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CHARACTERIZATION OF METALLOTHIONEIN ISOFORMS BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE UV AND ELECTROCHEMICAL DETECTION

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ABSTRACT

Four metallothioneins (MTs), rabbit liver MT (RL), MT-1 (RL 1) and MT-2 (RL 2) and horse kidney MT (HK), were subjected to reverse phase HPLC with on-line UV and electrochemical (EC) detection, the latter comprising of a graphite electrode. The EC detection is based on the direct oxidation of the thiols contained in the MT chains, into disulphides. MT samples were prepared at different pH values and eluted with a gradient of TFA and acetonitrile. The MTs are found to exhibit a different polymorphism and the various peaks differ in their detectability depending on the detection mode. RL 2 (one major peak) and HK (three peaks) have peak patterns which are stable with time and varying pH, all peaks being detected by both EC and UV modes. The two other MTs also exhibit peaks which are eluted within the same retention time range as the previously mentioned MTs and which give EC and UV signals (three peaks for RL 1 and four for RL), but which

also show some other peaks - eluted earlier - which are perfectly detected in UV but not or very little in EC. This latter information means that these species contain none, or very few, thiol groups. Stable with time at neutral and basic pH, the RL 1 and RL chromatograms are highly evolutional in acid pH with the formation of more of the non-EC detected species and a decrease in the doubly detected ones. We can therefore assume that the peaks detected by both EC and UV modes correspond to the original thiol containing isoforms, while those less hydrophobic of RL 1 and RL correspond to modified species, probably containing disulphide bonds which explains the lack of EC response at the graphite electrode. Since many MTs are highly reactive, this UV/EC combination appears to be a very good tool for a quick identification of reduced (thiolic) isoforms from oxidised (with disulphides) species.

INTRODUCTION

Metallothioneins (MTs) constitute a class of ubiquitous low molecular weight (6000-7000 Da) proteins, characterised by a high cysteine content (~ 30%), the lack of aromatic amino acids and the ability of binding metals such as zinc, cadmium, copper and mercury. The characteristics and properties of these molecules have been the subject of several monographies.[1-4] These metalloproteins probably play important roles in, at least, the homeostasis of some essential metals (Zn, Cu) and the detoxification of harmful (Cd, Pb) and excessive essential metals.[5] The metals are bound to the cysteine residues of the MT chains through thiolate complexes which are organised into two separate metal-binding clusters, α and β.[6,7] In most cases, MTs exist under various isoforms (isoMTs) that arise from genetic polymorphism encountered in many species.[5]

Based on their electrophoretic properties, two major isoforms of MT have been identified in mammals, MT-1 and MT-2, named after their order of elution by anion-exchange chromatography. They differ slightly in amino acid content and exhibit a single charge difference at neutral pH.[8] These two isoMTs are the products of two distinct genes.[9] Furthermore, many animal species generate various sub-isoforms of MT-1 and MT-2, therefore displaying significant microheterogeneity. However, the biological functions of individual isoforms remain unknown. Progress in this field is largely dependent on efficient separation and quantification of each isoMT, and therefore, on the development of analytical techniques providing a high degree of resolution.

Reverse phase high performance liquid chromatography (RP-HPLC), which separates compounds on the basis of their difference in hydrophobicity, has shown its significant ability to resolve additional isoforms of MT not previously separated by conventional techniques.[10-12]

Classically, RP-HPLC has been employed with UV detection at 214, 220 or 254 nm[10,12,13] and/or in combination with atomic absorption spectrometry (AAS) for quantification of metals.[11,14,15] Although sensitive, the HPLC-AAS technique is accurate only when the metal occupation is complete and homogeneous, which is not always the case.[13] Furthermore, applying this procedure obviously involves the need of a neutral buffer for both sample preparation and elution. However, in some situations, only acidic buffers are suitable for efficient separations, hence forbidding the AAS mode of detection. For instance, it has been shown that copper containing isoMTs cannot be adequately separated and detected by RP-HPLC at neutral pH.[15,16]

The measurement of UV absorbance is, by far, the most widely used mode of detection. It is a universal detector and, consequently, some commonly used elution buffers such as tris(hydroxymethyl)aminomethane (TRIS) can contribute significantly to the absorbance in the 200-220 nm range.[17]

Compared to this, the use of more selective detectors such as those based on electrochemistry could be more advantageous for applications that involve complex matrices such as those of biological origin. Electrochemical detectors (EC) are naturally highly selective because only the compounds, or functional groups, that undergo an electrochemical reaction at a particular potential will be detected. In the case of MT, this would bring information on the redox state of the sulphydryl groups contained in the structure. Until now, electrochemistry has been used to either quantify the total concentration of MTs in biological fluids through differential pulse polarographic (DPP) assays of thiol groups, based on the Brdicka reaction [18-20] or to study, in a more fundamental manner, the electrochemical behaviour of MTs (complexing properties of thiol groups, speciation)[21,22] However, to our knowledge, no study on electrochemical detection of MT isoforms via an on-line RP-HPLC-EC combination has ever been published. In a previous work, we studied the chromatographic behaviour of the trithiolic hexapeptide Lys-Cys-Thr-Cys-Cys-Ala (56-61) MT-1 intrinsic to the mouse liver MT structure, employing electrochemical detection.[23] We showed the advantage of using a coulometric mode in order to detect thiol groups at a porous graphite electrode according to the reaction $2\ R\text{-}SH \rightarrow R\text{-}SS\text{-}R + 2\ H^+ + 2\ e^-$ with a medium voltage of only 0.6 V. It was then possible to separate the reduced and oxidised forms of the peptide and to follow the evolution of the formation of intra- and intermolecular disulphide bonds.

In the present work, our main objective was therefore the study of the applicability of the same electrochemical system, along with UV detection, to identify and quantify the isoforms of four MTs, three from rabbit liver and one from horse kidney.

EXPERIMENTAL

HPLC Intrumentation and Procedure

A Kontron (Zurich, Switzerland) chromatograph was used, equipped with a dual piston pump Model 420, a gradient-former GF 425 and a HPLC 360 autosampler with an injection loop of 100 μL. The reverse phase column was a Hi-Pore RP 318 (250 x 4.6 mm), 300 Å pore size, 5 μm particle size (Bio-Rad Laboratories). The detection system includes a Coulochem Model 5100A electrochemical detector (ESA Inc., Bedford, MA, USA) set at a potential of 0.6 V, used with a Model 5011 analytical cell containing two coulometrically efficient porous graphite working electrodes and a Model 5020 guard cell. A UV capillary detector Model 433 (Kontron), set at λ = 230 nm, was also connected in series, before the electrochemical detector.

Buffer A was 0.1 % (v/v) trifluoroacetic acid, TFA, (pH = 3) and buffer B consisted of 0.1 % TFA in acetonitrile. MTs were eluted with a linear gradient of 10-30 % B in 70 minutes, at a flow rate of 1 $ml.min^{-1}$.

Chemical

Rabbit liver Cd, Zn MT (RL) and its purified isoforms MT-1 (RL 1) and MT-2 (RL 2), horse kidney Cd, Zn MT (HK), TFA (1 mL ampoules) were purchased from Sigma (St Louis, MO, USA). Acetonitrile was HPLC grade (Super gradient grade from Lab-Scan, Dublin, Eire). Ultra pure water was obtained from a Millipore Milli-Ro 10 Plus deionisation system followed by a Milli-Quater system (18 MΩcm resistivity) and a sub-boiling quartz distillation unit (Quartex SA, Paris, France).

All mobile phases were filtered through 0.22 μm Millipore membranes and continually purged with helium to remove dissolved oxygen. All experiments were carried out at room temperature with, unless otherwise stated, freshly prepared solutions.

RESULTS

MT Samples Prepared at Neutral and Basic pH

The MTs have been first prepared at neutral pH. The four proteins exhibit a different polymorphism, as shown in Figure 1. More stable baselines are generally obtained with the electrochemical detection where no shifting is oberved when the proportion of organic phase increases, which is sometimes the case for the UV detection. On the whole, UV and EC chromatograms present comparable morphologies in the cases of RL2 (Figure 1 b-b') and of HK (Figure 1 d-d'), while they display some different aspects for RL 1 (Figure 1 a-a') and RL (Figure 1 c-c'). *Nota bene:* in this text, peak numbering goes in the direction of increasing retention times, and labelling like H_2 stands for the height of peak 2.

-RL2 appears to be the least complex case, comprising one highly dominant peak (3 in Figure 1 b-b') eluted at a retention time (t_R) of about 41 minutes (Table 1), surrounded by several minor peaks (1, 2, 4). The height ratios of the various peaks are comparable on both EC and UV chromatograms (Table 2). One small UV peak, γ which is eluted earlier (t_R = 30 min), is not detected electrochemically.

- HK mainly exhibits three peaks (1, 2 and 3 between 36 and 44 minutes) on both chromatograms with a good resolution (R_{2-3} = 1.1). For this MT also, the peak height ratios are similar for both EC and UV modes (Table 2).

-RL I is more complex than the previous proteins (Figure la-a'). Four peaks (1-4) are clearly detected (37 min < t_R < 46 min) by both EC and UV modes with a good resolution (R_{1-2} = 0.85) and comparable height ratios except for the minor peak 3 (Table 2). Furthermore, the UV chromatogram exhibits, between 25 and 35 minutes, some smaller peaks (α,β,γ...) which are not or only slightly obvious on the EC picture.

- Both chromatograms of RL show two groups of peaks, group A (peaks 1, 2 and 3 at t_R < 35 min) and group B (one major peak, 6, and three minor peaks at t_R > 35 min, Table 1).

Considering A and B independently of one another, the peak height ratios in each group are comparable in both EC and UV (Table 2). On the contrary, when group A peaks are compared to those of group B, large differences occur between the two types of detection. Group A peaks clearly give much less intense EC than UV responses than those of group B.

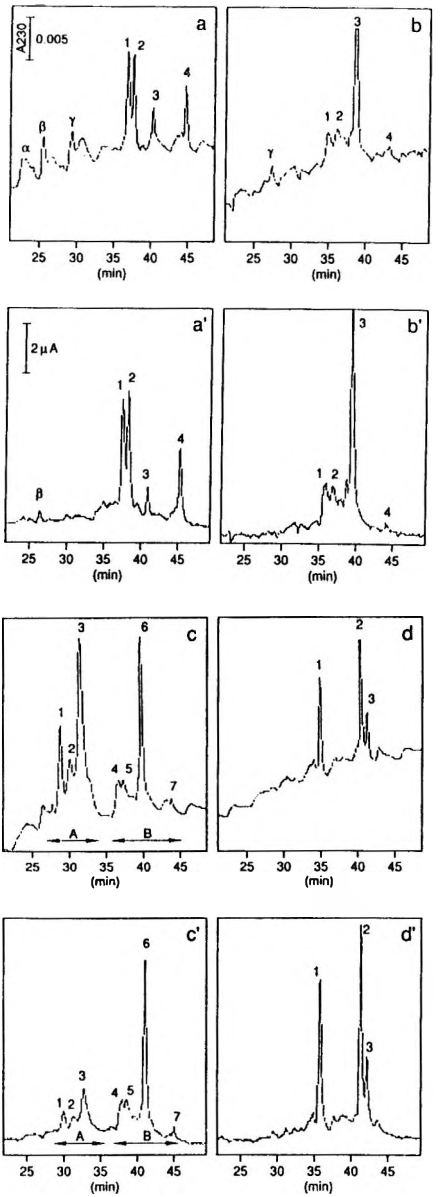

Figure 1. Reverse phase chromatograms of four metallothioneins prepared at neutral pH. a, b, c, d: UV detection (λ= 230 nm; 0.005 AUFS). a', b', c', d': EC detection (E = 0.6 V, gain = 700). a-a': RL 1 b-b': RL2 c-c': RL d-d': HK (C=500mg.l^{-1}). Elution: from 90% A (0.1%TFA)-10% B (0.1% TFA in acetonitrile) to 70% A-30% B in 70 min.

Table 1

Retention Times (Minutes) of all MT Peaks Detected by both UV and EC Detectors for Samples Prepared in Neutral and Acid Solutions*

	Peaks						
	α	β	γ	1	2	3	4
HK							
Acid pH	--	--	--	36.60±0.58	42.36±0.63	43.41±0.57	--
Neutral pH	--	--	--	36.77±0.72	42.36±0.71	43.47±0.70	--
RL 1							
Acid pH	23.260.83	26.11±0.84	29.94±0.78	37.70±0.63	38.66±0.60	41.14±0.56	45.60±0.57
Neutral pH	23.92±0.64	26.66±0.59	30.28±0.44	37.88±0.58	38.87±0.57	41.54±0.43	45.67±0.60
RL2							
Acid pH	--	--	29.71±0.58	37.60±0.48	38.58±0.40	40.72±0.58	44.33±0.31
Neutral pH	--	--	30.48±0.86	37.94±0.76	39.31±0.87	41.30±0.73	44.23±0.25

	Peaks						
	1	2	3	4	5	6	7
RL							
Acid pH	29.87±0.89	31.55±1.02	32.96±1.00	38.37±1.06	39.16±1.10	40.96±0.79	44.50±0.77
Neutral pH	29.81±1.06	30.76±0.90	32.49±1.04	37.88±1.38	39.44±1.14	40.93±1.00	45.32±0.97

* Respective peak numbering according to Figure 1.

Table 2

Electrochemical and UV Peak Height Ratios of the Four Metallothioneins Prepared at Neutral pH*

MT	Peak Height Ratios	EC	UV
	H_1/H_2	0.93	1.04
RL 1	H_1/H_4	1.57	1.64
	H_4/H_3	2.03	1.34
	H_3/H_1	4.05	3.86
RL 2	H_3/H_2	4.59	4.87
	H_3/H_1 (A-A)	1.73	1.82
	H_3H_2 (A-A)	2.3	2.73
RL	H_6/H_5 (B-B)	4.12	4.36
	H_6/H_3 (B-A)	3.28	0.97
	H_1/H_2	0.75	0.81
HK	H_1/H_3	1.95	1.95

* Respective peak numbering according to Figure 1.

Table 3

Ratios Between UV and EC Responses of Peaks of MTs Prepared at Neutral pH for the Given Detection Conditions*

Peaks	HK	RL 1	RL 2	Peaks	RL
--	--	--	--	1	13.1
--	--	--	--	2	10.3
--	--	--	--	3	13.8
1	2.7	3.6	3.5	4	3.9
2	2.7	3.2	3.9	5	3.8
3	2.7	5.1	3.3	6	4.1
4	--	3.4	4.1	7	4.1

* UV: λ = 230 nm, 0.005 AUFS; EC: E = 0.6V, gain = 700. MT respective peak numbering according to Figure 1.

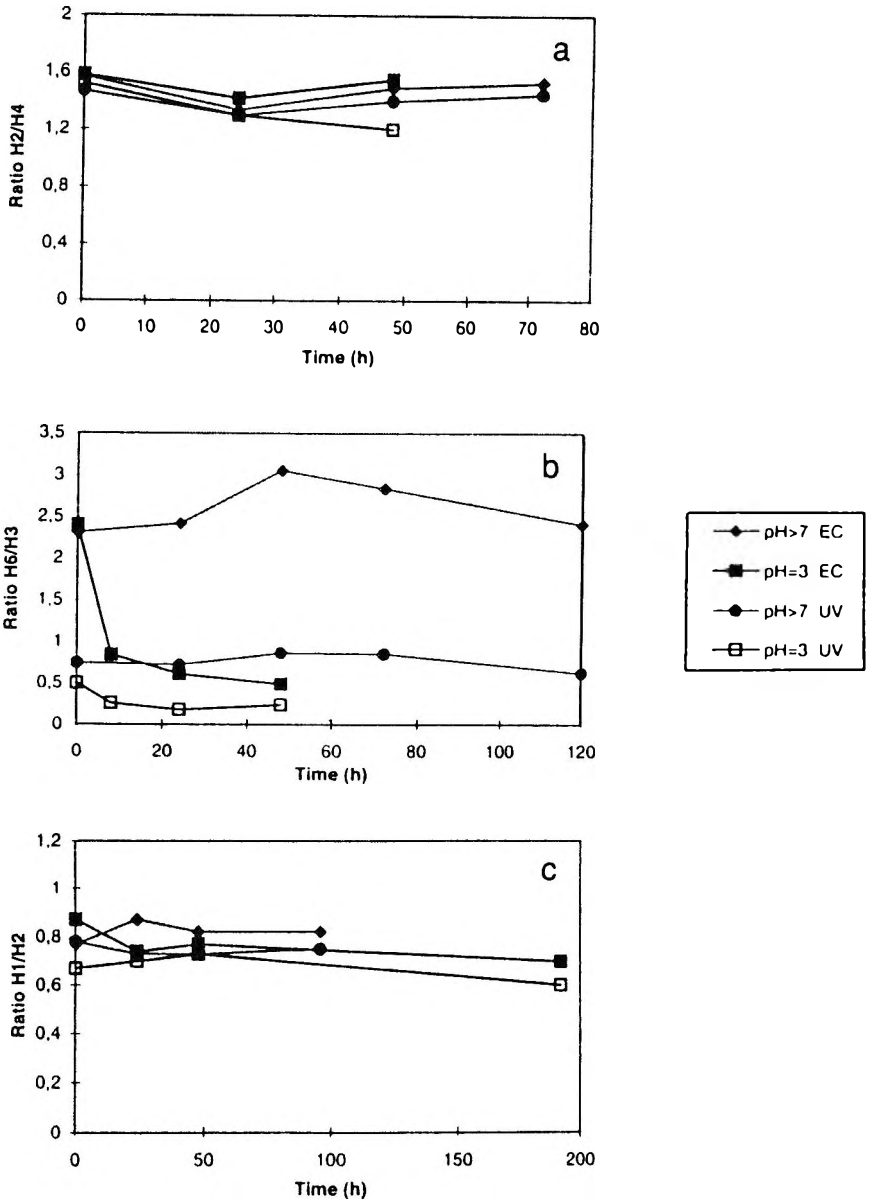

Figure 2. Variations in time of the UV and EC peak height ratios H_2/H_4 of the metallothionein RL 1 (a), H_6/H_3 of the metallothionein RL (b) and H_1/H_2 of the metallothionein HK (c) prepared at neutral and acid pH (respective peak numbering according to Figure 1).

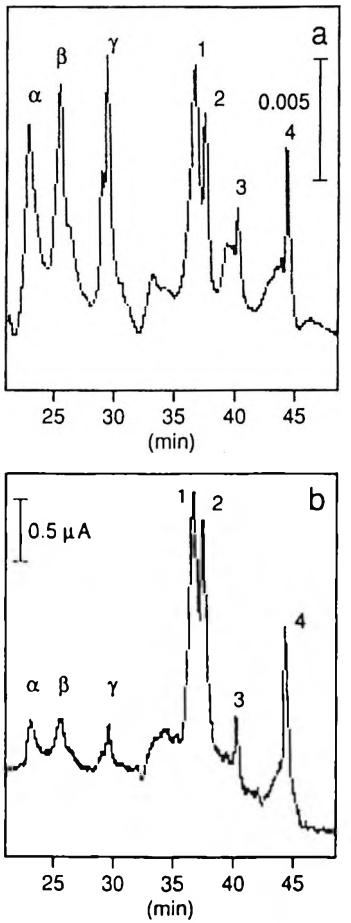

Figure 3. Reverse phase chromatogram of RL 1 prepared at acid pH. All other conditions as in Figure 1. a: UV detection b: EC detection.

Table 3 provides the ratios, for all MTs, between UV and EC heights for each peak under the given detection conditions. Peaks of RL 1, RL 2 and group B of RL obviously have the same UV/EC ratios comprised of between ~ 3.5 and 4, while group A peaks of RL show much higher values (> 10). As for HK, it has lower UV/EC ratios than the three rabbit liver MTs.

Prepared at pH 9, no major difference occurs in the morphology of any of the chromatograms. Retention times and peak ratios remain largely unchanged compared to neutral pH. Whatever the pH, neutral or basic, these separation

features are very stable with time. MT samples which had first been eluted immediately after preparation (t = 0, all results presented until now), have been re-subjected to separation at regular time intervals thereafter. No significant modification of the chromatograms of any of the MTs is observed. To illustrate this point, Figure 2 shows the evolution of the peak ratios H_2/H_4 of RL 1, H_6/H_3 of RL and H_1/H_2 of HK respectively. They remain perfectly constant even after three days at room temperature.

MT Samples Prepared at Acidic pH

At pH 3 and compared to what happened at pH 7, two types of behaviour occur: no appreciable changes are noticed for RL 2 and for HK (same number of peaks, retention times and peak ratios), while some significant changes other than that of the retention times, which do not vary (Table 1), are observed for RL 1 and for RL on both EC and UV chromatograms:

- for RL 1 (Figure 3a and 1a), the UV picture is characterised by a high increase of the α, β and γ peaks ($t_R < 35$ min) relative to the "main" peaks 1, 2, 3 and 4. In EC, the α, β, γ peaks remain rather negligible whatever the pH (Figure 3b and 1a'). However, despite the UV changes of the early peaks (α,β, γ), the height ratios of the main peaks (H_2/H_4 in Figure 2a), in both EC and UV modes, remain unchanged with time and are equal to the values obtained in neutral media .

- for RL, both EC and UV chromatograms, registered just after preparation, are rather similar to the neutral pH situation with quite stable group B/group A peak ratios (time = 0, Figure 2b). However, after a short time, one can observe a decrease of both EC and UV group B peaks (peak 6, Figure 4 a-b) and an increase of group A peaks (peaks 1 and 3, Figure 4a) in UV with a slight decrease in EC after 24 hours (Figure 4b). This results in an overall decrease of H_6/H_3 ratios in acid pH (Figure 2b). An example of a RL chromatogram after 24 hours in acid pH is shown in Figure 5.

Thus, in order to facilitate the attribution of the various peaks of RL, the elution of a mixture of RL 1 and RL 2 in an approximately 50/50 proportion (47% RL 1) has been carried out. This resulted in a chromatogram which was quite similar to the superimposition of RL 1 and RL 2 individual chromatograms (Figure 6), but which nevertheless showed an unexpected overincrease of the γ peak.

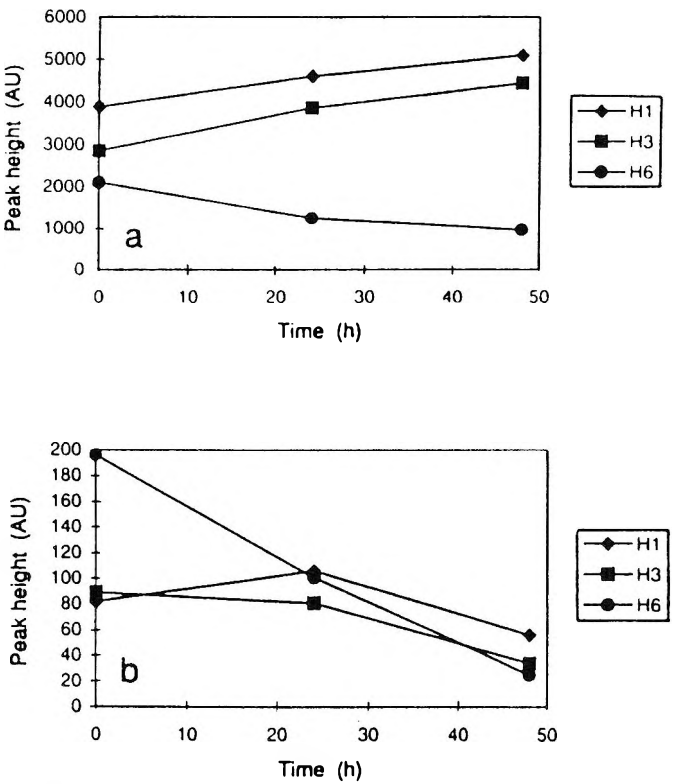

Figure 4. Variation in time of the height of the peaks 1, 3 and 6 (arbitrary units, AU) of RL prepared at acid pH. a: UV detection b: EC detection.

Variation of the EC and UV Responses with Concentration

Most of the previous experiments have been carried out with MT concentration of 500 $mg.l^{-1}$. We then checked that, for each MT, every EC and UV peak is linearly proportional to the MT concentration in the studied range of 20-700 $mg.l^{-1}$, with most of the correlation coefficients r being higher than 0.99. From these data, it becomes clear that the EC detector is capable, for instance, of detecting as low a content as about 0.5 μg of RL 1 (Table 4). It is possible to detect, in the absolute sense of the word, a much lower MT quantity in order to get a signal-to-noise ratio of at least 3, but we consider the values presented in the table as being more realistic limits of detection. In the working configuration presented here, the EC detector offers a sensitivity of 2 to 10 times higher than the UV.

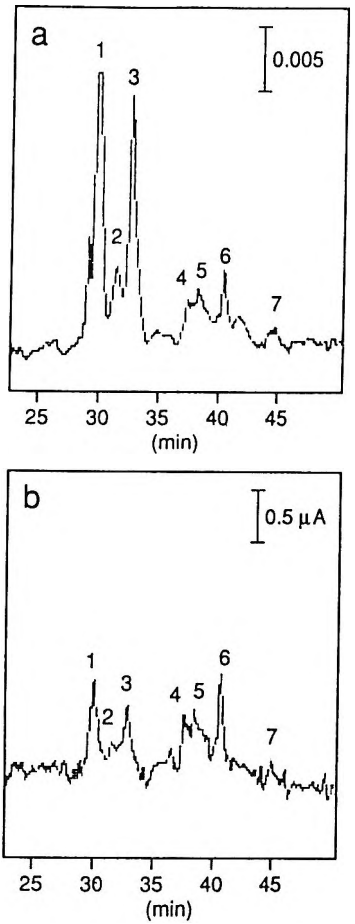

Figure 5. Reverse phase chromatogram of RL prepared at acid pH and kept at room temperature for 24 hours. All other conditions as in Figure 1. a UV detection b: EC detection.

DISCUSSION

The various metallothioneins submitted to a reverse phase HPLC separation show different peak patterns such as a variable number of peaks and a variable response depending on the detection mode (EC or UV). For all MTs which were studied, certain peaks are equally detected by both EC and UV with similar peak proportions.

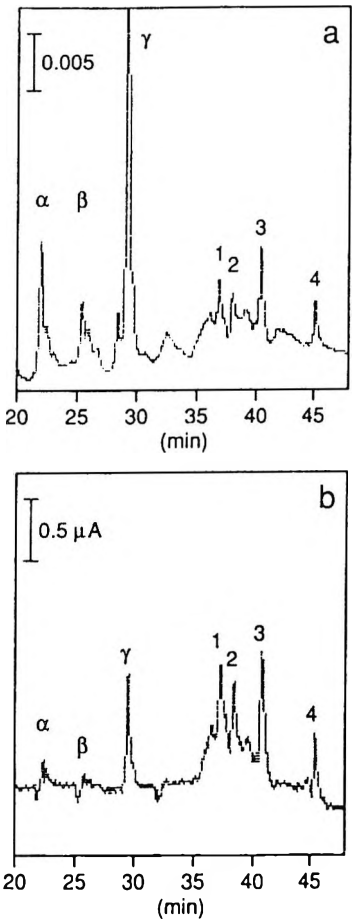

Figure 6. Reverse phase chromatogram of a mixture of RL 1 (47%) and RL 2 prepared at acid pH. All other conditions as in Figure 1.a: UV detection b: EC detection.

We can reasonably think of these peaks as corresponding to putative original (French: *originel)* isoforms of the metalloproteins.

From this point of view, horse kidney MT (HK) and rabbit liver MT-2 (RL 2) can be considered together, being relatively simple cases. HK exhibits three well resolved main isoforms and RL 2 one single dominant isoform (Figure 1), whatever the pH of the sample. Our HK results agree with those of Richards and Beattie,[24] also working on Sigma MT, who found three isoforms by capillary zone electrophoresis (CZE), and they improve their HPLC findings

Table 4

Detection Limits of the Four MTs According to the Mode of Detection, EC and UV

MT	Detection Limits (μg MT/100 μL) EC	UV
RL 1	0.5	1
RL 2	1	10
RL	2.5	15
HK	3	5

of two to three incompletely resolved peaks under acidic conditions. They also confirm our own CZE results which gave three largely dominant isoforms out of five peaks,[25] and they constitute a clear improvement of other works in which only two major isoforms were separated.[14,26] Our findings for RL 2 are also in agreement with most of the works published on this MT,[10,12,24] as well as with our CZE results.[25] Wan et al.[13] also found that MT-2 from rabbit kidney was composed of a single isoform. The separation features of these two MTs are stable with time at room temperature, being independent of the sample pH (Figure 2).

For the two other MTs, only the more hydrophobic species (peaks 1 to 4 of RL 1 and group B peaks 4 to 7 of RL, Figure 1) behave in an identical manner to those of RL 2 and HK: they are eluted by the same range of organic phase proportions and are detected by both EC and UV modes with similar peak signal ratios. The comparison of the retention times of the peaks of RL 1, RL 2 and RL (Table 1), together with that of their respective UV/EC ratios (Table 3), show that group B peaks of RL, peaks 4, 5 and 7 on one hand and peak 6 on the other hand correspond to isoforms of RL 1 and of RL 2 respectively. On the contrary, group A peaks (1, 2, 3) can hardly result directly from a simple contribution of either RL 1 or RL 2 (Table 1). Although not equal, we had found similar results in our work by CZE with UV detection in which one major peak of RL could not, indisputably, be attributed to either RL 1 or RL 2.

