3^- = 0.01 (Table 2).

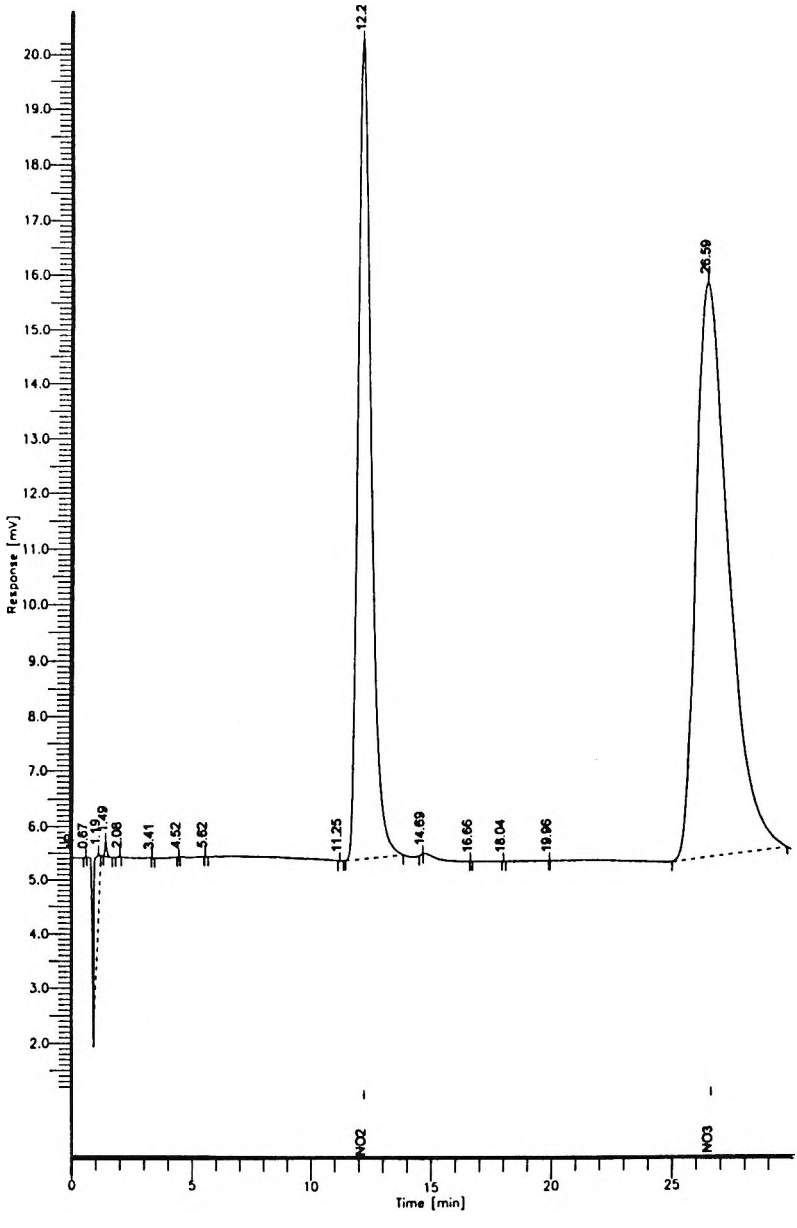

Figure 1. Chromatogram of HPLC Separation of NO_2^- and NO_3^- Anions: Standard.

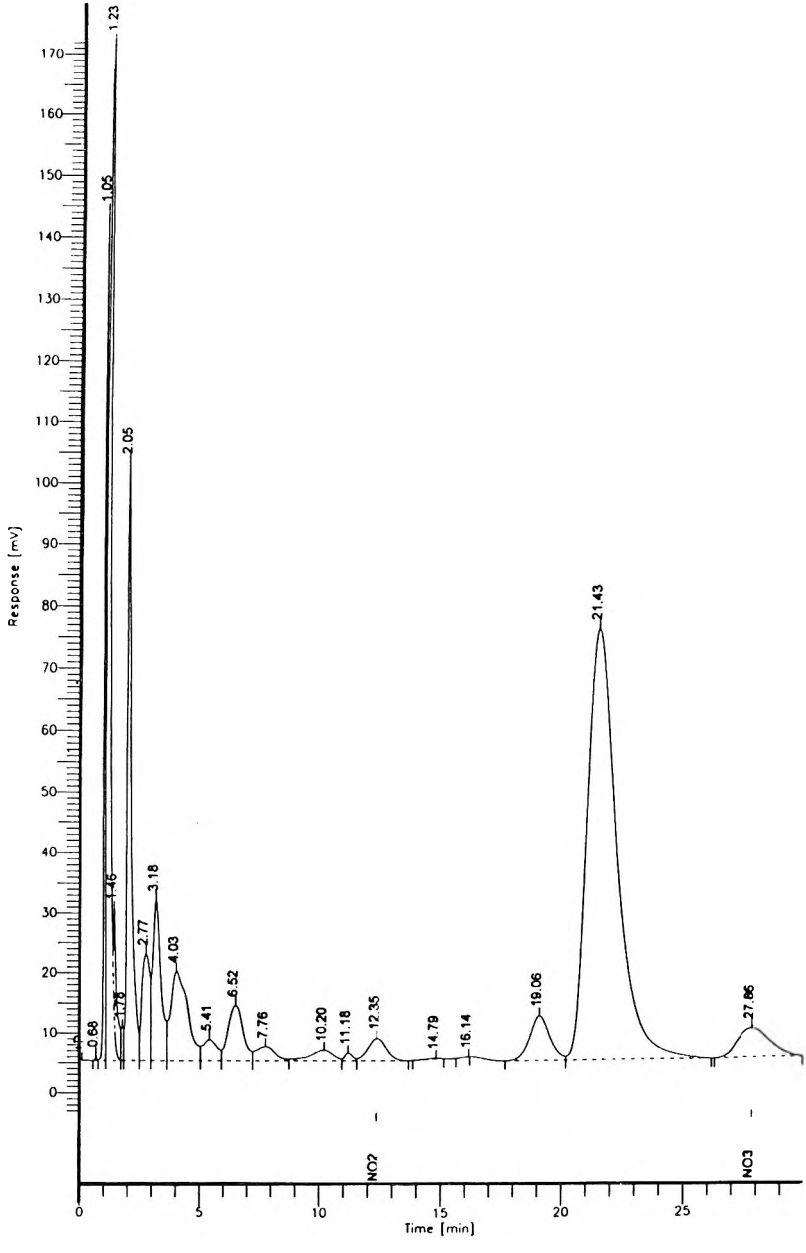

Figure 2. Chromatogram of HPLC Separation of NO_2^- and NO_3^- Anions: Control Human Plasma.

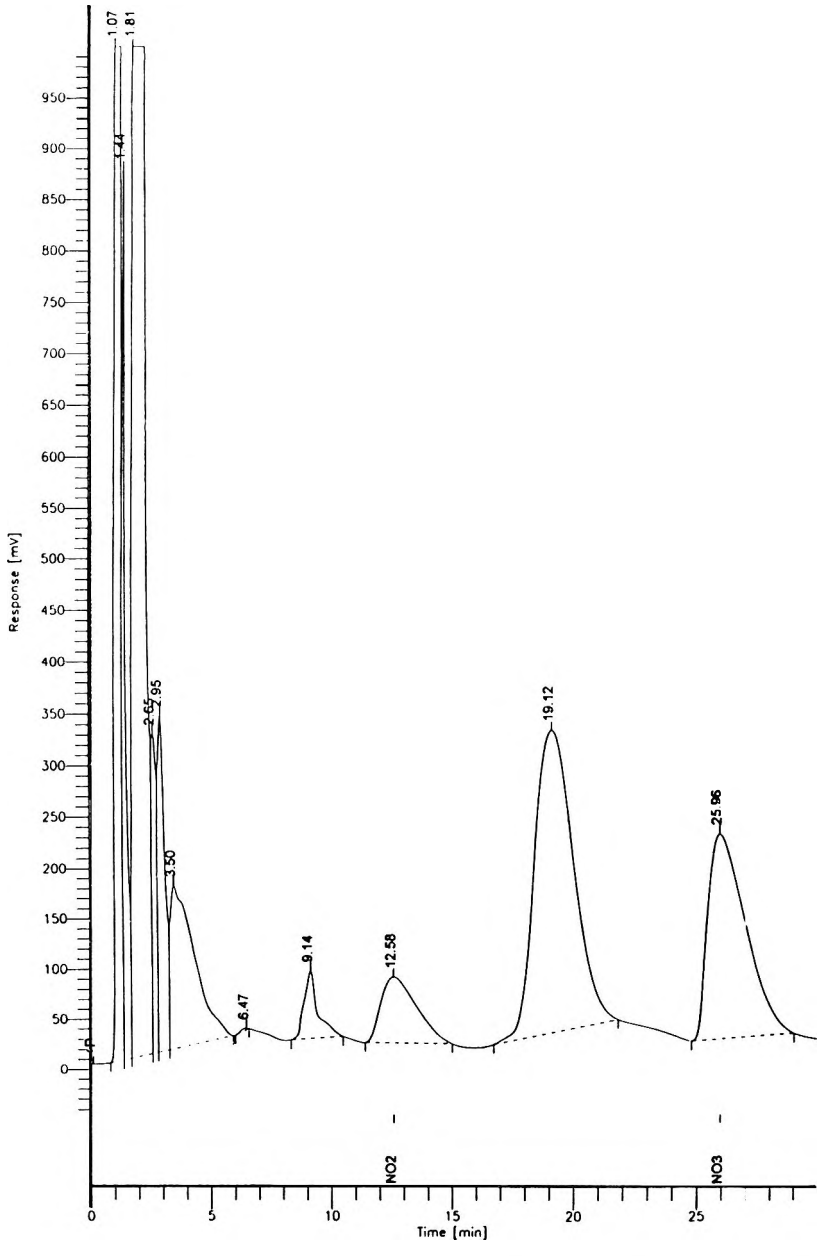

Figure 3. Chromatogram of HPLC Separation of NO_2^- and NO_3^- Anions: Control Human Plasma.

Table 1

Nitric Oxide Stable End Products Nitrite and Nitrate in Normal Human Plasm and Urine

	NO_2^- μmol/L	NO_3^- μmol/L	NO_2^-:NO_3^- Ratio
Plasma	3.1 ± 0.4	10.3 ± 0.7	0.3
Urine	380 ± 51	1345 ± 270	0.3

Table 2

Intercellular and Extracellular Nitrite and Nitrate in Human Macrophage Cell Line U-937

NO_2^- μmol/L/10^6Mϕ	NO_3^- μmol/L/10^6Mϕ	NO_2^-:NO_3^- Ratio	NO_2^- μmol/L/10^6Mϕ	NO_3^- μ,mol/L/10^6Mϕ	NO_2^-:NO_3^- Ratio
0.66 ± 0.08	0.68 ± 0.09	1	0.03 ± 0.01	3.03 ± 0.33	0.01

The average of recovery was 99.6 ± 0.9 % for NO_2^- and 99.4 ± 2.3% for NO_3^-. The precision of the assay was estimated with the coefficient of variation (Table 3). The precision of the assay over three consecutive days is shown on Table 4.

The intra-assay variabilities was calculated from concentrations of 31.2 μg/mL and 62.5 μ/mL of spiked plasma samples of NO_2^- and NO_3^- standards using the concentration determined for 6 samples that were analysed during the same analysis. The mean calculated concentration was 30.75 ± 0.25 μg/mL for NO_2^- and 30.28 ± 0.74 μg/mL for NO_3^- for the 31.2 μg/mL spiked samples, and 60.89 ± 0.21 μg/mL for NO_2^- and 59.61 ± 0.71 μg/mL for NO_3^- for the 62.5 μg/mL spiked sample. The percent coefficient of variation was 1.6%-6.0% and 0.8%-2.9% for the 31.2 μg/mL and 62.5 μg/mL spiked sample respectively (Table 5).

The inter-assay variability were calculated from the concentration of 15.6 μg/mL spiked samples of NO_2^- and NO_3^- determined during six assays run on consecutive day. The mean calculated concentration of 15.6 μg/mL spiked

Table 3

Recovery and Precision of NO_2^- and NO_3^- in Spiked Human Plasma Samples from Healthy Adults

	Concentration Spiked in Plasma μg/mL	Conc. Found in Plasma μg/mL	Coefficient of Variation %	Recovery %	Average Recovery %
NO_2^-	1.9	1.85 ± 0.02	2.16	97.37	
	3.9	3.92 ± 0.01	0.76	100.50	
	7.8	7.86 ± 0.03	1.02	100.77	
	15.6	15.31 ± 0.46	7.38	98.14	99.56 ± 0.94
	31.2	32.18 ± 0.59	4.54	103.14	
	62.5	60.89 ± 0.21	0.90	97.42	
NO_3^-	3.9	3.95 ± 0.05	0.23	101.28	
	7.8	8.44 ± 0.33	6.03	108.21	
	25.0	25.65 ± 0.62	3.48	102.60	
	62.5	59.95 ± 4.23	9.57	95.92	99.40 ± 2.30
	125.0	118.95 ± 4.05	4.58	95.08	
	225.0	210.00 ± 6.00	3.77	93.33	

Baseline amount in plasma samples before summplementation NO_2^- = 0.28 μg/mL; NO_3^- = 1.34 μg/mL.

Table 4

Precision Data for the NO_2^- Nno_3^- In Plasma Samples Spiked with 62.5 μg/mL of NO_2^- and NO_3^- Standards During Three Consecutive Days

Anion	Day	Mean Concentration μg/mL	Coefficient Variation %
NO_2^-	1	61.06 ± 0.22	0.87
	2	61.28 ± 0.19	0.70
	3	61.00 ± 0.21	0.87
NO_3^-	1	59.61 ± 0.71	2.90
	2	59.67 ± 0.69	2.85
	3	59.57 ± 0.70	2.87

Table 5

Intra-assay Variation (n = 6)

Spiked Concentration μg/mL	Mean Concentration	SD	Coefficient of Variation, %
NO_2^- 31.2 μg/mL	30.75 ± 0.25	0.50	1.63
NO_3^- 31.2 μg/mL	30.28 ± 0.74	1.82	6.01
NO_2^- 62.5 μg/mL	60.89 ± 0.21	0.52	0.85
NO_3^- 62.5 μg/mL	59.61 ± 0.71	1.73	2.90

samples was 15.22 ± 0.56 μg/mL for NO_2^- and 15.33 ± 0.37 μg/mL for NO_3^-. The percent coefficient of variation was 8.15 % and 5.87% for NO_2^- and NO_3^- respectively (Table 6).

Anion retention time and run times were alternated by changing eluent strength and flow rate without overlapping other anions. The individual specimen values of NO_2^- and NO_3^- were quantified by comparison to the external standard area. The added NO_2^- /NO_3^- standards in plasma appeared at the expected retention time and were 12.26 ± 0.09 min for NO_2^- and 27.01 ± 0.27 min for NO_3^- (Table 7).

The comparative analysis of the control plasma specimens revealed that there were no difference (p=0.1) for NO_2^- as detected by the Griess reaction [5.63 ± 1.02 μmol/L] and by our HPLC method [3.09 ± 0.36) μmol/L]. Also, there were no difference (p=0.2) for total NO_3^- /NO_2^- concentration detected by Griess-Saltzman reaction [17.28 ± 2.69 mol/L] and by HPLC [13.44 ± 0.63 mol/L] (Table 8). The HPLC technique had a sensitivity of 0.01 μmol/L separately for both anions.

DISCUSSION

High performance anion-exchange liquid chromatography in conjunction with a specific mobile phase of sodium chloride and sodium phosphate appears to represent a powerful and very sensitive method for the simultaneous separation and detection of NO_2^- and NO_3^- anions. This approach offers the advantages of rapid, simultaneous chromatographic detection, determination

Table 6

Inter-assay Variation (n = 6)

Spiked Concentration 15.6 μg/mL	Mean Concen. μg/mL	SD	Coefficient of Variation, %
NO_2^-	15.22 ± 0.56	1.24	8.15
NO_3^-	15.33 ± 0.37	0.90	5.87

Table 7

Retention Times of Spike NO_2^- and $NO3^-$ in Controlled Human Plasma

Retention Times (RT) in Minutes

	Mean Standard RT ± SE	Mean Plasma RT ± SE
NO_2^-	12.25 ± 0.04*	12.26 ± 0.09
NO_3^-	26.39 ± 0.15	27.01 ± 0.27

*p = ns for standard vs. plasma retention times.

Table 8

Comparison of the Griess Reaction to HPLC Determination of NO_2^- and Total NO_3^-/NO_2^- in Human Control Plasma

Anions	Griess	HPLC
NO_2^- μmol/L	5.63 ± 1.02^*	3.09 ± 0.36
Total NO_3^-/NO_2^- μmol/L	17.28 ± 2.69*	13.44 ± 0.63

^ = mean ± SE

*p = ns for Griess Reaction vs. HPLC

and the quantification of NO_2^- and NO_3^- anions at ambient temperature in biological fluids. We have found that this method worked well for intracellular and extracellular NO_2^- and NO_3^- detection in human biological samples. We were able to clearly separate simultaneously both anions in plasma, urine and macrophage cytosol/supernatant. It was necessary to develop this method for simultaneous determination of nitrite and nitrate anions, as present methods can not measure individually NO_3^- or NO_2 anions with high sensitivity.

Monitoring nitrite and nitrate generation could be of use in the monitoring of NO production,[22] or the status of NO-oxidation.[7] NO-production as well as NO-oxidation is associated with membrane receptor regulated oxygen consumption pathways.[23,24] An alternate pathway of oxygen consumption is for oxidative phosphorylation of ADP and P_i to yield ATP, where the electron-transport chain transfers electrons to oxygen in a series of exergonic reactions from cytochrome-c to cytochrome-aa_3 (cytochrome oxidase) and catalyzes the reaction of electrons and protons with molecular oxygen to produce water.[25] Nitric oxide generation,[22] and NO-oxidation which occurs when NO reacts with molecular oxygen producing NO_2 and NO_3^- [$2NO + O_2 \rightarrow 2NO_2$; $2NO_2$ 8 N_2O_4 -^{H2O} $\rightarrow$ NO_2^- + NO_3- + $2H^+$] or with superoxide anion generating NO_3^- [$NO + O_2^- \rightarrow NO_3^-$],[7] both are important steps in cell respiration receptor regulated pathways.[23,24] The production of NO by human monocyte in this paper represent basal value of NO release. The human monocytes can be classified into two different populations: a low-NO-producing and high-NO-producing monocytes. These differences likely result from the differential in vivo activation of these cells.[26]

Previous methods, have determined nitrate by colorimetric techniques where nitration of phenol-2,4-disulfonic acid or nitration of brucine yields an orange-brown solution, which can be determined colorimetrically . NO_2^- has been determined by the formation of 5-nitro-2,4-xylen-1-ol which with dilute sodium hydroxide forms a red salt. This involves, first, the reaction in acid solution of a primary amine such as sufanilic acid or sulfanilamide with nitrite to form a diazonium salt. The latter is then coupled to an aromatic amine to yield the red azo-die (diazonium salt) whose concentration can be determined in a colorimeter.[8]

Automated procedures for the analysis of nitrate via the reduction of nitrate to nitrite with a high-pressure cadmium column, are based on the Griess reaction. In the Griess reaction NO_2^- reacts with 1% sulfanilamide in 5% H_3PO_4 / 0.1% naphthalene-ethylenediamine dihydrochloride forming a purple azo dye. Using high performance liquid chromatography with a cadmium column all NO_3^- reduces to NO_2^- at high pressures through a column packed

with fine particles of copper-plated cadmium metal. The color of the product dye is developed by a 60°C water bath and followed by cooling at 0°C absorbance at 546 nm is detected by flow through a spectrophotometer and nitrate quantification determined.[9] Other methods use conversion of nitrate to nitrite with reductase and then nitrite is transformed to a chromophore via the Griess reagent with detection at 543nm and quantified spectrophotometrically.[27] Recently a kinetic cadmium reduction method has been developed, where NO_3^- is reduced to NO_2^- by copper-coated cadmium granules and nitrite determined by diazotization with sulfanilamide solution followed by N-naphthylethylene diamine.[16] Using ion-exchange HPLC column and a mobile phase of 50 mM H_3PO_4 with 2% (v/v) tetrahydrofuran at pH = 1.9 Romero and coworkers,[28] have been able to determine only NO_3^- anions in cytosol of Anacystis cells, but they could not determine NO_2^- anion by ion chromatography.

Previous investigators,[13] using ion chromatographic methods and an eluent solution of 0.75 mM $NaHCO_3$ and 2.2 mM Na_2CO_3 have been able also to determine only NO_3^- anions in serum and ocular fluid of healthy cattle, but nitrite could not be determined, and the detection limits was 0.01 mM/L Similar sensitivity limits have been reported in human plasma of 0.01 mM/L for NO_3^- anion which was in the concentration ranged from 0.03-0.12 mM/L (mean=0.06), but NO_2^- concentration were not detecable using a mobile phase of 50 mM NaH_2PO_4, 3 mM NaCl and 4 mM acetic acid at pH = 3.95.[17] Moreover, the sensitivity limits have been increased to 8 μmol/L for NO_3^- and 2 μmol/L for NO_2^- using ion-pair chromatography in rat plasma.[18]

Using anion exchange chromatography and direct UV-detection at a 210 nm wavelength we were able to determinate NO_2^- in our samples, but previous methods could not determine this anion in intracellular fluid and in plasma.[9,10,16,17,29] Human macrophages generates nitric oxide and the stable end products NO_2^- and NO_3^- anions, which primarily released immediately outside of cell to the intercellular space,[3-5,26] in that reason we found four times higher concentration of nitrate in extracellular fluid.

