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ENANTIOSELECTIVE HPLC DETERMINATION OF R AND S TRIMIPRAMINE IN HUMAN SERUM USING AN OCTYLDECYLSILANE COLUMN WITH β-CYCLODEXTRIN AS MOBILE PHASE ADDITIVE AND SOLID PHASE EXTRACTION

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ABSTRACT

A stereospecific HPLC method was developed for the analysis of the enantiomers of trimipramine in human serum. The assay uses amitriptyline as the internal standard and a C_{18} solid phase extraction column for serum sample clean-up. It is free of interference from desmethyl-trimipramine, 2-hydroxy-desmethyl-trimipramine and 2-hydroxy-trimipramine, the three major metabolites of trimipramine. Recoveries of 98.8% and 97.5% were obtained for the R and S enantiomers of trimipramine, respectively. Resolution of the enantiomers was obtained using an octyldecylsilane column with β-cyclodextrin as the mobile phase additive. The composition of the mobile phase was 80:20 v/v aqueous 10mM ammonium acetate buffer pH 4 (adjusted with acetic acid)- absolute ethanol containing 20mM β-cyclodextrin at a flow rate of 0.7mL/min.

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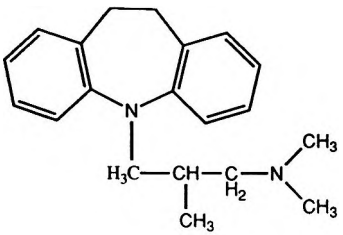
Linear calibration curves were obtained in the 25-400ng/mL range for each enantiomer in serum. The detection limit based on a S/N = 3 was 10ng/mL for each enantiomer in serum with UV detection at 220nm. The limit of quantitation for each enantiomer was 25ng/mL. Precision calculated as %RSD and accuracy calculated as % error were in the range 0.7-4.5% and 0.9-3.1%, respectively, for the R enantiomer and 0.7-5.1% and 0.4-4.4% respectively, for the S enantiomer. Separation of the three major metabolites of trimipramine was also investigated.

INTRODUCTION

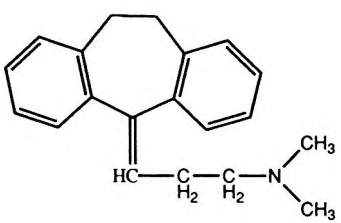
Trimipramine, 10,11-dihydro-N,N,β-trimethyl-5H-dibenz[b,f]azepine-5-propanamine is a dibenzazepine-derivative tricyclic antidepressant (TCA) commonly used for its antidepressant and anxiolytic activity (Figure 1). TCAs generally act by blocking the uptake of neuronal norepinephrine and serotonin in varying degrees.[1] Monitoring therapeutic levels of TCAs in serum as an adjunct to the clinical management of patients with depression is important since the side effects of these drugs are quite common and mainly dose related.[2]

Trimipramine and its three major metabolites, desmethyl-trimipramine, 2-hydroxy-trimipramine and 2-hydroxy-desmethyl-trimipramine, are chiral compounds with an asymmetric center at the side chain. Hydroxylation and desmethylation are the two major pathways of the metabolism of trimipramine (Figure 2). Although the chemical structure of trimipramine is closely related to other TCAs, it differs from many of them in some pharmacological aspects.[3] The most striking difference is the induction of supersensitivity of the noradrenergic system after long term treatment with trimipramine.[4] Differences in the physiological and behavioral effects induced by the two enantiomers have been demonstrated.[5] It has also been found that the enantiomers show different affinities for the D1 and D2 (dopamine), the $\alpha_{1A/B}$, α_{2A}, α_{2B} (noradrenaline) and the 5-hydroxytryptamine (5-HT_2) receptor subtypes.[3]

Cyclodextrins are the most commonly used chiral selectors in HPLC chiral separations. Native cyclodextrins such as α, β, and γ cyclodextrins are neutral natural cyclic oligosaccharides containing six, seven and eight glucose units, respectively. The shape of a cyclodextrin is similar to that of a truncated cone with a cavity that is hydrophobic within and hydrophilic outside. Enantiorecognition with cyclodextrins is a combination of inclusion phenomenon and additional interactions with the hydroxyl functional groups on the rim of the cyclodextrin.