Taking into account the intensity of the peaks of RL group B, it clearly appears that the contribution of RL 2 to total RL is much higher (~70%) than that of RL 1 (~30%), in agreement with our previous studies on the same MTs using size-exclusion and reverse phase HPLC, where we found RL being composed of 20-30% RL 1 and 70-80% RL 2.[27] The present results also agree with those we obtained by anion-exchange chromatography (TSK DEAE-650

S. data not shown). Our experiments carried out with mixtures made of RL 1 and RL 2 reinforce these conclusions, showing what could be, for instance, the features of a RL composed of 50% of each isoform 1 and 2 (peaks 1, 2, 3, 4 in Figure 6).

It is also to be noted that peak 3 of RL 1 and the small peaks 1, 2 and 4 of RL 2 (Figure 1) probably result from a cross contamination due to imperfect separation of RL 1 and of RL 2 from the original RL (1+2).

The first peaks of RL 1 (α, β, γ) and those of RL (group A: 1, 2, 3) pose more problems since they are well detected by UV but not at all, or with only very low response, by EC. Based on the retention times, we can observe that, while the first peak of RL (t_R= 29.8 min) coincides with the γ peak of RL 1 (t_R= 30.1 min), RL's third peak (t_R = 33 min) appears isolated, with no corresponding peaks on neither RL 1 nor RL 2 pictures (Table 1). Quite stable at neutral and basic pH, an acidic medium enhances the formation of the species α, β and γ of RL 1 (Figure 3) and of 1, (2) and 3 of RL (group A peaks, Figure 5). The formation of intra- or intermolecular disulphide containing compounds from the original reduced isoforms (all thiols being either complexed by metals when pH > 5, or protonated when pH < 3) could maybe explain these observations. Since the electrochemical detection is based on that of the thiols according to their oxidation in disulphides at the graphite electrode, thiols which were previously chemically oxidised will not be able to give an EC response anymore. Our UV-EC on-line detection system allows the registration of this chemical evolution. Let us consider the RL case. Initially, the UV chromatogram exhibits six to seven peaks, organised in two groups A and B, being independent of pH, of which three are dominant: 1-A, 3-A and 6-B. In EC, only the B peaks (6-B) give equivalent responses as in UV (Fig. 1c-c'). The A peaks might therefore correspond to species containing very few reduced thiols, giving low EC signals but "normal" UV absorbance. In acidic pH and at room temperature, the effect of time (air oxidation) is very striking: there is a decrease, both in UV and in EC, of the B peaks (disappearance of the SH containing isoforms) and an increase of A peaks in UV but not in EC (Figure 5): this being due to the formation of more disulphide containing molecules. The same occurs for RL 1 with its α, β and γ peaks, probably also corresponding to species with few remaining reduced thiols. Compared to RL 2 which does not show any significant changes with pH, time and temperature, RL 1 clearly appears to be much more unstable. A similar observation has also been made previously.[28] The fact that the oxidised forms are eluted earlier than the reduced ones is as expected. It is commonly observed on a reverse phase that, for a reduced (SH)/oxidised (SS) peptide pair, the capacity factor is larger for the reduced form of the peptide. This has been described for small peptides[23,29,30] and for a much larger thiol containing protein like

interleukin-2.[31] This could be attributed to a worse accessibility of the different amino acid residues to the solid phase when the peptide is oxidised (loop formation), therefore decreasing the overall hydrophobicity.

The original polymorphism we found for RL 1 (three isoforms - peaks 1 and 2 with close retention times and 4, more distant, Figure 1a-a') is in excellent agreement with the results of Klauser et al.[10] for the same MT and with those of Wan et al.[13] for rabbit kidney MT-1. Some other authors also working on rabbit liver MT-1 had found only one major isoform by RP-HPLC at neutral pH[12] or resolved two isoforms only by CZE and by RP-HPLC at acidic pH.[24] We had also previously stated that RL 1 was composed of two unequally abundant isoforms only, but this was found using a neutral buffer and a much shorter RP column.[27]

The chromatogram of the mixture RL1+RL2 (Figure 6) shows a major difference with that of the original RL (Figure 1 c-c')). The discrepancy does not arise from what we consider as being the putative original isoforms of RL (peaks 4 to 7), but from the chemically formed or modified ones, especially peak 3. This peak, which was previously qualified as isolated, has no corresponding peak on the chromatogram of RL1+ RL 2. This means that, from the point of view of the possibility of the various reduced isoforms to form disulphide bonds, there is no perfect reconstruction of RL by adding RL1 to RL 2, once these two have been separated. The primary structure determination of the reduced and oxidised species might provide a key to a more precise comprehension of this behaviour.

It is also important to note that RP-HPLC is capable of directly resolving more than the two main isoforms RL 1 and RL 2 of RL without their prior separation by anion-exchange chromatography, as has been reported.[14,24]

On the whole, this study demonstrates the differences in the degree of redox reactivity of the various metallothioneins, two of them, RL and RL1, being quite sensitive to pH change (neutral to acid).

From an operational point of view, two interesting conclusions may be drawn from this work. Firstly, the trifluoroacetic acid (TFA), although not yet widely used,[10,13,32] proves itself to be an excellent medium for MT isoform separation by RP-HPLC, eliminating peak co-elution that often occurs at neutral pH. Because of the acid pH, there is naturally a substantial decrease of the UV absorbance in the 200-220 nm range due to the dissociation of the metal-thiolate complexes.[33] Nevertheless, working at a detection wavelength of 230 nm, we still achieve UV limits of detection similar to those published by authors using a neutral buffer at 214 nm.[12] Furthermore, TFA is a volatile

buffer, which means that the various MT isoforms (original + modified) can be recovered, for further characterisation, by evaporation of the eluent with no salt formation, therefore, avoiding the problems encountered with phosphate. Secondly, the direct detection of thiols on the type of electrode that we have used does not require as high a potential as 1.0 V, with its inherent disadvantages, as has been stated several times:[34,35] a potential of about 0.6 V gives, in fact, an optimum signal-to-noise ratio, with a baseline reaching stability in a few minutes and a consistently low background current.

Overall, the use of the on-line WV-EC detection system constitutes a very good tool for MT isoform identification. In some cases, like that of RL 1 for instance, the UV mode used alone could overestimate the number of originally reduced isoforms. On the contrary, the electrochemical detection, employed alone, would provide the correct number of thiol containing isoforms (putative real polymorphism), but would not allow one to properly follow the chemical transformations of these species. Only the combination of the two detection modes, the universal UV and the more selective EC, can give access to the double information. To our knowledge, this work is the first one in which this type of electrochemical - coulometric- detection has been employed for MT isoform characterisation.

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SIMULTANEOUS HPLC ANALYSIS OF L-ASCORBIC ACID, L-ASCORBYL-2-SULFATE AND L-ASCORBYL-2-POLYPHOSPHATE

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ABSTRACT

L-Ascorbic acid is a difficult vitamin to quantitate by HPLC due to its low retention and poor resolution using the previously available column technologies. This is particularly evident in aquatic feed and meals, where various interferences deter the vitamin's accurate determination and quantitation. Furthermore, these matrices often contain other 'stabilized' forms of vitamin C, necessitating several determinations utilizing a variety of chromatographic methods.

An improved HPLC method is introduced, here, for the simultaneous separation of L-ascorbic acid, L-Ascorbyl-2-Sulfate and L-Ascorbyl-2-polyphosphate. This method utilizes two novel inert HPLC columns, Inertsil C_4 connected to an Inertsil C_{18}. The mixed phases, chemistry and packing of these columns have allowed improved separation of the earlier components (which include ascorbic acid) as well as that of the latter eluting ones (L-Ascorbyl-2-Sulfate and L-Ascorbyl-2-polyphosphate).

Various ion-pairing reagent concentrations, buffer ionic strength and pH were investigated to achieve the optimum mobile phase conditions necessary for a simultaneous and universal separation.

The final method carries out the analysis using one HPLC chromatographic system with ultra-violet detection, one mobile phase containing n-octylamine as an ion-pairing agent and the same column set for the simultaneous analysis of all the Vitamers. Percentage relative standard deviations for ascorbic acid were less than 0.5 % for the peak areas and retention times, minimum detection limits of less than 250 ppb with UV detection and recoveries in the 97 % range.

The method was then utilized to quantitatively asses bulk storage losses of commercial Ascorbyl-2-sulfate and Ascorbyl-2-polyphosphate at the typical tropical storage conditions of 37°C and 47% relative humidity.

INTRODUCTION

Vitamin C or *Ascorbic acid*, (C1), a white crystalline solid, is a water soluble micronutrient essential for normal growth and health. Various fish species and shrimp, guinea pigs and primates (non-human and human) cannot synthesize adequate ascorbic acid *in vivo*.[1] Lack of sufficient vitamin C in aquatic diets results symptoms such as poor growth, lordosis, loss of scales, reduced egg hatchability, impaired hydroxylation of collagen, internal fin hemorrhages and mortality.[2,3] Consequently, a dietary source of vitamin C is needed to meet their requirements. Ascorbic acid has been commonly added to the feed to supplement the diet. However, ascorbic acid is unstable in the feed and water as it is easily destroyed by oxidation.[4] The decomposition is accelerated in the presence of salts, moisture, heat and high pH. Substantial losses also result during feed manufacturing, storage, pelleting and extrusion.[5] Consequently, over the years, overages of ascorbic acid have been added to the diet to offset losses and permit vitamin C fortification at nutritional levels.

Stabilized forms of vitamin C have been introduced to permit fortification of feeds at cost effective levels. A variety of coating agents and ascorbic acid derivatives have been developed. These include silicone, oil and cellulose coatings as well as stearate, palmitate, sulfate and phosphate esters. The most effective derivatives to-date, recently being the focus of attention in the field of

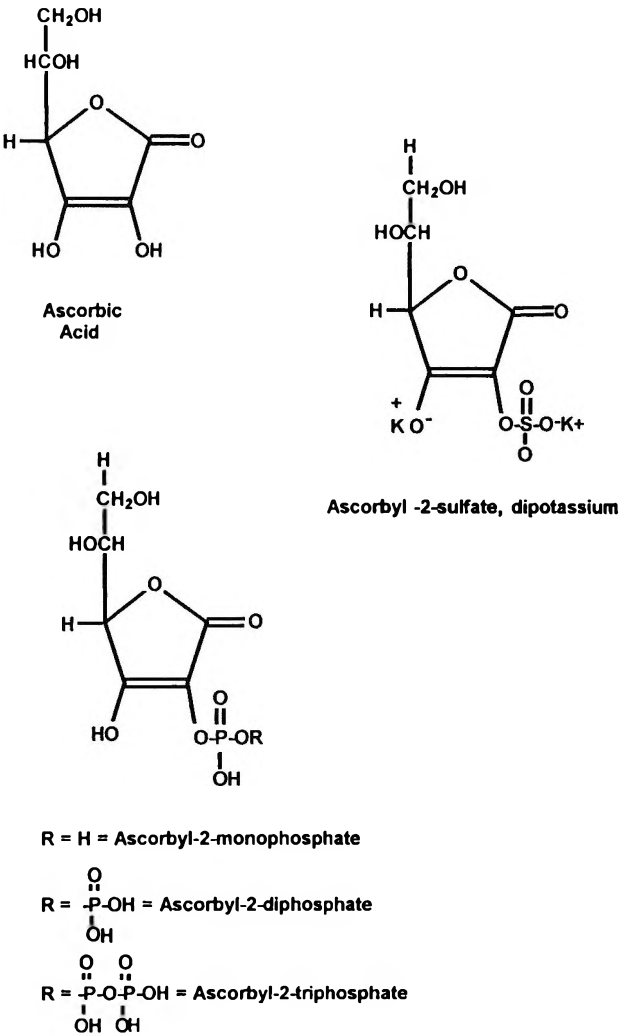

Figure 1. Chemical structure of vitamins.

scientific fisheries, are the 2-sulfate (C2) and 2-polyphosphate (C3) esters of L-ascorbic acid (Figure 1). The chemical and, therefore, the physical properties of these esters are quite different from ascorbic acid. For example, because of it's proven stability[6] and natural occurrence,[2,7] interest in L-ascorbyl-2-sulfate as a source of Vitamin C to enrich foods has grown.

The stability is the result of the protected OH-group at the C2 position of ascorbic acid by the sulfate ester group. When ingested, an enzyme known as ascorbate-2-sulfohydrolase converts the ascorbyl-2-sulfate back to ascorbic acid.[8]

New information,[9] showing that Ascorbyl-2-sulfate is absorbed directly into the lumen for further conversion into ascorbic acid, has further illustrated the mechanism of the vitamer's utilization. In order to assess the stability of the various commercially available vitamins, their potency, equivalency to ascorbic acid and ingestion, an accurate determination and quantitation is necessary. The various aquatic feeds and body part matrices has made this endeavor challenging. Various analytical methods have been available for the determination of ascorbic acid in particular. These have included titrimetric methods, derivatization reactions, enzymatic methods and chromatographic methods.[10-12] The most specific, quantitative, sensitive and rapid have been those methods utilizing High Performance Liquid Chromatography (HPLC) using reversed phase (C_{18} or ion paired C_{18}) and bonded phase (such as amine) columns.[13-15] Ultraviolet (UV) and electrochemical (EC) have been the two most commonly employed detectors.[5,16,17] Washko et al.[12] gave a good review of the earlier existing methods.

Since then, several attempts are worth mentioning. Felton and Halver[13] analyzed, simultaneously, for the three vitamers using C_{18}, n-octylamine ion-pair chromatography. Peak efficiencies were poor and, consequently, total peak resolution was not achieved. Wang et al. introduced enzyme shifting, which allowed the hydrolysis of all the ascorbyl-polyphosphate to ascorbic acid. The latter was analyzed using C_{18} with the ion pairing agent tetrabutylammonium phosphate. A column heater and electrochemical detection were necessary. Two years later, Wang and Seib[17] investigated acid catalyzed methanolysis to determine ascorbyl-2-sulfate alone as ascorbic acid. Hoffman et al.[19] compared three different chromatographic techniques for reliability, sensitivity and recovery for the separation of C1, C2 and C3.

Several extraction techniques (meta-phosphoric versus trichloroacetic acid), mobile phases (n-octylamine versus tetrabutylamine) and columns (reversed phase and ion exchange) were compared. Felton et al.[20] later attempted to eliminate the use of ion-pairing agents through the use of an Altima C_{18} column to separate ascorbic acid, ascorbyl-2-monophosphate and ascorbyl-2-sulfate. With only a difference of approximately two minutes between the vitamer peaks, the elution order was also reversed with C3 eluting after C2 and followed by C1.

Some of these methods seem to work only when the matrix is simple. For more complicated samples, such as fish feed, the methods suffered from poor resolution and peak efficiencies. This necessitated laborious, time consuming sample preparation, which, in turn, resulted in poor quantitation and reproducibility.

This work, an extension to the above attempts, uses the most optimal conditions, with major improvements in peak resolution and efficiency, leading to better quantitation and reproducibility. This is a consequence of the use of two different, novel columns. Inertsil C_4 and Inertsil C_{18}, in sequence. In addition, this method allows the simultaneous determination of all the vitamin C forms using the same column and HPLC conditions. This 'universal' method requires minimal sample preparation and overall analysis time.

MATERIALS

Apparatus

Two consecutive columns, one Inertsil C_4, followed by an Inertsil C_{18} (Metchem Technologies, Inc., Torrance, CA), each 15cm long, 4.6cm in internal diameter and 5μm particle diameter, were used in sequence for the separation. An LDC pump, autosampler, and spectrafocus detector (Thermo Separation Products, San Jose, CA) were employed. Centrifugation was performed using a Beckman J2-21 centrifuge (Beckman Instruments, Inc., Somerset, NJ).

Chemicals

The mobile phase is a solution made up of 0.1M anhydrous sodium acetate, the buffer (Fisher Scientific Co., Pittsburgh, PA), 174 uL/L n-octylamine, the ion pairing agent, (Sigma Chemical Co., St. Louis, MO), and 200 mg/L disodium (EDTA, chelates divalent metals which otherwise accelerates oxidation of the vitamins) (Fisher Scientific Co., Pittsburgh, PA). This solution is brought to pH 5 with glacial acetic acid (Fisher Scientific Co., Pittsburgh, PA). Acid phosphatase from potato (Boehringer Mannheim Corporation, Indianapolis, IN) is used for the enzyme shifting procedure, while dipotassium-ascorbyl-2-sulfate dihydrate, L-ascorbic acid (Sigma Chemical Co., St. Louis, MO), and L-ascorbyl-2-monophosphate magnesium salt (Wako Chemicals Inc., Richmond, VA) are used as the external standards. Meta-

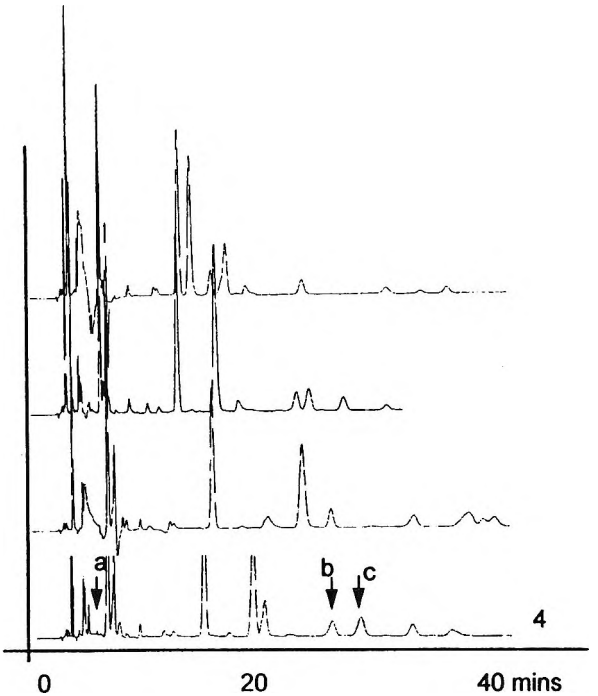

Figure 2. Effect of pH and ionic strength changes in the mobile phase. 1) pH 5.0, 0.15 M NaOAc; 2) pH 4.7, 0.1 M NaOAc; 3) pH 5.3, 0.1 M NaOAc; 4) pH 5.0, 0.1 M NaOAc (optimum separation). a = position of ascorbic acid, b = ascorbyl -2-sulfate, c = ascorbyl-2-monophosphate.

phosphoric acid (used to denature the proteins and prevent hydrolysis of the Lactone ring) and 1,4-dithiothreitol (DTT, Cleland's reagent, used to retard oxidation), (both from Aldrich Chemical Co., Milwaukee, WI) are essential components in the extraction and solvation solutions.

METHODS

Extraction Conditions of Aquatic Feeds

Upon receipt of the feed sample, the contents are stored in the freezer (-80°C) prior to analysis time. From each feed, two batches, 5 grams each, are

well ground and mixed using a mortar and pestle. Two 1.0 g samples are weighed (after reaching ambient temperature) into centrifuge tubes (record the exact weight), from each of the five-gram batches. This replication in sampling tests both the efficiency of vitamin mixing in the feed as well as the analytical extraction procedure.

The bags are then well sealed and stored at -80°C for any future re-analysis. To each 1.0 g add 20 mL of extraction solution. The extraction solution is prepared by mixing 1% metaphosphoric acid and 0.2% DTT to give a pH of approximately 2.16.

Doubly distilled, deionized water and high purity grade reagents are used throughout. Use of amber glass ware is recommended to minimize light exposure.

Each sample is placed in a sealed container and vortexed for 5 min or shaken for 5-10 minutes. A Tissumizer can be used, for approximately 30 seconds, if the sample is bulky. Note that excessive use of the Tissumizer may speed up the vitamin decomposition through the introduction of oxygen. Sonication is not recommended as it may heat up the sample. The samples are then centrifuged for 10 min at 13,000 rpm and -5°C.

The supernatant is consecutively filtered, first through 0.45μm, then 0.2μm acid resistant (e.g. PTFE) filters. Solid phase extraction (SPE) should be avoided if possible. The filtrate is then immediately chromatographed. Each sample is injected at least twice. Resultant areas are compared to those of the external standards.

Enzyme Shifting of Ascorbyl-2-Polyphosphate

When (and only if) ascorbyl monophosphate is detected, to 1 mL of filtrate add another 1.0 mL of acid phosphatase enzyme solution to initiate enzyme shifting. This method is adapted from Wang and Seib[17] and Maugle[21] with modifications. The solution is shaken and left in the dark for at least one hour before chromatography.

The enzyme solution consists of 4 mg/mL acid phosphatase enzyme dissolved in 0.1M sodium acetate (anhydrous) / 0.2% DTT solution (pH 5.0), and is prepared fresh. The final, higher pH on mixing the enzyme solution with that of the filtrate (pH ~3.5), was found to be more optimal for the enzyme.

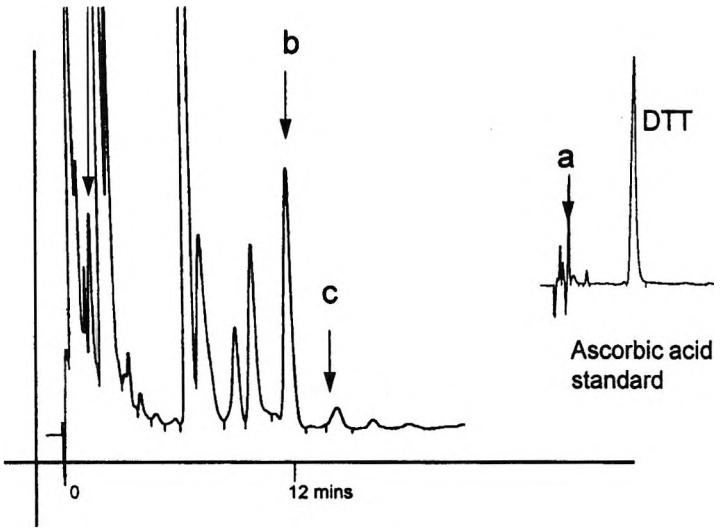

Figure 3. Separation of an aquatic feed using a Nova Pak C18 column, octylamine, EDTA, pH 5, 0.1M NaOAc. a= Ascorbic acid, b= Ascorbyl -2-sulfate, c= Ascorbyl-2-monophosphate.

Chromatographic Conditions

The column combination used in this work is used to improve the separation, especially for the early eluting peaks. Two Inertsil columns, a C_4 and a C_{18}, were connected in sequence, and equilibrated with the mobile phase (which contains the ion pairing agent n-octyl amine), along with the pumping system, overnight, before use. Once impregnated with the ion pairing agents these columns should be dedicated for this mobile phase only. In this way, reproducibility during the time of the analysis, and from day to day, is highly improved.

The ionic strength of the sodium acetate, and the pH of the mobile phase solution, were further manipulated to investigate enhanced resolution of the early eluting components, while maintaining acceptable analysis time.

For better reproducibility of the analysis, an isocratic mode was chosen at a flow rate of 1mL/min. Detection was carried out at 255 nm using a UV detector.

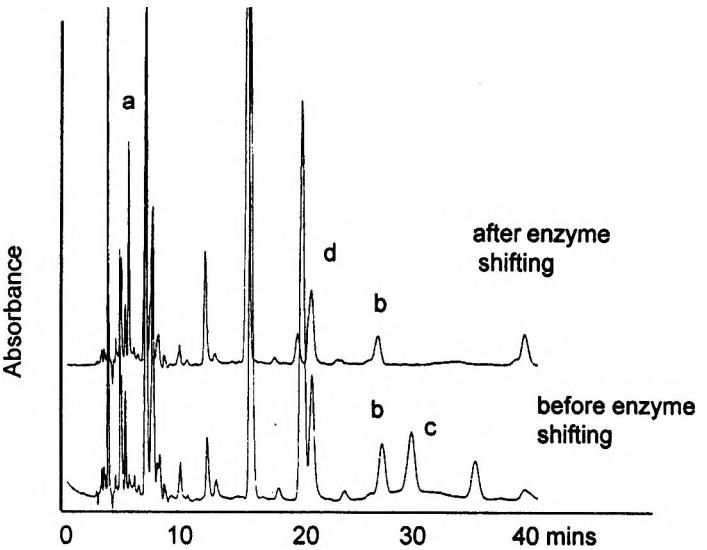

Figure 4. Separation of an aquatic feed using an Inertsil C4 and C18, octylamine, EDTA, pH 5, 0.1M NaOAc. a= Ascorbic acid, b= Ascorbyl -2-sulfate, c= Ascorbyl-2-monophosphate and d= DTT.

To effect a more rugged method, chromatographic conditions were chosen to allow for the more difficult feed matrices. Additionally, when sensitivity is required, this method can be easily adapted to glassy carbon electrochemical detection.

RESULTS AND DISCUSSION

The Chromatography

Increasing the pH of the mobile phase from 4.7 to 5.0 to 5.3, with manipulations of the amounts of glacial acetic acid, was found to increase the capacity factors of all the components, in turn increasing their retention times and their resolution (Figure 2). On the other hand, increasing the ionic strength via an increase in the sodium acetate concentration, resulted in a decrease in capacity factors and resolution (Figure2). Figure 3 shows a feed separation using a Waters Nova-pak C_{18} column, compared to that using the optimized conditions described by this method (Figure 4). Complete and clear

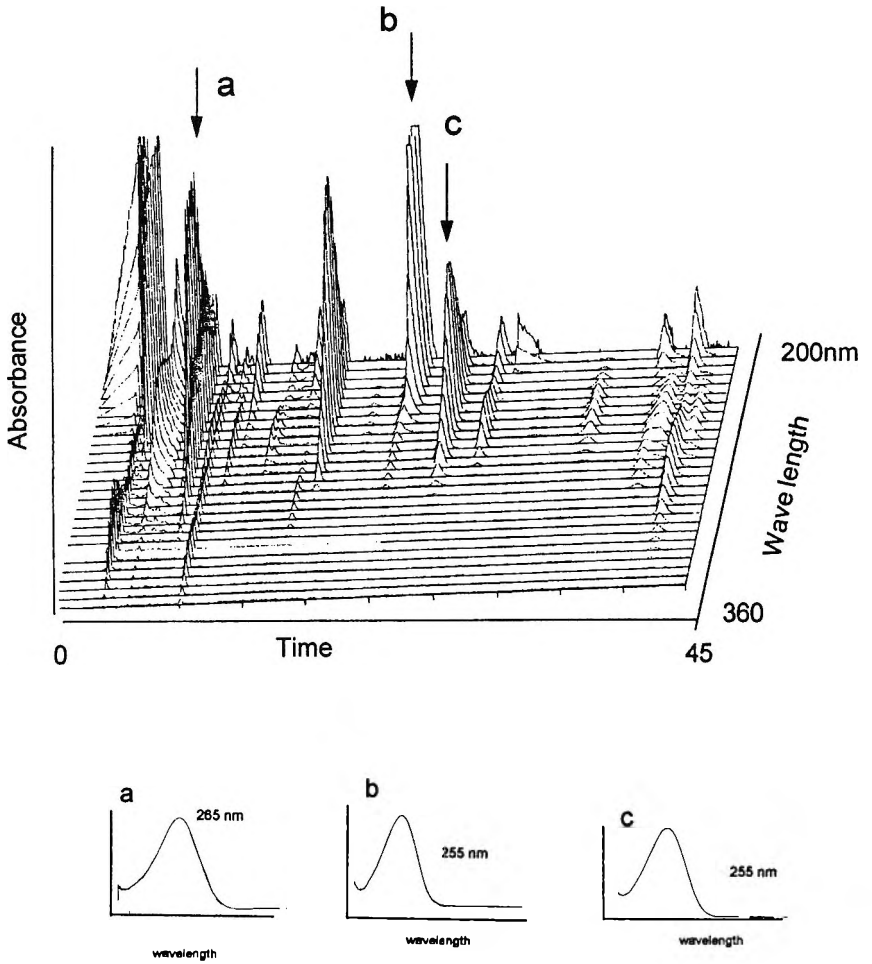

Figure 5. Three-dimensional plot for the aquatic feed sample. a = ascorbic acid, b = ascorbyl -2-sulfate, c = ascorbyl-2-monophosphate.

separation of the ascorbic acid peak is evident and repeatable, allowing a more accurate determination of the ascorbic acid content of the feed both before and after enzyme shifting of ascorbyl-2-polyphosphate. Ascorbyl-2-sulfate and ascorbyl-2-monophosphate are equally well resolved. The Inertsil C_4, being more polar, retains the more polar compounds, such as ascorbic acid, allowing better selectivity and, in turn, better resolution of the earlier eluting peaks. The

Inertsil C_{18} in conjunction, works to retain the more nonpolar compounds, in this case, the ester vitamins. Enzyme shifting, through the acid phosphatase hydrolysis of the phosphate moieties, quantitatively converted all the L-ascorbyl-2-polyphosphate to the equivalent amount of ascorbic acid. (Figure 4). This method eliminated the need for qualitative and quantitative determination of all the polyphosphate esters which are, with exception to the mono-ester unavailable as stable standards.