The novel aspect of our method is the use of the anion exchange chromatography with a specific mobile phase of an aqueous solution of 20 mM sodium chloride with 1 mM mono sodium phosphate at pH = 7, ambient temperature and a UV-wavelength of 210 nm, which allowed the simultaneous direct detection of intracellular and extracellular nitrite and nitrate with a sensitivity of 0.01 mol/L for both anions.

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THE EFFECTS ON SEPARATION OF CEPHALOSPORINS BY HPLC WITH β-CYCLODEXTRIN BONDED STATIONARY PHASE

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ABSTRACT

A β-cyclodextrin bonded stationary phase is used for the liquid chromatographic separation of cephalosporins. A complete separation of nine cephalosporins with methanol and tetraethylammonium acetate (TEAA) buffer solution has been demonstrated. It is also found that a 100% of TEAA buffer solution is suitable for the separation of cephalosporins containing a hydrophilic α-amino moiety.

The effects of pH, TEAA concentration, organic modifier concentration of mobile phase, and the column temperature on the retention are examined and discussed.

INTRODUCTION

Cephalosporins are β-lactam antibiotics possessing antibacterial activities against both gram-positive and gram-negative bacteria. Determination of cephalosporins in pharmaceutical dosage forms and biological fluids has been performed by microbiology analysis,[1] UV absorption,[2] thin layer chromatography,[3,4] ion exchange chromatography.[5,6] and capillary electrophoresis chromatography.[7,8] It has also been of considerable interest in the utilization of reverse phase high performance liquid chromatography (HPLC) for rapid separation and accurate determination of antibiotics.[9-16] Other HPLC techniques, such as ion-pairing,[17-19] microbore column,[20] postcolumn,[21] multi-phase column,[22,23] copolymer column,[24] and column-switching[25] have been developed recently.

The β-cyclodextrin (β-CyD) is an oligosaccharide of seven glucose units cyclized together to form a toroidal structure with a hydrophilic outerior face and a hydrophobic inner cavity. Since Armstong[26] developed the first high efficiency bonded β-CyD phase on 5 μm silica gel as the packing material in HPLC, many pharmaceutics have reported the separation and determination of drugs simultaneously by employing bonded β-CyD column. In this study, we describe for the first time the successful separation of a mixture of cephalosporins by HPLC with β-CyD bonded stationary phase.

Characteristics such as the effects of inclusion complex formation between the solutes and β-CyD cavity, the influence of pH, TEAA concentration, organic modifier concentration of mobile phase, and the column temperature on retention are investigated. The observed behavior of the compounds during chromatographic processes is also discussed.

EXPERIMENTAL

Materials

All cephalorsporins were purchased from Sigma (Louis, MO, USA). The HPLC solvents such as acetonitrile, methanol, and glacial acetic acid were obtained from E. Merck (Frankfurter, Germany). Tetraethylammonium acetate (TEAA), Tetrabutylammonium acetate (TBAA) and hexanesulfonic acid (HSA) were obtained from Aldrich (Milwaukee, WI, USA) and Fluka (Buchs, Switzerland), respectively. Distillated water was deionized twice before use.

Instrumentation

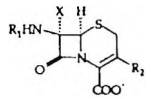
The liquid chromatographic system consists of a Waters Model 6000A pump connected to a U6K injector, a photodiode array detector model 990 set at 230 nm and a model 520 printer/plotter (Waters Associates, Milford, MA, USA). A model TCM temperature controller (Waters, MA, USA) was employed to adjust the temperature of the column from 30° to 55°C . The column employed was a 5 μm bonded β-CyD (250 mm x 4.6 mm i.d.) purchased from Advanced Separation Technologies Inc. (Whippany, NJ, USA). A model 112 pH meter (Photovolt, NY, USA) was used to measure the pH values of mobile phases.

Chromatographic condition

Buffers such as TEAA, TBAA, and HSA were prepared in water and glacial acetic acid was added until the desired pH was obtained. Mobile phase was prepared by mixing methanol with buffer solution and was degassed by bubbling helium into it for about 10 min. The mobile phase was filtered through a 0.45μ filter before use. Sample solutions were prepared by dissolving each drug in mobile phase to give a concentration of about 1 mg/mL. Typically, 10 μL of sample solution was injected. The flow rate was adjusted at 0.8 mL/min. The eluant was eluted and attenuated for full scale deflection at 2 units.

RESULTS AND DISCUSSION

The cephalosporins used in this work are listed in Figure 1. All compounds are β-lactam derivatives with C-7 amino substitutions or C-3 modified side chains. These compounds can be grouped in three types according to their structures. Type I, as compounds 1-5, containing a hydrophilic α-amino moiety gets stronger affinity in aqueous solution. Type II, as 7-ACA and 7ADCA, has the similar amino group at the C-7 position of β-lactam but with less polarity. Type III, as compounds 8-16, obtains bulky substituents at C-7 or C-3 side chain and gets different molecular sizes and polarity. Thus, separation of cephalosporins by HPLC with β-CyD bonded phase is a function of the formation of the inclusion complexes in the cavity of cyclodextrin that in turn would be primarily effected by many factors such as



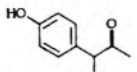
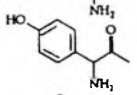
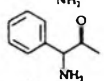
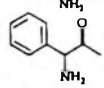
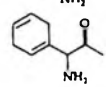
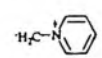
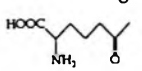
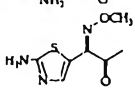
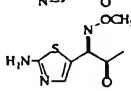
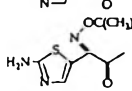
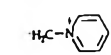
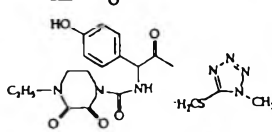
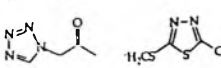
NO.	Name	R_1	R_2	X
1	Cefadroxil		$-CH_3$	H
2	Cefatrizine			H
3	Cefaclor		$-Cl$	H
4	Cephalexin		$-CH_3$	H
5	Cephradine		$-CH_3$	H
6	7-ACA	H	$-CH_2OCOCH_3$	H
7	7-ADCA	H	$-CH_3$	H
8	Cephaloridine			H
9	Cephalothin		$-CH_2OCOCH_3$	H
10	Cephalosporin C		$-CH_2OCOCH_3$	H
11	Cefotaxime		$-CH_2OCOCH_3$	H
12	Ceftizoxime		H	H
13	Ceftazidime			H
14	Cefoxitin		$-CH_2OCONH_2$	$-OCH_3$
15	Cefoperazone			H
16	Cefazolin			H

Figure 1. Structures of the analyzed cephalosporins.

Table 1

The Capacity Factors of Cephalosporins on β-Cyclodextrin Bonded Column with Cation and Anion (5mM) in MeOH/Buffer = 32/68, pH 3.6, Column Temperature 30°C.

		k'*		
No.	**Compound**	**TEAA**	**TBAA**	**HSA**
1	Cefadroxil	0.07	0.06	0.08
2	Cefatrizine	0.14	0.12	0.09
3	Cefaclor	0.14	0.13	0.12
4	Cephalexin	0.14	0.12	0.11
5	Cephradine	0.14	0.12	0.12
6	7-ACA	3.61	2.69	0.87
7	7-ADCA	2.49	3.01	0.65
8	Cefaloridine	0.81	0.59	0.47
9	Cephalothin	12.76	10.29	2.19
10	Cephalosporin C	7.23	6.57	1.52
11	Cefotaxime	8.04	7.26	1.87
12	Ceftizoxime	7.47	6.77	1.93
13	Ceftazidime	7.03	6.25	2.09
14	Cefoxitin	10.65	9.10	2.02
15	Cefoperazone	13.54	10.89	2.60
16	Cefazolin	16.65	12.96	2.46

*Capacity factor (k') = $t_1 - t_0/t_0$. t_0 is the retention time of unretained methanol, while t_1 is the average data in three times of retention time for each compound.

pH, ions, ionic concentration, organic modifier concentration of mobile phase, and the column temperature. This project plans to investigate these factors, so that a series of isocratic mobile phases can be developed.

Effect of Cation and Anion on Retention

Table 1 shows the retention behaviors of sixteen cephalorsporins in the mobile phase (MeOH/buffer = 32/68, pH 3.6) with adding different ion reagents. Addition of either cation or anion decreased the retention of Type II and III compounds. This is due to the added ions competing with solutes for

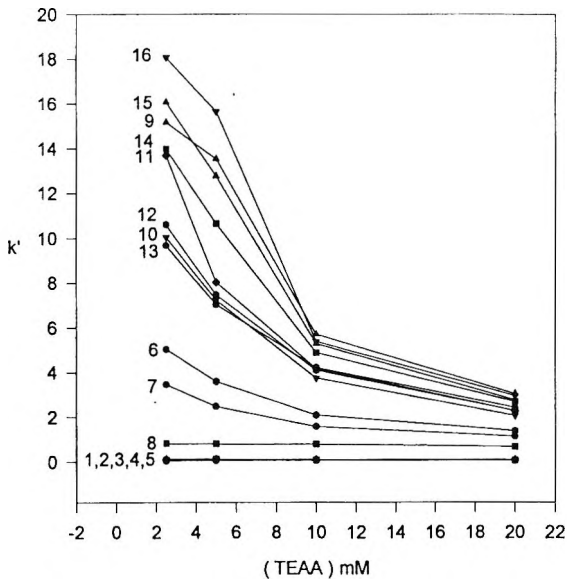


Figure 2. Effect of TEAA concentration on the capacity factors of cephalosporins. Chromatographic condition: Column, 250 x 4.6mm i.d. Cyclobond I; mobile phase, MeOH/TEAA buffer = 32/68, pH 3.6, column temperature 30°C; flow rate, 0.8 mL/min. Labels: 1, Cefadroxil; 2, Cefatrizine; 3, Cefaclor; 4, Cephalexin; 5, Cephradine; 6, 7-ACA; 7, 7-ADCA; 8, Cefaloridine; 9, Cephalothin; 10, Cephalosporin C; 11, Cefotaxime; 12, Ceftizoxime; 13, Ceftazidime; 14, Cefoxitin; 15, Cefoperazone; 16, Cefazolin.

the β-CyD cavity and thus renders the strength of inclusion complexation between the solutes and the β-CyD cavity weaker. By comparison, HSA has less separative selectivity than the other ion reagents, although it has more influence on retention. It also shows that the Type I compounds containing a hydrophilic α-amino moiety exhibit less retention and thus separation.

Effect of TEAA Concentration on Retention

The retention behaviors of cephalosporins over a wide range of TEAA concentration from 2.5 to 20 mM were studied. Figure 2 shows the capacity factors (k') of cephalosporins in mobile phase (MeOH/buffer = 32/68, pH 3.6) with different concentration of TEAA. A relatively fast decreasing in the k' values of Type II and III compounds is observed with increasing TEAA

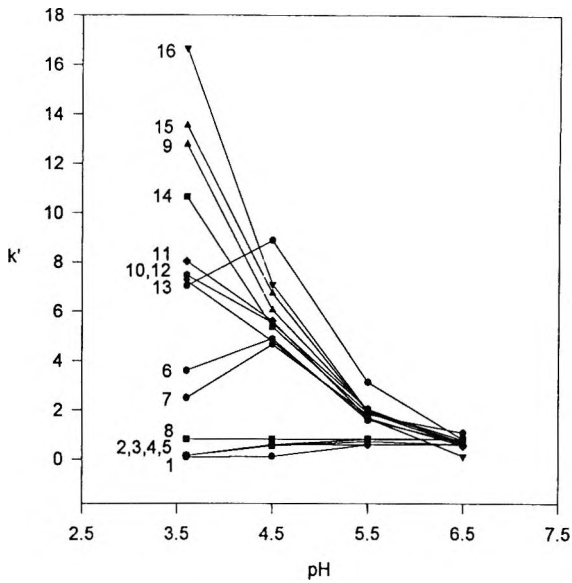


Figure 3. Effect of pH on the capacity factors of cephalosporins. Chromatographic condition: Mobile phase, MeOH/buffer = 32/68, TEAA = 5 mM, column temperature 30°C. Others are the same as in Figure 2.

concentration. The results indicate that higher concentration of TEAA in the mobile phase saturates the β-CyD cavities and causes the solutes to have less competition to form the inclusion complexation with β-CyD cavities resulting in faster decreasing on the retention of solutes. It is noted that in this case a suitable retention and selectivity is reached between 3 and 6 mM of TEAA in the mobile phase.

Effect of pH on Retention

The retention behaviors of cephalosporins with the same mobile phase (MeOH/5 mM TEAA = 32/68) at different pH values are indicated in Figure 3. The retention of Type I compounds has no significant changes at various pH values, while the Type III compounds except ceftazidime show the lower capacity factors with increasing pH. This can be explained by considering the effect of pH on the bonding strength and function of the solutes to the hydroxyl group of β-CyD. It had been reported that the OH^- ion exhibited a higher hydrogen bonding ability to the hydroxy groups of ROH molecules.[27]

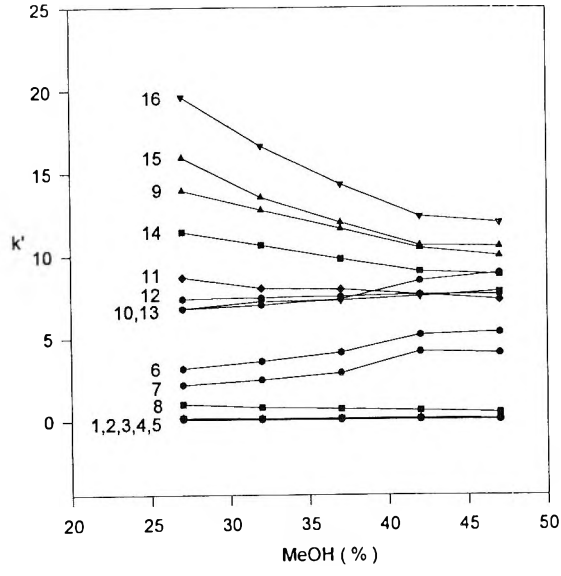


Figure 4. Effect of methanol concentration on the capacity factors of cephalosporins. Chromatographic condition: Mobile phase, TEAA = 5 mM, pH 3.6, column temperature 30°C. Others are the same as in Figure 2.

Therefore, the increase of OH^- ion concentrations by increasing pH of the mobile phase. The ions will compete with the carboxylate group of cephalosporin to interact with the hydroxy group of β-CyD. As a result, a decreased retention time accompaned with the increase of pH is observed. However, a variant retention model for 7-ACA (compound 6), 7-ADCA (compound 7), and ceftazidime (compound 13) is observed with increasing pH. This indicates that other factors that exercise influences on this β-CyD bonded stationary phase may be involved. The longest retention time appears near its isoelectric point, pH 4.5.

Effect of Organic Concentration on Retention

The effect of methanol concentration on the cephalosporin retention was also studied by changing the methanol-water ratio in the mobile phase. Figure 4 shows the typical plots of capacity factors *versus* the methanol contents from 27% to 47%. The TEAA concentration is 5 mM, and the pH of the mobile phase is 3.6 in all experiments. It is found that an increase in methanol content

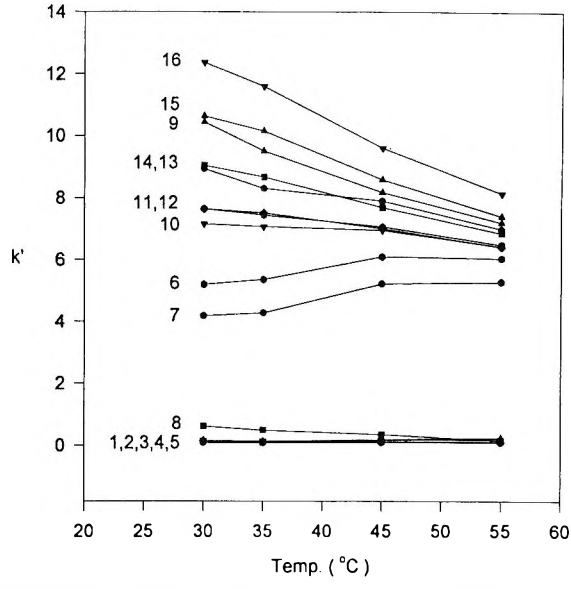


Figure 5. Effect of column temperature on the capacity factors of cephalosporins. Chromatographic condition: Mobil phase, MeOH/buffer = 42/58, pH 3.6, TEAA = 5mM. Others are the same as in Figure 2.

results in a decreased retention on Type III compounds. It is well explained from the cyclodextrin-binding studies that an increase in organic content in the mobile phase will weaken the strength of inclusion complexation between guest molecules and β-CyD.[28,29]

In contrast, for the Type II compounds and ceftazidime a medium increase on retention is obtained with an increasing of methanol content. This may be due to the stronger ion-pairing formation between solute and TEAA in the less aqueous content solution.

Effect of Temperature on Retention

The effect of column temperature on the retention behaviors of cephalosporins is also examined. As shown in Figure 5, the retention and resolution of Type III compounds is decreased as the column temperature increased. This result is due to the fact that the higher temperature weakens

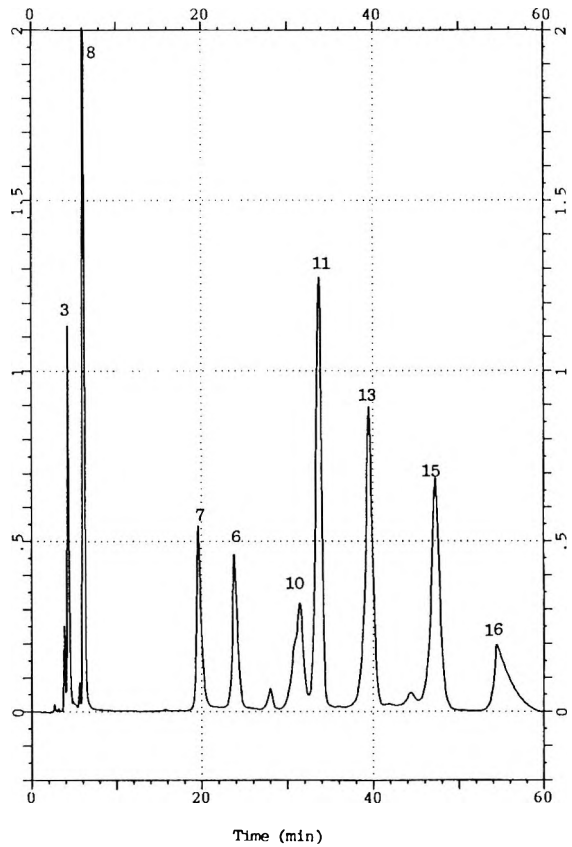


Figure 6. Chromatogram of the nine cephalosporins separated by using the optimized mobile phase of MeOH/buffer = 42/58, 5mM TEAA, at pH 3.6, 30°C, flow rate = 0.8 mL/min. Labels: 3, Cefaclor; 6, 7-ACA; 7, 7=ADCA; 8, Cefaloridine; 10, Cephalosporin C; 11, Cefotaxime; 13, Ceftazidime; 15, Cefoperazone; 16, Cefazolin.

the attraction strength between solutes and the β-CyD cavities. However, there is still a medium increasing retention for the Type II compounds at higher temperature.

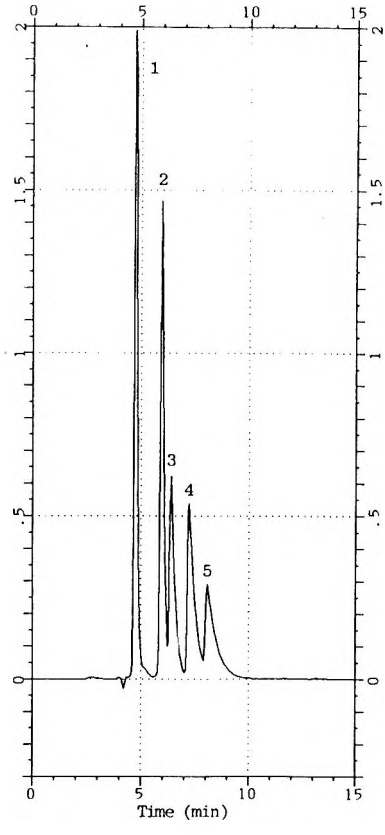


Figure 7. Chromatograms of the five cephalosporins containing a hydrophilic α-amino moiety separated by using 100% 5mM TEAA buffer solution at pH 3.6, 30°C, flow rate = 0.8 mL/min. Labels: 1, Cefaxroxil; 2, Cefatrizine; 3, Cefaclor; 4, cephalexin; 5, Cephradine.