TRIMIPRAMINE



AMITRIPTYLINE

Figure 1. Chemical structures of trimipramine and the internal standard amitriptyline.

Some of the earlier methods reported for the determination of racemic trimipramine in body fluids used gas chromatography[6-7] and high performance liquid chromatography.[8-11] Chiral separation of trimipramine enantiomers has also been reported.[12-16] The only method reported for the separation of trimipramine enantiomers and its major metabolites in serum used liquid-liquid extraction for sample pretreatment which was time consuming.[12]

This paper describes the separation and quantitation of trimipramine enantiomers in human serum and is free of interference from the three major metabolites, desmethyl, 2-hydroxy-trimipramine, and 2-hydroxy-desmethyl-trimipramine. The method involves solid phase extraction followed by separation on an octyldecylsilane column with β-cyclodextrin as the mobile phase additive and UV detection. The method is linear in the range 25-400ng/mL of each enantiomer.

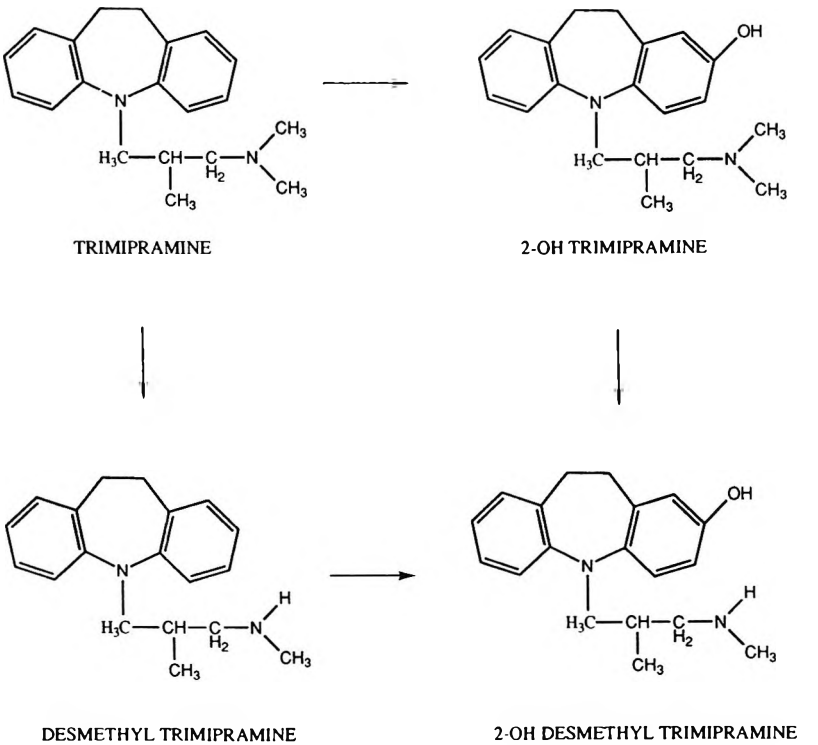


Figure 2. Major metabolic pathways of trimipramine in humans.

EXPERIMENTAL

Reagents and Chemicals

Trimipramine maleate was purchased from Sigma Chemical Co. (St. Louis, MO, USA). R and S trimipramine were a gift from Rhone-Poulenc Rorer Centre de Recherches (Vitry-Alforville, France). Desmethyl-trimipramine maleate, 2-hydroxy-desmethyl-trimipramine fumarate, and 2-hydroxy-trimipramine fumarate were kindly supplied by Dr. C. B. Eap of Hospital de Cerry (CH-1008 Prilly-Lausanne, Switzerland). Amitriptyline hydrochloride was obtained from Merck Sharp & Dohme (Rahway, NJ, USA). Absolute ethyl alcohol USP (200 proof) was purchased from Aaper Alcohol & Chemical Co. (Shelbyville, KY, USA) and ammonium acetate was obtained from J.T. Baker (Phillipsburg, NJ, USA). β-cyclodextrin (β-CD) was supplied by American

Maize Products Company (Hammond, IN, USA). All solvents were HPLC grade. Drug free human serum was obtained from Biological Specialty Corporation (Colmar, PA, USA). The C_{18}, C_8 and CN solid phase extraction columns (100mg/cc) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA).