Retention times, peak heights and peak areas for L-ascorbyl-2-sulfate, using this method, were found to have percent relative standard deviations (%RSD) of 1.8, 0.64 and 1.2, respectively (n=10), while those of ascorbic acid were 0.48, 1.25 and 0.28, respectively (Table 1) . Recoveries in feeds were 97%.

The identities of the components were confirmed using external standards and the instrument's fast scanning capabilities. UV spectra of the three vitamins, in the optimum mobile phase, were recorded on-line and are shown in Figure 5. From these spectra, an optimum wavelength of 255nm was chosen for the analysis.

The analytical method described has been shown to be very useful for separation of ascorbic acid from other components in aquaculture feeds. It has also been used for routine analysis and for determination of properties of the various forms of stabilized C vitamers as indicated below.

Using the above chromatographic method, it was possible to quantitatively assess the stability and, hence, potency of two commercial forms of vitamin C, ascorbyl-2-sulfate and ascorbyl -2-polyphosphate. L-ascorbic acid is well known to be highly soluble; however, once dissolved in water, it is subject to rapid oxidative degradation.

In order to retard oxidation during the time required for analysis, dithiothreitol (DTT) must be added or low ascorbic values will be obtained. Commercially available L-ascorbyl-2-polyphosphate (15% ascorbic acid equivalency) was found to be substantially insoluble in distilled water and seawater, floating to the top. Acidic solutions (1% metaphosphoric acid / 0.2% DTT with and additional 10% acetone) were required to effect complete solubility.

When exposed to conditions of high humidity (such as would be found in tropical feed mills, 47% relative humidity and 37°C), the stability of this ester was shown to progressively deteriorate reaching 18% loss of potency after 3

Table 1

Percent Relative Standard Deviations (n = 10)

Compound	% RSD Area	% RSD Height	% RSD Ret. Time
Ascorbyl-2-sulfate	1.2	0.64	1.8
Ascorbic acid	0.28	1.25	0.48

Table 2

Vitamer % Loss with Storage at 37°C, 47% Relative Humidity

Vitamin	Week 2	Week 3	Week 6
L-Ascorbyl-2-sulfate	1.7	0.1	4.1
L-Ascorbyl-2-poly-phosphate (15%)	4.7	18.4	34

weeks of storage (Table 2) and 34% loss in potency after 6 weeks bulk storage. L-Ascorbyl-2-sulfate dissolves readily and completely in both distilled water and sea water, but did not suffer a significant loss in potency when stored under the same conditions, losing only 4% in potency after six weeks storage (Table 2). This verifies the previous findings of Maugle[21] of the progressive loss in ascorbyl 2-polyphosphate potency after manufacture of an extruded salmon feed; this indicates that ascorbyl-2-sulfate provides a more cost-effective form of Vitamin C.

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MEASUREMENT OF 3,4 DIHYDROXYPHENYL ETHYLENE GLYCOL (DOPEG) IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

Usually, determination of DOPEG in plasma by EC-HPLC is always performed after extraction on alumina. This paper presents an extraction on boric acid gel, with good selectivity, reproducibility. In high sensitivity conditions (0.5 nA full scale) an autosampler is used and presents two major advantages: first, the possibility of storing samples in cold conditions to preserve stability of DOPEG, second the analysis of numerous samples in pharmacological studies, for example.

INTRODUCTION

Liquid chromatography with electrochemical detection (LC-ED) is very useful for the determination of catecholamines and 3,4 dihydroxyphenylethyleneglycol (DOPEG) after extraction always performed on alumina.[1,2]

In this paper, we describe a rapid procedure for the determination of DOPEG, a metabolite of norepinephrine (NE), by LC-ED after extraction on boric acid gel.

Indeed, DOPEG is a prominent intraneuronal MAO-A dependent metabolite of NE. For example, determination of DOPEG in plasma is a good biological marker to quantitatively estimate the inhibition of MAO-A in vivo, by antidepressor IMAO.[3,4] Because such pharmacological studies require numerous assays, an autosampler was used.

The main advantages of the method are the following: the extraction procedure of DOPEG from plasma is original and satisfactory, automation of the HPLC method was established with high reproducibility and sensitivity.

EXPERIMENTAL

Reagents and Chemicals

The boric acid gel (Affigel 601) was purchased from Biorad (California, USA). Acetic acid, sodium acetate and citric acid were of analytical-reagent grade from E. Merck (Darmstadt, Germany), EDTA, clorgyline, sodium octylsulfate and DOPEG were obtained from Sigma (St Louis, MO, USA).

Stock solution of DOPEG was prepared at a concentration of 5mg/mL in 0.1M perchloric acid and stored at -80°C for three months. The working standard containing (per mL) 2.5 ng in 0.75M acetic acid was prepared every day.

Extraction Procedure

The withdrawal of venous blood was performed in a tube containing 30 µL of 5% Na_2 EDTA and 20 µl of an IMAO-A (clorgyline 10^{-2}M). Blood was immediatly centrifuged at 1000 g for 15 min at 2-4°C, the plasma was collected and centrifuged at 11000 g, for 10 min and stored at -80°C (until extraction).

The extraction method was modified from Maruta.[5] Boric acid gel was first activated with successive acid and bases. Then 1 mL of plasma was extracted, DOPEG was desorbed with 200 µl of 0.75 M. acetic acid and an aliquot was injected into the HPLC system.

Apparatus and Chromatographic Conditions for Liquid Chromatography

The LC system consisted of one model 420 pump (Kontron Instruments SA, St Quentin-en-Yvelynes, France) and an autosampler 465 (Kontron) equipped with a refrigerated platinum and a 50 µl injection loop. The Nucleosil RP18 (5 µm) column (250 x 4.6 mm ID 5µm) was protected by a Brownlee RP18 precolumn (30 x 4.6 mm I.D). The mobile phase (pH 3.8 with glacial acetic acid) contained 50 mM of sodium acetate, 10 mM of citric acid, 50 mg/L of EDTA and 100 mg/L of sodium octyl sulfate. Before use, mobile phase was filtered on 0.45 µm and was continuously degased with helium gas during analysis. The flow-rate was 0.8 mL/min. DOPEG was detected with an amperometric detector (model 460 Waters, Millford, MA, USA) with a glassy carbon working electrode and a Ag/AgCl reference electrode at a potential of +700 mV. A microcomputer (Kontron) controlled the chromtographic system.

RESULTS AND DISCUSSION

Figure 1 shows the good resolution of DOPEG from a peak X present in standard solution and in plasma extract. Standard solution, prepared in acetic acid, is directly injected but acid desorbs an impurity (peak X) from the plastic tube. At such a sensitivity (0.5 nA full scale), it is not surprising to detect contaminants.

In plasma extract, impurity X has the same retention time as uric acid, that explains this large peak.

We can't replace plastic tubes in this method, because of the affigel extraction, but it's not a major problem, the contaminant doesn't interfere with DOPEG.

Alumina is commonly used by other authors for the plasma extraction of catecholamines and their metabolites. In this work, a boric acid gel (Affigel 601) was chosen. This gel is selective with an affinity for coplanar cis hydroxyl groups (cis-diols). Recovery (63 ± 3%) was determined after adding known amounts of DOPEG to a plasma treated in parallel.

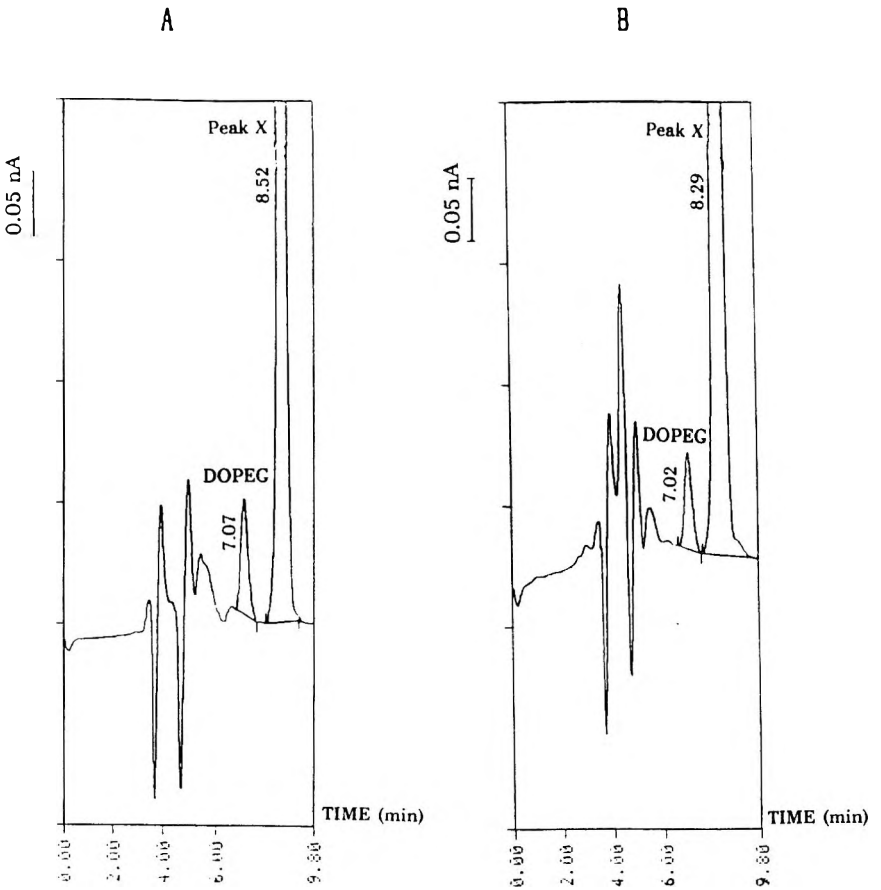

Figure 1. Chromatograms of (a) standard DOPEG (125 pg injected), peak X (impurity) and (b) plasma ; range : O.5 nA full scale ; chart speed 0.2 cm/min.

Calibration curve for DOPEG was established from 25 pg to 0.5 ng injected ($y = 7.0204\ x + 0.192$, $r = 0.999$). The inter-assay and intra-assay coefficients of variation ($n = 10$) were 8.3% and 6.2% respectively for DOPEG. The limit of detection was 1 pg injected.

An application of this method is given in Table 1. Two groups of healthy subjects of different age were tested in parallel and show significant difference in DOPEG. Average concentrations of DOPEG obtained by this method agree with those reported by other authors.[6,7,8]

Table 1

DOPEG Plasma Concentrations (Mean ± SEM) in Relation to Age (Mean ± SEM)

	Group 1 (n = 14)	**Group 2 (n = 11)**
Age (years) mean ± SEM	68 ± 0.6	23 ± 0.5
DOPEG (pg/mL) mean ± SEM	1104 ± 90.2	778 ± 59.0

This method offers good reproducibility and sensitivity. The use of an autosampler with refrigerated platinum in such electrochemical detection conditions (0.5 nA full scale) is very interesting and useful in pharmacological and pharmacokinetic studies.

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A SIMPLE DENSITOMETRIC METHOD FOR ESTIMATION OF POLAR AND NON-POLAR LIPIDS BY THIN LAYER CHROMATOGRAPHY WITH IODINE VAPOR VISUALIZATION

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ABSTRACT

A simple reflectance densitometric method for quantitation of polar and non-polar lipids was developed, by thin layer chromatography (TLC) or high performance TLC. The solvent systems used were a mixture of n-hexane:diethylether:glacial acetic acid (80: 20: 1, V/V) for non-polar lipids and chloroform: methanol:water (65:25:4, V/V) for polar lipids. After removal of solvents, lipid fractions were visualized by exposing the plate to iodine vapor. The plate was then covered with a glass plate and scanned at 365 nm. Different lipid fractions were quantitated by using appropriate reference standards. Iodine color is stable for at least three hours; the integrated area values of the lipid components are linear with their concentrations with a variation of 2 to 4 percent. The method of quantitation being nondestructive, the TLC plates may be used for further studies.

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INTRODUCTION

Lipids include a variety of compounds, ranging from simple glycerides to complicated gangliosides. Thus, separation, identification and quantitation of these compounds, when isolated from natural sources, is difficult. With modern chromatographic techniques, lipids may be separated into different classes and these individual lipid classes may further be fractionated into different subclasses and into individual components.

The number of fractions that can be obtained, thus, is quite substantial and the task involved in estimation of these individual components by classical methods is enormous. Phospholipids are generally estimated by measuring phosphorous content,[1] sterols and their esters by measuring sterol[2] and glycerides by IR-spectroscopy or chromic acid reduction[3] method, etc.

Estimation of amino groups, carbohydrates, amino sugars, sphingosine, etc. are also used to quantitate different types of lipids. However, these methods are manipulatively difficult and are generally used after separation of the components by chromatography.

Incomplete separation and poor recovery often leads to erroneous results. Thus, *in situ* quantitation by densitometry, after fractionation of the lipid components by TLC, was attempted by a number of laboratories.[4-13] Most of these methods involve charring with sulfuric acid, followed by reflection[4-7] or transmission[8-12] densitometry.

Scanning in fluorescence mode[4] was also tried. However, these methods were useful only over a narrow concentration range, due to non validity[7-16] or oversimplification[6] of the Kubelka-Munk equation.[13,14] Diffusion or washing out of the sample during reaction with chromogenic reagents also interferes with assay by densitometry.[15]

Visualization by iodine vapor is widely used for detection of lipids fractionated by thin layer chromatography.[15,17] Since iodine is physically absorbed by lipids and remains as a "solution",[17] Kubelka-Munk theory is applicable for these spots. However, this method could not be used for quantitation of lipids because iodine starts evaporating from the plate as soon as the plate is taken out of the iodine chamber. Spraying the plate with acetic acid, starch, cyclodextrin, etc., to stabilize the iodine color was attempted by different workers with limited success.[18-20] Also, these treatments may render the plate unsuitable for scanning densitometry.[15]

We have found that, if the chromatogram is covered with a glass plate immediately after exposure to iodine vapor, evaporation of iodine is delayed considerably and the plate can be scanned comfortably. We have standardized this method for quantitative analysis of lipids by scanning densitometry using a dual beam zig zag scanning densitometer. The results are described below.

MATERIALS

Apparatus

a) Spectrophotometer: Shimadzu UV-VIS double beam recording spectrophotometer, model UV-240 with OPI-4.

b) TLC Scanner: Shimadzu dual beam zigzag scanner, model CS-930.

c) Sample applicator: Camag Nanomat III with 100 nL nanopipette, 1 μL micropipette and holder and 20 μL micropipette.

d) Table centrifuge: Model Remi-8RC (Swing head, 5000 rpm).

e) TLC plate: (i) 20x20 cm silicagel 60 F-254 precoated plate with zone concentrating layers (E. Merck). Activated by heating at 110°C) for 1 hr before use. (ii) 20x20 cm laboratory coated plates with 0.4 mm thick layer of silicagel, E. Merck (60G:60HF_{254} :: 10:3,W/W) containing a 5cm wide sample concentrating zone of Kieselguhr G. Activated as above.

f) HPTLC Plate: 10x10 cm silicagel 60F-254 plates (Glass, E. Merck). Activated as above.

g) Developing Tank: Camag twin trough developing chamber.

Reagents

Phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, triolein and ergosterol were purchased from Sigma Chemicals, USA and squalene was a product of Fluka, AZ, Switzerland. The solvents used were of Lichrosolve (E. Merck) grade. Potassium dichromate (Analar) and ammonium molybdate were products of BDH (India) and ascorbic acid (GR) was from Sarabhai(M), India. Ground nut oil (arachis oil), cod liver oil and soyabean oil were purchased from a local market.

Standard Preparation

For non polar lipids (a) triolein (20 mg.) ergosterol (4 mg) and squalene (2 mg) were dissolved in 10 mL of n-hexane. Further dilutions of solution (a) were prepared by taking (b) 3 mL, (c) 2 mL and (d) 1 mL of (a), respectively, and making up the volume to 4 mL with n-hexane.

For polar lipids (e) 4 mg each of phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol were dissolved in 10 mL of chloroform. Further dilutions were prepared by taking (f) 3 mL, (g) 2 mL and (h) 1 mL of (e) and making up the volume to 4 mL with chloroform.

When not in use, the standard solutions were preserved at -20°C under nitrogen.

Sample Solution

About 100 mg each of ground nut oil, cod liver oil and soyabean oil were accurately weighed and taken in separate Teflon®-lined screw capped graduated test tubes and dissolved in 10 mL of n-hexane. The lipid solutions were preserved under nitrogen at -20°C when not in use.

Mobile Phase

(1) Non polar lipids: A mixture of n-hexane:diethylether:acetic acid. (80:20:1, V/V).
(2) Polar lipids : A mixture of chloroform:methanol:water, (65:25:4, V/V).

PROCEDURE

For assay by TLC-scanning densitometry, 1 μL each of test solutions and standard solution (c or g) were applied as separate, compact spots 10 mm apart on an imaginary line 15 mm (on the sample concentration zone) from the bottom of the plate. The plate was developed up to 15 cm in usual way in a filter paper lined tank, previously saturated with mobile phase 1 (non-polar lipids) or 2 (polar lipids).

For HPTLC, 100 nL of the sample was spotted 5 mm apart on a line 10 mm from the bottom of the plate and the plate was developed up to 5 cm. After development, the plate was dried in a current of warm air from a hair drier for

10 minutes. The different lipid spots were visualized by exposing the plates to iodine vapor in an iodine chamber (at 30°C) for 2 minutes. The plate was immediately covered with a scratch-free clear glass plate about 1 mm thick and of same size (a used precoated glass plate from which adsorbernts were carefully removed and was thoroughly cleaned, may be used). The edges of the sandwiched plates were sealed with an adhesive tape and the plate was scanned in the densitometer using reflection mode at the scanning wavelength of 365 nm, background correction wavelength of 650 nm, slit 0.4 mm x 0.4 mm (0.05 mm x 0.4 mm for HPTLC) in zig zag mode with auto zero mode on. Other parameters were set as given in the operation manual of the instrument. The amounts of the different lipid components were estimated from the area values obtained.

For assay by spectrophotometry after TLC , laboratory drawn plates (B) were used. The standard and the samples (20 μL) were applied as separate 10 mm-wide bands, 15 mm apart, and developed with solvent system 1 or 2, as the case may be. The different lipid components were visualized by exposing the plate briefly (30 sec) to iodine vapor, marked with a needle and iodine was allowed to evaporate. Adsorbent containing the different components were scraped into separate, scrupulously cleaned 15 mL glass centrifuge tubes. Glycerides and squalene were estimated by a chromic acid reduction method[3] using 0.25% potassium dichromate in 36N sulfuric acid. Sterols were estimated by a ferric chloride colorimetric method[2] and phospholipids were estimated by measuring inorganic phosphorus[1] after digesting the adsorbent containing the lipid fraction with perchloric acid. Suitable silicagel blanks were used in all cases.

To study the linearity of the integrated area values (from densitometer) with concentration of the different lipid fractions, standard solutions b, c, and d or e, f and g were chromatographed as described above and respective area values were determined.

RESULTS AND DISCUSSION

Absorption spectra of the lipid fractions (triglycerides), visualized by iodine vapor, showed an absorption maximum at about 365 nm. At 650 nm, the absorption was negligible (data not shown). Thus, we used 365 nm as scanning wavelength. Scanning at 650 nm may be used to compensate the noise caused by irregularities of the adsorbent layer when laboratory drawn plates are used. Precoated plates used by us did not require any such correction.

Table 1

Linearity of Concentrations of Different Lipids with Integrated Area Value Obtained by Densitometry after TLC and HPTLC

Lipid	Integrated Area*				Integrated Area*			
	Amt. Appl. (μg)	Average	SD	'r'	Amt. Appl. (μg)	Average	SD	'r'
Triglycerides	5	319686	24021	0.9991	0.5	9300	676	0.9992
	10	617990	27804		1.0	19403	1151	
	15	934956	37106		1.5	27901	2271	
Steroids	1	30576	3090	0.9973	0.1	2049	163	0.9944
	2	60481	1613		0.2	4127	158	
	3	91920	1650		0.3	6114	260	
Squalene	-	-	-	-	0.05	2296	95	0.9853
	-	-	-		0.1	4485	225	
	-	-	-		0.15	6657	502	
Phosphatidyl Inositol	2	20274	628	0.9980	0.2	2745	123	0.9983
	3	30681	570		0.3	4126	78	
	4	40828	732		0.4	5568	81	
Phosphatidyl Choline	2	54286	550	0.9995	0.2	12817	52	0.9919
	3	81430	670		0.3	17162	149	
	4	109169	1184		0.4	23168	913	
Phosphatidyl Ethanolamine	2	26406	543	0.9967	0.2	3523	59	0.9941
	3	40642	564		0.3	5023	69	
	4	52333	1028		0.4	7008	228	

* Average of 4 determinations.

When iodine vapor is used to visualize lipids on TLC plates, the background may also absorb iodine. This happens, generally, due to incomplete removal of developing solvents or contaminations of the plate by, e.g., laboratory fumes or simply by passive absorption iodine by silicagel. The colored background may cause a non-linear shift in baseline and error may occur in the integrated peak areas. In most of the cases, washing the plate by developing with methanol before activation and thorough drying of the plate after development reduces the background color. However, use of an auto zero system (background correction) also helps to rectify these problems.

Quantitative analysis by scanning densitometry is a very convenient method for assay of compounds fractionated by TLC. However, as was stated before, non-uniform density of the sample spot across the measuring beam and a nonlinear relationship between the sample concentration and optical density

Table 2

Stability of Lipid Iodine Color* on TLC Plates

Lipid Class	Time (minutes) 0	60	120	180
Phosphatidyl Inositol	27984	27350	26990	26961
Phosphatidyl Choline	53389	53041	53002	53106
Phosphatidyl Ethanolamine	19825	19287	19240	19195
Ergosterol	30991	-	31118	30893
Triolein	344108	-	348700	344316

* Measured as Integrated Area

Table 3

Assay Values[a] of Different Lipid Classes Determined by the Proposed and Conventional Methods

Sample	Lipid Class (b)	Proposed Method TLC Average	TLC SD	HPTLC Average	HPTLC SD	Conventional (c) Average	Conventional (c) SD
Arachis Oil	Sterols	21.9	7.8	21.0	1.0	21.7	1.0
	TG	641.7	14.4	659.1	15.6	652.4	10.9
Cod Liver	Sterols	26.4	0.9	26.6	1.0	27.2	0.9
	TG	818.7	37.2	820.0	25.7	788.7	11.2
Soya Bean Oil	Sterols	24.4	1.5	23.2	1.8	24.3	1.0
	TG	720.8	26.1	722.2	28.6	713.9	19.7
	PI	15.7	0.4	15.5	0.2	16.2	0.4
	PC	68.1	4.4	68.4	2.7	69.8	1.3
	PE	20.2	0.4	20.0	0.3	19.2	0.1

a. In mg/gg oil, average of 4 determinations.
b. TG: Triglyceride; PI: Phosphatidyl Inositol; PC: Phosphatidyl Choline; PE: Phosphatidyl Ethanolamine.
c. See text for procedures.

(obtained in reflectance or transmittance mode) often leads to erroneous results, particularity when area and amount of sample differ from those of the standard. To solve these problems, we scanned the whole spot with a narrow beam of

light and linearized the relationship between reflection absorption and concentration by using a working curve linearizer, programmed by a microcomputer on the basis Kubelka-Monk theory.[15,21,22]

Table 1 shows the relationship between integrated area values and concentrations of different lipid fractions. The area values are linear with amounts of lipids used. Relative percentages of different lipid components, computed directly from the area values for a sample, may differ from those obtained after estimation of individual components using respective standards.

This happens because different components absorb iodine with different intensities. Different migration distances also cause the intensity to vary. However, within same lipid classes, the integrated area values obtained by both methods are comparable.

Intensity of color of lipids visualized by iodine vapor decreases rapidly if proper precaution is not taken. In our method, where the TLC plate is covered with a glass plate and then scanned, the color is stable, at least, for 3 hours (Table 2) whereby quantitation by scanning densitometry is reproducible.

Table 3 shows the content of different lipid components of some naturally occurring lipids determined by the proposed and some conventional methods. It is apparent that results obtained by the proposed densitometric method are essentially the same as those obtained by the conventional methods.

Using appropriate reference standards, the fractionated lipid components can be precisely determined with a CV of about 2 to 4%.

Densitometry is manipulatively much easier and, since same method is used for estimation of all the lipids, estimation of different lipid classes is much more simplified. Use of iodine vapor is a non-destructive method. Thus, after quantitation, we could successfully identify the different lipid components, particularly the phospholipids by using ninhydrine, Dragendorff's and molybdenum blue[19] reagents successively on the same plate.

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RAPID DETERMINATION OF PAHs IN DRINKING WATER SAMPLES USING SOLID-PHASE EXTRACTION AND HPLC WITH PROGRAMMED FLUORESCENCE DETECTION

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ABSTRACT

A rapid, sensitive and selective method for determining 13 PAHs in drinking water samples using solid-phase extraction and HPLC with programmed fluorescence detection is developed. A solid-phase extraction method is described for preconcentrating the PAHs on Sep-Pak vac tC-18 cartridges. The volume of water analyzed was 1500 mL. The PAHs were eluted with ethyl ether, the eluates were evaporated to dryness and the residue was dissolved in methanol. The PAHs were analyzed on a Hypersil Green PAH column and a program of nine excitation and emission wavelength pairs were used. A mobile phase gradient of acetonitrile-water was used. It is possible to detect all the individual PAHs at very high sensitivity, at levels of ng/L. Recoveries were 60-96% for 12 PAHs at concentration levels of 2.33-48.7 ng/L with relative standard deviations in the range 0.4-10% (n=4). The method was applied to determine PAHs in tap-water and reservoir-water samples.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants that represent the largest class of suspected chemical carcinogens. PAHs can be formed from both natural and anthropogenic sources. PAHs are produced by the incomplete combustion and pyrolysis of fossil fuels among other organic materials. The carcinogenic and toxic nature of PAHs, specifically benzo(a)pyrene and dibenzo(ah)anthracene, have increased the need to detect these compounds. The presence of PAHs in polluted air has been extensively studied but water has received much less attention. The distribution of PAHs in different bodies of water is dependent on their sources and the PAH solubilities in water.[1-3]

The analytical procedures most often used for PAH determination include: **a)** isolation of PAHs from water by solid phase extraction (SPE), such as reverse phase silica, polystyrene-divinyl benzene,[4-7] or **b)** liquid-liquid extraction (LLE) with organic solvents such as cyclohexane, n-hexane or methylene chloride.[6,8-11] The analytical techniques used to separate and determine them are gas chromatography (GC) with flame ionization (FID) or mass spectrometry (MS) detectors[7,9] and reverse phase high performance liquid chromatography (RP-HPLC) with spectrophotometric (UV-VIS) or fluorimetric (FL) detection.[11-13]

Because of the poor chromatographic resolution of some PAHs pairs, such as fluorene-acenaphthene and chrysene-benzo(a)anthracene, and the very low PAH concentrations in water samples, the combination of off-line RP-SPE with RP-HPLC-FL is attractive.

In a previous paper we reported a sensitive, selective method for fluorimetric detection which allows ng/L concentration levels to be determined using the Hypersil Green PAH column.[14] In this paper, we present a HPLC method for determining PAHs in drinking water using solid-phase extraction. To detect the PAHs, the excitation and emission wavelengths were time programmed over the chromatogram.

EXPERIMENTAL

Apparatus and Materials

The chromatographic system consisted of the following components: a Milton-Roy CM 4000 high-pressure-gradient pump (Rivera Beach, FL); a Rheodyne 7125 with 20 μL loop injector (Cotati, CA); a Perkin Elmer LS 30 luminiscence spectrometer (Norwalk, CT) and a Milton Roy CI 4100 integrator. The column

used was a Hypersil Green PAH (100 x 4.6 mm) (5 μm particulate size) by Shandon (England); a P-Selecta Precisterm bath was used to maintain the column temperature below 22 °C (Barcelona, Spain). A Sep-Pak vac tC-18 (500 mg, Waters; Milford, MA) and Extra-sep C-18 (1000 mg, Phenomenex) (Torrance, CA) cartridges were used to extract and preconcentrate the PAHs from water samples. A vacuum flask (1000 mL, Pobel; Madrid, Spain); separatory funnels (100, 1000 mL, Pobel); a Barna-vacio vacuum pump and a Visiprep vacuum manifold system (Supelco; Bellefonte, PA) were also used. All PAHs solutions were prepared using a P-Selecta ultrasonic bath. Solvents used to prepare the mobile phase and sample eluates were filtered through nylon Lida membrane filters (Kenosha, WI) with 0.45 μm pore size. A P-Selecta Meditronic centrifuge capable of 4200 rpm (3700 g) was also used.