Chromatogram of Cephalosporins

Figure 6 shows the optimal separation of a mixture of nine cephalosporins in the mobile phase under the condition of MeOH/buffer = 42/58, 5 mM TEAA, and pH 3.6. In chromatography, the adjustment on retention of Type II compounds will be predominated by changing the methanol content or TEAA concentration rather than the other factors. For the Type III compounds, the conditions of pH 3.6, 3-5 mM of TEAA, and MeOH content ranged in 37-42%

are recommended. If the hydrophilic Type I compounds need to be separated, the less methanol content in the mobile phase is suitable for the purpose. Figure 7 demonstrates the typical chromatogram of these compounds.

In conclusion, the isocratic separation of a mixture of cephalosporins on cyclodextrin bonded column has been demonstrated. The effects of methanol and TEAA concentration in the mobile phase, pll, and column temperature on retention suggest that the inclusion complexes formation between The type III compounds and the cavities of β-CyD is more predominate than the Type I and II. It shows that the bulky substituents on C-7 or C-3 side chain of cephalosporins are the important factors for the separation. Although the separation of five hydrophilic Type I cephalosporins is intrigued, the separation performance can be improved by using 100% TEAA buffer solution as mobile phase.

ACKNOWLEDGEMENTS

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV DETECTION AND SCANNING UV CONFIRMATION OF CHLORAMPHENICOL IN FATTY LIVER

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ABSTRACT

A liquid chromatographic (HPLC) method has been developed to detect chloramphenicol (CAP) in fatty liver. After addition of water, samples were defatted with hexane partition and CAP was extracted by ethyl acetate. A solid-phase extraction protocol was used to recover CAP from the fatty liver extract. CAP was analyzed using a mobile phase of acetonitrile-0.005 M phosphate buffer, pH 7.9 (19:81), an ultraviolet detection wavelength of 278 nm and confirmation by scanning. The column used was a Nova-Pak C_{18} (150 x 3.9 mm). The limit of detection was < 1 ng/g in fatty liver and the mean recovery of CAP was found to be 71 % over the concentration range 5 - 20 ng/g. The analyte identity can be confirmed by the use of scanning detection and by LC/MS method for concentrations less than 10 ng/g.

INTRODUCTION

The breeding of palmipedes for fattened goose or duck liver is well established in France. Antibiotics may be used to treat diseases in palmipedes and the possible health risk to humans, presented by traces of antibiotics in fatty liver, has generated serious concern in the export and domestic markets.

To ensure that levels of antibiotics in fatty liver are within acceptable limits, monitoring of antimicrobial residues is realized by agar diffussion microbiological method.[1] But this method is not very sensitive for detection of CAP in fatty liver. CAP is one of the most controversial antibiotics because of its risk of provoking aplastic anaemia not related to the absorbed dose. The risk has led for several years to severe restrictions in the use of CAP. Since June 1994, CAP is not approved for food producing animals in the European Union.[2] Thus, there is a demand for development of techniques which are rapid, specific and sensitive for quantitative detection of CAP residues. Chromatographic methods are methods of choice for monitoring many antimicrobial agents and many high performance liquid chromatographic methods have been reported for the analysis of CAP in a number of matrices.[3-9] To date, there have been no published reports of HPLC analysis of CAP in fatty liver. So, the aim of this study was to develop a protocol to determine CAP in fatty liver from previous works.[10,11] For confirmation purposes, scanning UV detection may be used, but the analysis requires a high concentration in the detection cell to produce an accurate UV spectrum.

EXPERIMENTAL

Reagents and Chemicals

Acetonitrile (Merck, Darmstadt, Germany) was HPLC grade. Ethyl acetate distilled before use, methanol, chloroform, n-hexane and hydrochloride acid (Merck) were analytical grade. Diammonium hydrogen phosphate, anhydrous sodium sulfate and dipotassium hydrogen phosphate were obtained from Merck. HPLC-grade water was produced using a Milli-Q water purification system (Millipore, St Quentin en Yvelines, France). Chloramphenicol was obtained from Lepetit (Milan, Italy).

Bond Elut SPE cartridges (Silica gel, 3 cc), reservoirs 75 mL, adaptors for reservoirs and SPE vacuum manifold were obtained from Varian, Harbor City, CA, USA.

Diammonium buffer (pH 7.9) contained 0.005 M (0.66 g/L) of diammonium hydrogen phosphate. Phosphate buffer pH 10 contained 0.05 M dipotassium hydrogen phosphate.

Apparatus and Chromatographic Conditions

The HPLC system constisted of a Kratos Spectroflow 400 pump (Applied Biosystems, Foster City, CA, USA) connected to a Kratos Spectroflow 773 variable-wavelength detector operated at 278 nm and a Spectra-Physics SP 4290 integrator (Spectra-Physics, Fremont, CA, USA) coupled to a computer for data handling (Spectra-Physics Winner Software). A Focus multiwavelength scanning detector (Spectra-Physics) was also used. The analytical column was a 4 μm Nova-Pak C_{18} stainless-steel column (150 x 3.9 mm I.D., Waters Milipore) with a guard column (4 x 4 mm) packed with reverse phase material (Merck). The samples were injected with a Rheodyne 7125 injector with a 200 μL loop. The mobile phase consisted of acetonitrile - 0.005 M diammonium hydrogen phosphate buffer, pH 7.9 (19:81,v/v) and was delivered isocratically at a flow - rate of 1.0 mL/min. The mobile phase was filtered prior to use with a Millipore HPLC solvent system and 0.45 μm membrane Millipore filter.

Preparation of Standard Solutions

A 500 μg/mL CAP stock solution was prepared by dissolving 50 mg in 2 mL of methanol, then adjusting to 100 mL with water. After a 5 μg/mL intermediate solution, working standards (15, 30, and 60 ng/mL) were prepared by diluting the intermediate solution in water.

Sample Preparation

For spiking studies, fatty livers from untreated crammed ducks were used. Partially thawed fatty livers were homogenized with a scalpel. Approximately 3 g of fatty liver were accurately weighed in a centrifuge tube. To each control fatty liver, 1 mL of the working solution were added, resulting in a final concentration of 5, 10 and 20 ng/g. The sample was vortex-mixed and allowed to stand for 15 min before addition of 2 mL of water and extraction.

Extraction Procedure and Cleanup

Three g of cutted fatty liver were weighed in a 50-mL glass-stoppered centrifuge tube. A 3-mL volume of water was added and the tube was vortex-mixed for 1 min. Then, 15 mL of hexane was added and the tube was placed between the plates of a rotary stirrer Reax 2 (Heidolph, Germany) and stirred at 40 rpm for 10 min. After centrifugation for 5 min at 2400 g, the supernatant was discarded. A 12-mL volume of ethyl acetate was added and the tube was vortex-mixed for 2 x 1 min at 15 min interval. After centrifugation for 5 min at 4000 g, aliquot of the organic phase (8 mL) was decanted through glass wool topped by 1 g of anhydrous sodium sulphate which were then washed with 2 x 2 mL of ethyl acetate. To the combined extracts, 30 mL of hexane were added.

The combined filtrate was passed through the pretreated silica cartridge (10 mL of a mixture ethyl acetate-hexane) connected with a 75-mL reservoir. The flask and sodium sulfate were washed with 10 mL ethyl acetate-hexane (4:10, v/v). The cartridge was washed with 10 mL of hexane, then left under vacuum for 2 min in order to dry it. CAP was eluted from the cartridge with 5 mL of phosphate buffer pH 10, then extracted from the eluate with 20 mL ethyl acetate. After vortex-mixing and centrifugation for 10 min at 2400 g, 18 mL of the ethyl acetate phase was transfered into a round bottomed flask and evaporated to dryness with a rotary evaporator (Büchi, Switzerland). The oily residue was resuspended in 1.2 mL mixture of hexane - chloroform (1:1, v/v). After addition of 0.8 mL of water, the flask was stirred between the plates of the rotary stirrer for 5 min at 35 rpm. The mixture was transfered into a vial and centrifuged for 10 min at 3300 g.

Chromatography

The supernatant (aqueous phase) was injected by means of the loop injector for HPLC analysis. For confirmation, the HPLC system was coupled to a UV-VIS scanning detector. It was based on a comparison of the retention time and of the UV spectrum of the sample with those of standard CAP.

Assay Precision and Recovery

The recovery study was performed by adding 1 mL of CAP solution to 3 g of fatty liver. Six samples were prepared at each concentration (5, 10 and 20 ng/kg) and were not frozen before extraction. The area of the CAP peak was

Table 1

Recovery Data of CAP from Fatty Liver Fortified with CAP at Various Levels (n=6) and Repeatability of the Method

Concentration Added (ng/g)	Concentration Found (ng/g)	Mean Recovery	SD %	CV %
5	3.5	68.9	2.0	2.8
10	7.3	72.6	5.0	6.8
20	14.3	71.6	6.0	8.4

compared to the area of the peaks for identical amounts of standards solutions. A linear regression equation was obtained by plotting the peak areas corrected by the recovery against concentrations.

RESULTS AND DISCUSSION

The previous method employed to dose CAP in muscle, kidney and liver[10] cannot be used to determine CAP in fatty liver because interfering peaks appeared in blank sample chromatograms. A solid phase extraction step in silica cartridge was introduced to remove major interfering components. An additional washing step (liquid-liquid extraction with hexane) was also necessary to eliminate other lipophilic interfering components. Indeed, fat content in fatty liver represents about 50 to 60 % of the total weight. Dissolution of the final residue in water instead of the eluent allowed to increase the volume injected to 200 µl to improve the limit of detection. Indeed, the preconcentration which takes place on the top of the column prevents peak broadening. The chromatographic conditions specified here are the same as those proposed and discussed for the determination of CAP residues in muscle.[10] The mobile phase is only slightly more polar (81 % aqueous phase) to allow complete elution of interfering substances before CAP peak appearance. Figure 1 shows representative chromatograms of (A) a standard of CAP, (B) a blank fatty liver extract and (C) a CAP fortified fatty liver. They do not reveal significant interference at the retention time of chloramphenicol.

Table 1 gives the concentration examined, standard deviations, coefficients of variation and recoveries for CAP fortified fatty liver samples. A good recovery at all levels investigated and a low standard deviation for the

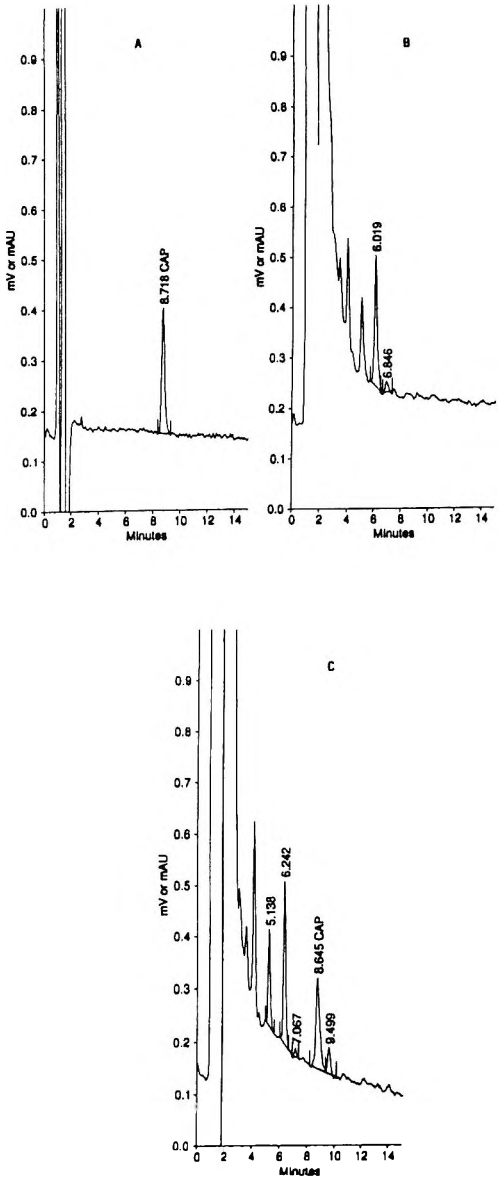

Figure 1. Chromatograms of (A) CAP standard solution (15 ng/mL), (B) a blank fatty liver sample and (C) a fatty liver sample spiked with 5 ng/g of CAP.

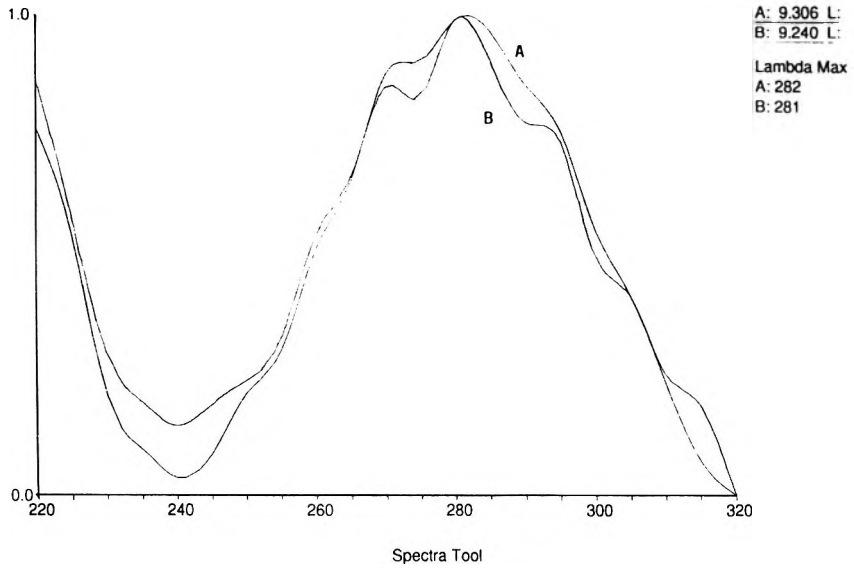

Figure 2. Ultraviolet spectra of (A) CAP standard solution (60 ng/mL) and (B) fatty liver extract spiked with 10 ng/g of CAP.

repeatability were attained. The mean recovery of CAP was found to be 71 % (SD = 4.5%) over the concentration range 5 - 20 ng/g. With this method, the peak area was linear against the dose added in the fatty liver (r = 0.998, n = 17). The coefficients of variation were less than 8.4 % (Table 1). The method can be considered as reliable for the intended application. However, some precautions have to be taken to avoid decrease of CAP concentration as it has been noted for calf liver.[10] The samples have to be stored at -20°C until analysis and never be frozen another time.

The limit of detection in the HPLC system using the UV detector was estimated from representative blank samples. It is equal to three times the peak-to-peak noise, i.e. 0.4 ng/g. The limit of quantification is estimated at the 0.8 ng/g level. The HPLC was also performed using scanning detection. CAP could be identified in samples with a CAP content above 8 ng/g. The CAP spectra obtained from liver samples are practically identical with that of standard CAP, including the wavelength of maximum absorption (in a range of 2 nm). A spectrum of the CAP peak of a fatty liver obtained with the scanning detector is presented in Figure 2.

The method was applied to control about 30 samples of fatty livers from different origin and species. All samples were found negative (< 0.8 ng/g). Some of these extracts were also analyzed by a particle beam HPLC - mass spectrometric method with negative ion chemical ionization.[12] This method which is applied with success at the laboratory for meat with a limit of detection of 0.5 ng/g has confirmed that samples were negative. CAP is metabolized in liver with a phase I metabolism involving oxidation, catalyzed by cytochrome P-450. Factors affecting liver function can, therefore, affect drug disposition as it can be noted by Soback[13] for some antibiotics. The metabolic pathways for CAP in the duck was recently reported by Cravedi[14] but no study was published about fattened duck.

In conclusion, this method has proved to be reliable and robust after testing by a second analyst. The procedure satisfies the quality criteria specified in Commission Decision 93/256/EEC[15] and possesses the required sensitivity to detect the improper use of CAP in the fatty liver-production industry in France and monitoring of CAP residues in imported livers. Further investigations will be programmed to validate the LC/MS method for the identification of CAP in fatty liver.

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SIMPLE AND RAPID ANALYSIS OF PEFLOXACIN, FENBUFEN AND FELBINAC IN HUMAN PLASMA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple and reproducible method for the simultaneous determination of the antimicrobial agent pefloxacin and the anti-inflammatory drug fenbufen with its metabolite felbinac in human plasma is described. It involves a two steps liquid-liquid extraction and separation using an Adsorbosphere SAX column with ultraviolet detection at 280 nm. 2-[4-(2'-

furoyl)phenyl]propionic acid is used as internal standard. The coefficients of variation are less than 5%. The lower limits of detection are 0.1, 0.3 and 0.1 μg/mL for pefloxacin, fenbufen and felbinac, respectively. This method was found to be applicable to pharmacokinetic and pharmacodynamic studies of each drug after the concomitant administration of pefloxacin and fenbufen.

INTRODUCTION

Fenbufen, or γ-oxo-[1,1'-biphenyl]-4-butanoic acid (Figure 1A), is a potent anti-inflammatory drug with analgesic properties, active by inhibition of prostaglandin synthesis.[1-5] Pefloxacin is a potent quinolone antibacterial agent; it is highly active in *vitro* against a broad spectrum of Gram-positive and Gram-negative organisms, including those resistant to β-lactam antibiotics.[6-9] Its chemical name is 1-ethyl-6-fluoro-1,4-dihydro-7-(4-methyl-1-piperazinyl)-4-oxoquinolinecarboxylic acid; its formula is shown in Figure 1B. Quinolone carboxylic acid derivatives, used in the treatment of several infections, are known to elicit epileptogenic neurotoxic effects. Several quinolones inhibit the specific binding of [^{3}H]GABA to synaptic plasma membranes from rat brain in a concentration-dependent manner. Since GABA is a major inhibitory neurotransmitter in the mammalian central nervous system, these results indicate that quinolones may induce seizures through the inhibition of postsynaptic GABA function.

Recently, it was reported that several patients had severe chronic convulsions during therapy with quinolones and fenbufen and it is reasonable to assume that, simultaneously administered, fenbufen enhanced the neurotoxic potency of quinolones via some pharmacodynamic interaction in the brain.[10] Many high performance liquid chromatographic methods have been developed for the determination of either pefloxacin[11,12] or fenbufen[13,14] in plasma, but none has described the simultaneous determination of both agents.

The interest in this group of drugs has prompted us to develop a simple and sensitive assay method for both these substances in human plasma, which could be applied to pharmacokinetic studies. The procedure, based on the use of the high performance liquid chromatography, allows accurate and precise results, and permits also the determination of felbinac or 4-biphenylacetic acid (Figure 1C), the major metabolite of fenbufen.

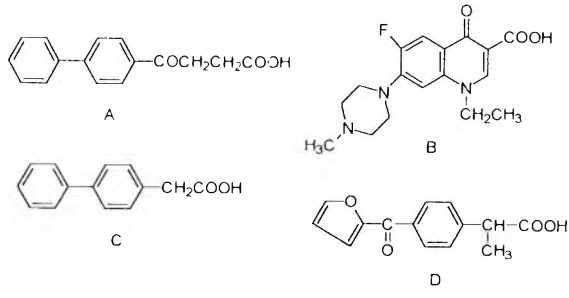

Figure 1. Chemical structures of fenbufen (A), pefloxacin (B), felbinac (C) and internal standard (D).

EXPERIMENTAL

Chemicals and Reagents

Fenbufen was purchased from Sigma (Sigma, St Louis, MO, U.S.A.). Felbinac was purchased from Aldrich (Aldrich Chimica, Milan, Italy). Pefloxacin was extracted from tablets. The internal standard (2-[4-(2'-furoyl)phenyl]propionic acid) (Figure 1D) was a gift of the Chair of Pharmacology of our University. HPLC-grade acetonitrile was obtained from Farmitalia-Carlo Erba (Farmitalia-Carlo Erba, Milan, Italy). Dichloromethane, diethylether and all other analytical-grade reagents (sodium hydroxide, sodium hydrogen phosphate and potassium dihydrogen phosphate) were purchased from Fluka Chemie (Fluka Chemie, Buchs, Switzerland). Water (HPLC-grade) was obtained by distillation in glass and passage through a Milli-Q Water Purification System (Millipore Corporation, Bedford, MA, USA).

Standard Solutions

Stock solutions of pefloxacin, fenbufen and felbinac were prepared by dissolving 10 mg of each compound in 10 mL of 0.01M sodium hydroxide. These solutions could be stored at -20°C for over 1 month with no evidence of decomposition. Stock solution of the internal standard was prepared by dissolving 20 mg of compound in 10 mL of methanol. Standard solutions, each containing the three drugs, were prepared with control human plasma in the concentration range of 0.2-10 μg/mL for pefloxacin, 0.5-30 μg/mL for

fenbufen, and 0.2-10 μg/mL for felbinac. For each solution the concentration of the other two drugs was kept constant at 5 μg/mL , and an aliquot (20 μl) of the internal standard stock solution was added. These standards were treated concurrently in the same manner as the samples to be analysed. The calibration curves were obtained by plotting the peak-area ratios of each drug to internal standard versus its concentration.