Instrumentation

The HPLC system consisted of a Beckman Model 110A pump (Beckman, San Ramon, CA, USA) and a Model 728 autosampler (Micromertics Instruments Corporation, Norcross, GA, USA) equipped with a 20μL loop. The detector was a Waters Millipore Model 481 LC Spectrophotometer (Milford, MA USA) and a Spectra-Physics Model 4270 integrator (Spectra-Physics, San Jose, CA, USA) was used to record each chromatogram and peak height responses. Separation of the analytes was achieved on a Prodigy 5μ ODS(3) 100Å column(150x3.20mm i.d., Phenomenex, Torrance, CA) equipped with a 0.2μ Opti-solv precolumn minifilter (Optimize Technologies, Portland, OR, USA).

The mobile phase consisted of 80:20 v/v aqueous 10mM ammonium acetate buffer pH 4 (adjusted with acetic acid)- absolute ethanol containing 20mM β-CD and it was delivered at a flow rate of 0.7mL/min. The mobile phase was filtered through a 0.45μm filter (Alltech Associates, Deerfield, IL USA) and sonicated prior to use. The column was operated at ambient temperature (23±1°C).

Preparation of Stock and Standard Solutions

Individual stock solutions of 100μg/mL of R and S trimipramine (maleate salts) calculated as trimipramine base and 100μg/mL of internal standard amitriptyline were prepared in 10mL volumetric flasks by adding 2mL of methanol followed by the addition of deionized water to volume. The solutions were kept refrigerated at 4°C. Stock solutions of 10μg/mL of racemic 2-hydroxy-desmethyl-trimipramine (fumarate salt), 2-hydroxy-trimipramine (fumarate salt) and desmethyl trimipramine (maleate salt) calculated as their free bases were also prepared and kept refrigerated at 4°C.

Appropriate dilutions of the R and S trimipramine stock solutions gave 5μg/mL solutions which were used for spiking blank human serum.

Preparation of Spiked Human Serum Samples

Accurately measured aliquots (10, 30, and 50μL of the 5μg/mL standard solutions) of R and S trimipramine diluted solutions were pipetted into a 1mL volumetric tube and evaporated. Then 10μL of the internal standard solution was added to the tubes and drug-free human serum added to volume and mixed well to give final concentrations of 50, 150, and 250ng/mL of each trimipramine enantiomer.

Assay Method

One milliliter of the spiked human serum samples were vortexed for 2min and then passed through a C_{18} Bond-Elut solid phase extraction (SPE) column attached to a vacuum manifold (Vac-Elut, Varian Sample Preparation Products, Harbor City, CA USA). The column was previously conditioned with 2x1mL of absolute methanol followed by 2x1mL of deionized water. After the application of the serum sample, the column was washed with 2x1mL deionized water and the analytes were eluted with 4x125μL of 10mM acetic acid in methanol and evaporated to dryness under a slow nitrogen stream. The residue was reconstituted in 1mL of mobile phase, filtered through a 0.2μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI, USA) and 20μL injected into the liquid chromatograph.

For absolute recovery experiments, spiked samples were compared to unextracted stock solutions. Drug peak height ratios were used to calculate the recoveries. Linear regression analysis of the peak-height ratios of each enantiomer to internal standard versus concentration of each enantiomer in the range of 25-400 ng/mL produced slope and intercept data which were used to calculate concentrations of R and S trimipramine in each serum sample.

RESULTS AND DISCUSSION

When cyclodextrins are used as chiral mobile phase additives in reversed phase liquid chromatography, the separation mechanism is thought to be a combination of inclusion phenomenon and the additional interaction of the analyte with the functional groups on the rim of the cyclodextrin. Among the factors that control the enantioseparation process are (i) differences in the stability/binding constants of the CD complexes, (ii) differences in the adsorption of CD complexes on the surface of the stationary phase, and (iii) differences in the adsorption of free solute molecules on the CD layer that is adsorbed on the surface.[17] Hence the differences in inclusion complex

strengths between solutes and the CD cavity, as well as differences in the interaction with the rim functional groups, can result in improved chromatographic separations.