Chemicals

Stock standard methanolic solutions of the PAHs with concentrations in the range $(10\text{-}1.0)\times10^{-4}$ M were prepared by dissolving the solids (Sigma; St. Louis, MO) in methanol. A working standard mixture was prepared by dilution of the stock standard solutions with methanol. HPLC purity acetonitrile, methanol and ethyl ether (Carlo Erba; Milan, Italy) were used. The other solvents and chemical reagents were also of HPLC purity. Water was purified with a Millipore Milli-Q system (Milford, MA).

Procedures

1. Water sample collection:

Tap-water samples were collected in the Faculty of Chemistry of the Universidad Complutense in Madrid city, and reservoir-water samples were collected from three reservoirs in Madrid (Picadas, La Pinilla and Valmayor). All samples were collected during the winter 1995, in amber bottles, previously cleaned with acetonitrile, in order to minimize photolytic decomposition, and were stored in a refrigerator at 4 °C from the time of collection until extraction. All samples were extracted immediately, within 24 hours of collection, and were then analyzed.

2. Sample preparation:

On receipt, the samples were filtered with nylon filters and extracted immediately with Sep-Pak vac tC-18 cartridges. The cartridges were previously conditioned with 6 mL of methanol, twice, and then with 6 mL of Milli-Q water, twice. 1500 mL of Milli-Q water containing the PAH mixture in the range 3.5-73

Table 1

Linear Gradient of the Mobile Phase

Time	Acetonitrile, %	Water, %
0.0	50	50
3.0	50	50
15.0	78	22
23.0	100	---
35.0	100	---
37.0	50	50

ng, or 1500 mL of water samples were preconcentrated with the cartridges at a flow rate of 50 mL/min. The cartridges were then dried in the vacuum system for 5 minutes, and then centrifugated at 1200 rpm for another 5 minutes. The adsorbed compounds were eluted from the Sep-Pak vac cartridges first with 3 mL, then with 1 mL of ethyl ether, at a flow rate of 1.5 mL/min.

The eluates were collected in a graduated glass tube and the solvent was evaporated by means of the vacuum manifold system; since solvent evaporation is an energy absorption process, in order to maintain the temperature constant at 20 ± 2 °C, the tube containing the eluate was inserted in a glass containing water at 20 ± 2° C. The residue was dissolved to 1 mL in methanol using the ultrasonic bath. This solution was filtered through nylon filters and analyzed by HPLC by injecting 20 μL into the HPLC system and applying the calibration procedure.

3. Calibration:

Standard solutions containing mixtures of the 13 PAHs were prepared at four concentrations levels in the range 0.4-150 μg/L. These solutions were analyzed by RP-HPLC with fluorimetric detection. In a previous paper, we have developed this chromatographic method.[14] The chromatographic parameters are the following: an acetonitrile-water mobile phase using the gradient is shown in Table 1, at a flow-rate of 1 mL/min at 22 °C.

Fluorescence detection was performed with time programming of excitation and emission wavelength pairs (the program is detailed in Table 2). The injection volume was 20 μL. The integrated peak areas were used to quantitate the PAHs. The mobile phase was degassed with helium. Figure 1 shows a chromatogram of a standard mixture of 13 PAHs.

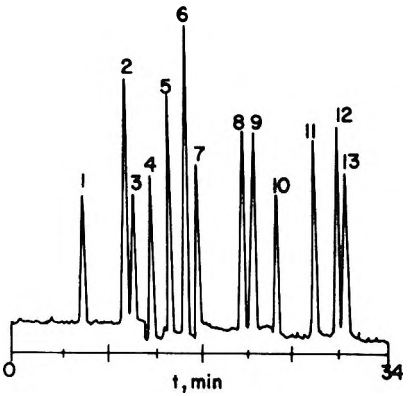

Figure 1: Chromatogram of a standard mixture of 13 PAHs. Conditions: Hypersil Green PAH (100 x 4.6 mm) column; Temperature, 22°C; Mobile phase, gradient of acetonitrile/water, see Table 1; Flow-rate, 1 mL/min; Fluorimetric detection, see Table 2; Injection volume, 20 µL. Peaks: 1, naphthalene; 2, acenaphthene; 3, fluorene; 4, phenanthrene; 5, anthracene; 6, fluoranthene; 7, pyrene; 8, B(a)a; 9, chrysene; 10, B(e)p; 11, B(a)p; 12, Db(ah)a; 13, B(ghi)p.

Table 2

Program of Excitation and Emssion Wavelength Pairs

Detected Compound	Time, s	λ_{ex}, nm	λ_{em}, nm
Naphthalene, Acenaphthelene			
Fluorene	0	280	324
Phenanthrene	720	250	365
Anthracene	840	254	402
Fluoranthene	920	285	465
Pyrene	990	270	390
B(a)a, Chrysene	1230	270	384
B(e)p	1410	290	390
B(a)p	1590	295	405
Db(ah)a, B(ghi)p	1755	290	418

B(a)a, Benzo(a)anthracene; B(e)p, Benzo(e)pyrene; B(a)p, Benzo(a)pyrene; Db(ah)a, Dibenzo(ah)anthracene; B(ghi)p, Benzo(ghi)perylene

Table 3

Influence of Eluent Type on the Recoveries of PAHs

	Recoveries %*									
PAHs	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Naphthalene	19	42	--	--	14	5.4	--	--	--	--
Acenaphthene	33	24	--	--	10	6.3	--	--	--	--
Fluorene	26	18	--	--	16	8.7	1.3	--	--	--
Phenanthrene	43	24	45	56	33	18	54	47	11	13
Anthracene	43	19	44	53	21	30	49	84	11	12
Fluoranthene	41	20	52	40	44	22	50	66	46	44
Pyrene	44	25	67	44	42	30	36	80	98	28
B(a)a	35	18	67	86	31	25	42	57	61	54
Chrysene	30	15	65	81	30	23	36	56	58	60
B(e)p	19	12	67	74	29	27	36	85	97	63
B(a)p	19	13	68	78	28	31	41	53	72	55
Db(ah)a	33	27	91	74	34	51	47	55	57	59
B(ghi)p	17	13	80	71	25	32	35	55	80	63

* Mean of two determinations

1, Acetontrile; 2, Methanol; 3, Methylene chloride, 4, Ethyl ether; 5, n-Hexane; 6, n-Pentane; 7, n-Pentane/Ethyl ether (0.6/10, v/v); 8, Methylene chloride/Ethyl ether (1/1, v/v); 9 and 10, Methanol/Ethyl ether (1/1 and 1/2, v/v, respectively).

RESULTS AND DISCUSSION

Optimization Of Solid Phase Extraction (SPE) Experimental Conditions

1. Selection of the eluent:

Various solvents were tested for PAH elution on SPE: acetonitrile, methanol, methylene chloride, ethyl ether, n-hexane and n-pentane, and the following mixtures (v/v): n-pentane/ethyl ether(0.6/10), methylene chloride/ethyl ether (1/1) and methanol/ethyl ether (1/1 and 1/2). 1 mL of eluent was used. The results are shown in Table 3. The best results, for the majority of PAHs, were obtained with methylene chloride and ethyl ether; the mean recoveries were 50% and 51% for methylene chloride and ethyl ether, respectively.

Table 4

Influence of Water Volume on the Recoveries of PAHs

PAH	Recoveries, %* Water Volume, mL							
	50	**100**	**250**	**500**	**1000**	**1500**	**2000**	**1000****
Naphthalene	--	--	--	18	25	20	--	--
Acenaphthene	17	32	42	59	57	65	73	19
Fluorene	35	51	55	70	68	72	91	36
Phenanthrene	64	74	82	80	76	96	75	60
Anthracene	51	53	68	70	64	71	73	85
Fluoranthene	67	78	81	77	78	95	94	86
Pyrene	42	60	72	66	64	89	46	65
B(a)a	57	65	76	74	71	95	79	87
Chrysene	58	66	75	71	70	76	73	87
B(e)p	47	57	63	65	64	85	60	82
B(a)p	46	41	52	57	51	67	56	92
Db(ah)a	39	45	50	53	54	66	66	57
B(ghi)p	38	39	39	48	46	60	38	55
Mean Recoveries	43	51	58	63	63	74	63	62

* Mean of four determinations. Relative standard deviations are in the range of 0.4 to 10.

** Values obtained by applying the clean-up procedure recommended in the 525 EPA method.

2. Selection of the eluent volume:

The recoveries are a function of the number and eluent volume of the desorption steps. Volumes in the range of 1-4 mL of the two solvents were tested. Where 50%, 53%, 68% and 64% has been the mean recoveries of 1, 2, 3 and 4 mL of methylene chloride, respectively, and 51%, 74%, 79% and 76% has been the mean recoveries of 1, 2, 3 and 4 mL of ethyl ether, it was observed that best results were achieved with 3 mL of ethyl ether, with a mean recovery of 79% (n = 2). However, to improve reproducibility the elution was carried out in two steps. First eluting with 3 mL, then with 1 mL of ethyl ether, collecting the two eluates in a tube. For the selection study of nature and volume of eluent, 100 μL of standard mixture of PAHs in the range 35-730 μg/L were eluted through the tC-18 cartridge.

3. Selection of volume of water sample flow through Sep-Pak:

To improve recovery of the PAHs, several volumes between 50 to 2000 mL of Milli-Q water containing amounts of PAHs in the range 3.5-73 ng were tested. Table 4 shows the results obtained. Volumes above 250 mL gave mean recoveries of over 50 %. The best recoveries are achieved with 1500 mL of Milli-Q water, recoveries being in the range 60-96% for 12 PAHs. The low recovery obtained for napthalene (20%) is due to its volatility especially during the concentration of the eluates by evaporation of the solvents. These results are similar to those reported in the literature.[15-18]

The relative standard deviations were in the range 0.4-10% (n=4). The results were compared with those obtained by applying the procedure recommended in the EPA 525 Method,[7] using 1000 mL of water, which also are shown in Table 4. In general, higher recoveries were obtained by applying our method, except for the following PAHs: anthracene, chrysene and benzo(a)pyrene.

4. Effect of PAH concentration in water samples:

1500 mL of water were spiked with 200, 100, 50 and 25 μL of PAHs standard mixtures at concentration levels of 35-730 μg/L. The PAH concentrations and the PAH recovery results are shown in Table 5. The best mean recoveries were 74% and 73% corresponding to the PAH concentration levels of 2.33 - 48.7 ng/L and 1.16 -24.4 ng/L, respectively. The relative standard deviations were in the range 0.4 - 19% (n=4).

Determination of PAHs in Drinking Water

The proposed method was used to determine the 13 PAHs in two types of drinking water sample: tap-water and reservoir-water. Three tap-water samples were analyzed; in these samples no PAHs were detected. On the other hand, three samples from three reservoirs, indicated in experimental section, were also analyzed. A chromatogram corresponding to sample 1 (Picadas reservoir) is shown in Figure 2; in the chromatogram peaks were detected at the retention times of the PAHs, the first being naphthalene and the last, chrysene.

The results corresponding to three reservoir-water samples are summarized in Table 6, which also shows those obtained by applying the EPA clean-up procedure. In general the results of proposed method are higher than those obtained by applying the EPA Method 525, because the recoveries are also smaller for the latter method. Relative standard deviations were determined from four replicates on the same

Table 5

Influence of PAH Concentration in the Study of PAH Recoveries from Water Samples*

PAHs	C_1, ng/L	R_1, %	C_2, ng/L	R_2, %	C_3, ng/L	R_3, %	C_4, ng/L	R_4, %
Naphthalene	97.4	11	48.7	20	24.4	16	12.2	–
Acenaphthene	48.0	73	24.0	65	12.0	45	6.00	29
Fluorene	22.6	66	11.3	72	5.65	78	2.83	30
Phenanthrene	14.7	60	7.33	96	3.66	95	1.83	72
Anthracene	12.0	66	6.00	71	3.00	54	1.50	46
Fluroanthrene	5.34	86	2.67	95	1.34	82	0.670	82
Pyrene	6.66	68	3.33	89	1.66	93	0.830	48
B(a)a	9.34	68	4.67	95	2.34	81	1.17	73
Chrysene	17.3	83	8.67	76	4.34	91	2.17	78
B(e)p	14.7	80	7.33	85	3.66	84	1.83	38
B(a)p	4.66	70	2.33	67	1.16	56	0.580	43
Db(ah)a	10.7	71	5.33	66	2.66	79	1.33	64
B(ghi)p	9.34	73	4.67	60	2.34	90	1.17	–
Mean Recoveries		67		74		73		46

Water volume: 1500 mL; R, %: percentage recovery.

* Mean of four determinations. Relative standard deviations are in the range of 0.4-19%.

C_1 , C_2, C_3 and C_4: PAHs concentration in spiked water samples.

sample; the values were between 3-15%. The amounts of PAHs in the samples are below the maximum permitted limits of 0.2 μg/L.[19] As can be seen in Table 6, the high molecular weight PAHs, such as benzo(e)pyrene, benzo(a)pyrene, dibenzo(ah)anthracene and benzo(ghi)perylene, which are the most toxic, were not detected. These results can be explained on the basis that these PAHs are very insoluble in water, there being a rough correlation between increasing molecular weight and decreasing solubility, and therefore it must be assumed that a large proportion of the PAH content of polluted water is adsorbed on suspended solids.[1,3]

CONCLUSIONS

The proposed procedure for the extraction and preconcentration of PAHs in water samples is faster than the EPA Method 525, due to the amount of packing material used in cartridges. Additionally, it affords a large reduction in the volume

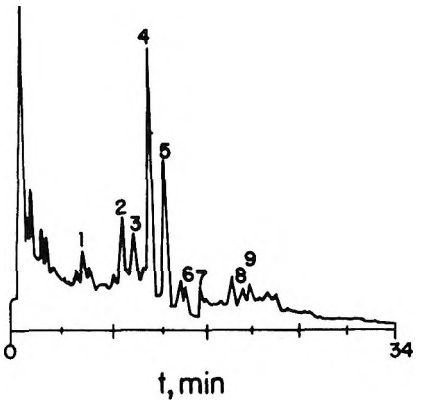

Figure 2: Chromatogram of a Picadas reservoir-water sample. Conditions: Hypersil Green PAH (100 x 4.6 mm) column; Temperature, 22°C; Mobile phase, gradient of acetonitrile/water, see Table 1; Flow-rate, 1 mL/min; Fluorimetric detection, see Table 2; Injection volume, 20 µL. Peaks: 1, naphthalene; 2, acenaphthene; 3, fluorene; 4, phenanthrene; 5, anthracene; 6, fluoranthene; 7, pyrene; 8, B(a)a; 9, chrysene.

Table 6

Determination of PAHs in Reservoir-Water Samples. (ng/L)*

PAH	Samples** 1		2		3	
	A	**B**	**A**	**B**	**A**	**B**
Naphthalene	4.8	3.8	--	--	--	--
Acenaphthene	3.6	2.2	2.3	--	13	--
Fluorene	2.0	1.8	1.3	3.6	6.7	--
Phenanthrene	5.1	3.1	5.6	4.4	2.8	0.9
Anthracene	1.2	3.5	0.1	0.1	1.1	1.8
Fluoranthene	0.04	0.06	0.1	--	0.6	0.1
Pyrene	0.1	0.3	1.1	0.8	0.8	0.2
B(a)a	0.1	0.4	--	--	1.1	0.4
Chrysene	0.3	0.4	--	--	1.0	1.2

*Mean of four determinations. Relative standard deviations are in the range 3-15%.
**Samples collected from three reservoirs in Madrid during the winter 1995:
1) PICADAS; **2)** LA PINILLA; **3)** VALMAYOR. **A**: Proposed method;
B: Applying the clean-up procedure recommended in 525 EPA Method.

of solvent required and, consequently, is very economical. The chromatographic method allows sensitive, selective determination of PAHs in drinking water samples at ng/L levels, due to use of the Hypersil green PAH column and the excitation and emission wavelength pairs program.

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A MODIFIED REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE MEASUREMENT OF HEPATIC CHOLESTEROL 7α-HYDROXYLASE ACTIVITY WITH A FILTER UV DETECTOR

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ABSTRACT

Hepatic cholesterol 7α-hydroxylase (CH7OH) is the first and rate-determining enzyme for the biosynthesis of bile acids from free cholesterol in the liver cells. It is important in the cholesterol metabolism, and in the formation of cholesterol gallstones in humans. A rapid reversed phase high performance liquid chromatographic (HPLC) procedure for the measurement of hepatic CH7OH was modified from an HPLC-spectrophotometric method (Chiang, J.Y.L., Meth. Enzymol., 206: 483-91,1991) and validated. A shorter column, a one-component mobile phase (100% acetonitrile), a higher flow rate, and a filter UV detector equipped with 254 nm wavelength were used in this modified procedure. The peaks of reaction products of 20α-, 7α- and 7β-hydroxycholesterol were resolved at baseline with retention times of 9, 10, and 11 min respectively. 20α-hydroxycholesterol was used as internal standard. A peak due to reaction product of 7α-hydroxycholesterol was validated by

retention time and spiked test. The linearity of the reaction product of 7α-hydroxycholesterol is up to at least 1250 pmole. Compared to the original procedure, this modified procedure is simpler (single vs binary component mobile phase), faster (22 vs 30 min of running time), and it does not require a variable wavelength UV detector, and it still retains the advantages of the original procedure.

INTRODUCTION

One of the key enzymes of cholesterol metabolism in the liver is cholesterol 7α-hydroxylase (CH7OH), which is the first and rate-determining enzyme for the biosynthesis of bile acids from free cholesterol in the hepatocytes.[1-3] This metabolic pathway is one of the two pathways for cholesterol excretion from liver. CH7OH has been shown to be a cytochrome P450 isozyme and it is down-regulated by bile acids returning to liver via enterohepatic recirculation.[4,5]

Three procedures have been used to analyze hepatic CH7OH activities: isotope incorporation method,[6] GC/MS method,[7] and high performance liquid chromatographic (HPLC)-spectrophotometric method.[8-11] The isotope method involves the use of radioactive material, [^{14}C]cholesterol, and it is interfered by endogenous substrate. GC/MS method is sensitive and specific but it needs an expensive instrument and special technical skills. HPLC-spectrophotometric procedure is a simple and reliable method and without the requirement of radioactive material and expensive instrument. However, it requires a variable wavelength UV detector to monitor response at 240 nm and it takes at least 30 minutes to have a chromatographic run. Here, a modified procedure of Chiang's HPLC-spectrophotometric method[11] was developed. This modified procedure uses a filter UV detector (254 nm), has a shorter running time and a simplified mobile phase.

MATERIALS

All reagents except those specified were obtained either from Sigma Chemical Co.(St. Louis, MO) or from Aldrich Co. (St. Louis, MO). 7α-hydroxycholesterol (7α-HOC) was from Steraloids (Wilton, NH). Cholesterol oxidase was bought from Calbiochem Co. (San Diego, CA). Absolute ethanol was from J.T. Baker Co. (Phillisburg, NJ). Acetonitrile and water were of HPLC grade (CMS, Houston, TX).

METHODS

HPLC System

The HPLC system (Waters) included an U6K injector, an M6000A solvent delivery system, an M400 absorbance detector (254 nm for sample analysis and 254/280 nm for the identification of a contaminant peak), an 820 Maxima work station, a reversed phase C_{18} column (Ultrasphere, 150X4.6 mm, ID. from Beckman Instruments, Fullerton, CA) and a Beckman C_{18} Ultrasphere guard column (45X4.6mm, ID.). Mobile phase is 100% acetonitrile, degassed by vacuum for 30 minutes and pumping at 1.0 mL/min flow rate during the first 12 minutes. The flow was increased to 3.0 mL/min between 12-13 min and then it was maintained until 21 min. It was changed back to 1.0 mL/min between 21 and 22 min for the next analysis. All analyses were performed in ambient temperature.

Preparation of Microsomes and Measurement of Enzyme Activity

Two rat livers were from Sprague-Dawley. Human liver biopsies (n=38) were obtained from subjects undergoing gastric bypass to treat their obesity. Patient consent was obtained and the protocol was approved by the Institutional Review Board of Louisiana State University Medical Center. Microsomal fraction was prepared from homogenized liver tissue by differential ultracentrifugation[10,11] and described briefly as follows. An aliquot of liver (0.5-1 g) was washed in isolation buffer once and minced with scissors to small pieces. Tissue pieces in isolation buffer were homogenized with Polytron and centrifuged at 10,000 g for 10 min twice to obtain supernatants. Microsomal fraction was isolated by centrifuging the collected supernatant at 105,000 g for 2 hours. The pellet was homogenized in resuspension buffer and recentrifuged at 105,000 g for 1 hr. Final pellet was homogenized in isolation buffer. Protein was assayed by Lowry's method[12] with bovine serum albumin as the standard. Enzyme activity was assayed by Chiang's method.[10,11] Microsomal fraction (0.5-1.0 mg protein) was incubated with cholesterol (10 mM) in buffer solution for 20 min at 37°C. Reaction was stopped with sodium cholate and 20α-hydroxycholesterol (20α-HOC) was added as an internal standard (IS). Cholesterol oxidase solution was added to change 7α-HOC and 20α-HOC to 7α-hydroxy-4-cholesten-3-one (7α-HCO) and 20α-hydroxy-4-cholesten-3-one (20α-HCO) respectively under 37°C for 10 min. The reaction mixture was extracted with 6 mL of petroleum ether three times. Petroleum ether extracts were pooled, dried under nitrogen in 37°C water bath and the residue was stored at -70°C until analysis. Residues were reconstituted with 100 uL mobile

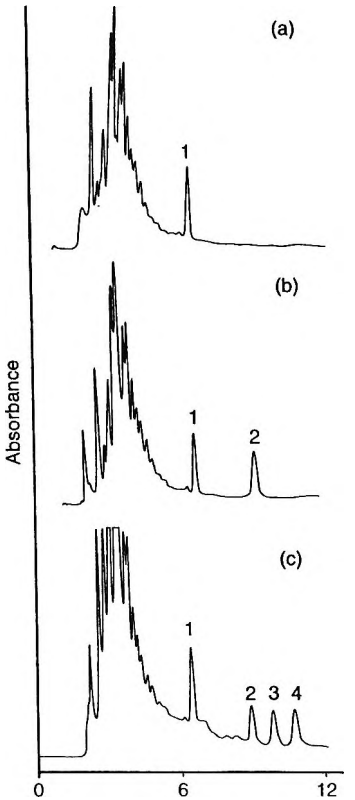

Figure 1. Chromatograms of a) reagent background, b) a sample of internal standard (20α-HOC), and c) a sample with 20α-HOC, 7α-HOC, and 7β-HOC added. Peak identification: 1= contaminant, 2= 20α-hydroxy-4-cholesten-3-one (20α-HCO), 3= 7α-hydroxy-4-cholesten-3-one (7α-HCO), and 4= 7β-hydroxy-4-cholesten-3-one (7β-HCO).

phase and aliquots (20-40 μL) were injected into HPLC for analysis. Both 7α-HCO and 20α-HCO reaction products have maximal absorption near 240 nm. Five calibration standards containing 7α-HOC (0-1250 pmole) with IS (2500 pmole each) added were constructed to evaluate linearity. Detectability of 7α-HCO was determined by injection of 20 uL of different dilutions of a calibrator (250 pmole). Enzyme activity of each sample was corrected with individual sample blanks, which measured the level of 7α-HOC at the 0 time of incubation.

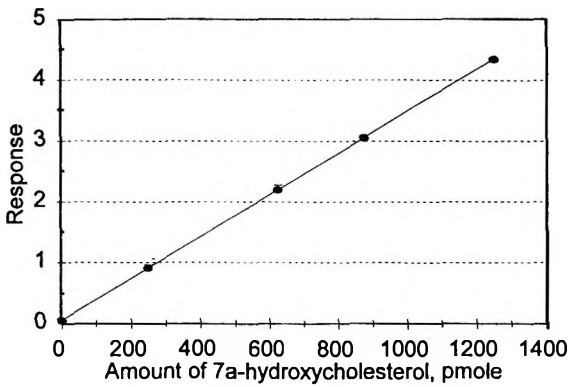

Figure 2. Calibration curve for 7α-hydroxycholesterol

Table 1

Comparison of this Method with Chiang's Method

Items	Chiang's Method	This Method
Column length	250X4.6 mm, ID.	150X4.6 mm, ID.
Mobile Phase	Acetonitrile methanol (70%:30%)	100% Acetonitrile
Flow rate	0.8-2.0 mL/min	1.0-3.0 mL/min
UV Detector/ Wavelength	Variable wavelength 240 nm	Filter 254 nm
Run time	30 min	22 min
Retention time for 20α, 7α, 7β-HCO	9, 12, 13 min	9, 10, 11 min

RESULTS AND DISCUSSION

The summary of this method compared to Chiang's is shown in Table 1. When the mobile phase of Chiang's procedure (acetonitrile:methanol = 7:3) was tested with a shorter column (150x4.6 mm, ID) and a flow rate of 1.0 mL/min, we could not obtain a similar separation. A contaminant peak coeluted with the peak of 20α-HCO. This contaminant peak is completely separated from 20α-HCO peak when 100% acetonitrile is used as mobile phase,

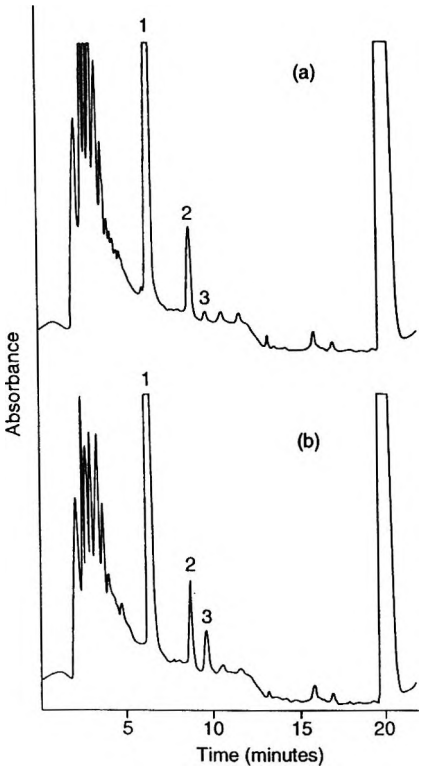

Figure 3. Chromatograms of a) CH7OH activity of a microsomal fraction from human liver sample, b) 7α-HCO spiked sample of a). Peak identification: 1= contaminant, 2= 20α-hydroxy-4-cholesten-3-one (20α-HCO), and 3= 7α-hydroxy-4-cholesten-3-one (7α-HCO).

(Figure 1). The contaminant peak has absorption at both 254 nm and 280 nm but peaks of 20α-HCO and 7α-HCO have absorption at 254 nm but not of 280 nm. This makes it being easily differentiated from 20α-HCO and 7α-HCO peaks. This contaminant is present in every sample including the reagent background and the source of it is not investigated.

The flow programming was optimzed in order to shorten the run time and still keep a baseline separation for 7α-HCO and IS. When the flow rate was of 2.0 mL/min the peaks of 20α-HCO and 7α-HCO were partially overlapped. Therefore, the flow rate of 1.0 mL/min was chosen for the first 12 min of the chromatographic run in this study. Selection of a shorter column and use of

100% acetonitrile as mobile phase also allowed us to increase the flow to 3.0 mL/min after 12 min to elute late peaks before 22 min without having high back pressure (pressure ≤ 3000 psi).

Under these modified conditions, we can have a chromatographic run of 22 min and it still has a baseline separation of peaks of 20α-HCO, 7α-HCO, and 7β-hydroxy-4-cholesten-3-one (7β-HCO) with retention times of about 9, 10, and 11 minutes respectively (Figure 1). 7β-HOC is an alternate IS in Chiang's method. The response of (7α-HCO)/(20α-HCO) is linear up to at least 1250 pmole of 7α-HOC (Figure 2). Detectability of 7α-HCO is 12.5 pmole (at S/N=5) by this procedure. This HPLC procedure was used to measure hepatic CH7OH in rats and humans. We obtained hepatic CH7OH activities of 6.0 and 8.8 pmole/min/mg protein in two rats and activities ranging from 0 to 16.4 pmole/min/mg protein (mean±SEM =4.02±0.59, n=38) in obese humans. These values are comparable to those of other studies.[2,7,11] Chromatograms of a human sample and 7α-HCO spiked human sample are shown in Figure 3. The spiked chromatogram confirms the identity of 7α-HCO peak in the sample.

Therefore, a modified HPLC procedure was developed to measure hepatic CH7OH in rats and humans. Compared to original procedure, this HPLC procedure is simpler (single vs binary component mobile phase), quicker (22 vs 30 minutes of chromatographic run), and it does not require a variable wavelength UV detector and still retains the advantages of the original procedure.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSES OF SULPHONAMIDES AND DIHYDROFOLATE REDUCTASE INHIBITORS. IV. RECOVERIES FROM THE STATIONARY PHASE, QUANTITATION AND SENSITIVITIES

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ABSTRACT

Of the twenty-two sulphonamides and three commonly used dihydrofolate reductase inhibitors investigated for reverse phase separation in previous studies, the recoveries of five representative drugs from the stationary phase have been examined. Sulphanilamide, sulfisomindine, sulphaquinoxaline, diaveridine and pyrimethamine were chosen. Peak areas off the analytical column were compared with those obtained by substitution with stainless steel tubing. After careful correction for flow rate differences where necessary, 100% recoveries were indicated. As a further check, Maloprim tablets were assayed for pyrimethamine and the analysed results were found to be (99.1 ±

2.9)%. Limits of detection (LOD) for aqueous standards varied from 0.67 (for sulphanilic acid) - 0.03 $\mu g\ mL^{-1}$ (diaveridine). The linear dynamic range extended from the LOD for each drug to >5 $\mu g\ mL^{-1}$.