Instrumentation and Chromatographic Conditions

The HPLC system consisted of a model 7125 sample injector Rheodyne (Rheodyne, Cotati, CA, U.S.A.) equipped with a 20μl loop, a Waters Associates constant flow reciprocating pump (model 510) with Data Module integrator model 740, and a Tunable absorbance detector model 486 (Milford, MA, USA). The separation system was on an analytical 250 x 4.6 mm I.D. anion-exchange Adsorbosphere SAX (5 μm particle size) column (Alltech Associates, Deerfield, IL, U.S.A.) protected by a 20 x 4.6 mm anion-exchange Vydac AXGU (10 μm particle size) precolumn (Separations Group, Hesperia, CA, U.S.A.). Separations were performed at room temperature and the detector set at 0.1 absorbance unit full scale. The mobile phase consisted of a mixture of acetonitrile, 0.1M phosphate buffer pH 7.0 (10:90, vol/vol). Phosphate buffer was filtered through an HA 0.45 μm filter, while the acetonitrile was filtered through a FA 0.5 μm filter (Millipore, Bedford, MA, USA). The mobile phase was prepared daily and delivered at a flow rate of 1.2 mL/min. Column eluate was monitored at 280 nm.

Extraction Procedure

Pefloxacin, fenbufen, felbinac and the internal standard were extracted from plasma using a simple two-steps extraction procedure. An aliquot (20μl) of the internal standard stock solution was added to 1.0 mL of plasma sample and mixed into a 16x150mm screw-capped tube. The mixture was vortex-mixed for 1 min with 4mL of dichloromethane-diethylether (80:20, vol/vol). The sample was centrifuged at 1000 x **g** for 10 min and the organic layer was separated and transferred into a second tube. Fresh dichloromethane-diethylether (4mL) was added to the first tube and the same extraction procedure was repeated twice. The organic phase was evaporated to dryness under a stream of nitrogen. 100μl of 0.01M sodium hydroxide were then added to the residue. The tube was shaken for 5 min and 20μl aliquots were used for HPLC analysis.

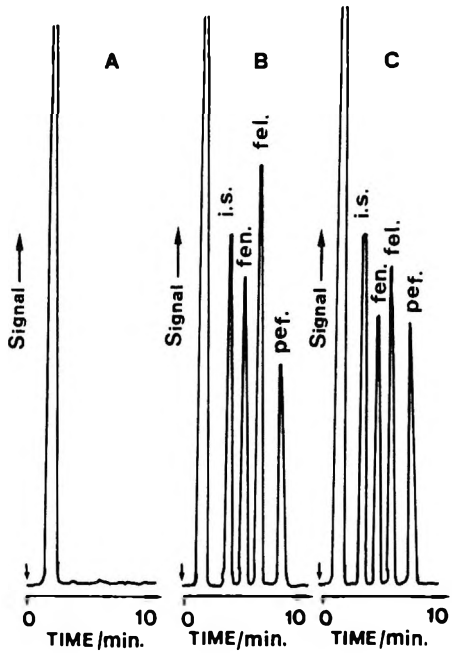

Figure 2. HPLC profiles of human plasma extracts. (A) drug-free human plasma; (B) drug-free human plasma spiked with 2.0 μg/mL of pefloxacin (pef.), 5.0 μg/mL of fenbufen (fen.), 3.0 μg/mL of felbinac (fel.) and 5.0μg/mL of internal standard (i.s.); (C) plasma sample from a volunteer (two hours after an oral dose of 400 mg of pefloxacin and 300 mg of fenbufen) containing 3.1 μg/mL of pefloxacin, 4.3μg/mL of fenbufen and 2.2 μg/mL of felbinac. Vertical axis: UV detector response (280 nm). Horizontal axis: retention time (min).

RESULTS AND DISCUSSION

Figure 2 shows a typical chromatogram of an extracted drug-free blank plasma (A), an extracted plasma sample (B), and a two hours post dose sample from a volunteer (C). The elution order is internal standard, fenbufen, felbinac and pefloxacin, respectively. The retention times for internal standard, fenbufen, felbinac and pefloxacin were 3.9, 5.3, 6.8 and 9.2 min, respectively. There were no interfering peaks detected in either the blank plasma sample or the plasma standard. The validity of the liquid chromatographic assay was established through a confirmatory study of calibration curves specificity, sensitivity, accuracy and precision. The calibration graphs were linear from 0.2

Table 1

Precision and Accuracy of Perfloxacin Calibration Standards in Human Plasma

Conc. (μg/mL)	N	Conc. Found (μg/mL)	SD	CV (%)	RE (%)
0.2	7	0.19	0.007	3.6	5.2
0.3	7	0.29	0.01	3.4	3.4
0.5	7	0.49	0.02	4.0	2.0
1.0	7	0.97	0.05	5.1	3.1
2.0	7	1.95	0.08	4.1	2.5
5.0	7	4.81	0.2	4.1	3.9
10	7	9.84	0.4	4.0	1.6

SD: standard deviation; CV: coefficient of variation; RE: relative error.

to 10 μg/mL for pefloxacin, from 0.5 to 30 μg/mL for fenbufen and from 0.2 to 10 μg/mL for felbinac, respectively. All correlation coefficients were more than 0.998. The mean of seven different calibration graphs yielded the following equations: $y = 0.041x + 0.264$, $y = 0.052x + 0.573$ and $y = 0.039x + 0.632$, where y is the peak area ratio of either pefloxacin, fenbufen and felbinac to the internal standard and x is the concentration of each drug (μ g/mL).

The precision and accuracy of the method was determined by preparing pools of plasma containing pefloxacin, fenbufen and felbinac at seven different concentrations. The values for pefloxacin, fenbufen and felbinac for each standard concentration were determined by nine repeated analyses, using spiked human plasma. Results are given in Table 1-3. The method was found to be reproducible and accurate. The coefficients of variation were less than 4.8% for pefloxacin (Table 1), 4.6% for fenbufen (Table 2) and 4.7% for felbinac (Table 3). The mean extraction efficiencies calculated by comparison of the peak-area ratios of the extracted samples with those of aqueous standards of same concentration, were 95, 96 and 95% for pefloxacin, fenbufen and felbinac. The extraction efficiency of the internal standard was 97%.

Table 2

Precision and Accuracy of Fenbufen Calibration Standards in Human Plasma

Conc. (μg/mL)	N	Conc. Found (μg/mL)	SD	CV (%)	RE (%)
0.5	7	0.48	0.01	2.1	4.1
1.0	7	0.95	0.03	3.1	5.2
2.0	7	1.92	0.05	2.6	4.1
5.0	7	4.9	0.15	3.0	2.0
10.0	7	9.57	0.3	3.1	4.5
20.0	7	19.4	0.85	4.3	3.1
30.0	7	29.0	1.4	4.8	3.4

SD: standard deviation; CV: coefficient of variation; RE: relative error.

Table 3

Precision and Accuracy of Felbinac Calibration Standards in Human Plasma

Conc. (μg/mL)	N	Conc. Found (μg/mL)	SD	CV (%)	RE (%)
0.2	7	0.19	0.01	4.7	5.2
0.5	7	0.48	0.02	5.1	2.0
1.0	7	0.96	0.05	5.2	4.1
2.0	7	1.95	0.08	4.1	4.1
3.0	7	2.92	0.14	4.7	2.7
5.0	7	4.78	0.18	3.7	4.6
10	7	9.88	0.35	3.5	1.2

SD: standard deviation; CV: coefficient of variation; RE: relative error.

The lower limits of detection, using a signal-to-noise ratio of three, were 0.1 μg/mL for pefloxacin, 0.3 μg/mL for fenbufen and 0.1 μg/mL for felbinac, respectively. The comparison of the peak-area ratios for pefloxacin, fenbufen and felbinac between fresh plasma samples and samples frozen at -20°C for a

period of four weeks showed no differences. The HPLC method described in this paper allows the simultaneous determination of pefloxacin, fenbufen and felbinac in human plasma. Since the inhibition of GABA receptor binding to synaptic membranes is dose dependent, it is useful to know the simultaneous concentrations of pefloxacin, fenbufen and felbinac. This method permits its application to pharmacodynamic studies of these compounds and will facilitate detailed investigations on the interactions between new quinolones and fenbufen.

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A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR A NOVEL SEROTONIN REUPTAKE INHIBITOR 5-CHLORO-2-[[2-(DIMETHYLAMINO)METHYL]PHENYL]BENZYL ALCOHOL (403U76)

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ABSTRACT

A sensitive and selective high performance liquid chromatographic assay using UV-detection was developed for the analysis of 403U76, a structurally novel serotonin reuptake inhibitor, and two demethylated metabolites, 515U88 and 336U76 in rat plasma. The compounds were isolated from plasma by liquid extraction followed by back extraction into 0.1N HCl. Extracts were chromatographed on an Apex I phenyl HPLC column cartridge (4.6 mm x 12.5 cm) using a mobile phase of 74% buffer (0.005M PIC-B5 low UV adjusted to pH 2.5 with 85% phosphoric acid) and 26% acetonitrile. The compounds of interest were detected at 210 nm with a lower limit of detection of 5 ng/mL. Overall precision of the assay for 403U76, 515U88, and 336U76, expressed as percent deviation of measured values from true values and percent coefficient of

variation, respectively, were $\leq$ 9.7 % at plasma concentrations between 5 and 500 ng/mL. This assay has been successfully used to analyze plasma samples from preclinical pharmacokinetic and disposition studies in rats.

INTRODUCTION

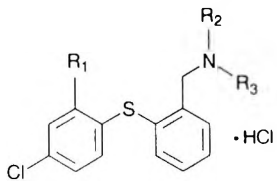
403U76, 5-chloro-2- [[2-[(dimethylamino) methyl]phenyl] thio] benzyl alcohol hydrochloride (Fig. 1), is a structurally novel serotonin reuptake inhibitor which is being studied as a potential antidepressant. Preclinical pharmacology indicated that 403U76 inhibits the neuronal uptake of serotonin (5-HT) and to a lesser extent, inhibits the neuronal uptake of norepinephrine (NE).[1] Consequently, 403U76 may be useful for the treatment of major depressive illness.[2-4]

403U76 is extensively metabolized and two metabolites have been identified in plasma as 5-chloro-2-[[2-[(methylamino) methyl] phenyl] thio]benzyl alcohol (515U88) and 5-chloro-2-[[2-[(amino) methyl] phenyl] thio]benzyl alcohol (336U76).[5] 515U88 is also a potent serotonin reuptake inhibitor while 336U76 has weak activity.[1] To provide analytical support for preclinical development of 403U76, a high performance liquid chromatographic (HPLC) assay was developed and validated for quantitation of 403U76 and its two metabolites in rat plasma. This assay has been used to monitor plasma concentrations during pharmacokinetic and disposition studies of 403U76 in rats.

MATERIALS AND METHODS

Materials

403U76, 515U88, 336U76, and the internal standard 516U88 (5-chloro-2-carbomethoxy-2-[(amino)methyl]diphenyl sulfide) were synthesized at Burroughs Wellcome Co., Research Triangle Park, NC. The chemical structures of 403U76, its metabolites, and internal standard are shown in Fig 1. Octane sulfonic acid, (PIC-B5, low UV) was purchased from Waters, Millipore Division, Milford, MA. Phosphoric acid (85%) was A.R. grade (Mallinckrodt, Paris, KY.) Hydrochloric acid (0.1N) was purchased from Fisher Scientific, Raleigh, NC. Acetonitrile, methyl-t-butyl ether and water were HPLC grade (Omnisolv, EM Science, Cherry Hill, NJ).



Compound	R_1	R_2	R_3
403U76	CH_2OH	CH_3	CH_3
515U88	CH_2OH	CH_3	H
336U76	CH_2OH	H	H
516U88	$COOCH_3$	H	H

Figure 1. Structure of 403U76, its metabolites, and internal standard 516U88.

Instrumentation

A Hewlett Packard HP-1090 HPLC system (Hewlett Packard, Avondale, PA) was used in conjunction with an Apex I phenyl analytical cartridge (4.6 mm x 12.5 cm) (Jones Chromatography, Litton, CO) for the separation of 403U76 and its metabolites. A precolumn cartridge containing the same bonded phase was coupled directly to the analytical cartridge. The analytical cartridge was maintained at 45°C within the heated column compartment of the HPLC. Samples were injected onto the column with a HP-1090 autoinjector and the analytes were quantitated with a Waters 486 variable wavelength detector set at 210 nm. The HPLC mobile phase was 26% acetonitrile and 74% buffer (0.005M PIC-B5 adjusted to pH 2.5 with 85% phosphoric acid). With a flow rate of 1 mL/min, the retention times of 403U76, 515U88, 336U76 , and the internal standard were approximately 12.8, 9.9, 8.3, and 14.9 min, respectively. Chromatographic data acquisition, peak-area analysis and data reduction were performed on a HP-Chemstation (Hewlett-Packard, Avondale, PA).

Preparation Of Standards And Solutions

Analytical standard solutions of 403U76, 515U88 and 336U76, (100 µg/mL) and the internal standard (50 µg/mL) were prepared in methanol. The hydrochloride salts of each compound were used and weights were corrected such that concentrations represented the free base. Analytical standards were diluted with methanol to prepare working stock solutions containing 5-500 ng/mL of 403U76, 515U88 and 336U76. Calibration standards were prepared by evaporating aliquots of the methanol solutions under nitrogen in a Zymark Turbo Vap LV (Zymark, Hopkinton, MA) followed by the addition of 1 mL of rat plasma. Quality control (QC) samples were prepared in a similar manner at concentrations of 5, 50, and 500 ng/mL. These samples were frozen at -80°C and replicate samples (n=2) were assayed with each analysis of experimental samples.

Extraction Procedures

Plasma samples (1 mL) and calibration standards (5-500 ng/mL) were spiked with internal standard (50 µg/mL of 516U88) and extracted with 4 mL of methyl-t-butyl ether. Samples were shaken for 15 min and then centrifuged at 1000 x g for 5 min. The tubes were immersed in a dry-ice acetone bath to freeze the aqueous phase, and the organic phase was transferred to a graduated tapered test tube containing 150 µL of 0.1N HCl. The tubes were shaken for 15 min, and centrifuged at 1000 x g for 5 min. The acid fraction was pipetted into autoinjector vials and a 50 µL aliquot was injected onto the HPLC column.

Assay Validation

To determine the intra-day accuracy and precision of the assay, replicate (n=3) plasma samples at seven different concentrations (5-500 ng/mL) were analyzed using SAS JMP® statistical software (SAS Institute, Cary, NC). The peak area ratios of each compound, and the internal standard, were fitted to different regression models; namely, an unweighted least-squares regression, a weighted least-squares regression (1/c, $1/c^2$ or 1/variance) and a least-squares regression of the logarithmically-transformed data. Residual plots were generated for each regression model and the best fit determined, based on the variance observed over the concentration range. For each compound, the residual plot from least-squares regression of the logarithmically-transformed calibration curve data showed equal variances over the concentration range. Therefore, this regression model was used to calculate the concentrations of

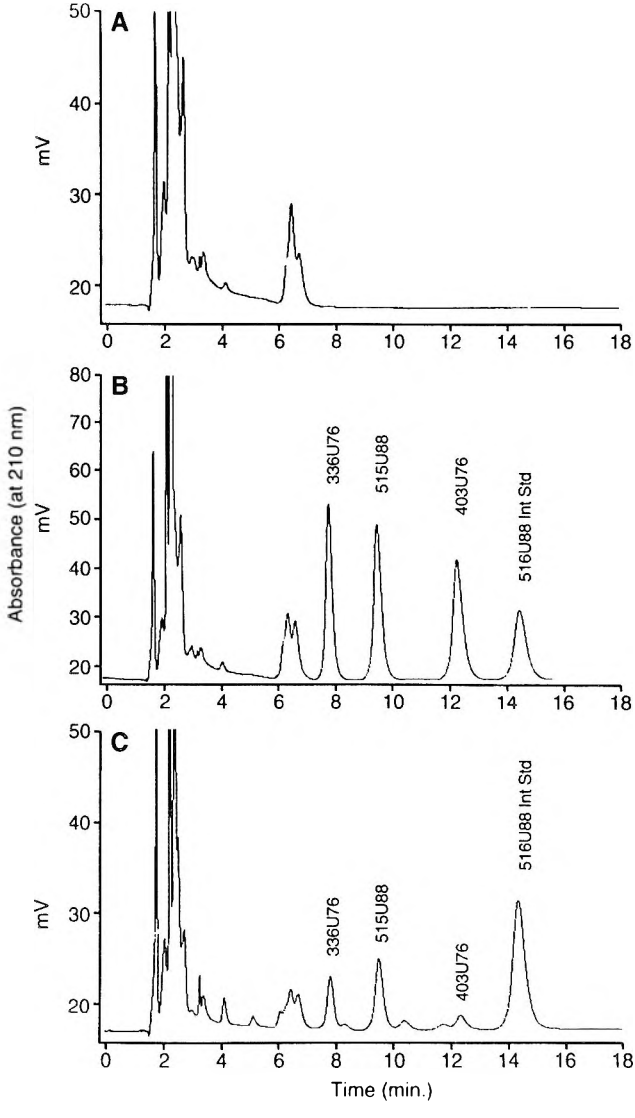


Figure 2. HPLC chromatograms of extracted rat plasma samples. A: blank plasma. B: plasma containing 500 ng/mL of 403U76 (peak 1), 515U88 (peak 2), 336U76 (peak 3) and50 µg/mL internal standard 516U88 (peak 4). C: actual plasma sample collected from a rat dosed with 403U76.

each compound in unknown samples. Precision was estimated from the standard deviation expressed as the percentage of the mean (% coefficient of variation, CV). Accuracy was calculated as the percentage difference between the mean calculated concentration and the amount added (% bias). To determine the inter-day accuracy and precision of the assay, spiked plasma pools were prepared at three concentrations (5, 50 and 500 ng/mL) of each compound. The pools were separated into 1 mL aliquots and frozen at -80°C. Duplicate samples from each pool were analyzed with each calibration curve over a 7-day period to assess the inter-day accuracy and precision of the assay. An analysis of variance (ANOVA) was performed using SAS JMP® statistical software (SAS Institute, Cary, NC) to determine assay precision.

The stability of 403U76, 515U88, 336U76, and internal standard 516U88 in rat plasma stored at -80°C was determined. Plasma was spiked with 25 and 500 ng/mL of each compound. Replicate samples (n=2) were analyzed immediately and at various times after storage at -80°C. The stability of each compound was also determined in standard solutions stored at -20°C and in plasma samples after five freeze-thaw cycles.

The extraction efficiency of the assay was determined by comparing the peak areas for each compound in extracted standards with those obtained by injection of unextracted standards.

The pharmacokinetics and disposition of 403U76 and its metabolites were studied in male and female rats after an oral (15 mg/kg) and intravenous (5 mg/kg) dose of ^{14}C-403U76.[5] Venous whole blood samples were collected at various times after dosing and plasma was separated by centrifugation. Plasma samples were frozen at -80°C until analyzed.

RESULTS AND DISCUSSION

A sensitive and selective HPLC assay using UV-detection was developed for the analysis of 403U76 and its two demethylated metabolites in rat plasma. The assay is specific for 403U76 and its metabolites with no interfering peaks in chromatograms of control plasma (Fig. 2). Fig. 2 also shows a chromatogram of a calibration standard (50 ng/mL) and a chromatogram of extracted plasma from a rat dosed with 15 mg/kg 403U76. The lower limit of quantitation for each compound was 5 ng/mL plasma.

Table 1

Intra-day Precision and Accuracy for the Analysis of 403U76, 515U88 and 336U76 in Rat Plasma

Compound	Mean Conc. Added (ng/mL)	Mean Conc. Found (ng/mL)	CV (%)	Bias (%)
403U76	5	4.7	8.5	-6.4
	10	10.5	4.4	4.8
	20	22.1	9.3	9.5
	50	50.5	0.5	1.0
	100	101.2	1.2	1.2
	250	247.7	2.5	-0.9
	500	481.1	0.7	-3.9
515U88	5	4.8	4.8	-4.2
	10	10.4	4.9	3.8
	20	20.3	4.9	1.5
	50	50.8	0.5	1.6
	100	102.0	1.7	2.0
	250	242.8	1.1	-3.0
	500	497.2	0.2	-0.6
336U76	5	4.8	9.7	-4.2
	10	10.2	6.1	2.0
	20	20.3	1.8	1.5
	50	51.7	1.1	3.4
	100	103.1	1.0	3.0
	250	246.9	1.6	-1.3
	500	483.4	1.2	-3.4

Replicate analyses (n=3). Precision was derived from the standard deviation and is expressed as the percent coefficient of variation. Accuracy is expressed as bias of the theoretical concentration.

Table 2

Inter-day Precision and Accuracy for the Analysis of 403U76, 515U88 and 336U76 in Rat Plasma

Compound	Mean Conc. Added (ng/mL)	Mean Conc. Found (ng/mL)	CV[a] (%)	Bias (%)
403U76	5	4.9	3.7	-1.2
	50	53.0	1.5	5.7
	500	477.1	2.3	-4.8
515U88	5	5.3	5.9	4.9
	50	50.9	2.4	1.7
	500	499.9	2.9	0.0
336U76	5	5.1	7.3	2.3
	50	48.6	4.4	-2.8
	500	539.9	3.5	7.4

Replicate analyses (n=14) for low, medium and high concentrations.
Precision was derived from the standard deviation and is expressed as the percent coefficient of variation. Accuracy is expressed as bias of the theoretical concentration.
[a] Inter-day precision was derived using an Analysis of Variance (ANOVA).