The enantiomers of trimipramine were successfully resolved on a nonporous octyldecylsilane column in our laboratory with β-CD as the mobile phase additive.[18] The bulky nature of trimipramine forms inclusion complexes with native β-CD and the side chains interact favorably with the CD rim. It is important to note that trimipramine enantiomers failed to separate with hydroxypropyl-β-CD because of the lack of any hydrogen bonding functional groups at or near the chiral center of the analyte with which to interact with the hydroxypropyl groups on the derivatized CD.

The nonporous column would not successfully resolve desmethyl trimipramine, one of the metabolites of trimipramine from the parent trimipramine despite modifications of the components of the mobile phase. A Prodigy ODS(3) column was, therefore, investigated because of its reported success of using the technique of β-CDs as mobile phase additives for chiral separation.[19] The Prodigy column successfully resolved the enantiomers of trimipramine and desmethyl-trimipramine. The mobile phase composition was 80:20 v/v 10mM ammonium acetate buffer pH 4 (adjusted with acetic acid)-absolute ethanol containing 20mM β-CD.

The influences of β-CD, ethanol and ammonium acetate buffer concentrations on the resolution of trimipramine and the three metabolites were investigated. Typically, the use of sodium or potassium phosphate buffers failed to resolve any of the analytes. An increase in the concentration of ethanol reduced retention times for all the analytes, but resolution was either reduced or lost completely as in the case of 2-hydroxy-desmethyl trimipramine and 2-hydroxy trimipramine.

The method is free of interference from human serum containing trimipramine and the three metabolites. Good resolution was obtained for the enantiomers of desmethyl trimipramine, partial resolution for the enantiomers of 2-hydroxy trimipramine, and no resolution for the enantiomers of 2-hydroxy-desmethyl trimipramine. Typical HPLC chromatograms for both blank human serum and serum spiked with 150ng/mL of each enantiomer of trimipramine and the three metabolites and the internal standard are shown in Fig.3.

The two more polar metabolites, 2-hydroxy-desmethyl trimipramine and 2-hydroxy trimipramine had shorter retention times and were not well resolved on the Prodigy column compared to the less polar desmethyl trimipramine.