INTRODUCTION

In the previous papers in this series, the reverse phase retention behaviour of twenty-two sulphonamides(SFA) and the three commonly used dihydrofolate reductase inhibitors(DHFR) have been examined. The objective was to see if a full separation of the 25 drugs could be achieved and HPLC could be used for broad screening purposes. Phosphate buffers were adopted throughout. In general, sulphathiazole (ST) and sulphapyridine (SP) coelute. Also, in the middle of the chromatograms, sulphameter (SM), sulphamoxole (SAM), sulphamethazine (SMAZ), diaveridine (DVD) and sulphamethizole (SMIZ) were only partly separated under the majority of conditions investigated throughout the extended study.[1-3] These five compounds were generally tightly bunched, usually included more than one coelution and often overlapped with the next compounds to elute. In methanol modified mobile phases,[1] in the optimum pH range of 2.7 and 3,[4,5,1] the best gradient separated 19 of the drugs and 2 hydrolysis products with $R_s \geq 1$[6] and 2 more drugs with $R_s \approx 0.9$. ST and SP had $R_s \approx 0.65$, and, SAM and SMAZ were not separated. Higher pHs are generally unfavourable and lead to excessively congested chromatograms. However, due to differences in $pK_{a,2}$ values, SM, SAM, SMAZ, DVD and SMIZ were baseline resolved from each other at pH 6.5. At low phosphate concentrations (0.001 M), the last 10 drugs can be eluted almost perfectly. At higher phosphate concentrations (0.01-0.1 M), the front end of the chromatogram is improved.

Acetonitrile modified mobile phases provided some promising selectivity differences, but these were countered by other losses of resolution.[2] Attempts to incorporate these beneficial differences into MEOH gradients were unsuccessful. Returning to MEOH modified mobile phases, combined flow and solvent programming resolved the first 13 compounds, including the seldom separated ST and SP ($R_s \approx 1.1$).

In the third stage,[3] the effects of a competing base (tertiary butyl ammonium phosphate) were not found to be helpful. However, with the exception of SAM and SMIZ, ion pairing (heptane sulphonic acid at 0.5 and 1.0 mM) allowed the separation of all other pairs of compounds with $R_s \geq 0.9$. The result is clearly superior to any previous HPLC separation.

In summary, the simultaneous separation of all 25 drugs was not achieved. However, most combinations of most of the drugs were separable under some conditions.

The final stage of this study is concerned with quantitation. In spite of the long recognised problems of irreversible adsorption in GSC, related phenomena in LSC (albiet to a lesser degree), tailing and band-broadening on silica and silica-based phases in all areas of chromatography and monumental efforts to produce deactivated silica surfaces and polymeric coatings to cover the silica support, it is often presumed in reverse phase HPLC (RPLC) that what is injected will be detected. In more recent times, it has been accepted that SFE recoveries of non polar substances from solid matricies (for example, polycyclic aromatic hydrocarbons from urban dust, fly ash and river sediment)[7] are greatly enhanced by the presence of co-solvents such as MeOH which compete for active surface sites. And for some of the analytes, recoveries were still poor. For polar analytes, the situation is far worse. Above 10% MeOH in supercritical CO_2, 90% recoveries of SFA from sand were readily achieved.[8] Greater than 90% recoveries were difficult and recoveries only very slowly and asymptotically approached upper limits with increasing time or severity of extraction conditions (pressure, % co-solvent). One hundred per recoveries were never obtained.

In our initial investigations of the separation of SFA and DHFR (using an alternative stationary phase)[5], losses of both classes of analytes occurred. In the case of the SFA, large (46-73%) losses occurred, but the amounts seemed to vary randomly with respect to elution order and the polarity of the SFA. The reduced amounts of the SFA eluted in a normal fashion, in comparable amounts in successive runs. As far as could be determined, the DHFR were not eluted, irrespective of the number of injections or the strength of the mobile phase. Only in the presence of EDTA did the DHFR elute, and then, at the solvent front in amounts dependent upon the concentration of EDTA. In view of the negligible effect of the EDTA upon the retention of the SFA and the retention of DHFR on alternative reverse phases, the implication apppeared to be that the mechanisms of the 'irreversible adsorption' and the reverse phase retention were different. This was interpreted to mean, that EDTA underwent equilibrium chelation with metal impurities responsible for the 'irreversible adsorption' and formed soluble complexes with the EDTA that had a high charge to mass ratio, thus preventing reverse phase retention.

In view of all of the above, the presumption of 100% elution of any of the SFA or DHFR from any silica-based stationary phase would be unwise. However, this is seldom directly checked. Alternative strategies are usually adopted.

The recovery of drugs from complex liquid media such as biological fluids (urine, serum) or tissues is a demanding task and recently published assays for the SFA and DHFR[9-20] are representative of the experimental methods practised generally. Spiking of otherwise identical, but uncontaminated matricies provides a method of estimating recoveries to validate the combined work-up, extraction and liquid chromatographic analytical procedures. Alternatively, where recoveries are not measured, the determination of unknowns relative to spiked, like matrices, removes many of the sources of potential error. All of the assays mentioned above have used spiking. Standard additions - routinely used in atomic absorption but seldom used in LC[10] - would clearly provide a higher level of protection from more subtle effects possible due to an imperfect match of matricies. The (frequent) use of internal standards (IS),[11-13,15,18,20] goes much of the way to fulfilling this role and to compensate for systematic variations in instrumental conditions. However, no IS can perfectly match the target analyte so that there will always be some element of residual risk. An example of this potential problem, is provided by SFE data for the SFA.[8] Recoveries from sand and spiked, homogenised tissues varied greatly between the five SFA investigated. Furthermore, the dependence of recoveries upon extraction variables (%MeOH, pressure, time and matrix) was not the same for each SFA. SMIZ was by far the hardest of the five SFA to extract from sand and might therefore be expected to be most likely to give rise to problems associated with adsorption on the silica support. Recoveries of sulphamethoxazole (SMOX) from sand were far more sensitive to temperature than for the rest. Both of these were difficult to recover from fortified homogenate on sand at temperatures near optimal for SMOX, thus indicating SMOX to be most subject to matrix interactions. As the presumption of 'like physico-chemical behaviour' is made of an IS, detailed knowledge of the interactions of an IS and the target analytes with the intended environments is highly desirable. But this is not generally the case. The usual guidelines of identical functionality and minimal size difference for an IS seem essential. The combination of matrix matching and internal standardisation is likely to eliminate the vast majority of sources of error, especially when combined with checks of system equilibration and reproducibility via repetition studies.

One such set of circumstances that could give rise to difficulties, is the measurement of a target analyte in quantities near the limit of detection (LOD), especially with the use of a new column. Even for small losses on the stationary phase, positive results would become false negatives. In this region of the LOD, a small shift in the LOD might not be considered of significance. Due to differing physico-chemical properties, the use of an IS would not necessarily help. In this study, we therefore directly examine recoveries from the stationary phase.

EXPERIMENTAL

Chemicals and Solutions

All chemicals and solution preparation (stock solutions 25 ng/μL of each SFA and DHFR in 4% methanol and mobile phases) were prepared as described previously.[1,3] The full names and abbreviations for all of the analytes were given in Part I of this study.[1] Structures of all of the drugs have been listed previously.[21]

Maloprim tablets, containing a specified 12.5 mg of pyrimethamine (PYR) each, were purchased from a local pharmacy. Five sample solutions were prepared from separate, single tablets. Each tablet was dissolved in methanol by vigorous shaking and sonication and then diluted to 100 mL with methanol (solution A). Various dilution procedures were tried, but the one that yielded the largest peak areas was adopted. Twenty mL of solution A was blown down to dryness with nitrogen, re-dissolved with sonication in two mL of methanol and made up to 100 mL with Milli-q water (25 ppM). Five independent standards containing approximately 25 ppM PYR were prepared by dissolution of PYR solid in methanol with sonication and dilution to the same 2% methanol Milli-q water. All PYR solutions were filtered through a Millipore 0.2 μm HA filter prior to injection.

For the determination of detection limits, the 25 ng/μL, 4% MEOH standard solutions of the drugs were then diluted further to prepare final concentrations of the SFA and DHFR at exactly 15, 9, 5, 2.5, 1, .5, .25 and .2 ng μL^{-1}. Additional methanol was added to maintain a final 4% MEOH concentration.

Instrumental Configuration

A full description of the modified Varian (Walnut Creek, CA, USA) LC with split flow and packed capillary columns has been previously described.[1] Figure 1 is a schematic of it. A is the packed capillary and B is the parallel conventional column for the diversion of the majority of the mobile phase which was pumped at 1.00 mL min^{-1}. This was the normal configuration as used for all analyses. To check recoveries from the stationary phase, A was replaced with an equal length (30 cm) of 0.13 mm id stainless steel tubing (sst) and B was modified to achieve the same flow rate (~ 6 μL min^{-1}) through the detector. This was ultimately achieved by bending a piece of the same id sst

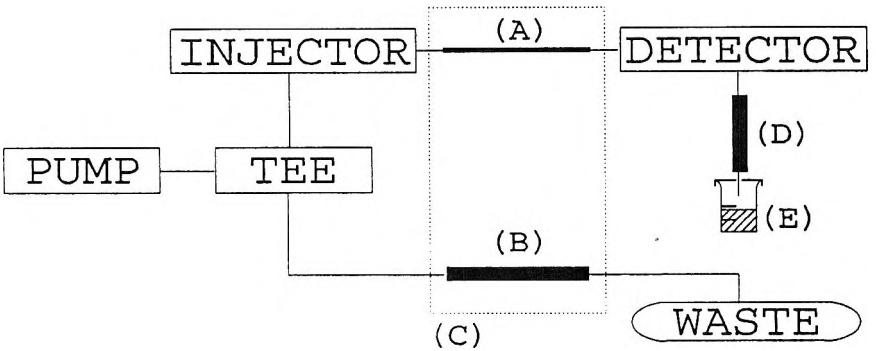

Figure 1. Instrumental configuration for the study of recoveries from the stationary phase. 1. For the analytical system: (A) 0.35 mm i.d. x 300 mm packed capillary column; (B) 4 mm i.d. x 300 mm column; (C) column oven set to 31°C; (D) 4 mm i.d. x 150 mm column; (E) measuring cylinder for flow rate determination. 2. For the alternative configuration without retention: (A) 0.13 mm i.d. x 1/16" o.d. stainless steel tubing; (B) as 2(A) except sharply bent to provide a constriction; (C), (D) and (E) as above.

and hammering it flat until the desired result was obtained. A reduced pumping rate of 0.15 mL min^{-1} was required and the resultant measured flow rate through the flow cell was 5.7 μL min^{-1}. The detector was set at 270 nm.

Chromatography

The protein C_{18} columns were 30 cm stainless steel, 0.35 mm i.d. and 0.48 mm o.d. and were packed by Varian. The stationary phase was the Separations Group Vydac IDI-TP 5 μm ± 1 μm (75%) silica with surface area 80 m^2/g, pore volume 0.63 cm^3/g and average diameter of 330 Å. The C_{18} bonded phase was TMS capped with a total carbon loading of 6-7%.

All experiments were performed at a column oven temperature of 31 Celcius, at flow rates of approximately 6 μL min^{-1} through the detector. For the recovery studies from the stationary phase and the analysis of Maloprim, isocratic elution was utilised. The mobile phase was 75% (0.001 M phosphate buffer, 0.5 mM with respect to heptane sulphonic acid (HPSA), pH 2.95) and 25% methanol. In the case of the detection limits, the best previously determined gradient (Table 5, Part III of this study)[3] was employed. It is a complex gradient, pH 2.95, 0.001M phosphate with the percentage of methanol

varying from 0-70%. The 1 mM HPSA aqueous phase was substituted by an otherwise identical aqueous phase 0.5 mM with respect to HPSA over the 8-20 minute interval. Precise flow rates were determined by timed collection of the eluent over several hours in a semi-sealed environment.

RESULTS AND DISCUSSION

Recoveries from the Stationary Phase

Of the 25 drugs examined in this study, it was desirable to pick a representative group for the recovery studies. As potential problems with the two classes of compounds might be expected to be different,[5] it was necessary to choose analytes from each class. Also, losses can occur due to a variety of mechanisms. For example, in an unfortunate choice of sample filters it was found that the 22 SFA were removed in progressively larger amounts in proportion to their elution time. For the small, more polar, early eluting drugs there appeared to be little or no loss. The intermediate eluters gave peaks about half the size to those from an unfiltered solution and the late eluters were absent from the chromatograms.[22] Hydrophobic interactions were clearly implicated, so the size range of the analytes must be represented in any recovery study. To represent this range of sizes/polarities, three SFA were chosen: sulphanilamide (SAN, early eluting), sulfisomindine (SISM, intermediate eluter) and sulphaquinoxaline (SQ, late eluter). Although only three DHFR were used in the current study, the problems previously observed with their losses have been more severe.[5] Hence, the earliest eluter in the group (diaveridine, DVD) and the late eluter (pyrimethamine, PYR) were included.

Isocratic elution was chosen not only to minimise the turnaround time between runs, but also to ensure that random fluctuations in the solvent composition and thus, minor shifts in the wavelengths of maximum absorbance and variations in the molar absorptivity were kept to absolute minima. Isocratic elution is also necessary in order to be able to make unequivocal corrections for flow rate fluctuations. For the fixed pumping rates adopted (1.00 mL min^{-1} for the normal configuration with the packed capillary and conventional columns in parallel), it was observed that the actual (measured) flow rate varied slightly. This was usually only between 5.8 and 5.9 μL min^{-1} from day to day and generally stayed constant once established each day. However, when the columns were replaced with sst the closest measured flow rate obtainable was 5.9 μL min^{-1}.

The ramifications of variations in flow rate are twofold. There is firstly the changed residence time in the detector, and secondly, an altered volume of mobile phase able to flush the automatic injector loop in the programmed interval. At the average, measured flow rate of 5.9 μL min^{-1}, one loop volume of 1.0 μL is pumped through the analytical side of the split-flow system in 0.17 minutes. Checks of the variation in peak areas with the injection interval showed that virtually complete delivery of the loop contents was achieved by an injection interval of 0.19 min. (1.1 μL). Any trace of analytes after that time would be better left in the loop. Minor variations in the amount of sample delivered from the loop would be compensated for by calibration for flow rate whereas, longer injection intervals and peak tails could decrease the reproducibility due to the high level of uncertainty associated with the integration of the extended part of peak tails.

The effect of flow rate upon peak area was determined by varying the pumping rate. To achieve this the columns were removed and replaced with the sst. As there was no retention, the peak shapes for all compounds were the same and the choice of analyte was immaterial. SAN was used. There is a simple linear relationship between the set pumping rate (spr/mL min^{-1}) and the actual flow rate (afr/μL min^{-1}) through the detector,

$$afr = -1.289 + 46.79spr$$

and the correlation coefficient is 0.9979. The variation of the SAN peak areas $(A_{SAN})/10^3$ with afr is given in Figure 2. (The differently shaded points represent different numbers of measurements.) Via SigmaPlot V4.1 the relationship was found to be:

$$A_{SAN} = (23.68afr^2 - 352.8afr + 1644)x10^3 \quad (1)$$

Table 1 shows the quintuplicate peak area measurements and the means and standard deviations for each of the five selected analytes. Column 2 contains the areas measured after elution from the packed capillary, corrected to a flow rate of 5.9 μL min^{-1} with the aid of equation 1, where necessary. Column 3 shows the raw data obtained from samples passed through the sst at 5.7 μL min^{-1} and in column 4 are the equivalent data after correction to 5.9 μL min^{-1}. Comparison of columns 2 and 4 shows that each mean falls within the range of (the other mean + its standard deviation (sd)), and generally, a long way within one sd.

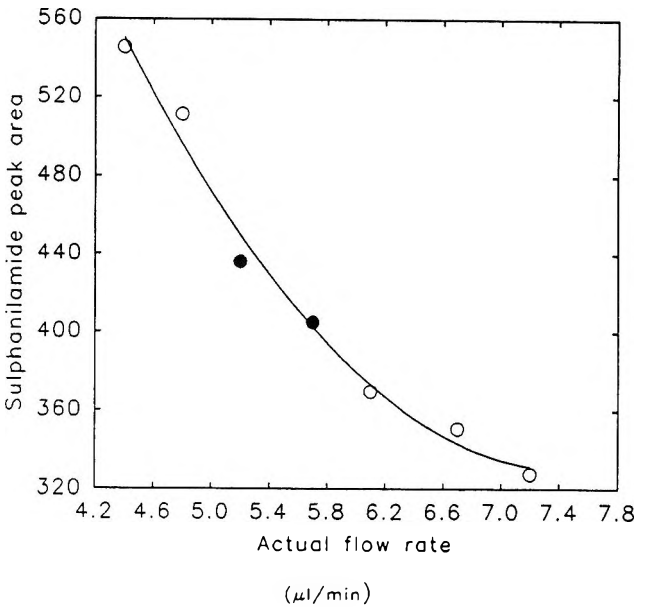

Figure 2. Variation in the detected area of sulphanilamide ($/10^3$) as a function of the flow rate.

The application of a t-test or some other statistical process such as the analysis of variance (for the two variable factors of 'column' and drug), ultimately depends upon the null hypothesis. That is, that the two sets of data are spread about the same population mean. When a test is done and the null hypothesis is rejected, difference outside of random variation is proven at some confidence level.[23] On the other hand, if the null hypothesis is accepted the converse conclusion does not follow. The sets of data are not proven to be 'the same'. For sets of data that are progressively more divergent, it may be shown that differences continue to have a higher (percentage) significance. Again, the converse is not true.

It is not possible to demonstrate a statistical difference between the data of columns 2 and 4 for the normal range of p values (or percentage confidence levels: 0.1-0.005 (or 90-99.5%). The null hypothesis is proven in all cases.

To demonstrate the 'sameness' of two sets of data is not simple. In the end, random variations dictate that sets of data will not be the same and degrees of sameness are not addressed. Statistics is largely concerned with the

Table 1

Peak Area Measurements after Retention on the Analytical Column and Without Retention*

	Peak Areas		
		Stainless Steel Tubing	
Compound	**Capillary**	**Measured**	**Corrected**
Sulphanilamide	388362	407487	285529
	386132	417252	394768
	384420	405707	383845
	389545	407206	385264
	381544	406776	384857
	386001 ± 3182[a]	**408886 ± 4726**	**386853 ± 4471**
Sulphisomidine	467804	497087	470301
	466405	495643	468935
	470666	500775	473791
	460979	494481	467836
	460488	486801	460570
	465268 ± 4419	**494957 ± 5138**	**468286 ± 4861**
Sulphaquinoxaline	299665	320729	303446
	304768	329106	311372
	306701	326718	309113
	316376	319389	302179
	305826	328391	310696
	306667 ± 6073	**324867 ± 4498**	**307361 ± 4256**
Diaveridine	221326	240419	227464
	230881	240913	227931
	229731	230123	217723
	221177	229056	216713
	223689	239175	226207
	225361 ± 4641	**235937 ± 5841**	**223224 ± 5527**

Table 1 (continued)

Peak Area Measurements after Retention on the Analytical Column and Without Retention*

	Peak Areas		
		Stainless Steel Tubing	
Compound	**Capillary**	**Measured**	**Corrected**
Pyrimethamine	237628	247182	233862
	228964	232063	219558
	223312	241491	228478
	227177	242366	229306
	226321	239667	226752
	228680 ± 5403	**240554 ± 5499**	**227591 ±5203**

* All areas are corrected to a standard flow rate using Equation 1.

[a] Average ± s.d.

definition of legitimate difference. In an attempt to demonstrate sameness, we have calculated confidence intervals (ci) associated with approximately the lowest confidence levels for inclusion of the population mean. For SISM, it is possible to go as low as the 90% confidence level to ensure that the mean area measurement off the column still lies within the ci off the sst, and vice versa. This means that for the distributions implied by the means and sd's for SISM, there is only a 90% confidence of this range including the (overall) population mean. However, the mean from the alternate measurement on SISM is still within the range. In the case of the other drugs, the approximately lowest ci [24] which includes the alternative mean (and vice versa) have levels of confidence of 80% for (DVD), 70% for (SAN and PYR) and 65% for (SQ). This sort of concordance between the two sets of data for each drug when there is such doubt about inclusion of the population mean, is interpreted to indicate a high degree of agreement.

Careful inspection of the data in Table 1 does reveal some systematic variation. For the three SFA, the areas of the compounds off the sst are all marginally larger (SAN, +0.2%; SISM, +0.6%; SQ, +0.2%) than those off the analytical column. The reverse is true for the DHFR (DVD, -0.9%; PYR, -0.5%). However, these differences are so far within the sd's that it must be

concluded that there were not detectable losses of any of the drugs on the stationary phase. There appears not to be any significant dependence upon drug type or elution order and therefore polarity.

It should also be noted that no effort was made to ensure the similarity of peak shapes in the comparison and indeed the adoption of a constant isocratic regime over all analytes ensured that the recoveries for most drugs were based upon dissimilar peak widths. As has been found by others,[25] peak shape is immaterial provided the flow rates and mobile phase are constant, and that integration is 'complete'.

Analysis for Pyrimethamine

As a check of the recoveries, we have chosen to analyse for pyrimethamine (PYR). This drug was chosen since the DHFR appeared to be more susceptible to loss than the SFA. As residue analysis was beyond the scope of this particular study, a pharmaceutical product (Maloprim) was chosen.

The exact concentrations of the PYR standards (in mg L^{-1}), the area counts and the response factors (in mg L^{-1} per unit area count / 10^{-5}) of the five independent standards were 25.06, 323805 and 7.739 for std. 1; 25.10, 324810 and 7.728 for std. 2; 24.92, 320534 and 7.775 for std. 3; 25.96, 326382 and 7.954 for std. 4; 25.38, 322803 and 7.862 for std.5. Hence, the mean response factor ± sd (σ_{n-1}) was (7.812 ± .095) x 10^{-5}. Using this mean and the five solutions of a Maloprim tablet, the mean recovery (relative to the stated 12.5 mg per tablet) and the overall sd were (99.1 ± 2.9)%.

Within recent years there have been few determinations of the SFA and DHFR reported in pharmaceuticals. Three of these include a CE determination with β-cyclodextrin modifier,[26] one HPLC determination with amperometric detection[27] and an MECC analysis employing an internal standard.[28] The recovery rate obtained above is well within the agreement found between the analytical result and stated composition as found in these other studies. It also supports complete recovery from the stationary phase, in the case of PYR.

The precision of this recovery study requires some comment. It is obvious from the response factors quoted above, that there is a systematic trend underlying the random fluctuations in values. The RSD is 1.2%. As the afr was of the order of 6 μL min^{-1}, several hours were required to collect an appropriate volume for measurement with reasonable certainty. The problem with this was that there was only an average rate determined. Fluctuations in

the flow rate could not be ascertained. As an alternative check of the flow rate, the (slow) drip rate was timed and correlated with the volumetric flow rate. This was then used as a quick daily check of the system. However, we did not analyse the range of drip rates about the correlated mean values. Hence, it is possible that one of the significant contributers to the RSDs observed would be minor variations in the flow rate. The minimum possible decrements in the spr were 0.01 mL min^{-1}. From 0.15-0.14 mL min^{-1} at the pump, the afr changed from 5.7-5.4 μL min^{-1} and the areas recorded for SAN increased from 404993-436095; about 7.7%. Minor changes in this range could easily account for the 1.2% RSD. There are two possible sources of flow rate variation. The first is the pump. The second is derived from the experimental design because the restrictor column (D in Figure 1) was not housed in the oven, thus allowing the mobile phase to change in temperature and viscosity and thus, alter the flow rate (according to ambient conditions) before measurement.

For the solutions of the Maloprim tablets, the peak areas varied in a similar fashion to the response factors for the standards, but over a larger RSD (2.6%). Dissolution inconsistencies may also contribute. For a greater level of precision, it would be necessary to enclose the restrictor column in the oven and intersperse the standards and samples, draw a trend line from the standards and take response factors from that line of best fit. A dynamic on-line flowmeter and a study of dissolution kinetics would also enhance the technique. However, there was no point in further refinement for the current study.

Detection Limits and the Linear Dynamic Range

In order to establish approximate values for the limit of detection (LOD) for each drug and to investigate the linear dynamic range (LDR) for the micro scale LC system, the range of standards chosen was concentrated at the dilute end (0.2, 0.5, 1.0, 5.0. 15.0 and 25.0 ppm).

LOD is defined as the minimum concentration or amount of analyte that can be detected with reasonable certainty for a given analytical procedure.[29] The problems associated with the accurate definition of detection limits and its confusion with other concepts in trace analyses has been reviewed.[30] The current standard method for estimating the detection limit in an analytical procedure is when the peak height of the analyte is three times the standard deviation of the baseline noise.[29-32]

The detection limits are thus determined by baseline noise fluctuations and the width of the eluting band. A reduction in both of these factors will greatly improve the detection of trace amounts of analytes. Hence, gradient

elution which provides narrow peaks thoughout the entire run was employed. Baseline noise was determined as the height of the largest noise fluctuation in a pre-selected chart time interval.[30] The 60 minute analysis time for the IPC gradient run was divided into four chart time interval sections. In each of these sections, the height of the largest noise fluctuation was measured. Hence, the noise value used in the signal-to-noise ratio (S/N) calculation for each analyte peak was based upon which chart interval section the analyte peak emerged within and the attenuation of the chromatogram.

Using the low concentration data points for each drug, the approximate 'concentration LODs' in ppm are: SNAC, 0.67; SCP, 0.32; SP, 0.29; SDZ, 0.28; ST and SMP, 0.27; SMOX, 0.25; SMAZ, 0.22; SMRZ and SST, 0.20; SAM, 0.19; SG, SISM and SMIZ, 0.18; SM, 0.16; PST, 0.12; TMP, 0.11; SAN, SISX, SDIM and PYR, 0.10; SAC and SB 0.08; SQ, 0.07; DVD, 0.03.

For a comparison of these LOD for the micro LC system with values for a conventional LC system, it is necessary to find data obtained by UV absorption at similar wavelengths. Only two of the recent studies fit this prescription. Using 4.6 mm id columns, LODs obtained for aqueous standards in ppm are: SMOX, 0.035 (271 nm, 20 μL injected),[19] and, SG, 0.08; ST, 0.07; SP, 0.05; SMOX, 0.04; SDZ, 0.03 (260 nm, 50 μL injected).[10] In the case of SMOX, the agreement between these two studies is notable. Compared to the micro system, the sensitivity of the conventional system is approximately 2, 4, 6, 6 and 9 times that of the micro system, for the compounds in the order listed. Some of this variation from compound to compound is due to the different monitoring wavelengths. However, the difference is clearly about half an order of magnitude. This is a clear demonstration of the higher concentration sensitivity of conventional systems which arises from the compatibility with larger sample sizes. As the micro injector used in this study delivered only 1 μL of sample, there were 20- and 50-fold advantages in sensitivity in the studies using the conventional systems, respectively. Since gradient elution was used in each of these studies, an analysis of peak widths would be necessary to determine the effect of this factor in the relative sensitivities of the micro and conventional systems. The restricted radial diffusion in the micro system would play an ameliorating role with respect to the sample size advantage of the conventional system. However, with vastly different sample sizes, much of the advantage of the greater mass sensitivity of the micro column is lost. Plots of peak height or S/N ratio versus concentration clearly show curvature below 15 ppm of the analytes but are linear to beyond 5 ppm. Insufficient data points were used to be more definite. Thus, the LDR is limited and extends only from the 'concentration LODs' for each compound to about 5 ppm; ranges of only 1-2 orders of magnitude, generally between 25 and 50.

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EFFECT OF SOME PARAMETERS ON ENANTIOMER SEPARATION OF EPHEDRINE, METHAMPHETAMINE AND SELEGILINE USING HPLC WITH β-CYCLODEXTRIN STATIONARY PHASE

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ABSTRACT

The influence of different parameters (mobile phase composition - pH, organic solvent, salt nature and concentration; flow rate, injected amount and temperature) on enantiomeric separation of three pharmacologically important compounds (ephedrine, methamphetamine and selegiline) was studied using β-cyclodextrin stationary phase.

The evaluation of effect of these parameters allowed to optimize condition for optical purity determination. The following experimental conditions were chosen for separation of racemic mixture: stationary phase - ChiraDex, 5 μm; column - LiChroCart 250 × 4 mm I.D.; mobile phase - 500 mmol triethylamine/l with H_2SO_4 in water, pH = 3.5, flow-rate - 0.8 mL/min; detection - UV absorption at 206 nm; temperature - ambient.