The intra-day precision and accuracy of the assay for 403U76, 515U88, and 336U76 in rat plasma are shown in Table 1. The intra-day %CV was 9.7 or less for 403U76 and its metabolites over the concentration range of 5-500 ng/mL. The % bias was less than 9.5% for all compounds. The inter-day accuracy and precision of the assay, measured daily over a 14 day period, is shown in Table 2. The inter-day % CV was 7.3 % or less and the % bias was 7.4% or less for all three compounds.

The extraction efficiencies for 403U76, 515U88, and 336U76 over the concentration range of 5-500 ng/mL are shown in Table 3. The mean extraction efficiencies were 99.5, 102.1 and 102.9% for 403U76, 515U88 and 336U76, respectively.

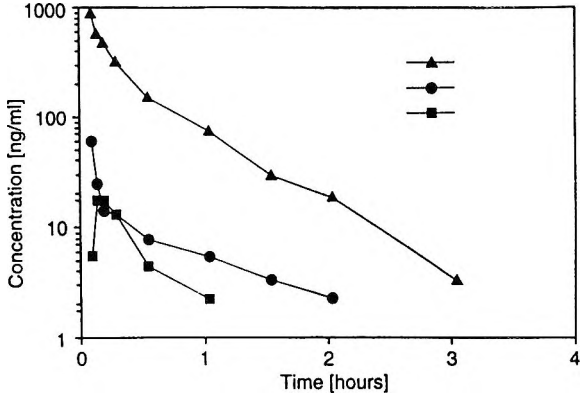


Figure 3. Plasma concentration profile for 403U76 (▲)515U88 (●) and 336U76(■) in rats following administration of a 5 mg/kg single intravenous dose of 403U76.

Table 3

Extraction Efficiencies for the Analysis of 403U76, 515U88 and 336U76 in Rat Plasma

Compound	Conc. (ng/mL)	Recovery (Mean ± S.D.) (%)
403U76	5	973 ± 12.0
	50	105.9 ± 4.8
	500	95.4 ± 3.1
515U88	5	105.2 ± 9.7
	50	101.3 ± 4.1
	500	99.9 ± 3.1
336U76	5	102.3 ± 14.9
	50	98.6 ± 5.1
	500	107.9 ± 3.8

Replicate analyses (n = 14) for low, medium and high concentrations.

The stability of 403U76 and its metabolites in plasma stored at -80°C was evaluated. No degradation of either 403U76, 515U88 or 336U76 was detected in plasma samples stored for at least 56 weeks at this temperature. In addition, no degradation of either compound was detected in plasma samples after five freeze-thaw cycles.

This assay was used to quantitate 403U76 and its metabolites from a rat pharmacokinetic and disposition study.[5] As an example of the utility of the assay, a plasma concentration versus time curve for each compound following an intravenous dose of 5 mg/kg of ^{14}C-403U76 to rats is shown in Fig. 3.

In summary, this assay allowed for the quantitation of 403U76, and its metabolites 515U88, and 336U76 in rat plasma and provides evidence that the method can be used to support preclinical pharmacokinetic and disposition studies.

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DETERMINATION OF DIHYDRO-5,6-DEHYDROKAWAIN IN RAT PLASMA BY HPLC AND ITS PHARMACO-KINETICS APPLICATION

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ABSTRACT

A simple high performance liquid chromatographic method was developed to study the pharmacokinetics of dihydro-5,6-dehydrokawain (DDK) in the rat plasma. After addition of an internal standard (osthole), plasma was deproteinized by acetonitrile for sample clean-up. The drugs were separated on a reverse phase column and detected by UV detection at a wavelength 281 nm.

Acetonitrile-water-diethylamine (50:50:0.1, v/v/v, pH 3.0 adjusted by orthophosphoric acid) was used as a mobile phase. It was applied to the pharmacokinetic study of DDK in rats after a dose of 5 mg/kg intravenous administration. A biphasic phenomenon with a rapid distribution followed by a slower elimination phase was observed from the plasma concentration-time curve.

INTRODUCTION

Alpinia speciosa K. Schum. (Zingiberaceae) is used as a folk medicine in Taiwan for the treatment of vomiting, dyspepsia and gastric ulcers. Dihydro-5,6-dehydrokawain (DDK, Fig. 1) was isolated from the root of *Alpinia speciosa* rhizoma.[1] It has been demonstrated that DDK has a protective effect on various experimental gastric ulcers (30-250 mg/kg), and can markedly inhibit gastric secretion (30-200 mg/kg).[2-4] The biologically active constituent was reported to have antiplatelet action due to the inhibition of thromboxane A_2 formation.[5] It was recently reported that DDK possesses an analgesic effect via non-opiate pathway.[6] Determination of kawain and its derivatives have been reported by gas chromatography-mass spectrometry.[7] However, the spectrum identification of DDK from plasma and its pharmacokinetic properties have not been studied. In this work, we developed a high performance liquid chromatographic (HPLC) method with photodiode-array and UV detection to determine the concentration of DDK in rat plasma and its related pharmacokinetic profile.

MATERIALS AND METHODS

Chemicals And Reagents

DDK and osthole (internal standard)[8] were extracted from the rhizome of *Alpinia speciosa* and *Angelica pubescens*, respectively. Identification and purity of DDK and osthole were compared with an authentic compound by ^{13}C-NMR (Bruker, Germany), infra-ray and HPLC/photodiode-array detection (Fig. 2). Acetonitrile and orthophosphoric acid (85%) were obtained from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore Corp., Bedford, MA, USA) was used for all preparations.

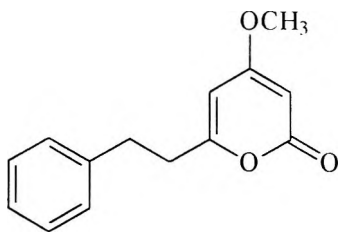

Figure 1. Chemical structure of dihydro-5,6-dehydrokawain (DDK).

Apparatus And Chromatography

The HPLC system consisted of an autosampler (SIC model 23, Tokyo, Japan), a variable wavelength UV-VIS detector (Soma, Tokyo, Japan) and a chromatographic pump (Waters model 510). Separation was achieved on a reverse phased Nucleosil 5C8 column (250 x 4 mm, particle size 5 μm, Macherey-Nagel, Duren, Germany). The mobile phase was acetonitrile-water-diethylamine (50:50:0.1, v/v/v; pH 3.0 adjusted by orthophosphoric acid), and the flow rate was 1.0 mL/min. DDK was monitored at a wavelength of 281 nm throughout the experiments. The system was operated at room temperature (25°C).

Animals

Male Sprague-Dawley rats (250-300 g) were obtained from the Laboratory Animal Center at the National Yang-Ming University. These animals were specifically pathogen free and kept in our own environmentally controlled quarters (temperature maintained at 24 ± 1 °C and with 12:12 light-dark cycle) for at least 1 week before use. Standard laboratory food and water was available continuously, except when food was withdrawn 18 hours prior to experimentation.

Blood Sampling

Rats were anesthetized with intraperitoneal urethane 0.8 g/kg. Additional dosages of 0.08 g/kg urethane was given when the rat showed signs of awakening during the experiment period. Blood samples (0.3 mL) were collected via cardiac puncture[9,10] at interval of 2.5, 5, 10, 15, 20, 30, 45, 60, 90,

120 and 180 min after intravenous administration of DDK (5 mg/kg). Data from these sample were used to construct pharmacokinetic profiles by plotting concentration of DDK in plasma versus time.

Treatment Of Plasma Samples

Each blood sample collected was transferred to a heparinized microcentrifuge tube and centrifuged at 8,000 *g* for 3 min (Eppendorf Model 5402, Germany). The resulting plasma (0.1 mL) was then mixed with 0.2 mL of acetonitrile containing osthole (2 μg/mL) as an internal standard. The denatured protein precipitate was separated by centrifugation at 8000 *g* for 3 min and a 20 μL aliquot of the supernatant was directly injected into the HPLC. The same sample handling process was used for recovery and precision determination.

Recovery

Plasma samples were spiked with DDK at concentrations ranging from 0.1 to 2 μg/mL. The resulting peak area ratio (DDK : internal standard) were compared to the standards prepared in acetonitrile.

Precision

Precision over the entire working dose range was determined by replicate analyses of plasma samples (n=4) spiked with three different concentrations (0.1, 1, or 2 μg/mL) of DDK. To determine intra-assay variance, quadruplicate assays were carried out on the same samples at different times during the day. Inter-assay variance was determined by assaying the spiked samples in quadruplicate on days 1, 2, 4, and 6 after spiking. Coefficients of variation (CVs) were calculated from these values.

Pharmacokinetic Analysis

A calibration curve was constructed based on the analysis by HPLC of various concentrations of DDK spiked in rat plasma. The concentrations of DDK in rat plasma after i.v. administration was determined from the peak area by using the equation for linear regression from the calibration curve. All data

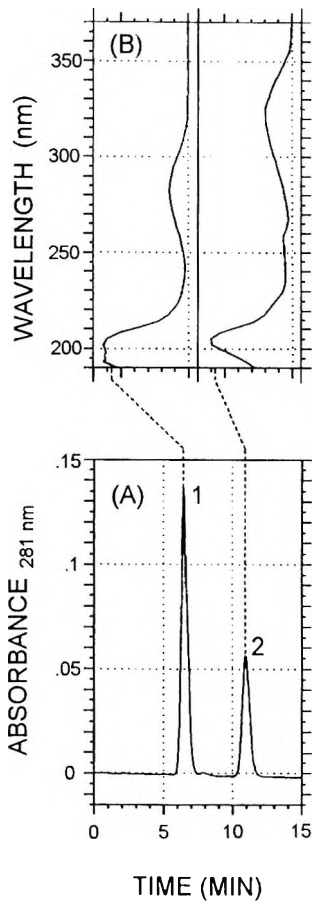

Figure 2. Chromatogram (A) and UV spectra (B) of authentic DDK and osthole (internal standard), measured by a photodiode-array detector (Waters, Model 990). 1: DDK, 2: osthole.

were subsequently processed by the computer program PCNONLIN (SCI Software Inc., Lexington, KY). The data for the area under the curve of concentration in plasma versus time ($AUC_{0\text{-}inf}$) were calculated by the trapezoidal method and extrapolated to infinite time.

RESULTS

Under the conditions described above, the retention times of DDK and osthole (internal standard) were found to be 6.49 and 11.62 min, respectively (Fig. 2A). The main characteristic spectral data obtained in the mobile phase were shown as an absorption maxima at 203 and 281 nm for DDK and at 203 and 323 nm for osthole (Fig. 2B).

The recoveries of DDK from rat plasma were found to be 93.19, 93.27, and 95.85 % for the concentrations 0.1, 1, and 2 μg/mL, respectively. The reproducibility of the method was also defined by examining both intra- and inter-assay variabilities. The intra-assay CVs for DDK at concentrations of 0.1, 1, and 2 μg/mL were 6.64, 6.48, and 4.82%, respectively, and the inter-assay CVs for DDK at the same concentrations were 7.33, 5.97, and 4.98%, respectively.

To determine the linearity and the detection limit of the HPLC method, rat plasma samples spiked with six different concentrations of DDK (0.05-5 μg/mL) were analyzed. The peak area ratios (DDK to osthole) were linearly related to the concentration of drug and the equation for the regression line for DDK was found to be $y = 2.4361x - 0.0568$ (r^2=0.999).

The limits of the lowest concentration on the standard curve which can be measured with acceptable accuracy (C.V. <20 %). The lower practical limit of quantification was 0.05 μg/mL. Under the procedure described above, the detection limit for DDK, at a signal-to-noise ratio of 3, was 0.01 μg/mL in rat plasma.

Fig. 3(A) shows the chromatogram of blank rat plasma. No discernible peaks were observed within the time frame in which DDK and osthole were detected. Fig. 3(B) shows the chromatogram of rat plasma spiked with DDK (0.5 μg/mL) and internal standard. Fig. 3(C) shows the chromatogram of DDK (0.61 μg/mL) sample obtained 20 min after i.v. administration of DDK (5 mg/kg) to a rat.

The data from the dose fit best into a two-compartment open model by the computer program PCNONLIN. The following equation applies into a two-compartment pharmacokinetic model:

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

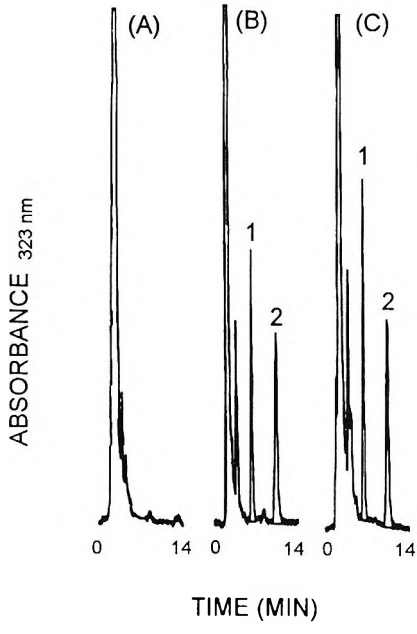

Figure 3. Chromatograms of DDK in rat plasma: (A) blank plasma. (B) spike DDK (0.5 μg/mL) and osthole (internal standard). (C) plasma sample 20 min after a 5 mg/kg i.v. administration of DDK (0.61 μg/mL). 1: DDK; 2: osthole.

In equation 1, A and B are the concentration (C) intercepts for fast and slow disposition phases, respectively, and α and β are disposition rate constants for fast and slow disposition phases, respectively. The K_{12} and K_{21} are micro rate constants between the central and peripheral compartments, and K_{10} as the elimination rate constant. The distribution half-life ($t_{1/2,\ \alpha}$) and elimination half-life ($t_{1/2,\ \beta}$) of DDK as shown in the initial phase and terminal phase of the plasma concentration-time curve was determined by the equation of $0.693/\alpha$ and $0.693/\beta$, respectively. Analysis of data after i.v. injection of DDK at 5 mg/kg yields equations 2 (and Fig. 4), respectively:

$$C = 1.79e^{-0.18t} + 0.92e^{-0.017t} \qquad (2)$$

The pharmacokinetic parameters, as derived from these data and calculated by PCNONLIN program, are shown in Table 1.

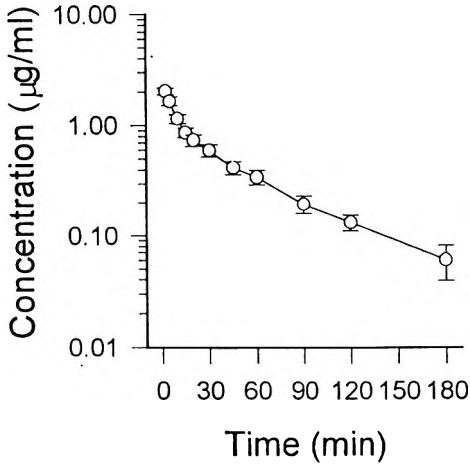

Figure 4. Plasma concentration-time curve after i.v. administration of DDK in rats at dose of 5 mg/kg.

DISCUSSION

The complementary use of the photodiode-array detection for identifying the analyzed compound by its retention time / wavelength absorption is more reliable than the UV detector which gives only the retention time.[9-12] The photodiode-array detection allowed for observation of the full UV spectrum of each peak as it eluted from the chromatographic column. Hence the detection of other components could also be observed.

A statistical nonlinear regression program was accessed through the JANA and PCNONLIN programs for the kinetic analysis. The pharmacokinetic models (one *vs.* two compartment) were compared according to Akaike's information criterion (AIC)[13] and Schwartz criterion (SC)[14] and with minimum AIC and SC values were regarded as the best representation of the plasma concentration time course data. A two-compartment open model with elimination from the central compartment was proposed and validated through the program to explain the apparent biphasic disposition of DDK in rat plasma after iv administration.

The noncompartmental method for calculating disposition parameters of DDK are based on the theory of statistical moments.[15] The area under the concentration curve of a plot of the product of concentration and time versus time from zero time to infinity is often referred to as the area under the moment

Table 1

Pharmacokinetic Parameters of DDK in Rats after 5 mg/kg, Intravenous Administration.

Parameters	Estimate
A, μg/mL	1.79 ± 0.18
B, μg/mL	0.92 ± 0.14
α, 1/min	0.18 ± 0.025
β, 1/min	0.017 ± 0.002
K_{10}, 1/min	0.042 ± 0.003
K_{12}, 1/min	0.081 ± 0.014
K_{21}, 1/min	0.076 ± 0.013
Vol, 1/kg	1.87 ± 0.10
$t_{1/2,\alpha}$, min	4.18 ± 0.62
$t_{1/2,\beta}$, min	44.60 ± 5.73
Cl, 1/kg/min	0.079 ± 0.009
AUC, μg min/mL	67.56 ± 8.09
AUMC, μg min^2/mL	3710 ± 740
MRT, min	52.79 ± 5.02

Data are expressed as mean ± SEM. Cl: clearance. See text for other abbreviations.

curve, AUMC.[15] The ratio of AUMC to AUC for DDK is a measure of its mean residence time (MRT).[16] MRT calculated after iv administration is the statistical moment analogy to drug elimination half-life. After administration of DDK (10 mg/kg, iv), MRT and $t_{1/2,\beta}$ were 44.60 and 52.79 min, respectively (Table 1).

Like half-life, MRT is a function of both distribution and elimination. Whereas, half-life tell us the time required to eliminate 50% of the dose, MRT_{iv} tells us the time required to eliminate 63.2% of the dose.

In conclusion, the present method allows a high selectivity and reliability. The relative simplicity permits its use for pharmacokinetic studies. Analysis of data after i.v. injection of DDK at 5 mg/kg yields a two-compartment pharmacokinetic model.

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SEPARATION AND CHARACTERIZATION OF SURFACTANTS BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

The separation of different types of surfactants of all four groups - anionics, nonionics, cationics and amphoterics, has been achieved by single development on silica plates. Ethanol and a mixture of ethanol with water have been used as the developer instead of various mixtures of much more toxic solvents (chloroform, benzene etc.). A new mode of detection with water has been applied among known agents of detection such as UV light (254 nm and 366 nm) and iodine. Anionic surfactants have shown characteristic white spots with gas bubbles after development with ethanol and detection with water. As auxiliary methods in classification of surfactants by charge type colour reactions, obtained by indicator dyes such as methylene blue, KJ_3,

and bromphenol blue, have been used as simple detection tests. The reliability of the recommended TLC method has been tested on 28 different surfactants, for which the R_F values, forms and colours of spots, and gas bubbles have been determined.

INTRODUCTION

Since most of the commercial surfactant products are mixtures of several components, special separation methods are required for their identification, quality, and pollution control. Thin layer chromatography (TLC) is often used in separation of surfactants because of its simplicity, efficiency, and low material costs.

Systematic separation of surfactants by TLC has been described by many authors. Bey[1] studied different solvents, adsorbents and reagents for the detection in the separation of surfactants according to their ionic character and functional groups. König[2] used ion exchangers in pre-separation of different classes of surfactants into groups, and then used TLC to separate the compounds of one class. Frahne, Schmidt and Kuhn[3] separated the groups of electrochemically different surfactants used in textile finishing processes by electrophoresis and TLC. They used general indicating reagents for the uniform loaded groups and special reagents for the functional groups, sometimes in connection with micro splitting reactions. Hellmann[4] did interesting work by separating anionic, cationic and nonionic surfactants from waste waters and sludges by multiple chromatography on silica plates. He decomposed the complexes of cationic and anionic surfactants on thin layer before being separated. Hellmann[5] has described the application of TLC by selective mobile phases in separation of numerous groups of substances of polar and nonpolar character, among them surfactants in water, waste water and sludges. The identification was done by combination of TLC and IR. Hohm[6] introduced stable fluorescence indicators such as Thioflavin S and Primulin under long-wave UV light. Henrich[7] applied six chromatographic systems on hundred and fifty surfactants on commercial cleaning agents. He has done the reflexion spectra absorbing in UV area. Kruse, Buschmann and Cammann[8] separated different types of surfactants by four-fold development on silica plates.

This paper describes the separation of different groups of surfactants (anionic, nonionic, cationic, amphoteric) by HPTLC on silica plates. A new mode of detection on the thin layer with water is introduced after single development with ethanol.

Table 1

Anionic and Amphoteric Surfactants Investigated

Product Name	Manufacturer	Chemical Composition
Anionics:		
Cocoa soap	Lab	Sodium laurate
Na diheptyl sulfosuccinate	Merck	Sodium diheptyl sulfosuccinate
Na n-dodacyl sulfate	Merck	Sodium n-dodecyl sulfate
Texapon TN 25	Henkel	Sodium laurilether sulfate
Rewopol NOS-5	Rewo	Sodium nonylphenolpentaethoxy sulfate
Geronol ACR 9	Geronazzo	Disodium nonylphenolnona ethoxy sulfosuccinate
Emulgator RF-9P	Kutrilin	Sodium nonylphenolnonaethoxy phosphate
Hostapur SAS 60	Hoechst	Alkane sulfonate
Rhodacal 70 B	Rhone Poulenc	Calcium dodecylbenzene sulfonate
Amphoterics:		
Rewoteric AM B13	Rewo	Cocoamidopropyl betaine
Rewoteric AM KSF 40	Rewo	Sodium cocoamphopropionate

EXPERIMENTAL

Reagents

All chemicals used were of p. a. grade and were obtained from Merck, Darmstadt. As the developers, absolute ethanol, 95 %, and 90 % ethanol were used. As the reagents of detection, iodine and distilled water were used.

Samples

The surfactants used were of p. a. grade, Merck, commercial products of different producers and the samples made in the laboratory. The composition of the samples was controlled by IR and ^{1}H-NMR spectra. Tables 1 and 2 show the investigated surfactants with their commercial names, manufacturers, chemical composition and charge type.

Table 2

Nonionic and Cationic Surfactants Investigated

Product Name	Manufacturer	Chem. Composition
Nonionics:		
Dehydol 3	Henkel	Fatty alcohols $C_{12}C_{14}$ + 3 EO
Brij 35	Merck	Fatty alcohols C_{12} + 23 EO
Tenzilin AA 65	lab	Fatty alcohols $C_{16}C_{20}$ + 11 EO
Tenzilin 1086	lab	Unsaturated fatty alcohols $C_{16}C_{18}$ + 3EO
Tenzilin 676	lab	Stearic acid + 20 EO
Tenzilin 65UR	lab	Castor oil +40 EO
Rokafenol 9	Rokita	Nonylphenol + 9 EO
Marlophen 814	Huls	Nonylphenol + 15 EO
Marlophen 830	Huls	Nonylphenol + 30 EO
NF + 100 EO	lab	Nonylphenol + 100 EO
Synperonic OP 40/70	ICI	Mixture of octylphenol with 40 and 70 EO
Span 20	Atlas	Sorbitan monolaurate
Dehypon LS 54	Henkel	Fatty alcohols $C_{12}C_{14}$ + 5EO + 4 PO
Cationics:		
N-cetyl-N,N,N-trimethyl ammonium bromide	Merck	N-cetyl-N, N, N-trimethyl-ammonium bromide
N-dodecylpyridinium chloride	Merck	N-dodecylpyridinium chloride
Lorocid	Kutrilin	Benzyldodecyldimethyl-ammonium chloride
Arquad 12.50	Akzo	Dodecyltrimethylammonium chloride

Chromatography

Chromatographic equipment: HPTLC silica plates with fluorescence indicator, Merck: 10 x 10 cm, No. 5629; 10 x 20 cm, No. 5642. Microsyringe for TLC, Hamilton, 5 μL, No. 7005. Double chromatographic chambers,

Camag (23.5 x 8.2 x 22.5 cm and 13.0 x 5.5 x 13.4 cm). UV lamp with wavelength 254 nm and 366 nm, Camag, UV-cabinet II. Glass Petri dish, diameter = 24.0 cm. Photocopy machine, Canon, NP 1215.

Chromatography was performed on 10x10 cm and 10x20 cm silica plates for HPTLC. The silica plates were prewashed with 95 % ethanol. The samples of the surfactants were solved in ethanol + water 1:1, v/v mixture to give concentrations of 50 mg/mL. The injection for TLC was used to apply the surfactant solutions 1μL (50 μg) at the point of exactly 1 cm above the edge of the plate. The chromatograms were developed up to 6.5 cm height. The developing time was about half an hour.

As the reagents of detection UV light 254 nm and 366 nm, iodine vapour and water were used. The plates were exposed to iodine vapour in closed chambers overnight at room temperature. Iodine chromatogram was visualized under VIS and UV light, photocopied, and the plate was dipped into distilled water in a big Petri dish. The spots with gas bubbles can be observed on the water chromatogram. The number, the form, the colour and R_F values of the spots are to be observed on the water chromatogram under VIS and UV light.

Detection Of Surfactants By Charge Type[9]

Determination of charge type of surfactants was done in an ordinary way described in the literature. Anionic surfactants were detected by colour reaction with methylene blue, nonionics with KJ_3 by colour and reaction of precipitation, and cationics with bromphenol blue.

RESULTS AND DISCUSSION

By following the described separation method it is possible to achieve complete separation of the four classes of surfactants, as well as the separation of single members in the class.

Before chromatographic procedure, tests for the detection of surfactants by charge type were done.

After detection of the charge type, development with 95 % ethanol as the best proven developer follows (among absolute, 95 %, and 90 % ethanol.). The development in 90 % ethanol has not given satisfactory results; the front has been spilt due to the high percentage of water, and the nonionic surfactants

Table 3

Description of the Chromatograms and R_F Values of Some Investigated Anionic Surfactans, Which Gave the Water Chromatograms After Developing With 95 % Ethanol. HPTLC Plates Merck No. 5629

	Detection				
	$UV_{254\ nm}$	Iodine		Water, VIS	
Surfactant	**R_F**	**R_F**	**Form**	**R_F**	**Form, Colour, Gas Bubbles**
Coco soap	---	0.88	round	0.89-0.46	drop, white, bubbles
Na diheptyl sulfosuccinate	---	0.91	round	0.91	round, white, bubbles
Na n-dodecyl sulfate	---	0.89 0.71	round round	0.88	ellipse, white, bubbles
Rewopol NOS-5	1.00-0.80	0.92-0.62	pearls	0.92	ellipse, white, bubbles
Hostapur SAS 60	---	0.85	ellipse big	0.83	ellipse, white, bubbles
Rhodacal 70 B	0.98-0.82	0.88	square, big	0.85	ellipse, white, bubbles

Forms of spots: Round, ellipse, drop, tail, pearls. --- The spot is not visible. Bubbles: Bubbles of gas are coming out of the spot after dipping of the plate into the water.

have not shown such a clear pearl finger print with iodine. (Pearl finger print means the characteristic spots with difference in mass 44 - mass of ethylene oxide).

Tables 3 and 4 show R_F values of investigated surfactants after developing with 95 % ethanol, appearance of the spots under UV light 254 nm, appearance of the spots after reaction with iodine and water, and observation of gas bubbles on some spots after dipping of the plate into distilled water.

Table 4

Description of the Chromatograms and R_F Values of Some Investigated Nonionic Surfactans, Which Gave the Water Chromatograms After Developing With 95 % Ethanol. HPTLC Plates Merck No. 5629

Surfactant	Detection $UV_{254\ nm}$ R_F	Iodine R_F	Iodine Form	Water, VIS R_F	Water, VIS Form, Colour, Gas Bubbles
Tenzilin 676	---	0.71-0.35 0.14	pearls	0.17-0.00	dark
Marlophen 830	0.25	0.31-0.00		0.32-0.00	dark
NF + 100 EO	0.09	0.12-0.00		0.11-0.00	dark tail
Synperonic OP 40/70	0.18	0.26-0.00		0.23-0.00	dark tail

Forms of spots: Round, ellipse, drop, tail, pearls. --- The spot is not visible.
Bubbles: Bubbles of gas are coming out of the spot after dipping of the plate into the water.

At first, the developed chromatograms have been examined under UV light of 254 nm wave length and there were differences between aromatics and compounds with unsaturated bonds regarding others (unsaturated alcohols, acids, glycerides). Under 366 nm only ethoxylated castor oil can be seen. Then the plates stayed in the iodine chamber over night at room temperature. The next day the plates were covered with glass and photocopied. After that the plate with iodine chromatogram was dipped into distilled water in a big Petri dish while the new chromatogram occured by reaction of water and surfactants on the thin layer. The spots of different colours appeared, with gas bubbles coming out of some of them. The water chromatogram is characteristic for individual and types of surfactants.

The anionic surfactants: sulfates, sulfonates, phosphates, succinates and sulfosuccinates, give white spots with R_F values nearly 0.90 with gas bubbles on the water chromatogram.

Alkoxylated surfactants developed with absolute and 95 % ethanol and detected with iodine give characteristic pearls similar to the pearls described by Bürger[10] given with the developer ethylacetate-acetic acid- water (7.5:1.5:1.5). Low ethoxylated compounds have higher R_F values. Nonylphenols with a higher degree of ethoxylation from 15 moles EO have R_F 0.70 and below, so nonylphenol with 100 moles EO has R_F 0.09. After dipping the plates into the water, high ethoxylated compounds show black/brown spots from the start up to a certain height dependent upon the degree of ethoxylation.

Cationic surfactants give R_F values below from 0.50 and can be seen on the iodine chromatogram under visible light. They can be also seen at the start on the water chromatogram under UV light 366 nm, while others are not visible. Some of them show other spots (grey, white, etc.) after dipping of the plate into the water.

Amphoteric surfactants stay at the starting point, but they differ from cationics because of the special form of spots on the iodine chromatogram, such as gymns. Amphoteric surfactants can be seen also on the water chromatogram under UV light 254 nm, while they are not visible under 366 nm.

In some cases, such as Na dodecyl sulfate, Texapon TN 25 and Emulgator RF-9P (anionic surfactants), it is recommendable to do the developing in absolute ethanol, and perform identification by comparing two water chromatograms. Texapon TN 25 shows three white spots on the water chromatogram with gas bubbles after developing with absolute ethanol, and Na dodecyl sulfate only one. Phosphate, Emulgator RF-9P, after developing with absolute ethanol and dipping of the plate with iodine chromatogram into the water, has shown a white round spot with gas bubbles $R_F = 0.11$. It makes a difference by comparison with high ethoxylated compounds, because they show black/brown tails at the starting position. The length of the tails is inversely proportional to the degree of ethoxylation.

In this paper, single development has been described with simple low toxicity solvent ethanol or mixtures of ethanol with water. This represents an ecological advantage for the analyst, because, in the previous procedures, more toxic solvents such as chloroform, benzene, etc. have been used. The iodine chromatograms can be photocopied. The advantage of the described chromatographic method is also in the use of water as a new, simple and nontoxic agent in the additional detection and characterization process. Consequently, except for the efficient separation of surfactants of different chemical composition, the advantages of this method are its simplicity, low cost and ecological privilege for the analyst.

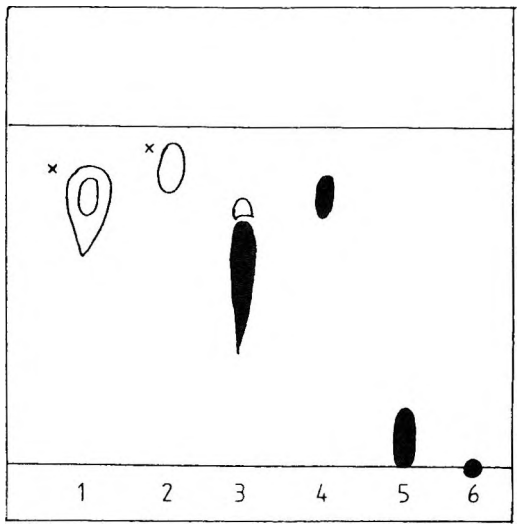

Figure 1. Water chromatogram of some tensides under UVlight 254 nm and after exposure to iodine vapour. Silicagel plate Merck No. 5629. Samples: 50 µg. Developer: 95 % ethanol.
1. Coco soap
2. Rhodacal 70 B
3. Rokafenol 9
4. Dehypon LS 54
5. Arquad 12.50
6. Rewoteric AM KSF 40
White spots signed with x give gas bubbles.

CONCLUSION

A new method for the detection of surfactants on the thin layer has been discovered and described here to assist in identification of surfactants. The anionic surfactants (sulfates, sulfonates, phosphates, succinates, sulfosuccinates) have given white spots with evolution of gas bubbles. High ethoxylated compounds have migrated in the form of black/brown tails from the start. Amphoteric surfactants differ from other groups of surfactants because they stay at the starting point and can be seen such as dark dots, after the reaction with water, under UV light 254 nm.

It has been shown that the absolute and 95 % ethanol can be successfully applied in separation of different surfactants.

As the agents of detection, UV light 254 nm and 366 nm, iodine, and water have been used. The iodine and water chromatograms have been examined under visible light and under UV light 254 nm and 366 nm.

The iodine chromatograms have been photocopied before dipping the plates into the water (Fig. 1, for example).

Experience of the chromatographer, as well as auxiliary tests for the detection of surfactants by charge type, are required. A combination of other analytical methods, such as ion exchange and TLC/IR is recommended. This method also represents a contribution to the decrease of use of toxic chemicals the analyst uses at his work.

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SIMULTANEOUS HPLC DETERMINATION OF CHOLESTEROL, α-TOCOPHEROL, RETINOL, RETINAL AND RETINOIC ACID IN SILICONE OILS USED AS VITREOUS SUBSTITUTES IN EYE SURGERY

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ABSTRACT

A reverse phase high performance liquid chromatographic method for the simultaneous determination of cholesterol, α-tocopherol, retinol, retinal and retinoic acid in silicone oils, used as vitreous substitutes in eye surgery, is reported. The mixture components are removed from the matrix with recoveries close to 97% by solid-phase extraction with silica cartridges and subsequently separated on a C_8 column, using two different mobile phases consisting of acetonitrile/ammonium acetate and methanol/water that are passed sequentially.

The compounds are detected by UV-Vis spectroscopy at two different wavelengths (210 and 350 nm) that are also set sequentially. The detection limits thus achieved range from 0.19 to 7.48 $\mu g\ mL^{-1}$.

INTRODUCTION

Researchers have long strived to find a substitute for vitreous, which is indispensable for successful treatment of complex retinal detachments. So far, silicone oil appears to be the most suitable and least troublesome choice for this purpose.[1] However, some authors have noted the risk of complications such as cataracts, corneal opacity, glaucoma, *etc.* that arise from the operation itself or from the silicone or some impurity it may contain.

Nakamura et al.[2] studied the eye toxicity of low-molecular weight substances present in silicone oil and suggested the need to remove them prior to implantation of the oil.[3] Also, because of its chemical nature, silicone oil can dissolve fat-soluble compounds while residing in the vitreous cavity, as pointed out by Refojo et al.[4] who reported the presence of cholesterol and retinol, in addition to a large number of unidentified compounds. The identification of such compounds and their determination is of great interest, with a view to expanding available knowledge on the interaction of silicone oil with its receptacle (the vitreous cavity) and optimizing procedures for the treatment of retinal detachment (one of several uses of silicone oil). This entails the prior extraction of the compounds to be identified from the oil and their subsequent analytical determination.

There appears to be only a single reported procedure for the extraction of cholesterol and retinol from silicone oil,[4] and none for the other compounds. From other types of matrices, these compounds are extracted preferentially by saponification,[5-11] which can result in degradation of the extract, or by direct solvent extraction,[4,12-20,23,25] solid-phase extraction[21,22] or supercritical fluid extraction.[23,24] These procedures usually employ fat-soluble anti-oxidants (e.g., hydroxytoluene butylate, ascorbic acid, pyrogallol) in order to reduce potential losses through oxidation.

There is also no single available method for the determination of all the above mentioned compounds simultaneously. There are, however, a number of methods for the separation and quantitation of some, most of which rely on either gas chromatography,[11,18,25] gel permeation chromatography,[11,25-27] or high performance liquid chromatography in its normal phase mode with an amino or silica column[7-10,17,19,22,29] or the reverse phase mode with a C_8 or C_{18} column.[5-7,12-16,19,24,26,28] Detection is preferentially done by UV-Vis[4,6,7,10,11,13,15-17,22,24,26] or fluorescence spectroscopy,[4,6-10,27] but can also be electrochemical,[5,14] chemiluminescent,[29] refractometric[19] or even by measurements of diffuse radiation.[28]

Based on the above considerations, we developed a reverse phase HPLC method with UV detection at two different wavelengths which allows the determination of cholesterol, α-tocopherol, retinol, retinal and retinoic acid in ophthalmic silicone oils from a single chromatogram.

EXPERIMENTAL

Apparatus and Chromatographic Conditions

The chromatographic instrumentation consisted of a Hewlett-Packard HP-1050 liquid chromatograph (Waldbronn, Germany) furnished with a multi-solvent partitioning pump, a UV-Vis variable wavelength detector and an integrator.

The column used was a 15 cm x 0.46 cm ID Zorbax C_8 from Jones Chromatography (Lakewood, CO) packed with particles of 5 μm diameter. Samples were injected via a Rheodyne 7125 injector (Berkeley, CA) furnished with a 20-μl loop.

The compounds of interest were eluted with either of two solvents. Eluent A consisted of 72:25 acetonitrile/0.2M NH4OAc and Eluent B of 95:5 methanol/water. The former was passed at a rate of 2 mL/min for 10 min and followed by a linear gradient of the latter for 1 min, which was maintained at 1.5 mL/min until the end of the run.

For detection, a wavelength of 350 nm was used for the first 14 min; it was subsequently changed to 210 nm.

Figure 1 shows the chromatogram obtained for a mixture of standards. Retention times were highly reproducible between chromatograms. The coefficients of variation obtained in 10 consecutive runs with the standards ranged from 0.25% for α-tocopherol to 2.16% for retinoic acid.

Reagents

The silicone oil used was supplied by IOBA-Domilens (Barcelona, Spain) in a 2000 cs viscosity. All solvents used were HPLC grade. Acetonitrile, methanol, *n*-hexane and *n*-propanol were purchased from Scharlau (Barcelona, Spain). Methylene chloride was supplied by Merck (Darmstadt, Germany).

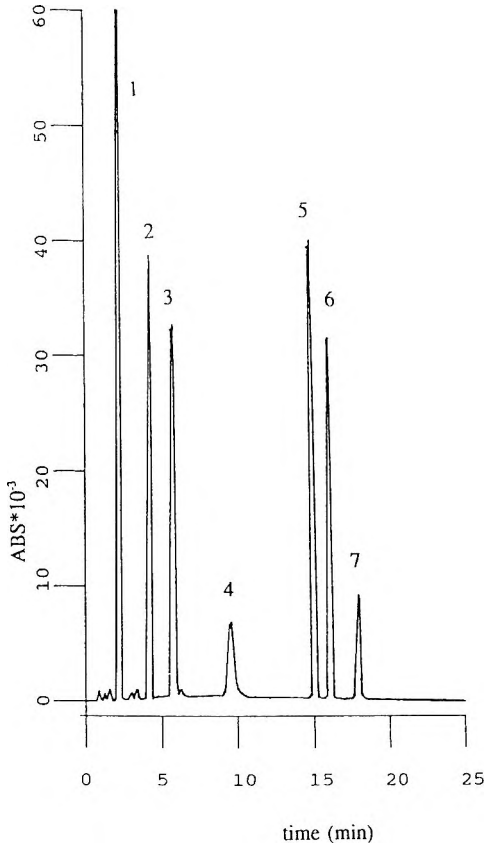

Figure 1. Chromatogram obtained from a silicone oil spiked with a mixture of standards (0.01 mg mL^{-1}). Peaks: 1=Retinoic acid, 2=Retinol, 3=Retinal, 4=Retinol acetate, 5=Cholesterol, 6=α-Tocopherol, 7=α-Tocopherol acetate .

The water was purified by passage through Compact Milli-RO and Milli-Q water systems from Millipore (Milford, MA).

Ammonium acetate, sodium acetate, ammonium hydrogen phosphate, anhydrous sodium sulphate and all other chemicals used to prepare the buffers were analytical-reagent grade and provided by Merck.

Retinoic acid, retinol, retinal, α-tocopherol, cholesterol, retinol acetate, α-tocopherol acetate (used as internal standards), and hydroxytoluene butylate (BHT, used as anti-oxidant) were supplied by Sigma Chem Co. (St Louis, MO).

Individual solutions of the analytes were prepared at a 0.1 mg mL^{-1} concentration in *n*-hexane (0.2 and 1 mg mL^{-1} for retinol and BHT, respectively). The solutions were degassed and stored refrigerated (-18°C) in topaz vials. Working-strength standards were prepared from these solutions by diluting 25 μL of 1 mg mL^{-1} BHT, 25 μL of 0.2 mg mL^{-1} and 25 μL of 0.1 mg mL^{-1} in *n*-hexane.

Reagent Stability

Because the compounds to be determined were labile and prone to oxidation, heat and UV radiation, we studied the temporal effect of light and heat on them. From the results obtained, it was decided that the standards could be used for 21 days with no appreciable alteration, provided they were kept refrigerated and in the dark.

We also studied changes in the compounds caused by the addition of variable amounts (0 - 100 μg/mL) of hydroxytoluene butylate (an anti-oxidant). It was observed that the presence of BHT extended the service life of the standards (a BHT concentration of 50 μg mL^{-1} preserved them unaltered for at least 60 days).

Calibration

Calibration was done with 7 different concentrations of the mixed standard that were added to 1 g of purified silicone oil. After stirring in a vortex mixer, (Fisher Scientific, Pittsburgh, PA), the mixture was passed through an SPE Si-Bond Elut cartridge (Varian, Harbor, CA) packed with 1 g of solid phase that was pre-activated with 5 mL of *n*-hexane. The compounds of interest were eluted with 0.5 mL of methanol and injected into the chromatograph following passage through 0.45-μm Millipore filters (Millipore, Bedford, MA). All samples were prepared and injected at least in triplicate in order to calculate coefficients of variation and the chromatographic reproducibility.

Sample Preparation

Prior to extraction, water was removed as follows: 1 g of silicone oil was mixed with 2 mL of methylene chloride, stirred in a vortex mixer for 2 min and centrifuged at 3000 G. Then, the supernatant was withdrawn with a Pasteur pipette. The procedure was repeated twice more. The sample was subsequently

passed through a 0.45 μm filter and the filtrate heated at 50°C and exposed to a helium stream for 30 min. Finally, the internal standards and BHT were added (retinol acetate 5 μg, α-tocopherol acetate 2.5 μg and BHT 25 μg) and the procedure described above for the standards was applied.