The separation of minor (1%) enantiomer in excess of major one can be improved using flow-rate 0.2 mL/min and thermostated column (20°C for methamphetamine and selegiline, 5°C for ephedrine).

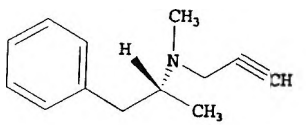
INTRODUCTION

The importance of chirality in the natural world is well known. Many biologically important compounds show optical activity. The interest in the separations of chiral compounds has been growing rapidly over the past few years. The chromatographers in the different fields of work (pharmacy, agriculture, biotechnology, etc.) have to solve the problem of the enantiomer separation. For example, many pharmaceutical substances exhibit chirality and their enantiomers often have different pharmacological effects or different levels of activity. That is why the regulatory authorities require information about properties of individual enantiomers as well as about analytical techniques used for their separation. In the International Symposium on Purity Determination of Drugs (Stockholm, Sweden, 6 – 8 December 1993), Sven-Erik Hillver pointed out that "the presence of a non-wanted enantiomer could, in principle, be considered as any other impurity and hence, normal regulatory requirements and guidelines would be applicable."[1]

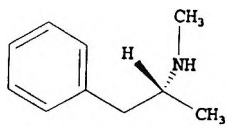
Among other separation techniques (CZE,[2] GC[3] etc.) the high performance liquid chromatography (HPLC) is also widely used for the optical isomer recognition.[4,5]

Different ways can be used in chiral separation by HPLC, such as the derivatization, the chiral additive in a mobile phase or the chiral stationary phase.

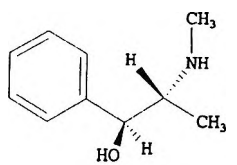
As the stationary phases, bonded cyclodextrins (native or derivatized) are often used.[4,5] In this work, native β-cyclodextrin stationary phase in the reversed phase chromatography mode has been tested for the enantiomer separation of three basic drugs - ephedrine (EP), methamphetamine (deoxyephedrine) (MAP) and selegiline (SEG) - *Deprenyl*, *Jumex*, (for structures see Fig. 1). These compounds are widely used or, especially in the case of MAP, abused for their pharmacological effects. They also represent different steps of pharmaceutical synthesis. The EP is a starting compound, MAP is an intermediate and SEG, (*R*)-(–) isomer, is a final product used as antidepressant and antiparkinsonian.



SEG



MAP



EP

Figure 1. Structures of studied drugs: SEG - selegiline, N-(1-phenylisopropyl)-N-methyl-N-propinylamine; MAP - methamphetamine, deoxyephedrine; EP - ephedrine, phenyl-2-methylamino-1-propanol.

The enantiomers of studied drugs were separated by HPLC after derivatization with chiral agents e.g. EP derivatized with (*S*)-(+)-1-(1-naphthyl)-ethyl isocyanate,[6] MAP derivatized with GITC or FLEC,[7,8] MAP and EP derivatized with GITC or FLEC,[9,10,11] where GITC is 2, 3, 4, 6-tetra-*O*-acetyl-β-D-glukopyranosyl isothiocyanate and FLEC is (+)- or (–)-enantiomer of 1-(9-fluorenyl)ethyl chloroformate.

The chiral stationary phases of Pirkle-type,[12] cellulose-type,[13,14] for MAP and cellulose-type[15] for MAP and EP were also used. The separation on cyclodextrin stationary phase for MAP was published.[16]

We successfully used a native β-cyclodextrin stationary phase for the separation of optical isomers of all named drugs. The influence of the different experimental parameters (pH, nature and concentration of salt, temperature etc.) on the separation was studied and evaluated in detail. Optimization of the enantiomer recognition has led to the method for the minor isomer determinations in the excess of the major one.

Concerning the practical application, we were looking for one mobile phase that would allow routine analyses of all three drugs with 1% level of minor isomer. It should be stated that for this purpose we did not search for an optimum of enantiomeric separation of individual compounds that can be, in general, different for each of them.

EXPERIMENTAL

The chromatographic work was carried out by a liquid chromatograph Spectra Physics (pump SP 8700, UV/VIS detector SP 8440, all Spectra-Physics, San Jose, CA, USA). UV absorption chromatograms were recorded at 206 nm and 258 nm respectively. The chromatographic station CSW version 1.0 (DataApex, Prague, Czech Republic) was used for chromatogram acquisition and handling. The samples were injected by a 10 μL syringe (Hamilton, Reno, NV, USA) in a manual 7125 injector equipped with a 10 μL loop (Rheodyne, Cotati, CA, USA).

The separations were performed using a 250 × 4 mm I.D. ChiraDex 5 μm LiChroCart column (E. Merck, Darmstadt, F. R. Germany). The temperature of the column was controlled with the precision ± 0.1°C using a glass water jacket and a laboratory water thermostat equipped with a freon cooler. The flow rate was changed among 0.2 - 1.0 mL/min (see results and discussion).

The mobile phases were prepared by volume by volume mixing of components. As these components, HPLC grade acetonitrile and tetrahydrofuran (E. Merck, Darmstadt, F. R. Germany), UV grade methanol (Lachema, Brno, Czech Republic) and salt solutions were used. The salt solutions were prepared by dissolution of adequate amounts of salt or triethylamine (TEA) in redistilled water and pH was set up by corresponding acid concentrated - CH_3COOH, HCOOH, H_3PO_4 or diluted - $H2SO_4$, HNO_3, $HClO_4$ (20% or 5% (v/v) solutions). The abbreviation TEAS is used for combination of TEA with H_2SO_4 and mentioned concentration is related to TEA. All chemicals used for this purpose were of analytical grade.

Table 1

Chromatographic Parameters (See Text) Versus pH, Salt Cation (M^+), Salt Concentration (c) and Temperature (T) for Ephedrine (EP), Methamphetamine (MAP) and Selegiline (SEG)

Studied Parameter		$k_{c,rel}$			$\alpha_{c,rel}$			S_{rel}		
		EP	MAP	SEG	EP	MAP	SEG	EP	MAP	SEG
pH	3.5	0.59	0.80	0.87	0.73	1.00	0.83	0.53	1.00	0.69
	4.5	0.65	0.84	0.92	0.71	0.90	0.79	0.58	0.95	0.91
	5.5	0.77	0.92	1.00	0.69	0.78	0.67	0.66	0.90	0.84
M^+	Na^+	0.87	0197	1.00	0.76	0.67	0.64	0.82	0.80	0.79
	NH_4^+	0.86	0.96	0.99	0.77	0.71	0.67	0.82	0.84	0.81
	TEA^+	0.73	0.86	0.90	1.00	0.94	0.87	0.90	1.00	0.97
C (nmol/L)	50	0.81	0.96	1.00	0.76	0.80	0.82	0.71	0.89	0.95
	100	0.82	0.96	1.00	0.81	0.83	0.84	0.76	0.93	0.98
	150	0.82	0.96	1.00	0.82	0.85	0.84	0.77	0.95	0.96
	250	0.82	0.96	1.00	0.87	0.87	0.85	0.82	0.97	0.99
	500	0.80	0.95	0.99	0.95	0.90	0.84	0.89	0.99	0.97
	750	0.80	0.94	0.99	1.00	0.91	0.82	0.93	1.00	0.95
T (°C)	5	0.83	0.95	1.00	1.00	0.84	0.81	1.00	0.97	0.98
	10	0.81	0.95	0.99	0.93	0.81	0.77	0.91	0.92	0.92
	15	0.80	0.94	0.98	0.87	0.78	0.74	0.84	0.87	0.87
	20	0.78	0.93	0.97	0.82	0.75	0.71	0.76	0.84	0.82
	25	0.76	0.92	0.96	0.76	0.72	0.68	0.69	0.79	0.77
	30	0.75	0.90	0.95	0.71	0.70	0.65	0.62	0.75	0.73

Stationary Phase: ChiraDex, 5μm. Column: LiChroCart 250 x 4 mm I.D. Mobile phase: for pH - 500 mmol TEA/l with H_2PO_4 in water, for M^+ - 500 mmol cation/l with H_2PO_4, pH = 3.5, for c - TEA with H_2SO_4, pH = 3.5 and for T - 500 mmol TEA/l with H_2SO_4, pH = 3.5; flow-rate 0.8 mL/min. Detection: UV absorption at 206 nm. Injecton: 10 μL, 0.1 mg each enantiomer/mL water. Temperature: for pH, M^+ and c ambient.

Hold-up volumes were determined by triplicate injections of water (10 mL) with the detection wavelength 200 nm, mobile phase methanol : water = 40 : 60 and flow-rate 0.8 mL/min. Mean values of two retention times were used for calculation of retention factors.

The hydrochlorides of all studied enantiomers - (*1S,2R*)-(+)-EP, (*1R, 2S*)-(−)-EP, (*R*)-(−)-MAP, (*S*)-(+)-MAP, (*R*)-(−)-SEG and (*S*)-(+)-SEG) - were gifts of Farmakon, Olomouc, Czech Republic. The contents of the minor enantiomer in major one was undetectable using evaluated methods. The concentration of each enantiomer in the stock solution was 2 mg/mL of redistilled water.

RESULTS AND DISCUSSION

Starting information on the retention of the studied substances was obtained on the base of some preliminary experiments with mobile phases methanol - phosphate buffer (50 mM-$Na_2P\ O_4$, pH 3.5 or 7.5 with H_3PO_4). It was found out that for pH 3.5 the compounds can be eluted from the column only with the buffer solution without any organic solvents. For the evaluation of separation quality we used separation factor S (1),[17] resolution R_s (2)[18] and relative values $k_{c,sel}$ (6), $a_{c,sel}$ (7) that characterize the contribution of capacity and selectivity respectively to the separation of studied enantiomers.

$$S = (k_2 - k_1)/(k_2 + k_1 + 2) \qquad (1)$$

where k_1 and k_2 is a capacity factor of compound with lower and higher retention respectively.

$$R_S = 1/4 \cdot n^{1/2} \cdot (a_{1,2} - 1)/a_{1,2} \cdot k_2/(k_2 + 1) \qquad (2)$$

where $a_{1,2}$ is a relative retention and n is a number of theoretical plates. In this work, the resolution was used for the good separated peaks ($R_s > 1.1$), in other cases the total quality of separation was estimated with the use of separation factor S and with the consideration of the peak tailing. The other applied parameters were

$$k_c = k_2/(k_2 + 1) \qquad (3)$$

$$\alpha_c = (\alpha_{1,2} - 1)/\alpha_{1,2} \qquad (4)$$

where k_c and α_c means respectively the contribution of capacity and selectivity to the resolution.

$$k_{c,rel} = k_c / k_{c,max} \quad (5)$$

$$\alpha_{c,rel} = \alpha_c / \alpha_{c,max} \quad (6)$$

$$S_{rel} = S/S_{max} \quad (7)$$

where $k_{c,max}$, $\alpha_{c,max}$ and S_{max} are the maximum values of k_c, α_c and S from the evaluated data set.

The Effect of pH and Organic Solvent

We evaluated the effect of pH in the range of column stability (from 3.0 to 7.5). Table 1 shows the impact of pH change from 3.5 to 5.5 where we used a mobile phase without organic solvent. As can be predicted, $k_{c,sel}$ is increasing (most for EP). The studied drugs are basic compounds and owing to protonization the retention is lower for lower pH. Hydroxyl group of EP decreases and propargyl group of SEG increases the retention of these compounds in comparison to MAP. For all experimental pH values, the drugs are eluted in the order of EP, MAP and SEG.

The selectivity is improving (higher $\alpha_{c,sel}$, Table 1) with pH diminishing but only slightly for EP. The combined effect of capacity and selectivity appears in S. At pH 5.5 S_{rel} shows the deterioration of separation for SEG and MAP but the improvement for EP. It means that for the first two drugs the decrease of selectivity is not compensated by increase of capacity. For EP the higher capacity factor is the cause of the better separation. Because the anion optimization (see below) led to the increase of capacity (the critical parameter in EP separation), it was possible to apply pH = 3.5 as optimum for all drugs. We can also expect the better robustness of method in pH = 3.5. The small change in acidity does not mean so high change of the retention as it could occur in pH = 5.5 (impact of equilibrium of protonized and non-protonized form).

The effect of pH on the separation near the optimum with the optimal salt nature and concentration (TEAS, 500 mmol/L) is small. The resolution is relatively good for all enantiomer couples (1.33 - 1.38 - 1.33 for EP, 1.46 - 1.50 - 1.48 for SEG and 1.55 - 1.57 - 1.56 for MAP in order of pH - 3.0, 3.5, 4.0).

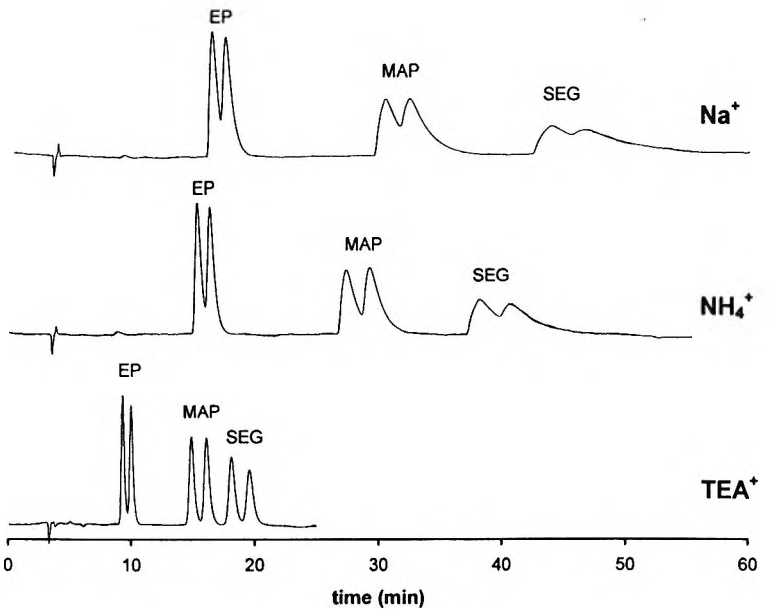


Figure 2. Chromatogram of ephedrine (EP), methamphetamine (MAP) and selegiline (SEG) for different cations of salt in a mobile phase. Stationary phase: ChiraDex, 5μm. Column: LiChroCart 250 x 4 mm I.D. Mobile phase: 500 mmol cation/l with H_2SO_4, pH = 3.5; flow-rate 0.8 mL/min. Detection: UV absorption at 3\206 nm. Injection: 10μL, 0.1 mg each enantiomer/mL water. Temperature: ambient.

To keep retention in approximately the same level for different pH values (3.5, 4.5, 5.5, 6.5 and 7.5) the methanol was added to the mobile phase in appropriate amount (in dependence on pH). These experiments led to the same conclusion about pH optimum as described above.

For pH = 3.5 and optimal salt nature as well as concentration, the effect of organic solvents (methanol, acetonitrile, tetrahydrofuran) was evaluated. In all cases the retention, but also the separation, was decreasing. That is why the organic solvents were not used in the mobile phases and this parameter was not optimized in detail.

We can conclude that protonized forms of drugs show a good selectivity of separation from following the differences in the formation of diastereomeric inclusion complexes, and a lower capacity in comparison with non-protonized forms, owing to the lower stability of the charged molecule complexes.

The Effect of theNature of the Salt

For pH optimum the impact of three cations (Na^+, NH_4^+ and TEA^+) in combination with PO_4^{3-} or SO_4^{2-} respectively was studied. In the case of PO_4^{3-}, the choice of optimal cation (TEA^+) was made on the base of peak shape. The use of TEA^+ allows one to attain the better peak shapes especially for SEG and MAP. The differences in $k_{c,sel}$, $\alpha_{c,rel}$ and S_{rel} are more evident for combination of cations with SO_4^{2-} that was found as the best for our purposes (see below).

The parameters are changing in the order Na^+ - NH_4^+ - TEA^+, $k_{c,sel}$ is decreasing, $\alpha_{c,sel}$ and S_{rel} are increasing (Table 1). Again (as for pH), the decrease of capacity contribution for EP is higher in comparison with other two drugs, but in this case the increasing selectivity compensates a loss in $k_{c,sel}$. TEA^+ offers the best separation in the shortest time in comparison with the other two tested cations, where peak tailing also contributes to the deterioration of the enantiomer recognition (Fig. 2).

We can suppose that basic compounds separated as cations compete with cations of salt in the cyclodextrin cavity occupation as well as in the interaction with the hydroxyl groups. This competition leads to the decrease of drug retention with the cation change (Na^+ - NH_4^+ - TEA^+) as well as to the peak shape improvement in the same order. In the same sense the "salting out" effect (the highest for Na^+, the lowest for TEA^+) acts on the retention.

In the next step the nature of anion was optimized in combination with TEA^+. Its concentration was 150 mmol/L for univalent and 100 mmol/L for bivalent anions, to keep approximately constant ionic strength.

The anions can be arranged in the succession by the increasing drug retention - $ClO_4^- < CH_3COO^- < NO_3^- < HCOO^- < PO_4^{3-} < SO_4^{2-}$. In this order $k_{c,sel}$ is increasing for all studied compounds (Fig. 3) owing to the increasing hydratation of anions. Thc graph of $\alpha_{c,rel}$ is slightly more complicated. The best separation for SO_4^{2-} was found out. For this anion in comparison with the others, the EP shows the highest $k_{c,rel}$ as well as $\alpha_{c,rel}$, MAP and SEG show highest $k_{c,rel}$ but not $\alpha_{c,rel}$. However, the capacity increase has higher impact on the separation than decrease of selectivity, as seen from S_{rel} graphs.

The studied compounds have the absorption maximum around 206 nm that is by order higher than side maximum at 258 nm. It was used in anion optimization experiments together with higher injected amount.

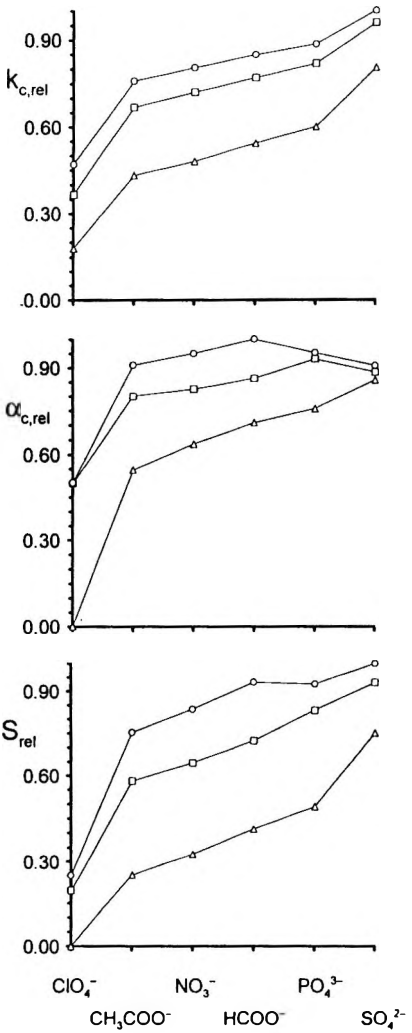


Figure 3. Chromatographic parameters (see text) versus anion of salt for ephedrine (Δ).methamphetamine (□) and selegiline (○). Mobile phase: 500 mmol TEA/L with acid of correpsonding anion, pH = 3.5. Detection: UV absorption at 258 nm. Other conditions as in Figure 2.

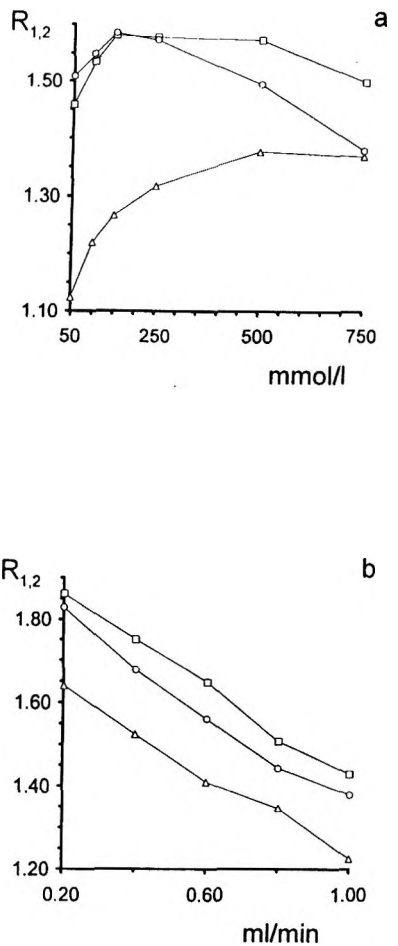


Figure 4. Resolution versus salt concentration (4a) and versus mobile phase flow-rate (4b) for ephedrine (Δ), methamphetamine (□) and selegiline (○). Mobile phase: 4a - TEA with H_2SO_4, pH = 3.5. Other conditions as in Figure 2.

Some studied anions (ClO_4^-, CH_3COO^-, NO_3^-, $HCOO^-$) in used concentration did not allow the detection at 206 nm due to too high mobile phase absorptivity. For the enantiomeric separation the injected amount of drugs is very important (see below). The detection at 206 nm allows its decrease.

Anion SO_4^{2-} shows the good behaviour in both cases (detection as well as chromatographic separation) and is the most convenient one. It was used in mobile phases for verification of above optimized parameters (pH, cations) as well as in the following optimization steps.

The Salt Concentration

The dependencies of the $k_{c,rel}$, $\alpha_{c,rel}$, S_{rel} and R_s on the salt concentration are shown in the Table 1 and in Fig. 4a, respectively. The contribution of the capacity change to the resolution is not as important as the change of selectivity, especially for EP. The resolution that is also dependent on efficiency, after starting improvement, becomes smaller for all three compounds. The difference is in the size and starting concentration of decline (Fig. 4a).

For the next experiments the TEA^+ concentration 500 mmol/L was chosen (optimum for EP). The optimum for SEG and MAP lies around 150 mmol/L. However in both cases the resolution at 500 mmol/l is higher in comparison to EP and at the same time close to 1.5 (1.57 and 1.50 for MAP and SEG respectively).

The concentration effect on capacity can be explained by cation and anion contribution. The capacity becomes higher with the increase of SO_4^{2-} concentration but lower with the increase of TEA^+ concentration. The result is a small change in the capacity.

The Effect of Mobile Phase Flow-Rate

The flow-rate impact on $k_{c,rel}$, $\alpha_{c,rel}$ and S_{rel} is minor and the change of these parameters is more probably due to the reproducibility of measurements. The resolution is then effected by the change of efficiency. For all compounds the resolution of enantiomers is decreasing with higher flow rate (usual trend in HPLC) (Fig. 4b).

The increase of peak areas (typical for concentration detectors) and peak heights (increase of efficiency) with the flow-rate decrease contributes to the detection improvement. On the other side, in the same direction, the analysis time is increasing.

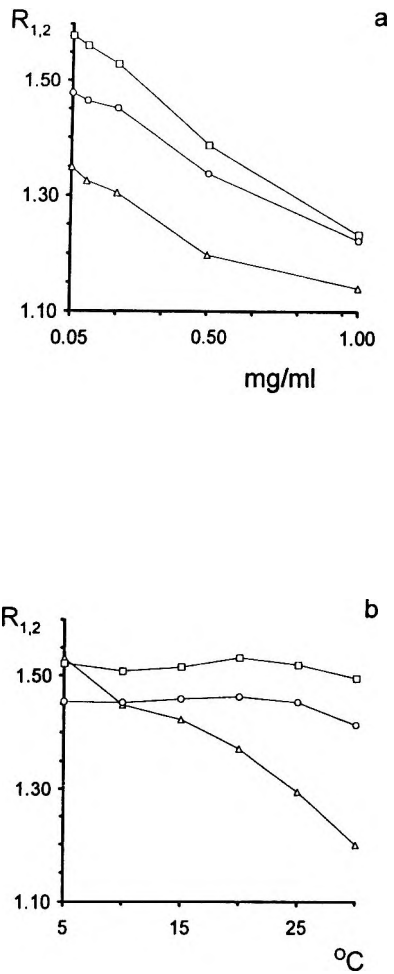


Figure 5. Resolution versus racemate concentration in injected solution (5a) and versus temperature (5b) for ephedrine (Δ), methamphetamine (□) and selegiline (○). Mobile phase: 500 mmol TEA/l with H_2SO_4, pH = 3.5. Other conditions as in Figure 2.

The Effect of Injected Amount

The possibility to use lower detection wavelength (more sensitive detection) led to the test of injected amount impact on the resolution. Only neglected changes of $k_{c,rel}$, $\alpha_{c,rel}$ and S_{rel} in the whole studied range were

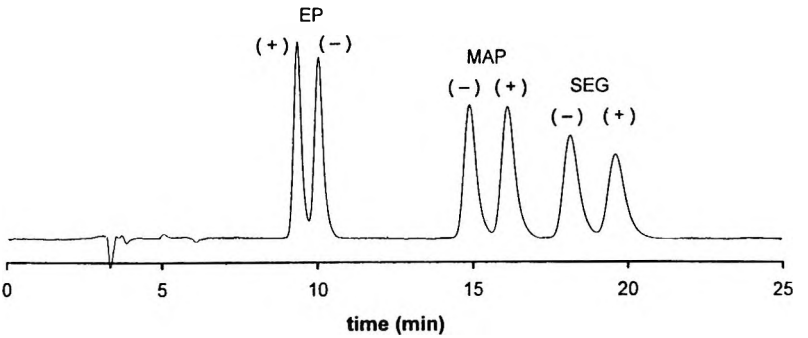


Figure 6. Separation of racemic mixtures of ephedrine (EP), methamphetamine (MAP) and selegiline (SEG). Mobile phase: 500 mmol TEA/l with H_2SO_4, pH = 3.5. Other conditions as in Figure 2.

observed. The change of resolution is more important (Fig. 5a). We can conclude that the change of number of theoretical plates (decrease of peak tailing) is the main factor contributing to the resolution improvement with the decrease of injected amount. The highest amount (for good detection) with a satisfactory resolution i.e. 0.2 mg racemic mixture/mL was selected as optimal. The importance of sensitive detection (see above discussion about the anion optimization) that allows diminishing of injected amount is evident.

The discussed effect can be explained by the nonlinearity of separation isotherm in the studied concentration range. With the injected amount decrease, we move to or closer to, the linear part of isotherm. It means the peak shape improvement, the decrease of peak tailing.

The Effect of Temperature

The effect of temperature was studied in the range 5 - 30°C. With decreasing temperature, we can see the increase of $k_{c,rel}$, $\alpha_{c,rel}$ and of course S_{rel} for all studied drugs (the highest for EP)(Table 1). The lower temperature is more convenient for the formation of the inclusion complexes.

Increase of complex stabilities (capacity factor increase) but also increase of difference in complex stabilities of the enantiomeric pairs (selectivity increase) is evident.

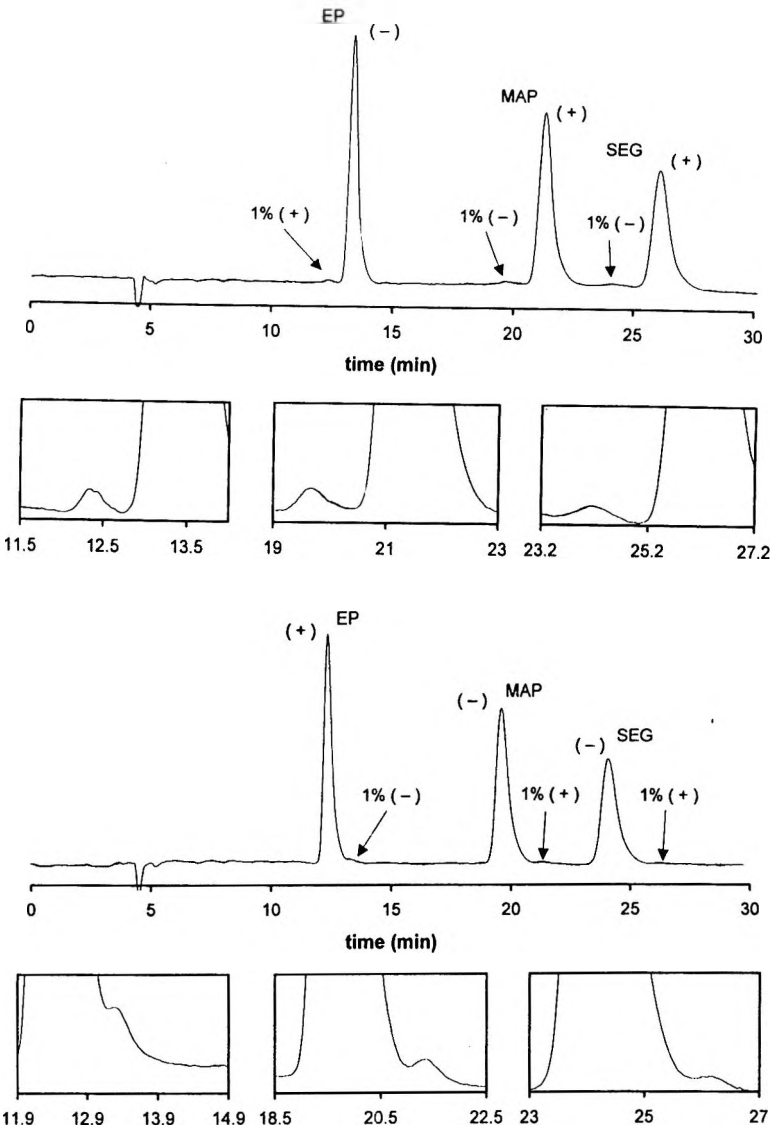


Figure 7. Separation of minor isomers of ephedrine (EP), methamphetamine (MAP) and selegiline (SEG). Mobile phase 500 mmol TEA/l with H_2SO_4, pH = 3.5; flow-rate 0.6 mL/min. Injection: 10μL, injected solution - 0.1 mg major and 0.001 mg minor enantiomer/mL water. Other conditions as in Figure 2.