RESULTS AND DISCUSSION

Chromatographic Conditions

Wavelength

While some of the compounds studied exhibit native fluorescence, we opted for UV-Vis detection as it allowed all of them to be identified with no derivatization. From the absorption spectra for the analytes it was noted that they exhibit absorption maxima at different wavelengths. Thus, retinal, retinol and retinoic acid can be determined at 350 nm, where they possess a high absorbance. On the other hand, cholesterol and α-tocopherol do not absorb at this wavelength. Therefore, even though some authors detect α-tocopherol at 290 nm, we chose to use S = 210 nm, where both this compound and cholesterol absorb maximally.

Chromatographic column

We tested both normal and reverse phase columns, loaded with different functionalities to separate the analytes. Initially, we used 25 cm x 0.46 cm ID IB-Sil-Silica columns and 15 cm x 0.46 cm ID Spherisorb amino-modified columns, both from Phenomenex (Torrance, CA) and packed with particles of 5 μm. The two types of columns were used with various mobile phases consisting of hexane/*n*-propanol mixtures. The results thus obtained were unsatisfactory (retinol and α-tocopherol could never be separated). We also tested various C_{18} columns, but also in vain, since retinal and retinol could not be resolved in a reasonably short time (see Table 1, which shows the retention times for these compounds). We then switched to a C_8 column, which afforded the separation of all the analytes in a fairly short time and was, therefore, adopted for subsequent experiments.

Mobile phase

The mobile phase was optimized as regards both composition and the chromatographic conditions leading to maximal resolution.

Table 1

Retention Time Obtained for Retinol and Retinal in Different Columns.*

Column	Retinol	Retinal
Lichrosorb KAT, 10μm (12.5x0.4cm)	1.40	1.41
Pinkerton 5μm (25x0.46cm)	2.89	2.88
Hypersil SAS, 5μm, C_{18}, (25x0.46cm)	3.08	3.13
T-Bondapak, 10μm, C_{18}, (30x0.39cm)	5.16	5.26
Hypersil ODS, 5μm, C_{18}, (13x0.32cm)	7.49	7.64
Spherisorb ODS-1, 5μm (15x0.46cm)	11.97	13.73
Bonclone, 10μm, C_{18}, (30x0.39cm)	12.91	13.40
Sphere ODS, 10μm, (20x0.46cm)	15.08	15.96
Novapak C_{18}, (15x0.39cm)	17.12	18.49
Lichrospher 100 rp-18, 5μm (25x0.46cm)	19.47	20.38
Spherisorb ODS-2, 5μm (25x0.46cm)	25.91	30.19

* Mobile phase, MeOH:H_2O (90:10); flow rate, 1mL/min.

Regarding composition, we initially considered using methanol/water mixtures that were tested in variable proportions. The mixture afforded the separation of cholesterol, α-tocopherol and α-tocopherol acetate (see Fig. 2.a), but failed to resolve retinol and retinal, which gave strongly overlapped

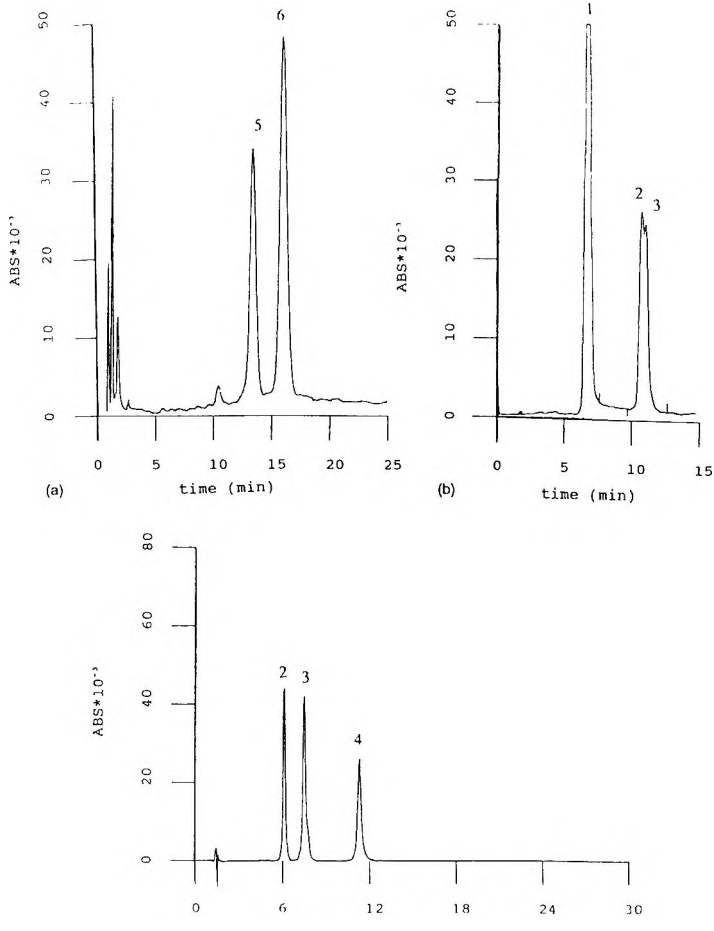

Figure 2. Chromatograms of a silicone oil, spiked with a mixture of standards, in different mobile phases. Conditions: column, Zorbax C_8 (5μm, 15 cm x 0.46 id); flow rate 1mL/min. Peaks: 1=Retinoic acid, 2=Retinol, 3=Retinal, 4=Retinol acetate, 5=Cholesterol, 6=α-Tocopherol, 7=α-Tocopherol acetate . **2a.** Mobile phase: Methanol-water (85:15); **2b.** Mobile phase: Methanol-water (92.5:7.5); **2c.** Mobile phase: Acetonitrile-water (85:15).

chromatographic peaks (Fig. 2.b) whatever the methanol content in the mixture. We then tested acetonitrile/water mixtures, which resolved retinol, retinal and retinol acetate; however, retinoic was eluted almost immediately and the other compounds, with adequate retention times, could not be resolved

Table 2
Variation of Analytes Recovery as a Function of Hexane Volume used to Dissolve the Samples

n-Hexane (ml)	Retinoic Acid	Retinol	Retinal	Cholesterol	α-Tocopherol
10	93.93	57.64	15.34	54.67	14.77
5	94.42	93.38	16.33	77.19	19.83
2	94.83	94.53	20.58	78.85	27.64
1	95.63	101.25	97.85	98.99	99.65
0.5	94.85	95.55	41.13	89.50	60.75

from one another (See Fig. 2c). We considered altering solute retention by changing the ionic strength. Of all the salts tested for this purpose, ammonium acetate provided the best results; while the most strongly retained compounds could not be resolved, the retention of retinoic acid was indeed increased. Studying the influence of the ammonium acetate concentration on solute retention, it was observed that the retention of retinoic acid increased with increasing NH_4OAc concentration up to 0.2M, with no appreciable effect on the other compounds. Above 0.2M, the peak for retinoic acid was considerably broadened, so such a concentration was adopted for further experiments. The pH was found to have no appreciable effect on the separation, so the value provided by the salt solution was accepted.

The inability to obtain a mixture allowing all the analytes to be resolved and the fact that two of the mixtures tested afforded the full resolution in two groups, led us to consider using them in sequence. In order to optimize resolution, the best proportion of organic modifier in both was established. For this purpose, we first changed the proportion of acetonitrile and examined its effect on the retention times of the analytes. As can be seen in Fig. 3a, increasing the amount of acetonitrile in the mobile phase decreased the resolution. We chose a proportion of 75%, which resulted in acceptable separation and elution in a fairly short time. The methanol-water mixture was then optimized similarly.

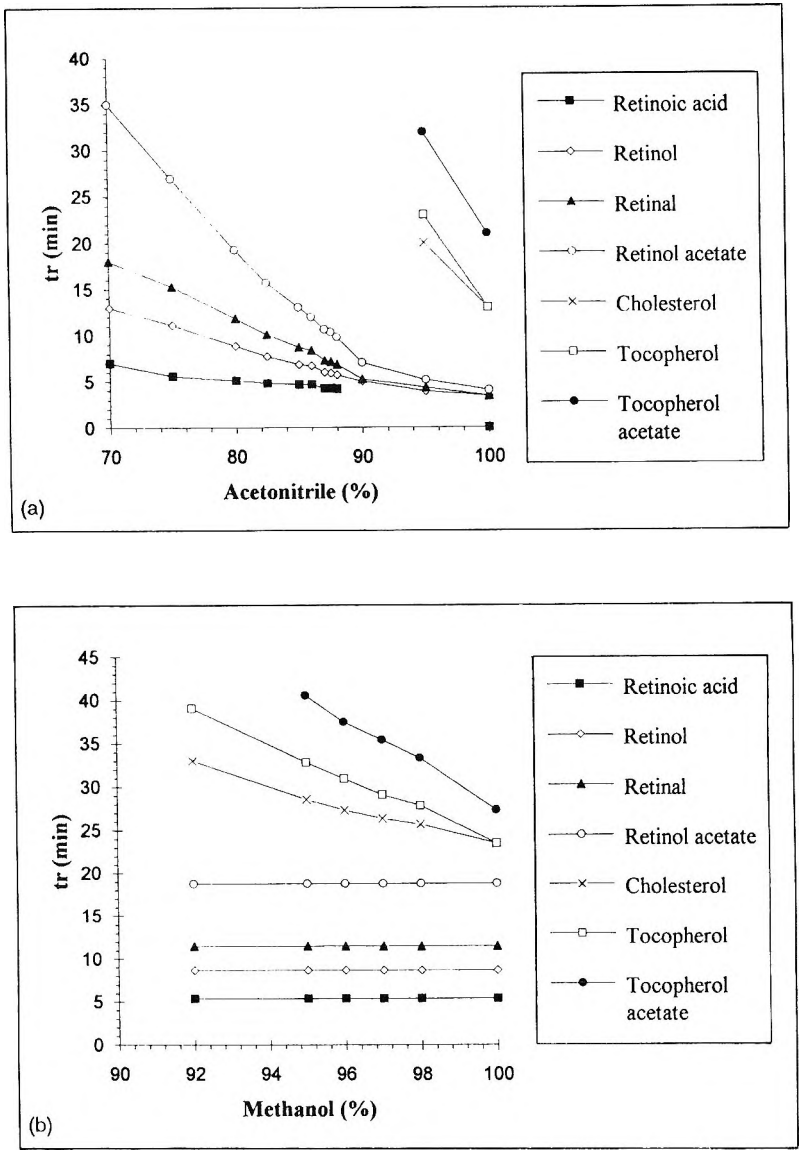

Figure 3. Plots of t_r values versus percentage of organic modifier. Flow rate, 1mL/min. **3a.** Variation of the percentage of Acetonitrile; Mobile phase ACN-NH_4Ac; **3b.** Variation of the percentage of methanol; Mobile phase ACN:NH_4Ac 0.2 M (75:25) during 10min and MeOH:H_2O at 11min.

To this end, a 75:25 acetonitrile/NH_4OAc mixture was passed for 10 min and then replaced with a methanol/water phase of variable composition. As can be seen from Fig. 3b, 95% MeOH ensured acceptable separation in a reasonably short time.

We also examined the influence of the mobile phase flow-rate and column temperature on the separation and found increases in both variables to decrease the retention times for all the solutes. Increasing the flow-rate shortened the analysis time, so 2 mL/min was adopted for the acetonitrile/NH_4OAc mixture and 1.5 mL/min for the methanol/water mixture.

Extraction/Clean-up

Prior to analyses proper, the silicone oil samples required developing a procedure for extracting the analytes from the matrix. We evaluated two choices for this purpose: liquid-liquid and solid-liquid extraction.

Liquid-liquid extraction

We first attempted extraction with solvents such as methanol, acetonitrile, isopropyl alcohol and acetone, all of which dissolved the analytes but not the silicone (except acetone, which dissolved the latter slightly). The procedure used was the same in every instance: an amount of 1 g of purified oil was spiked with known amounts of the analytes and the internal standards (all in *n*-hexane in order to facilitate dissolution in the sample) and the solvent was then removed in a rotary evaporator. Next, 10 mL of extractant was added with stirring for 10 min, the two phases were separated and the solvent was concentrated to dryness. The resulting residue was dissolved in methanol and filtered, and the filtrate was injected into the chromatograph.

All the solvents provided very high recoveries (about 97%) at every concentration tested except for isopropyl alcohol, which recovered only *ca.* 50% of the analytes. We then studied the influence of experimental variables potentially affecting the extraction process, *viz.* the extractant volume, addition of salts, pH and agitation time. Recoveries hardly changed above a volume of 10 ml, which was, therefore, adopted since larger volumes made the sample cumbersome too handle. The addition of salts had no appreciable effect. As regards pH, a neutral or alkaline medium had no effect on the separation, whereas an acid medium was detrimental as it caused retinol and retinal to decompose.

Table 3

Recovery and Reproducibility for Analytes, from Spiked Silicone Oil Samples, after Solid-Liquid Extraction (n=7). Detection and Quantification Limits, Applying the Method with Solid-Liquid Extraction

	Retinoic Acid	Retinol	Retinal	Choles-terol	α-Tocoph-erol
%Recovery	94.34	100.45	96.05	97.97	98.70
%CV	1.26	0.99	1.61	0.85	0.95
Detection limit (μg/ml)	0.0792	0.0514	0.2350	7.4789	0.6049
Quantification limit (μg/ml)	0.2641	0.1712	0.7835	24.9295	2.0163

In order to test the above procedure, several identically treated samples were injected into the chromatograph. But, the results were scarcely reproducible, possibly because of the thermolabile nature and ready oxidation of the analytes, facilitated by some of the conditions used in sample preparation procedure.

Solid-liquid extraction

We tested various extraction cartridges, loaded with different functionalities. We initially tried C_{18} cartridges. Samples were spiked with the analytes, supplied with 10 mL of methanol and agitated in a vortex mixer for 2 min. The two phases were then separated and the organic one was passed through the cartridge, previously activated with a solvent (methanol, acetonitrile or isopropyl alcohol). Next, the cartridge was dried with a nitrogen stream and eluted. In no case was full retention of the compounds achieved, so the packing was replaced with a more polar material: silica. Thus, we used Si-Bond Elut cartridges that were also pre-activated with various solvents and loaded with samples treated in different ways. Based on the results, the highest retention was obtained by activating the cartridge with 5 mL of *n*-hexane. As regards sample preparation, if 1 g of silicone oil was spiked with standards dissolved in *n*-hexane, the volume of solvent used had a dramatic effect on analyte recovery, as shown in Table 2 (1 mL of *n*-hexane resulted in the highest recoveries for all the analytes). The cartridge was then desiccated and the analytes eluted with 0.5 mL of methanol (larger volumes did not raise recoveries and resulted in superfluous dilution).

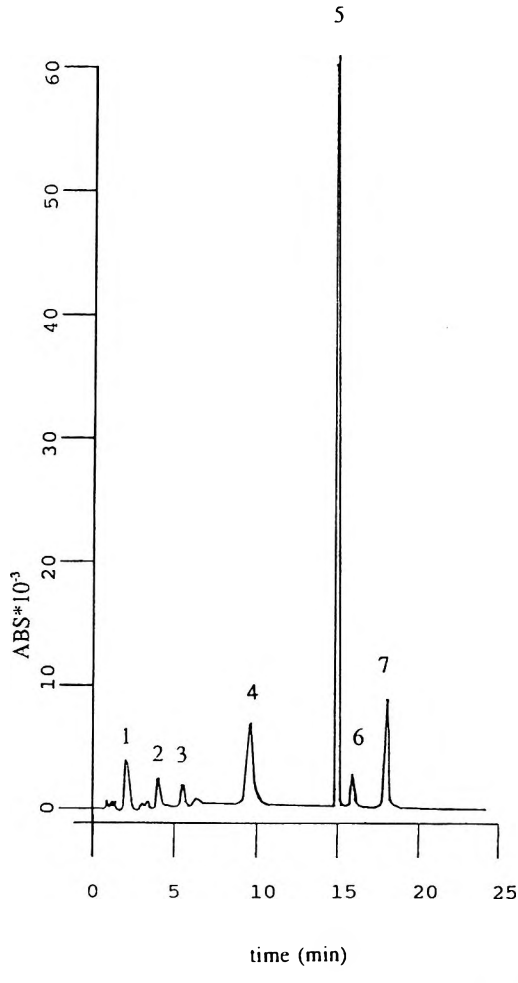

Figure 4. HPLC chromatogram of a silicone oil sample extracted from patient's eye, after surgery treatment. Peaks: 1=Retinoic acid, 2=Retinol, 3=Retinal, 4=Retinol acetate, 5=Cholesterol, 6=α-Tocopherol, 7=α-Tocopherol acetate .

The reproducibility of the procedure was tested by applying it to samples spiked with variable concentrations of all the analytes in the range 0.15-20 1g mL^{-1}. As can be seen from Table 3, the reproducibility was quite good throughout the concentration range studied.

Table 4

Results Obtained Applying the Proposed Procedure to Samples of Silicone Oil Extracted from Patients (μg/mL)

Sample	Retinoic Acid	Retinol	Retinal	Cholesterol	α-Tocopherol
1	0.03	0.16	0.02	33.96	0.08
2	0.02	0.08	0.02	53.78	0.11
3	0.03	0.17	0.14	61.98	0.12

Quantitation

The analytes were quantified in spiked samples subjected to the above-described treatment using the internal standard method. Calibration curves were obtained from replicate injections of a fixed volume of 20 μL containing the analytes at concentrations up to 50 mg mL^{-1} and were linear for all the compounds.

Table 3 gives the detection and quantification limits obtained, as calculated from the expression $3\sigma_{y/x}/s$ and $10\sigma_{y/x}/s$ (where $\sigma_{y/x}$ is the deviation from the fitting and s the slope of the calibration curve).

The proposed method was validated by using the standard addition method to spiked samples containing variable amounts of the analytes. Comparing the results obtained by applying both methods it was observed that the two sets are quite consistent.

Sample Analysis

The proposed method was applied to the determination of the analytes in 3 samples of silicone oil obtained from patients in which they have been implanted to repair retinal detachment. Figure 4 shows the chromatogram obtained for one of the samples. The results are given in Table 4. As can be seen, the compounds underwent some dissolution, which was maximal for cholesterol and minimal for retinoic acid.

CONCLUSIONS

The proposed method is a straightforward, expeditious means for the simultaneous determination of cholesterol, α-tocopherol, retinol, retinal and retinoic acid in silicone oils used as substitutes for vitreous in eye surgery. The sequential use of two different eluents and two wavelengths for detection results in high sensitivity and reproducibility. Also, subjecting the samples to solid-liquid extraction with silica cartridges affords analyte recoveries close to 97%.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SOME ANTIARRHYTHMIC DRUGS IN HUMAN SERUM USING CYANOPROPYL DERIVATIZED SILICA PHASE

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ABSTRACT

A high performance liquid chromatographic determination of antiarrhythmic agents: propafenone (PPF) with its principal active metabolite 5-hydroxypropafenone (5-OHPPF), amiodarone (AD) with an active metabolite desethylamiodarone (DEAD), mexiletine (MEX) and diltiazem (DILT) in human serum is presented. The compounds are separated on Supelcosil LC-CN column at ambient temperature under isocratic conditions. For PPF, 5-OHPPF, MEX and DILT analysis the mobile phase: CH_3CN : H_2O : 0.5 M KH_2PO_4 (360:620:20 v/v) is pumped at a flow rate of 1.8 mL/min and the ultraviolet detection is performed at 210 nm. AD and DEAD are eluted with stronger mobile phase: CH_3OH : CH_3CN : H_2O : 0.5 M KH_2PO_4 (480:136:360:24 v/v) at a flow rate of 1.5 mL/min and detected at 240 nm. Sample preparation is based on simple liquid-liquid

extraction from acidified serum for AD and DEAD and on liquid-liquid extraction from alkalized serum followed by back extraction for PPF, 5-OHPPF, MEX and DILT analysis. Sensitivity of the assay was set at 5 ng/mL for PPF and MEX and at 10 ng/mL for 5-OHPPF, DILT, AD and DEAD. Coefficient of variation for intra- and interassay precision was below 10% for all the tested compounds in all the tested concentrations. The method is convenient for therapeutic monitoring of those four antiarrhythmics. Described assay may be easily adapted for the determination of verapamil, gallopamil, quinidine, flecainide and a few beta-blockers.

INTRODUCTION

Propafenone (PPF), amiodarone (AD) and mexiletine (MEX) are well-known antiarrhythmic agents, diltiazem (DILT) is a calcium channel blocker used in ischemia, hypertension and also in supraventricular arrhythmias.[1-4] Narrow therapeutic range with large, especially evident for PPF, intersubject concentration variability (in part associated with CYP2D6 mediated polymorphic metabolism), severe side effects as well as often occurring interactions render monitoring serum concentration very helpful in the therapy with these drugs.[4-8] Recently, in some patients the PPF-MEX combination was found effective for suppressing of arrhythmias.[9-11] For some antiarrhythmics i.e. flecainide, quinidine, lidocaine, procainamide, N-acetylprocainamide immunological assay kits are commercially available for therapeutic drug monitoring. However, our drugs of interest are quantified by chromatography and HPLC is often used. Many methods have been described for the determination of PPF, AD, MEX and DILT alone or with several of their metabolites.[12-28] The assay established in our laboratory has been done for serum monitoring of antiarrhythmics saving both cost and labour. Presented assay puts us also in the way of determining the main active PPF metabolite, 5-hydroxypropafenone (5-OHPPF) which is also the marker of individual metabolic activity co-segregated with CYP2D6 function.[5,29]

MATERIALS

The pure substances of PPF hydrochloride, 5-OHPPF hydrochloride, N-depropylPPF fumarate and an internal standard LU41616 {2'-[2-hydroxy-3-(3"-hydroxy-3"-methylbuthylamino)-propoxy]-3-phenylpropiophenone hydrochloride} were obtained from Knoll (Ludwigshafen, Germany). MEX

hydrochloride, its p-hydroxy (KOE 2127), hydroxymethyl (KOE 2259) and their corresponding alcohol metabolites (KOE 2618 and KOE 2619) were from Boehringer Ingelheim (Ingelheim, Germany). DILT hydrochloride with its deacetyl metabolite as well as AD, DEAD and L8040 hydrochlorides were supplied by Sanofi-Winthrop (Gentilly, France).

Stock solutions of PPF, 5-OHPPF, MEX, DILT, LU41616, AD, DEAD and L8040 (1 mg/mL) were obtained by dissolving appropriate amounts of their hydrochlorides in methanol (except DILT - in 0.01 M HCl) and were stable for at least six months when stored at 4°C in the dark.

HPLC grade acetonitrile and diisopropyl ether were from Merck (Darmstadt, Germany), methanol, hexane and KH_2PO_4 were from JT Baker (Deventer, Netherlands) and water was obtained from BDH (Poole, England). All other chemicals were of analytical grade.

METHODS

An HPLC isocratic system (LKB, Bromma, Sweden) consisted of a pump (model 2150), injector with 50 μL loop (model 7125, Rheodyne, Cotati, CA, USA), variable wavelength UV monitor (model 2141) and integrator (model 2221). The separation of compounds was made on Supelcosil LC-CN (150x4.6 mm, 5 μm) column protected with Supelguard LC-CN (20x4.6 mm, 5μm) precolumn (Supelco, Bellefonte, PA, USA) at ambient temperature. The weaker mobile phase: CH_3CN : H_2O : 0.5 M KH_2PO_4 (360:620:20 v/v) for PPF, 5-OHPPF, MEX and DILT quantification was pumped at a flow rate of 1.8 mL/min. Detection was accomplished at 210 nm. The stronger mobile phase: CH_3OH : CH_3CN : H_2O : 0.5 M KH_2PO_4 (480:136:360:24 v/v) for AD and DEAD analysis was pumped at a flow rate of 1.5 mL/min and detection was set at 240 nm.

Sample Preparation for PPF, 5-OHPPF, MEX and DILT

A 0.4 mL of serum was transferred into a 15 mL glass centrifuge tube, then mixed with 200 ng (20 μL) of LU41616 methanol solution (internal standard) and with 50 μL of 10% Na_2CO_3 solution. Next, 4 mL of diisopropyl ether was added and the sample was vigorously shaked for 4 min. After centrifugation and freezing at -20°C the organic layer was quantitatively transferred into a 10 mL conical glass test tube to which 100 μL of 0.01 M HCl

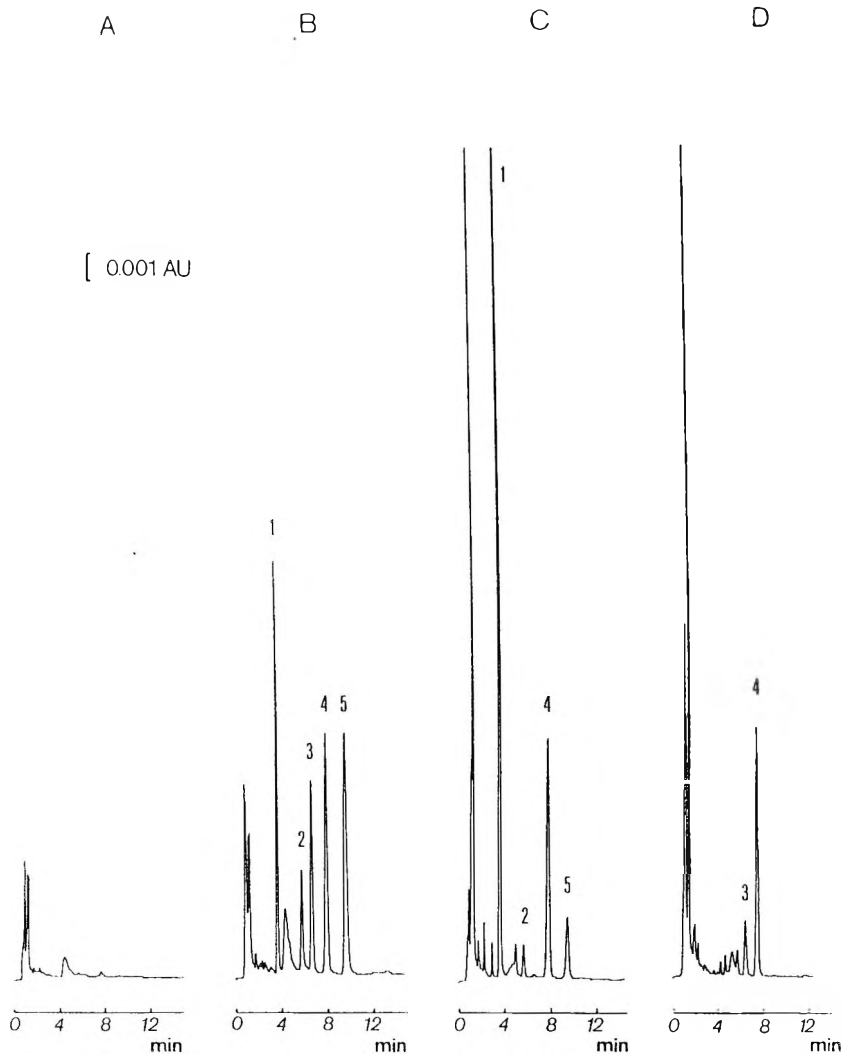

Figure 1. Chromatograms of the serum samples extracted as for PPF, MEX and DILT in Methods: A - drug-free serum; B - drug-free serum spiked with test mixture to obtain the concentrations: PPF - 500 ng/mL, 5-OHPPF - 250 ng/mL, MEX - 500 ng/mL, DILT - 250 ng/mL, LU41616 - 500 ng/mL; C - serum sample from patient treated with PPF and MEX, containing 143 ng/mL of PPF, 85 ng/mL of 5-OHPPF and 2311 ng/mL of MEX; D - serum sample from patient treated with DILT, containing 96 ng/mL of DILT; 1 - MEX, 2 - 5-OHPPF, 3 - DILT, 4 - LU41616 (internal standard), 5 - PPF.

were added. Then the tube was carefully vortex-mixed for 45 s using micro-shaker, centrifuged and the organic phase was removed to waste. The aqueous residual was evaporated to dryness in a water bath at 56°C under a stream of argon. The dried extract was then reconstituted in 100 μL of a mobile phase and a 50 μL aliquot was injected onto the column.

Sample Preparation for AD and DEAD

A 0.2 mL of serum was transferred into a 15 mL glass centrifuge tube, then mixed with 500 ng (20 μL) of L8040 methanol solution (internal standard) and with 100 μL of 0.5 M KH_2PO_4 solution. Next, 4 mL of hexane was added and the sample was shaked for 3 min. After centrifugation and freezing at -20°C the organic layer was quantitatively transferred into a 10 mL conical glass test tube and evaporated to dryness in a water bath at 37°C under the stream of argon. The dried extract was then reconstituted in 100 μL of methanol and a 50 μL aliquot was injected onto the column.

Calibration

Working solutions for calibration and controls were prepared from the stock solutions by an adequate dilution in methanol. Working solutions were added to drug free serum to obtain the concentration levels of: 20, 100, 250, 1000, 4000 ng/mL for PPF; 40, 200, 500, 1000, 4000 ng/mL for MEX and 20, 50, 100, 250, 500 ng/mL for 5-OHPPF and DILT and 50, 200, 500, 1000, 4000 ng/mL for AD and DEAD. The following procedures were as described above for sample preparation.

RESULTS AND DISCUSSION

PPF, 5-OHPPF, MEX, DILT and LU41616 were well separated from the biological background at retention times of: 9.3, 5.6, 3.5, 6.4 and 7.7 min, respectively (Figure 1). In described chromatographic conditions we did not observe any interference with metabolites of analyzed drugs available for tests which were found at retention times: p-hydroxyMEX and hydroxymethylMEX at 2.2 min, their alcohols at 1.6 min, deacetylDILT at retention time of 4.5 min and N-depropylPPF at 4.9 min (Table 1). Because of co-eluting peak of endogenous origin we did not calibrate the assay for determination of

Table 1

Retention Data for Interference Studies

Compound	Relative Retention Time (Internal Standard = 1.00)	Capacity Factor
MEX	0.45	3.4
5-OHPPF	0.73	6.0
DILT	0.83	7.0
LU41616 (internal standard)	1.00	8.6
PPF	1.21	10.6
HydroxymethylMEX-ol + p-hydroxyMEX-ol	0.21	1.0
HydroxymethylMEX + p-hydroxyMEX	0.29	1.8
DeacetylDILT	0.58	4.6
N-depropylPPF	0.64	5.1
Theophylline	0.17	0.6
Phenacetine	0.22	1.1
Aprobarbital	0.22	1.1
Atenolol	0.22	1.1
Nadolol	0.25	1.4
N-acethylprocainamide	0.25	1.4
Procainamide	0.26	1.5
4-Hydroxydebrisoquine	0.26	1.5
Pentobarbital	0.26	1.5
Hexobarbital	0.27	1.6
Sotalol	0.27	1.6
N-desmethylclobazam	0.31	2.0
Acebutolol	0.32	2.1
Mephenytoin	0.34	2.3
Metopolol	0.36	2.5
Clobazam	0.36	2.5
Celiprolol	0.38	2.6
Debrisoquine	0.39	2.8
Diazepam	0.39	2.8
Lidocaine	0.40	2.9
Prazosin	0.43	3.1
Progesteron	0.55	4.3
Quinidine	0.55	4.3

Table 1 (Continued)

Retention Data for Interference Studies

Compound	Relative Retention Time (Internal Standard = 1.00)	Capacity Factor
Propranolol	0.66	5.4
Bupranolol	0.69	5.6
Flecainide	1.13	9.9
Verapamil	1.38	12.3
Gallopamil	1.42	12.6
Desethylamiodarone	4.91	46.3
Amiodarone	7.23	68.6

Analytical conditions as described in Methods for PPF, 5-OHPPF, MEX and DILT analysis.

N-depropylPPF and deacetylDILT which similarly to pharmacologically inactive MEX metabolites are all of minor clinical importance. However, the goal of the presented assay is the fact that in case of PPF parent drug monitoring is linked with the estimation of individual CYP2D6 activity.

Several cardiac agents and other drugs were checked for possible interference with the method. Their solutions were injected onto the column and detected under the described analytical conditions. Retention data for tested compounds are presented in Table 1. None of these drugs interfered with the assay. Additionally, the extraction procedure was established as efficient (75-90%) for quinidine, verapamil, gallopamil, flecainide and a few beta-blockers (acebutolol, propranolol, metoprolol). After only small changes in mobile phase composition the assay may be adapted for the determination of these cardiac drugs. On the other hand, celiprolol was extracted in 40%, atenolol and sotalol were practically unextractable.

AD, DEAD and L8040 were all eluted "far away" after other tested drugs (Table 1). That was the reason for introducing the stronger mobile phase composition. After that modification AD, DEAD and L8040 were also completely resolved and separated from the biological background at retention times of: 6.7, 5.9 and 8.0 min, respectively (Figure 2).

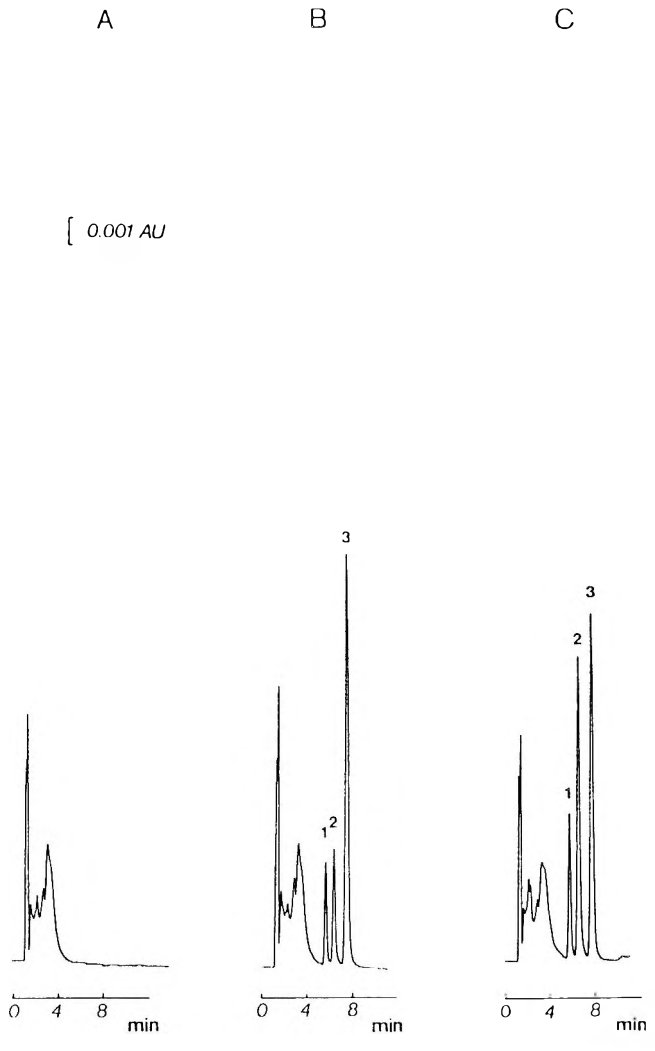

Figure 2. Chromatograms of the serum samples extracted as described for AD in Methods: A - drug-free serum; B - drug-free serum spiked with test mixture to obtain the concentrations: AD - 500 ng/mL, DEAD - 500 ng/mL, L8040 - 2500 ng/mL; C - serum sample from patient treated with AD, containing 1403 ng/mL of AD and 790 ng/mL of DEAD; 1 - DEAD, 2 - AD, 3 - L8040 (internal standard)

The calibration curves were obtained by analyzing four serum samples for each tested concentration. The curves were linear and described by the following equations: y=0.00244x-0.0405 (r=1.0000) for PPF; y=0.00135x-0.0115 (r=0.9993) for 5-OHPPF; y=0.00160x-0.0259 (r=1.0000) for MEX; y=0.00249x-0.0075 (r=1.0000) for DILT; y=0.00056x-0.0183 (r=1.0000) for AD and y=0.00040x-0.0069 (r=0.9999) for DEAD.

The absolute extraction recovery was analyzed by comparing the peak areas for extracted calibration standards with those obtained from direct injection of equivalent quantities of standards. Satisfactory recovery was acquired for all the compounds i.e. 90.2% (89.3-92.6) for PPF, 78.2% (72.5-84.2) for 5-OHPPF, 88.9% (85.9-94.2) for MEX, 80.2% (76.3-83.9) for DILT, 93.3% (88.3-96.9) for AD and 63.5% (62.3-64.9) for DEAD being stable for the concentrations covering the calibration range (data in parentheses). The alkalization of serum sample to pH 10.5 using Na_2CO_3 solution was chosen because of its value for efficient PPF, 5-OHPPF, LU41616 and MEX extraction. The back-extraction step decreased the chromatographic interference from serum. In contrast, our early findings showed that simple one-step extraction with hexane from the acidified serum gives good results for AD and DEAD analysis what is in opposition to the findings of Arranz Pena et al.[25] Some investigators noted the phenomenon of DILT hydrolysis occurring when pH>9 was achieved during analytical procedure.[12-14] In our experiment DILT was extracted in about 80 % with good repeatability and we keep considering such a recovery satisfactory for quantification of this drug. We did not observe any significant influence of the preparation time up to 3 hours from the start of the analytical procedures on extraction recovery. Both internal standards were extracted in a high percent i.e. LU41616 - 90.8 ± 4.4 % (n=20) and L8040 - 96.1 ± 5.6% (n=20).

The precision of the assay was examined using the data from calibration for intraassay and analyzing standard samples in duplicates on 4 different days for interassay precision. Coefficient of variation (CV) for all the tested concentrations was always below 10%. Detailed information is presented in Table 2. The sensitivity of the method (signal-to-noise ratio 3:1) was set at 5 ng/mL for PPF and MEX and at 10 ng/mL for 5-OHPPF, DILT, AD and DEAD.

Verbesselt et al.[28] gave a valuable script for HPLC assays of 12 antiarrhythmics (including PPF, MEX and AD) applying the same apparatus, solid-phase extraction system and general rules but different analytical

Table 2

Precision of the Method (n=4)

	Concentration Added [ng/mL]	Intraassay Factor Determined*	CV [%]	Interassay Factor Determined*	CV [%]
	20	0.046 ± 0.0034	7.51	0.046 ± 0.0045	9.84
	100	0.214 ± 0.0072	3.37	0.212 ± 0.0106	5.01
PPF	250	0.556 ± 0.0187	3.36	0.555 ± 0.0095	1.71
	1000	2.355 ± 0.1062	4.51	2.371 ± 0.0974	4.11
	4000	9.734 ± 0.1667	1.71	9.577 ± 0.1402	1.46
	20	0.027 ± 0.0014	5.61	0.027 ± 0.0017	6.38
	50	0.053 ± 0.0027	5.11	0.056 ± 0.0034	6.10
5-OHPPF	100	0.123 ± 0.0064	5.18	0.122 ± 0.0025	2.05
	250	0.312 ± 0.0146	4.69	0.319 ± 0.0264	8.27
	500	0.672 ± 0.0249	3.70	0.680 ± 0.0153	2.25
	40	0.061 ± 0.0022	3.65	0.062 ± 0.0015	2.43
	200	0.304 ± 0.0068	2.26	0.305 ± 0.0217	7.12
MEX	500	0.758 ± 0.0144	1.91	0.780 ± 0.0310	3.97
	1000	1.546 ± 0.0595	3.85	1.607 ± 0.1150	7.17
	4000	6.371 ± 0.1651	2.59	6.116 ± 0.2170	3.55
	20	0.046 ± 0.0033	7.22	0.043 ± 0.0040	9.51
	50	0.115 ± 0.0043	3.76	0.108 ± 0.0081	7.52
DILT	100	0.239 ± 0.0149	6.26	0.237 ± 0.0049	2.08
	250	0.615 ± 0.0439	7.13	0.603 ± 0.0578	9.59
	500	1.236 ± 0.0681	5.51	1.226 ± 0.0573	4.68
	50	0.020 ± 0.0018	9.19	0.020 ± 0.0007	3.41
	200	0.097 ± 0.0026	2.66	0.100 ± 0.0027	2.66
AD	500	0.254 ± 0.0056	2.19	0.259 ± 0.0047	1.81
	1000	0.527 ± 0.0061	1.16	0.541 ± 0.0121	2.23
	4000	2.213 ± 0.0241	1.09	2.268 ± 0.0427	1.88
	50	0.019 ± 0.0019	10.23	0.019 ± 0.0006	3.22
	200	0.075 ± 0.0065	8.64	0.076 ± 0.0027	3.50
DEAD	500	0.198 ± 0.0173	8.70	0.188 ± 0.0110	5.83

Table 2 (continued)

Concentration Added [ng/mL]	Intraassay Factor Determined*	CV [%]	Interassay Factor Determined*	CV [%]
1000	0.375 ± 0.0028	0.73	0.379 ± 0.0292	7.72
4000	1.591 ± 0.0545	3.42	1.574 ± 0.0467	2.97

*Factor determined - the ratio of peak area of analyzed compound to peak area of internal standard.

conditions for particular drugs. Additionally, of some importance may be large (1 mL) sample volume and for PPF analysis also insufficient (25 ng/mL) limit of quantitation. The analysis of PPF, DILT and AD simultaneously with DEAD on LC-CN column was also described by Mazzi,[27] unfortunately, the sensitivity of that assay was poor (50 ng/mL for PPF, AD, DEAD and 20 ng/mL for DILT).

The presented method provides the possibility of simultaneous measurement of PPF, its 5-hydroxymetabolite, MEX and DILT on LC-CN column. After changing the mobile phase followed by only twenty minutes system washing, detection wavelength change and, obviously, applying different extraction procedure we are successfully able to determine AD with DEAD using the same HPLC system. Described procedure may be the basis for establishing the analysis of verapamil, gallopamil, quinidine, flecainide and beta-blockers.

Concluding, the paper presents simple assay which may be recommended for therapeutic monitoring as well as for pharmacokinetic studies on PPF, AD, MEX and DILT.

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