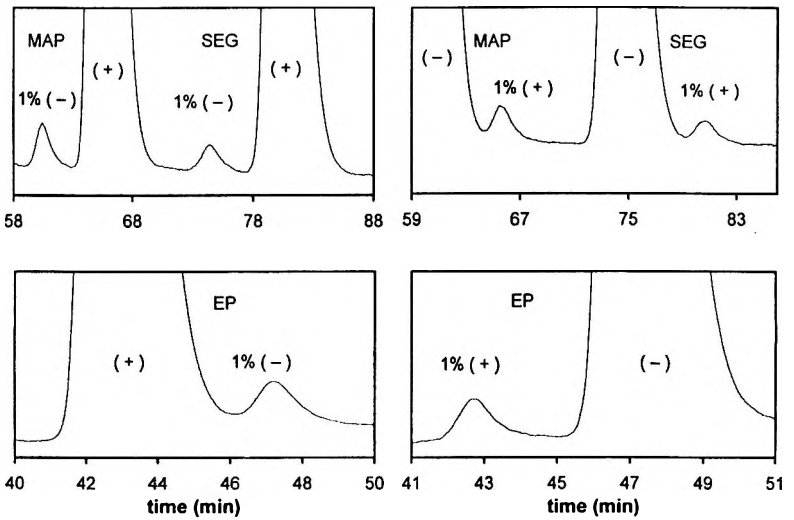


Figure 8. Separation of minor isomers of ephedrine (EP), methamphetamine (MAP) and selegiline (SEG). Mobile phase: 500 mmol TEA/l with H_2SO_4, pH = 3.5; flow-rate 0.2 mL/min. Temperature: 20°C for MAP an SEG, 5°C for EP. Injection: 10μL, injected solution -0.1 mg major and 0.001 mg minor enantiomer/mL water. Other conditions as in Figure 2.

The resolution is increasing in whole studied temperature range for EP but not for MAP and SEG (Fig. 5b). The difference between S_{rel} and R_s dependencies can be explained by the decrease of efficiency at lower temperatures (the decrease of diffusion, increase of mobile phase viscosity). From a practical point of view, the increase of working pressure for the same flow-rate in lower temperatures should not be forgotten.

Application of Optimized Method

The optimization procedure led to the method that allows analysis of racemic mixture (Fig. 6) as well as the determination of 1% of a minor enantiomer (10 ng injected) in the excess of a major one (Fig. 7). Other improvements can be reached by decreasing flow-rate and temperature (Fig. 8), of course with the longer analysis time.

The column was used more than 100 hours. During this time the efficiency and peak symmetry became worse but enantiomer separation was still acceptable. The column washing, after daily work, as well as the use of a precolumn is highly recommended.

The method validation for the analysis of pharmaceutical substance (SEG) is in progress.

CONCLUSION

The evaluation of the effect of studied parameters (pH and composition of mobile phase, injected amount etc.) allowed one to optimize the chiral separation on cyclodextrin stationary phase . Each parameter contributes to the final result. Some starting knowledge (about retention, detection, injected amount) is very useful, e.g. very high injected amount can make the separation impossible but also can lead to the omission of influence of other optimized parameters (some improvement of separation can not be recognized). The described approach can also be applied to the solution of a chiral separation of other compounds using b-cyclodextrin stationary phase.

An optimized method allows optical purity determination of all studied compounds using the same mobile phase that is useful for the routine control of production process. The presence of minor enantiomer below 1% can be found out.

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DYNAMIC BEHAVIOUR OF C_{18} HPLC COLUMNS BY STIMULUS-RESPONSE ANALYSIS PART II: DETERMINATION OF DISPERSION COEFFICIENTS VIA PECLET NUMBERS

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ABSTRACT

In this study, the dispersion coefficients of acetonitrile, ascorbic acid, patulin, 5-hydroxymethylfurfural, 2,4-dichlorophenoxy- acetic acid, 4-chlorophenoxyacetic acid and ß-naphtoxyacetic acid are determined by using the stimulus-response technique. The system was pulse stimulated with 10 µL of each tracer with definite concentrations at the flow rate of 1 mL/min. The resulting chromatograms, so called response "C" curves, were obtained for each tracer, individually. The Peclet numbers of each tracer were calculated and evaluated to get dispersion coefficients. The relative dispersion coefficients, based upon the non interacting tracer Acetonitrile (D: 1.83×10^{-6} cm^2/s), were calculated as follows; acetonitrile: 1.00, ascorbic acid: 3.72, patulin: 1.34, 5-hydroxymethylfurfural: 1.68, 2,4-dichloro-phenoxyacetic acid: 2.32, 4-chlorophenoxyacetic acid: 3.23 and ß-naphtoxyacetic acid, 2.70.

INTRODUCTION

Stimulus-response methods have been well established in chemical engineering processes for measuring rate and equilibrium parameters such as mass transfer coefficients, diffusivities and adsorption rate constants.[1-3] The moments of the response curves to pulse inputs have been extensively used in the analysis of packed bed systems.[4-7]

The stimulus-response approach can also be used to investigate the dynamic interaction between mobile and stationary phases for high performance liquid chromatography (HPLC) systems. Two C_{18} HPLC columns have been examined using this technique, in order to compare their dynamic behaviours by us.[8]

Acetonitrile has been used as non-interacting tracer through the columns and than, Peclet number, which is the basic indication of the dynamic flow characteristic of the column and dispersion coefficients, have been determined.[8] The dispersion coefficients, calculated based upon the observed Peclet numbers, may give us an idea about how an analyte particle is retained in the column for a definite chromatographic condition.

In this study, the stimulus-response technique was employed to get the dispersion coefficients of acetonitrile, patulin, 5-hydroxymethylfurfural (5-HMF), ascorbic acid, 2,4-dichlorophenoxyacetic acid (2,4-D), 4-chlorophenoxyacetic acid (4-CPA) and ß-naphtoxyacetic acid (BNOA). The column was pulse stimulated with 10 µL solution of each tracer and the responses of the column were measured individually.

EXPERIMENTAL

High Performance Liquid Chromatograph

Varian Star model liquid chromatograph was used. It was equipped with a Rheodyne model 7161 six-way injection valve, 10 µL loop, and a Varian model 9050 variable wavelength UV-VIS detector set at 276 nm for patulin and 5-HMF, 254 nm for ascorbic acid and 270 nm for 2,4-D, 4-CPA and BNOA to obtain the highest sensitivity. Varian model 4400 integrator was used with a chart speed of 2 cm/min to record the resulting chromatograms.

Columns

A column (ShimPak®) supplied from Shimadzu, made of stainless steel, 250 x 4 mm (id), packed with C_{18} octadecyl groups (5 µ), operated at ambient temperature, was used.

Mobile Phase

Mixture of water:acetontirile (95:5, v/v) filtered through a regenerated cellulose acetate membrane (0.45 µ) and degassed ultrasonically, was used as the mobile phase with the flow rate of 1.0 mL/min.

Test Tracers

Chemicals used were all obtained from Merck, Germany. Acetonitrile was HPLC grade and used without any further purification. Patulin and 5-HMF solutions were prepared in pH 4.0 water to a concentration of 2 µg/mL. Ascorbic acid solution, at a concentration of 10 µg/mL, was prepared dissolving an appropriate amount of ascorbic acid in water. It was then stabilised adding 1 mg of dithiothreitol for each mL of solution. 2,4-D, 4-CPA and BNOA solutions were prepared in a mixture of isopropanol:water (75:25, v/v) to a concentration of 1 µg/mL.

Stimulus-Response Analysis

The system was "pulse" stimulated by injecting 10 µl of acetonitrile as inert tracer or the other test tracers, separately, to the mobile phase at flow rate of 1 mL/min to determine the reference response of the column. Then, the column was stimulated with interacting tracers, individually. The column responses, which are so-called "C" curves, were determined by following the absorption of the tracers in the eluent stream. In each case, the absorption was detected by a UV-VIS detector. All the experiments were carried out at ambient temperature.

RESULTS AND DISCUSSION

The response "C" curves obtained in the stimulus-response experiments for the chemicals used in that group of study was given in Figure 1. It is clearly

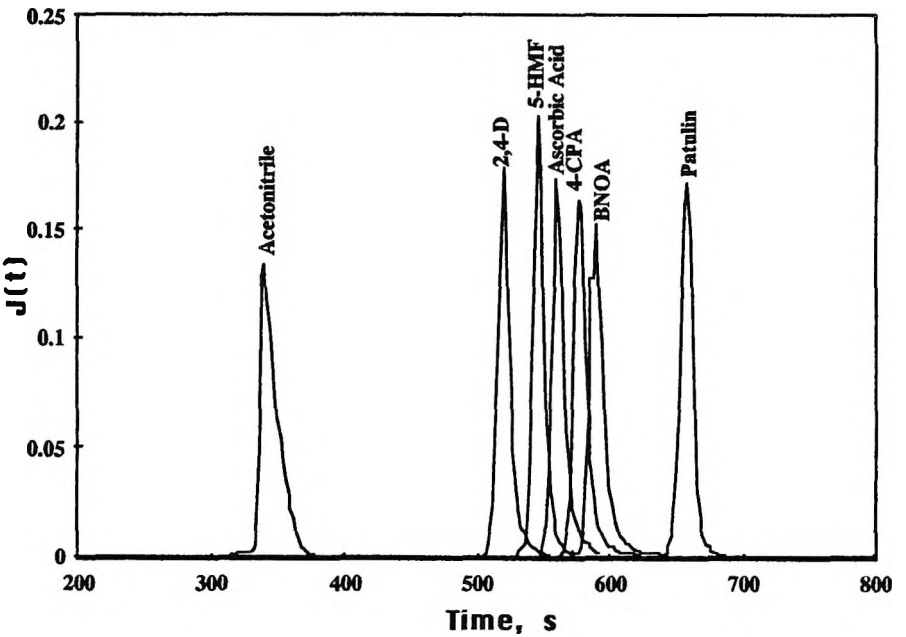

Figure 1. Response "C" curves of pulse-stimulated ShimPak® column for different chemicals

observed that, the non interacting tracer "acetonitrile" leaves the column first, than the others follow it, according to their interactive behaviour with both RP-HPLC column packing material and the mobile phase. It is well known that, due to interaction of chemicals with column packing material, the response of the column for a particular compound would delay, but such an interaction does not cause any loss of material. Therefore, the total amount of tracer injected to the column could be collected to obtain "C" curves. The area under the "C" curves was calculated and normalized to be unity, according to the analysis method given elsewhere.[9]

The principle mathematical analysis method, so called "parameter estimation by cybernetic moment technique", to obtain the individual Peclet numbers, N_{Pe} from "C" curves are given in our previous paper.[8] The equation describing the Peclet number is:

$$N_{Pe}=\frac{U\,d_p}{D} \qquad (1)$$

where:

N_{Pe} : Peclet number, dimensionless
U : interstitial velocity, cm s^{-1}
d_p : particle diameter, cm
D : dispersion coefficient, cm^2s^{-1}

The response "C" curve which belongs to each chemical, was evaluated to obtain Peclet numbers, and by utilising Equation 1, the dispersion coefficients were calculated. The relative dispersion coefficients based upon the non interacting tracer Acetonitrile was also calculated, and the results are given in Table 1.

It is very well known that, Peclet number is the ratio of the mass transfer by bulk flow to mass transfer by diffusion. The non interactive tracer "acetonitrile" is carried through the column by the mobile phase, but due to its' non interactive behaviour with column packing material, the dispersion through the pores becomes less effective in transportation of molecule. Therefore, the dispersion coefficient of acetonitrile ($D=1.83x10^{-6}$ cm^2/s) is found as the minimum of all others. Patulin, which is a mycotoxin and 5-HMF have similar chromatographic properties, and therefore, separation of patulin from 5-HMF is difficult as recorded in the literature.[10-11]

The dispersion coefficients of patulin and 5-HMF are found to be $2.45x10^{-6}$ cm^2/s and $3.08x\ 10^{-6}$ cm^2s, respectively. Those values are closed to each other which confirms the difficulty of separation. 2,4-D, 4-CPA and BNOA, which belong to a group of chemicals called "phenoxycarboxylic acids", have also shown similar interactive behaviours with the column packing material as expected, due to their similar aromatic chemical structures. It is thought, that the separation of these acidic compounds may not be easy using a common solvent mixture like water:acetonitrile (95:5, v/v) as can be seen easily from their dispersion coefficients and response "C" curves in Figure 1.

The dispersion coefficient of the final compound, ascorbic acid, was found to be $6.80x10^{-6}$ cm^2/s and that value is considerably greater than that of phenoxycarboxylic acids'. This can be explained by the molecular structure of ascorbic acid which extends polarity of molecule.

As conclusion, it can be said that, the stimulus-response analysis of several compounds to determine their mass transfer behaviour through the RP-HPLC column may give a brief idea about the separation efficiency of the column for a defined chromatographic condition. The preestimation of the

Table 1

Peclet Numbers (N_{pe}), Dispersion Coefficients (D) and Relative Dispersion Coefficients (Rel D) for Various Chemicals

Tracer	N_{pe} (Dimensionless)	D cm^2/s	Rel D (Dimensionless)
Acetonitrile	36.259	1.83×10^{-6}	1.00
Patulin	27.077	2.45×10^{-6}	1.34
5-hydroxymethylfurfural	21.529	3.08×10^{-6}	1.68
2,4-dichlorophenoxylacetic acid	15.645	4.24×10^{-6}	2.32
β-naphtoxyacetic acid	13.420	4.94×10^{-6}	2.70
4-chlorophenoxyacetic acid	11.225	5.91×10^{-6}	3.23
Ascorbic acid	9.746	6.80×10^{-6}	3.72

separability of some complex mixtures can be investigated by suggested technique. Studies concerning the generalization of this dynamic test approach are still under investigation.

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QUANTITATIVE ANALYSIS OF BROMOCRIPTINE MESYLATE IN TABLET FORMULATIONS BY HPLC

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ABSTRACT

A rapid, specific and reliable high performance liquid chromatographic assay of bromocriptine mesylate in tablets has been developed. Reverse phase chromatography was conducted using a mobile phase of 0.05 M ammonium acetate and acetonitrile, (40% v/v) pH 5.6 and detection at 240 nm. The % recovery and coefficient of variation from six placebo tablets containing 2.5 mg of bromocriptine mesylate were 100.65 and 0.456 respectively. Replicate regression analyses of three standard plots in the concentration range 0.5 - 10 mcg/mL obtained on three different days gave a correlation coefficient >0.9997 and the coefficient of variation of the slopes <0.1%. The assay was precise within day and between days as indicated by ANOVA test. The percentage recoveries from 10 replicate tablets of two commercial brands was 99.4 and 102.8% of the label amount and their coefficients of variation were 1.51 and 1.07%.

INTRODUCTION

Bromocriptine mesylate is 2-Bromo-12'-hydroxy-5'a-isobutyl-2'-isopropylergotaman-3',5',18-tione, a dopamine receptor against and a prolactin inhibitor.[1]

Few methods have been developed for the determination of bromocriptine in pharmaceutical dosage forms, using liquid chromatography-mass spectrometry[2,3] and HPLC method.[4,5]

The purpose of this study was to develop a simple, direct, precise and convenient HPLC assay for the quantitation of bromocriptine mesylate in tablet formulation, by passing several tedious steps involved in other assay methods.

EXPERIMENTAL

Chemical and Reagents

Bromocriptine mesylate[6] and propyl paraben[7] were used without further purifications. Acetonitrile,[8] methanol[8] and water were HPLC grade. All other chemicals and reagents were U.S.P. or A.C.S. quality and were used as received.

Instrumentation

A water HPLC systems[9] was used consisting of the following components: Model 45 pump, the WISP model 710 B autosampler, the model 481 UV detector set at 240 nm at 0.02 AUFS, the model 730 data system. Chromatographic separation was accomplished using C_{18} column, 8 mm i.d. x 10cm μBonda pack C_{18} column with 10μm packing.

Chromatographic Conditions

The eluting medium consisting of 40% v/v of 0.05 M ammonium acetate and acetonitrile pH 5.6 with glacial acetic acid, was prepared and degassed by bubbling helium gas for 5 min. prior to use. Column equilibrium with the eluting solvent was established by pumping the mobile phase at a rate of 0.2 mL/min. overnight. The flow rate was set at 0.8 mL/min. during analysis. The chromatogram was recorded and integrated at a speed of 0.2 cm/min.

Internal Standard

A stock solution of propyl paraben containing 10 mg in 100 mL methanol was prepared weekly and stored at 4°C.

Standard Solution of Bromocriptine Mesylate

A stock solution of bromocriptine mesylate was prepared by dissolving 2.5 mg of bromocriptine mesylate in 25 mL methanol (protected from light) water. Nine aliquots equivalent to 0.5, 1, 2, 3, 4, 6, 8, 9, and 10 mcg of bromocriptine were added to 1 mL volumetric flask. After the aliquot of the internal standard equivalent to 4 mcg was added, the flasks were brought to volume by acetonitrile and thoroughly mixed. Twenty μL of the standard solutions was injected onto the column for analysis. The peak area ratio of the drug: internal standard were plotted against the standard bromocriptine concentrations. Least square linear regression analysis was performed to determine the slope, y-intercept, and the correlation coefficients of the standard plots.

Sample Preparation

Individual tablets were pulverized using a mortar and pestle, and completely transferred to 25 mL volumetric flask (protected from light). The volume was adjusted with methanol and the flask was mechanically shaken for five min. Five mL of the solution was removed into a centrifuge tube and centrifuged at 3000 r.p.m. for 5 min. Eighty μL was transferred to a one mL volumetric flask containing 40 μL of propyl paraben stock solution, and diluted to the volume with acetonitrile. Twenty μL was loaded into the sample loop for chromatography. Ten replicate commercial tablets of bromocriptine mesylate were analyzed for statistical evaluation of the assay.

Quantitation

The amount of bromocriptine per tablet was determined from the following equation:

$$Q = [R/A + B] \times \text{dilution factor}$$

where Q is the mg bromocriptine per tablet, R is the peak area ratio (drug/internal standard), A is the slope of the calibration curve and B is the y-intercept.

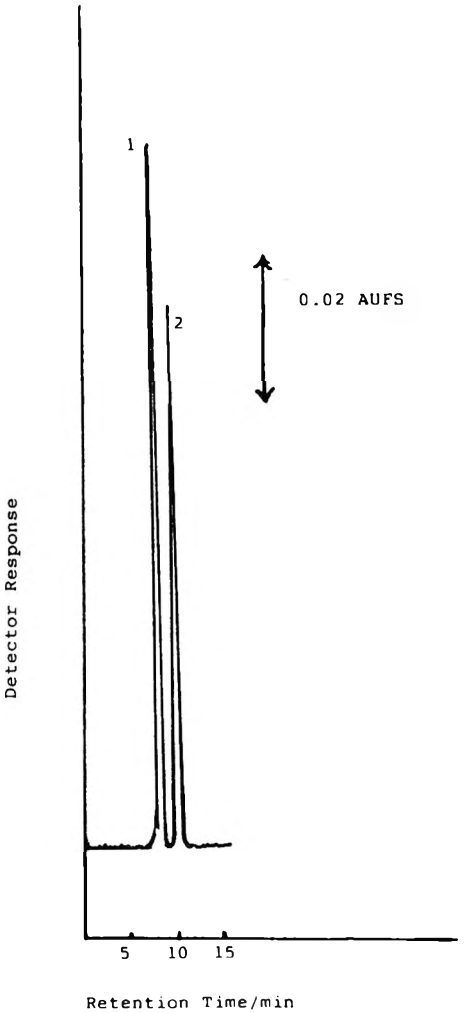

Figure 1. Chromatogram of Bromocriptine mesylate tablet.
Key: 1. Propylparaben, 2. Bromocriptine mesylate

Recovery of Bromocriptine Mesylate from the Fabricated Placebo Tablets

The reference tablets containing 2.5 mg of bromocriptine and 50 mg each of starch and lactose were prepared and subjected to the described HPLC assay to measure the accuracy and precision.

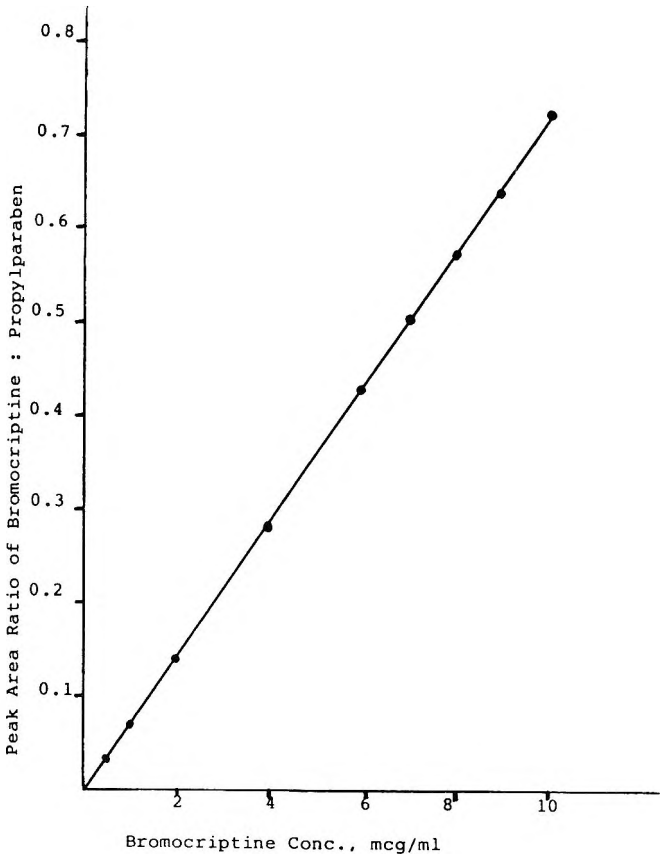

Figure 2. Standard Calibration Plot of Bromocriptine

RESULTS AND DISCUSSION

Figure 1 shows typical chromatograms obtained following analysis of bromocriptine mesylate in tablets. Using the chromatographic conditions described, bromocriptine mesylate and propyl paraben were well separated and their retention times were 9.73 and 7.9 min., respectively. For both compounds, sharp and symmetrical peaks were obtained with good baseline resolution and minimal tailing, thus, facilitating the accurate measurement of the peak area ratio. No interfering peaks were found in the chromatogram due to tablet excipients. Figure 2 shows a calibration plot for the peak area ratio

Table 1

Regression Analyses of the Three Standard Plots of Bromocriptine

Standard[a]	Slope[b]	Intercept[b]	Correlation[b] Coefficient
1	0.072778	-0.0004	0.99981
2	0.070020	-0.0013	0.99890
3	0.077200	-0.0006	0.99900

a) obtained in 3 different days
b) the mean of 3 determinations at each drug concentration

of varying amounts of bromocriptine (0.5-10 mg/mL) to a constant amount of propyl paraben (4 mcg/mL). The plots were linear (r=0.9998) and the regression analysis of the data gave the slope and intercept as:

$$Y = 0.0727 x - 0.0004$$

where Y and X are the peak area ratio and bromocriptine concentration, respectively. Three replicate analyses of bromocriptine at concentration of 0.5 - 10 mcg/mL were performed at three different days over one week period. The results of this evaluation are summarized in Table 1. The average correlation was higher than 0.9990 and the coefficient of variation of the slopes of the three lines was <0.1%. Analysis of variance of the data showed no detectable difference in the slopes of the three standard plots ($F=4.09$, $P > 0.01$). The similarities in the slopes and the high correlation coefficients indicate that the assay possesses excellent reproducibility and linearity. Thus, the method should be accurate and precise within the assay day as well as between assay days.

Precision and Accuracy

Six placebo tablets containing 50 mg each of lactose and starch and 2.5 mg bromocriptine were assayed for four consecutive days for intra and interday precision studies. The average recovery shown in Table 2 was (2.504 mg) with the coefficient of variation 1.49%. Estimation of day to day and within day precision were calculated by ANOVA test. The calculated F values, $F_{0.05}$ (5, 15) = 0.5637 and $F_{0.05}$ (3, 15) = 1.0257 were smaller than the table values $F_{0.05}$

Table 2

Analysis of Variance for Intra- and Inter Day Precision

Day/Assay	1	2	3	4	5	6
1	2.555	2.457	2.570	2.52	2.482	2.515
2	2.510	2.455	2.495	2.542	2.477	2.517
3	2.540	2.523	2.477	2.567	2.442	2.512
4	2.465	2.547	2.522	2.507	2.462	

Mean = 2.504 mg SD = 0.0374 CV% - 1.49

Anova Test

Source of Variation	DF	Sum of Squares	Mean of Squares	F Ratio	P
Within day	5	0.0025796	0.0005159	0.56368	0.05
Between day	3	0.007824	0.0015648	1.02570	0.05
Error	15	0.022882	0.00152548		
Total	23	0.0335856			

(5, 15) = 2.44 and $F_{0.05}$ (3, 15) = 2.24, respectively. Thus, it was concluded, that there was no significant difference for the assay which was tested within day and between days.

Recovery

Table 3 shows the average recovery by the placebo tables containing 2.5 mg bromocriptine and 50 mg each of lactose and starch. The average recovery was % 100.56 and its relative standard deviation was 0.456.

Analysis of Bromocriptine Tablets

Table 4 presents the results obtained from the analysis of bromocriptine mesylate commercially available. The mean percent recoveries were 99.4, 102.8.

Table 3

Recoveries From Spiked Placebo Tablets

Bromocriptine, mg added	mg Recovered	% Recovery
2.5	2.524	100.80
2.5	2.505	100.20
2.5	2.497	99.88
2.5	2.530	101.20
2.5	2.525	101.00
2.5	2.520	100.80
Mean	2.5160	100.65
SD	0.0115	0.459
CV	0.456	0.456

Table 4

Analysis of Dosage Form of Bromocriptine

Sample	n[a]	Mean % Recovery	SD	%CV
A	10	99.4	1.5	1.518
B	10	102.8	1.1	1.070

The stability indicating nature of the assay has not been demonstrated in this study, since no sign of degradation was observed by TLC after subjecting the drug solution (pH 3 and 9) at 70°C for 2 hr which, was also evident, from the absence of any additional peaks in the chromatograms.

CONCLUSION

The HPLC method developed in this study has the advantages of simplicity, precision and convenience. It also allows for the direct determination of bromocriptine. Therefore, the method should be useful for routine analytical and quality control assay of bromocriptine in dosage forms.

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STUDY OF THE COMPETITIVE BINDING OF TBA AND CP IONS ON C_{18} SURFACE

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ABSTRACT

The adsorption behavior of tetrabutylammonium (TBA) and cetylpyridinium (CP) cations was studied on Hypersil 5-ODS sorbent. After establishing that CP has about 5-fold stronger affinity to the C_{18} surface than the structurally different, symmetric, spherical molecule of TBA, the adsorption from their common solution was also examined. The data of "competitive binding" seem to show that TBA should have certain selective binding sites on the C_{18} surface from which it can not be displaced by the CP, even in the case where the latter is present in a 4-fold excess in the mobile phase.

The maximal coverage was found to be 52.9%(CP) and 12%(TBA), i.e., every second C_{18} group is covered by CP cations and only 1/8th of C_{18} groups may interact with TBA. The accessible, proton releasing silanol content, related to the total C_{18} content of the column, was found to be very low.

INTRODUCTION

The retention determining role of the stationary phase concentration of ion pairing agent is a generally accepted view in reversed phase ion pair (RPIP) HPLC.[1-6] Correspondingly, the binding to the C_{18} surface of the different IP'ing agents was the subject of several works.[7-15] In this respect, numerous structural types of the cationic and anionic IP'ing agents were examined during the last decades, but only a few compounds are widely used in the daily practice of RPIP HPLC routine and research.

From the cationic IP'ing agents the cetyltrimethylammonium, CTMA(Chloride=cetrimide) tetrabutylammonium, TBA (bromide, sulfate or dihydrogenphosphate) are the most often used IP'ing substances. Although the adsorption onto the C_{18} surface under different experimental conditions was examined for both of these agents (CTMA[9,10,14], TBA[13,15]) a comparative study on the binding property of the two substances has not been yet performed. Such an examination offered interesting observations by the fact that TBA and CTMA represent two different structural subtypes of the quarternary ammonium IP'ing compounds. While TBA is a symmetrically substituted "spherical" cation, the cetyl chain makes CTMA asymmetric.

Assuming that the binding on the C_{18} surface takes place through one of the butyl radicals (TBA) and the cetyl group (CTMA), it can be expected that the nitrogen, i.e., cationic head, facing towards the mobile phase, will have quite different interacting (hydrophobic,electrostatic) properties due to the different volume, carbon content, etc., of the environmental trimethyl and tributyl moieties.

In this work, instead of CTMA as asymmertric quarternary ammonium compound, cetylpyridinium, CP (chloride) was applied. This compound emerged as IP'ing agent in a work of Bidlingmeyer[16] and was studied by Budvári-Bárány et al.[17] in a dynamically modified silica-based RP system. The aromatic nucleus content, resulting in UV activity (i.e., UV detectability) and enabling the molecule to certain selective interactions, may be the source of potential advantages of CP over CTMA being applied as an IP'ing agent or functioning, in case of cationic or neutral solutes, as a displacing modifier.

Since the chromatographic selectivity of TBA or CP is largely determined by the quality and concentration of the anchoring cationic head, a study of the competitive binding of TBA and CP on C_{18} (i.e., when they are present simultaneously at the same eluent) seemed for us—also technically—an attractive task. While the breakthrough of TBA was signaled by refractometry, the pyridine moiety (i.e., UV activity) of CP allowed the application of dual (UV spectrophotometric-refractometric) detection for selectively revealing the breakthrough of CP and the selective binding sites on the C_{18} surface.

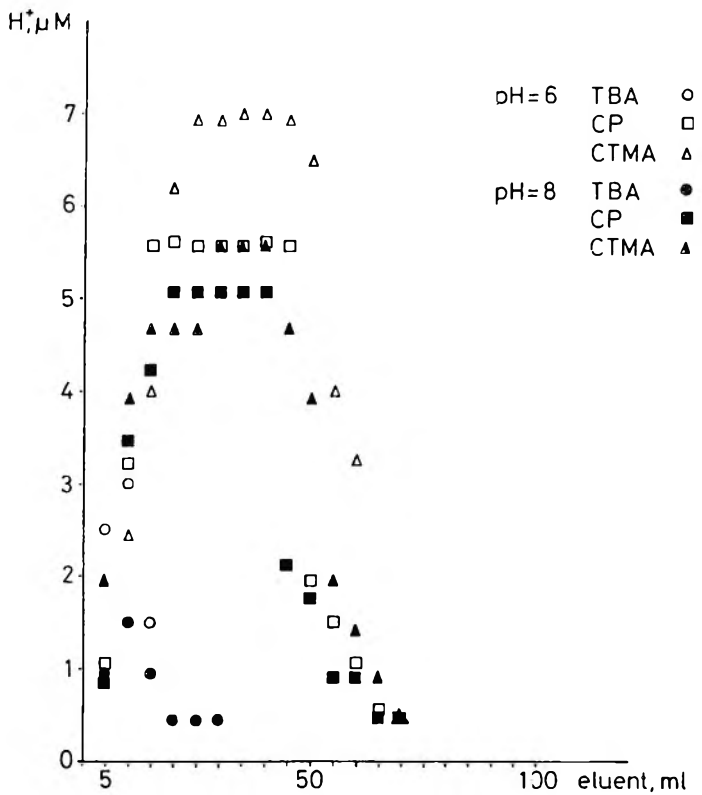

Figure 1. The amount (μM) of proton released by the IP'ing reagents on Hypersil ODS column (sorbent content: 2.5 g = 425 m^2; see, also, Table 4).

EXPERIMENTAL

Chromatography

The HPLC apparatus comprised an ISCO pump Model 2350 (USA), combined with a Valco injector unit (10 μL loop). An ISCO variable wavelengh absorbance detector (230-800 nm) was used.

For plotting the breakthrough curves of TBA and CP (CTMA), a Waters differential refractometer, Model R401, was employed. For the breakthrough determination, in case of CP, dual (refractometric-UV spectrophotometric) detection was used. The description of the equipment is available in a previous work.[18] The equipment units, subsequent to the pump, were thermostatted at

$25^0C \pm 1^0C$ (Ultrathermostat MLW type U2C, Freital, Germany). The breakthrough curves and the chromatograms were recorded, the data handling was effected by a Hewlett Packard integrator Model 3396 Ser II.

The C_{18} sorbent, Hypersil 5-ODS (Shandon), particle size 5 μm, was packed in a stainless steel column (250 x 4.0 mm I.D., BST, Budapest, Hungary).

As mobile phase, sonically degassed and filtered mixtures of acetonitrile and aqueous phosphate buffer solutions (pH = 3.0; 6.0; 8.0) containing different amounts of TBA and/or CP was applied. All ready-made eluents contained sodium chloride in an amount to adjust the ionic strength to 0.1. Each data of binding or retention was calculated as an average of two or three parallel runs. The column void time was signalled by the solvent peak of acetonitrile. Following testing, the columns were brought to the initial state by washing with a 90:10 mixture of water-acetonitrile (200 mL) and then with acetonitrile (100 mL). In case the column was loaded by an eluent with higher pH (6.0 and 8.0) a prewash with a 10:90 mixture of acetonitrile-phosphoric acid (pH = 2.5) was performed.

The eluent flow rate was 1.0 mL/min. The effluent was monitored at the wavelength of maximum absorption for each compounds.

Materials

Cetylpyridinium chloride monohydrate, 98%, Aldrich, Tetrabutyl-ammonium hydrogen sulfate, 97%, Aldrich; Buffer solutions at the pHs 3.0 6.0 8.0 were prepared by mixing the proper volumes of 0.067 M aqueous solutions of potassium dihydrogenphosphate and sodium hydrogenphosphate (KH_2PO_4,Na_2HPO_4,$2H_2O$, analytical grade, Reanal, Budapest); the pH of the solutions were tested by potentiometry with an accuracy ±0.02 unit. Acetonitrile for HPLC, Chemolab, Budapest. Water, deionized,double distilled. Sodium chloride 99.99%, Aldrich. Potassium iodomercurate(II), K_2HgI_4, Merck. Model substances (Table 5) met the requirements of the Hungarian Pharmacopeia. From their 0.01 % methanolic solution 10 μL was injected.

RESULTS & DISCUSSION

Table 1 contains the data of binding of the TBA and CP by two Hypersil ODS columns of different batches. The binding data were obtained by the usual evaluation of the breakthrough curves. Prior to each breakthrough experiment, the column was purged with the method which was described in the experimental section.

Table 1

The Amount of Adsorbed Ion Pairing Reagent, μM

	Column I			Column II		
	5*	**10***	**20***	**5***	**10***	**20***
TBA	93	125	144	102	120	144
CP	600	624	635	581	597	624
I**	6.5	5.0	4.4	5.7	5.0	4.3

Stationary phases: I-II: Hypersil 5-ODS (Shandon); Mobile phase: Phosphate buffer 0.067 M (pH = 3) - acetonitrile 90: 10 + TBA or CP, I = 0.1 (NaCl); * IP'ing reagent conc'n in the mobile phase mM; ** CP/TBA.
NOTE: The adsorbed amount of ion pairing reagents at pH 6 and 8 varied within ± 10% compared to the values at pH 3.

Table 2

Ratio of the Adsorbed Amount from CP-TBA Common Solution

		Ratio of CP/TBA Concentrations in Eluent, mM							
		5/5	**10/10**	**20/20**	**10/5**	**20/5**	**20/10**	**5/10**	**5/20**
pH 3	Cp[b]	572	615	609	592	600	594	600	558
	TBA[b]	93	96	114	74	59	81	105	102
	CP/TBA	6.2	6.4	5.3	8.0	10.2	7.3	5.7	5.4
pH 6	Cp[b]	538	552	528	567	618	576	546	541
	TBA[b]	84	90	96	78	69	78	106	93
	CP/TBA	6.4	6.1	5.5	7.3	9.0	7.4	5.2	5.8
pH 8	Cp[b]	570	554	567	540	555	516	545	540
	TBA[b]	83	90	114	70	62	75	104	120
	CP/TBA	6.9	6.1	5.0	7.7	9.0	6.9	5.2	4.5

Stationary phase: Hypersil II 5-ODS (Shandon); Mobile phase: Phosphate buffer 0.067 M - acetonitrile 90:10 + CP or TBA; I = 0.1 (NaCl); [b]Bonded amount, μM.

The breakthrough for TBA, signalled by the refractometric detector, strictly coincided with the point where continous monitoring of the effluent by Mayer's reagent (aqueous solution of K_2HgI_4) began to show a positive response. In this way, with a chemical indication of the breakthrough, we could establish, with a great accuracy, that the effluent practically did not contain TBA at all before the breakthrough: the limit of visual detectability (appearance of white turbidity) 0.8 μg/mL.

The breakthrough of CP was signalled by both detectors. The values in Table 1 show that CP has an affinity about 5-fold stronger than that of TBA towards the ODS surface. It may also be seen that the adsorbed amount of TBA fairly depends upon the TBA concentration in the mobile phase, while CP binding seems quasi-independent of the mobile phase concentration. As a consequence, I^x (CP/TBA binding) values decrease parallel to the increasing of the mobile phase concentration of TBA and CP.

Table 2 shows the binding ratio CP/TBA (I^x) when the two reagents are present in a common eluent in different concentration ratios, and also, the pH influence on the binding is shown. What is to be seen, at 1:1 ratios with increasing concentrations is that I^x decreases down to 4.9 from 6.4. This experience allows the assumption that TBA should have some selective binding sites on the C_{18} surface and these sites cannot be occupied by the CP; this reasoning seems to be confirmed by the fact that CP, although the bound amount of TBA somewhat decreases, cannot substitute for it. The coverage under the experimental conditions, i.e., the number of CP and TBA groups/nm^2, is rather low (see Table 3). Only half (52.9%) of the C_{18} chains may be considered as covered (interacting) with CP cations, and only 1/8th (12%) of the C_{18} groups are occupied by TBA cations.

Table 4 and Fig. 1 show the data for silanol content, which was found accessible and proton releasing by CP and TBA under the experimental conditions used. In this experiment, for the sake of comparison, CTMA was also involved. The calculations (see the scheme under Table 4) were based upon our assumption and other authors' findings[19] that CP, CTMA & TBA displace K^+ ions at the sites the latter occupied during the preconditioning of the column. In these experiments, the pH of the effluent was monitored in 5 mL portions. Summing up the pH changes caused by the different eluent portions, the total amount of protons released by CP(CTMA) or TBA was calculated. The released proton amount was taken equivalent to the accessible and proton releasing silanol content. The data indicate a silanol content which is much lower than might be expected from the findings, i.e., that residual silanol content on the silica C_{18} surface may reach 50% of that of the parent silica. Although total silanol content was not measured in this work, the silanol content found to be accessible and proton-releasing by CP or TBA was very low, compared to the

Table 3

Bonding Density

	CP	TBA
Coverage μM/Column (2.5 g)	635	144
/g (170 m^2)	254	57.6
/m^2	1.49	0.34
Coverage, group/nm^2	0.90	0.2

Coverage by ODS (Hypersil) 2.8 μM/m^2 (=1.7 $C_{18}H_{37}$/nm^2).
Coverage CP/ODS: 0.53; TBA/ODS: 0.12.

Table 4

Accessible, Proton Releasing Silanol Content

	SiOH/nm^2	
Ion	**pH = 6**	**pH = 8**
K	0.004	0.007
CP	0.066	0.060
TBA	0.010	0.007
CTMA	0.087	0.070

$$\sum_{n}^{0}\left[H^{+}\right]M / 1.5 \bullet 10^{-3} = M$$

$$\frac{M}{10^{-6}} = \mu M / \text{column}$$

$$\frac{\mu M / \text{column}}{2.5} = \mu M / g$$

$$\frac{\mu M / g}{170} = \mu M / m^2$$

$$\frac{\mu M / m^2 \bullet 6.02 \bullet 10^{-17}}{10^{18}} = H^{+} / nm^2 = SiOH / nm^2$$

literature data for residual silanols (~1.2 silanol group/nm^2). The relative coverage by accessible and proton releasing silanols at pH = 8 comes from the binding density data for CP and TBA (Table 3) divided by the number of accessible and proton-releasing silanols: CP/SiOH = 15; TBA/SiOH = 28.

Table 5

Cationic Ion Pairing Reagents' Influence on the Retention Time (min) of Cationic Compounds

Compound	0.1 M Phosphate Buffer (pH=3) - Acetonitrile				
	------ 95:5 ------			90:10	80:20
		TBA 2.5 mM	CP 5 mM		
Norephedrine	10.2	3.2	1.9	5.2	2.7
Ephedrine	15.2	3.6	2.0	6.8	3.0
Homatropine	33.9	5.5	2.4	10.6	3.3
Atropine	104.8	11.2	2.3	23.1	4.6
Codeine	27.4	4.4	1.7	7.7	2.8
Ethylmorphine	88.4	10.2	1.8	18.9	4.0
Procaine	19.1	3.0	1.6	6.8	3.0
Tetracaine	∞	∞	5.3	∞	39.3

These results provide numerical data for estimation of the contribution of salt formation in the silanophil effect. The latter was found to be almost negligible under the experimental conditions employed, confirming the decisive role of other polar interactions (H-bond, complex formation, etc.) in the silanophil effect of basic (amine) compounds.

Table 5 illustrates some potential practical aspects of applying CP or TBA as a displacing organic modifier in RP-HPLC. The model compounds in Table 5 represent four pairs of pharmaceutical amine compounds being homologs or structurally related derivatives. The reasonable applicability of such systems most clearly manifests itself in case of tetracaine which cannot be eluted (t_r>100 min) in a mobile phase with 10% acetonitrile content, while the addition of 0.005M CP results in a rapid elution with excellent peak quality in an eluent with only 5% of acetonitrile.

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ANALYSIS OF ORGANOCHLORINE PESTICIDES IN PLAIN MILK USING DIRECT INJECTION ON AN ISRP COLUMN, WITH COLUMN SWITCHING

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ABSTRACT

A simple and rapid procedure for extraction and separation from aldrin, DDT, endrin, heptachloro- and methoxychloro-organochlorine pesticides in raw milk has been developed by direct injection into an HPLC system without pretreatment of the samples, using an ISRP column.

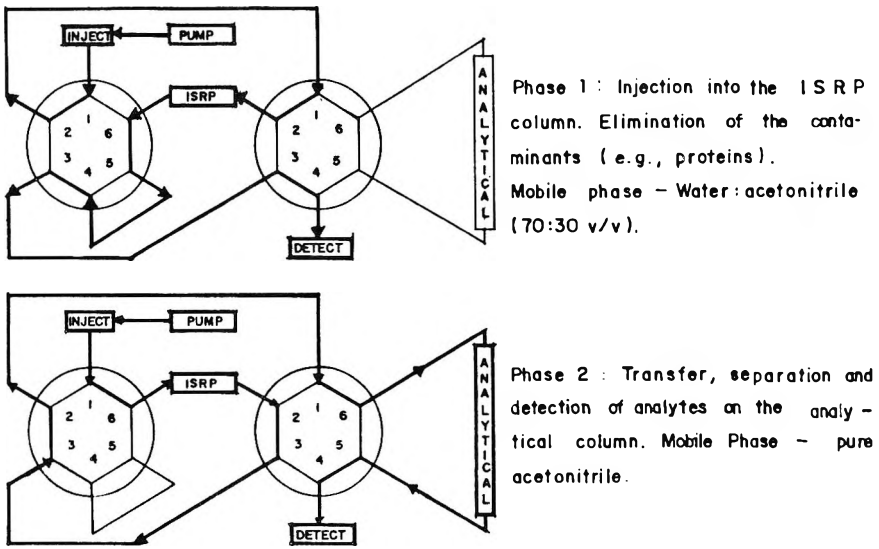

Figure 1. Schematic Representation of the HPLC column switching system.

INTRODUCTION

The determination of organochlorine pesticide residues in milk has always presented problems because the most common approach has required total extraction of fat, together with lipophilic compounds, including organochlorine pesticide residues.[1] The analytical methods generally involve initial solvent extraction of lipids, proteins and contaminants. The subsequent separation of pollutants from lipids often presents considerable problems in the analyses of organochlorine pesticides, partitioning between solvents of different polarities,[1] precipitation of proteins[2] and chromatography using aluminium oxide,[3] silica gel and florisil[4] have been employed.

These traditional methods are time consuming and expensive because of the high cost of the solvents and adsorbents. In contrast to such conventional extraction procedures, on-line extraction and clean-up procedures have been described using normal solid-phase extraction[5,6] which allows a significant decrease in the number of manual operations involved, but has the disadvantage of requiring large amounts of solvents.

The employment of steam distillation, solvent extraction at normal pressure has been described for recoveries of organochlorine compounds in water samples,[7] but require various operations to obtain pure samples.

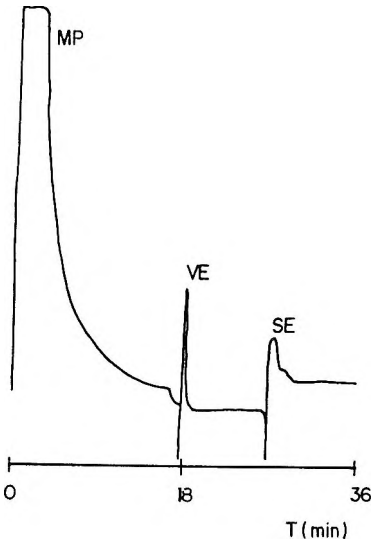

Figure 2. Chromatogram of the plain milk blank. MP = milk proteins; VE = valve effect; SE = solvent effect.
Chromatographic conditions: Extraction column - HSA-Si-C_{18} (30 x 4.6 mm), mobile phase - water:acetonitrile (70:30 v/v); analytical column - ODS-Hypersil (30 x 4.6 mm), mobile phase - acetonitrile; flow rate 1.5 mL/min, room temperature, detection = UV at 220 nm; injection volume - 20 μL.

Various extraction methods have been mentioned and, depending on their concentrations, the final analyses of organochlorine contaminants are performed by electron-capture GC[1,3-10] or GC-MS.[6,8,10]

The development of internal surface reversed phase (ISRP) silica materials by Pinkerton[11,12] for serum, milk and urine injection assays of drugs by HPLC have been successful for analysis of biological samples.

The use of human serum albumin (HSA), immobilized on the surface of silica, has also been employed for the resolution of drug enantiomers.[13,14]

This work describes a procedure for the extraction and separation of organochlorine pesticides by direct injection into an HPLC system, without treatment of samples using a new ISRP column.

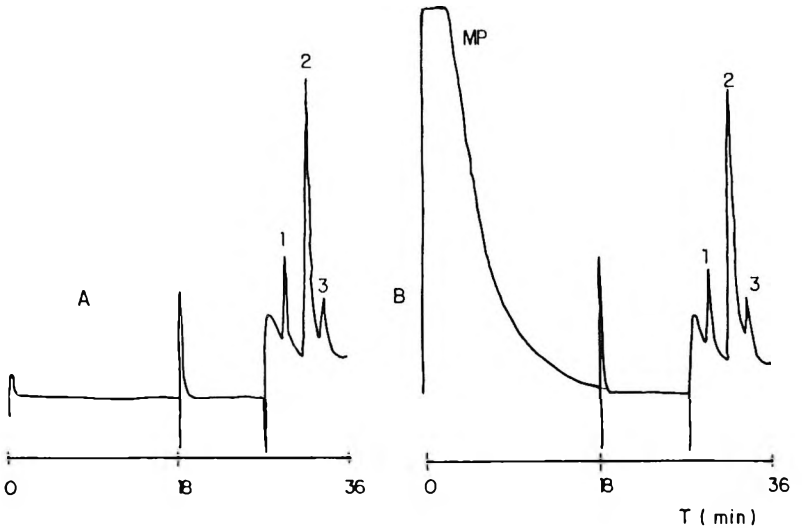

Figure 3. Chromatograms of the first group of pesticides.
(A) A standard sol'n of 50 μg/μL each of Endrin (1), DDT (2), and Aldrin (3). (B) Plain milk fortified with 50 μg/μL for Endrin (1), DDT (2), and Aldrin (3); MP = milk proteins. Chromatographic conditions as in Fig. 2.

EXPERIMENTAL

Chemicals and Solvents

Acetonitrile (HPLC grade) was obtained from Sharlau (ICS, Lapeyrousse Fossat, France). The standards (aldrin, DDT, endrin, heptachloro- and methoxychloro-) organochlorine pesticides, were obtained from Polyscience Corporation (Niles, Illinois, USA). The human serum albumin was obtained from Sigma Chemical Company (St. Louis, MO, USA), the trichlorooctadecylsilane, sodium cyanoborohydride and 25% (v/v) glutaraldehyde solution were obtained from Aldrich Chemical (Strasbourg, France). The pure water was prepared with an Elgastat UQH II (Cofralab, Bordeaux, France). The silica gel Merckosorb (pore diameter 60 Å and particle size 10μm) was obtained from Merck (Germany).

The natural raw milk was obtained from a grocery store in Bordeaux, France and was diluted with mobile phase [water:acetonitrile (70:30 v/v)] to 50% (v/v) and 25% (v/v) milk solutions. Stock standard solutions were prepared by dissolving known amounts of aldrin, DDT, endrin, heptachloro in acetonitrile to obtain 50 and 100 μg/μL.

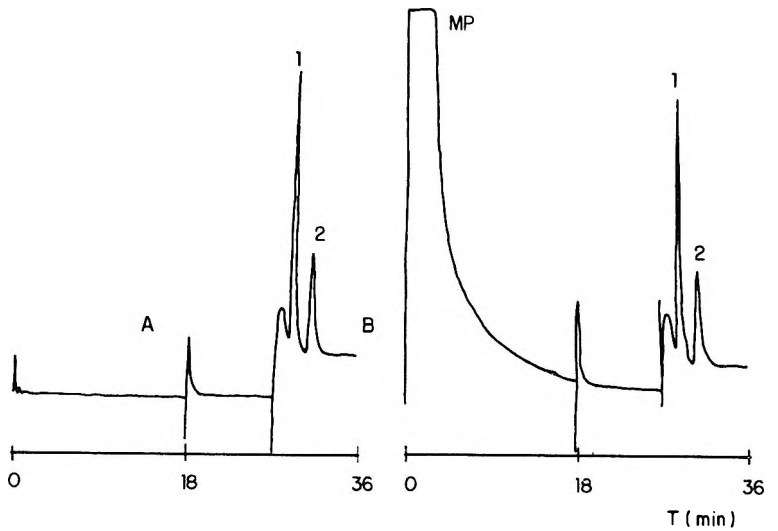

Figure 4. Chromatogram of the second group of pesticides.
(A) A standard sol'n of 50μg/μL each of Methoxychlor (1) and Heptachlor (2). (B) Plain milk fortified with 50μg/μL of Methoxychlor (1) and Heptachlor (2); MP = milk proteins. Chromatographic conditions as in Fig. 2.

Fortification of Milk Sample

The milk sample was fortified by adding 100 μL aliquots of standards aldrin, DDT, endrin, heptachloro and methoxychloro (100 μg/μL) solution into 100 μL aliquots of 50 % (v/v) milk solution, resulting in a milk sample at 50 μg/μL for aldrin, DDT, endrin, heptachloro and methoxychloro.

Chromatouraphic System and Conditions

The HPLC system consisted of a Philips Model 4015 pump, Philips Model 4025 Multi-Wavelenth UV-Vis detector (Atl, Bobigny, France) and a Kipp & Zonen BD 40 recorder (Enraf-Nonius, Gagny, France). The extraction of the proteins was carried with a ISRP-C_{18} column (30 x 4.6 mm), synthesized according to the reported protocol[15] and chromatographic separations were carried out on an ODS-Hypersil (30 x 4.6 mm, 3 μm) column, (Hewlett Packard) according to the schematic representation in Figure 1, maintained at room temperature, at a flow-rate of 1.5 mL/minute.

The initial mobile phase composition was water:acetonitrile (70:30 v/v). After 18 minutes, the switching valve (Model 7000-Rheodyne-AII Tech), was opened and the mobile phase was changed to HPLC grade acetonitrile.

The system was equilibrated at the initial mobile phase composition for 15 minutes before injecting the next sample. A manual injector (Model 7125-075 fitted with a 20 μL loop, Rheodyne, Cotati, CA,USA) was used for direct injection into the ISRP column. The detection of the eluted organochlorine pesticides was carried out at 254 nm with a UV-Vis detector.

RESULTS AND DISCUSSION

The employment of an HSA-C_{18} ISRP column with 60 Å pore and 10 μm particle size showed good results in the extraction of milk proteins, due to two factors. First, the milk proteins are not adsorbed by human serum albumin immobilized on the surface of the silica gel,[13,14] and, second, the milk proteins are large molecules that are not able to get into the small pore silica.

Various sample solutions were tried for direct injection: plain milk, and milk spiked with 50 % and 25 % (v/v). The 25 % (v/v) sample solution showed the best results, considering the time for the in-line column extraction and the minimized clogging of the HPLC system or column.

Figure 2 shows the extraction of milk proteins in 18 minutes, which is followed by the baseline disturbance at 18 ± 0.3 minutes, caused by the valve change, and at 28 ± 0.5 minutes, caused by the solvent change. The same effects were observed in Fig. 3 (A and B chromatograms).

The mobile phase, water:acetonitrile (70:30 v/v) gave good results, a separation without interference, with the use of these two columns (one for pre-concentration and the other for separation) with column switching (Fig. 3, A chromatogram).

The use of HPLC grade acetonitrile as the carrier for the organochlorine pesticides from the ISRP to the analytical column gives the best results, permitting the aldrin, endrn, and DDT separation, (see Fig. 3, A and B chromatograms) and the methoxychloro and heptachloro separation, (see Fig. 4, A and B chromatograms) when employing the analytical column mentioned.

On the ISRP column the extraction of these organochlorine pesticides was excellent, with recoveries of 50 ~g/ml additions to the milk samples beig 99.3 % (n=5). Elimination of any sample treatment, as described by others (1-10) make the procedure much simplier, and quicker to do, and, above all, minimizes sample loss. Such a facile method shoul appeal to many laboratories doing such analyses of pesticides.

ACKNOWLEDGMENTS

The authors thank the Foundation of Support the Search of Sao Paulo (Brazil) and the Aquitaine Regional Council of Bordeaux (France), for their financial support.

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J. LIQ. CHROM. & REL. TECHNOL., 19(19), 3231-3236 (1996)

LIQUID CHROMATOGRAPHY CALENDAR

1996

DECEMBER 17 - 20: First International Symposium on Capillary Electrophoresis for Asia-Pacific, Hong Kong. Contact: Dr. S. F. Y. Li, Dept of Chemistry, National University of Singapore, Kent Ridge Crescent, Singapore 119260, Republic of Singapore.

1997

MARCH 16 - 21: PittCon '97, Atlanta, Georgia. Contact: PittCon '97, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

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

MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California. Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California. Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994, USA. Tel: (517) 496-6491; FAX: (517) 496-6824.

MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY. Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd, Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois. Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York. Contact: T. Noce, Rust Envir & Infrastructure. 12 Metro Park Rd, Albany, NY 12205, USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas. Contact: J. H. Rigby, Chem Dept, Wayne State Univ., Detroit, MI 48202-3489, USA. Tel: (313) 577-3472.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th St., NW, Washington, DC 20036, USA.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.ord.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218.

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MARCH 1 - 6: PittCon '98, New Orleans, Louisiana. Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA. Email: reglmtgs@acs.org.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Res Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, Tenn 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2003

MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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MARCH 13 - 17: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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Journal of Liquid Chromatography & Related Technologies
P. O. Box 970210
Coconut Creek, FL 33097

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

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NOTE: Failure to adhere to the following guidelines will delay publication of a manuscript.

1. The preferred dimensions of the printed area of a page are
   6" (15.2 cm) width by 8.5" (21.6 cm) height.
   Use Times Roman 12 point font, if possible.

The general organization of the manuscript should be:

Title
Author(s)' names and full addresses
Abstract
Text Discussion
References

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

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

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

3. **Abstract**: The heading **ABSTRACT** should be typed boldface, capitalized and centered, 2 lines below the addresses. This should be followed by a single-spaced, concise abstract. Allow 2 lines of space below the abstract before beginning the text of the manuscript.

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

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGMENTS

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

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

5. The **first line of each paragraph** within the body of the text should be indented a half inch.

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

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

Following are acceptable reference formats:

Journal:

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

Book:

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

Chapter in a Book:

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

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

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used. **Hand-drawn characters are not acceptable.**

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

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2. All text should be **typed single-spaced**.

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

4. **Tables** should be typed as part of the text, but in such a way as to separate them from the text by a 2-line space above and below the table. Tables should be inserted in the text as close to the point of reference as possible. **A table may not be longer than one page**. If a table is larger than one page, it should be divided into more than one table. The word **Table** (followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

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

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J. LIQ. CHROM. & REL. TECHNOL., 19(19), (1996)

Contents Continued

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CONTENTS

(continued on inside back cover)

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