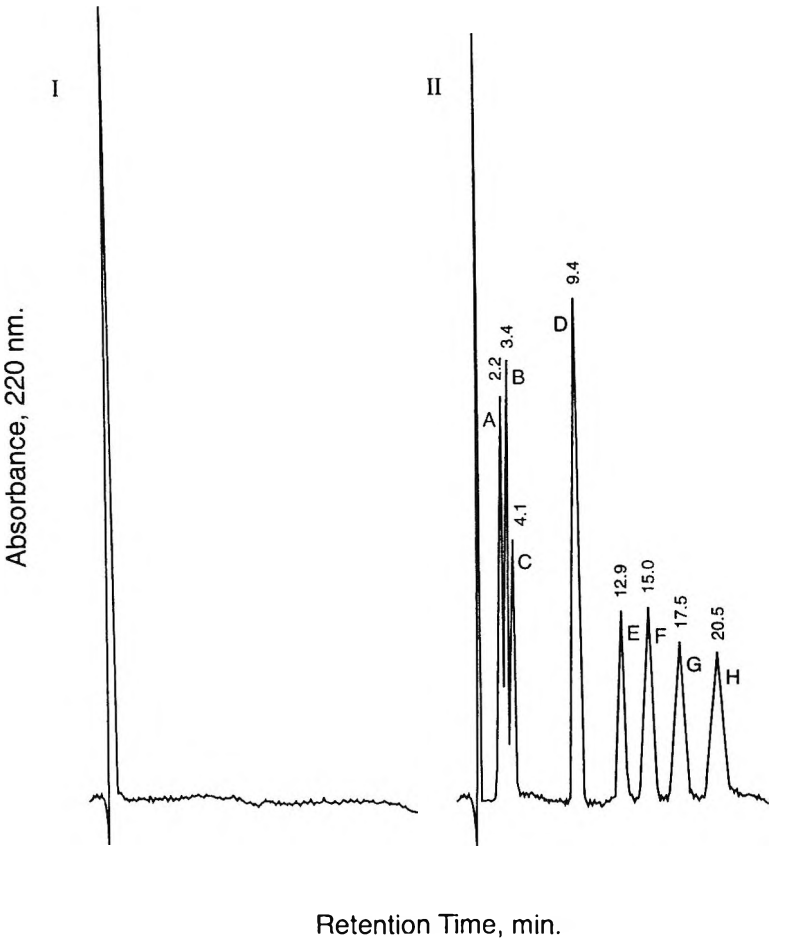


Figure 3. Typical chromatograms of (I) blank serum (left) and (II) serum spiked with 50ng/mL of each enantiomer of trimipramine, desmethyl-trimipramine, 2-hydroxy-desmethyl-trimipramine, 2-hydroxy-trimipramine and the internal standard. Peaks: A and C = 2-hydroxy-trimipramine, B = unresolved racemic 2-hydroxy-desmethyl-trimipramine; D = internal standard; E = R-trimipramine enantiomer; F = S-trimipramine enantiomer; G and H = desmethyl-trimipramine.

Modifying the composition of the mobile phase to 95:5v/v 10mM ammonium acetate buffer pH 4 (adjusted with acetic acid)- absolute ethanol enabled 2-hydroxy-desmethyl trimipramine and 2-hydroxy trimipramine to be completely resolved (see Fig.4).

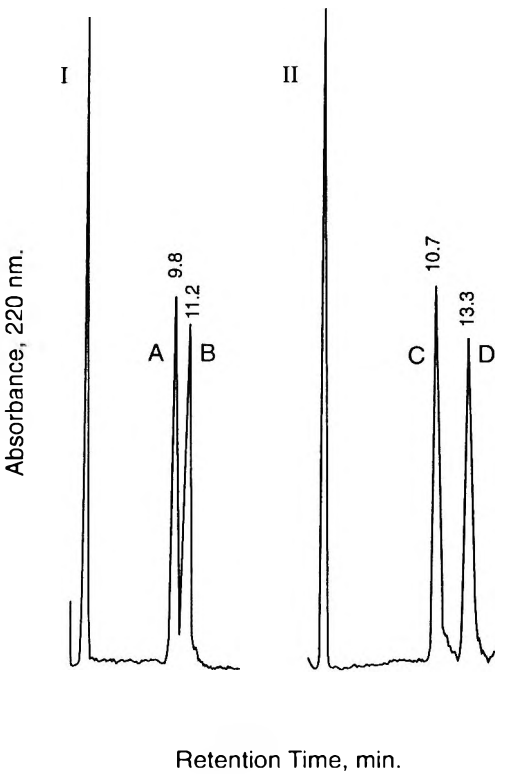


Figure 4. Separation of (I) 2-hydroxy-trimipramine enantiomers A and B and (II) 2-hydroxy-desmethyl-trimipramine C and D on the Prodigy ODS(3) column with mobile phase of 95:5v/v 10mM ammonium acetate buffer pH 4(adjusted with acetic acid)-absolute ethanol containing 20mM β-CD.

To decrease sample preparation time, a solid phase extraction (SPE) procedure was developed. Initial solid phase extraction experiments using ethylsilane, octylsilane, and cyanopropyl cartridges gave less than 70% recoveries of the two enantiomers. The octadecylsilane sorbent was selected because it allowed excellent recoveries of trimipramine enantiomers and the internal standard. Absolute recoveries of >98% (n=3) were obtained for each enantiomer with 10mM acetic acid in methanol. Addition of 10mM acetic acid to the methanol ionized the trimipramine at an acidic pH and hence reduced the affinity of the drug for the bonded phase silica. Amitriptyline was selected as the internal standard based on its structural similarity to trimipramine and also its similar extraction behavior to trimipramine (recovery of 99%).

Table 1

Accuracy and Precision Data for Trimipramine Enantiomers in Spiked Human Serum

Analyte	Conc. Added (ng/mL)	Conc. Found[a] (ng/mL)	Error (%)	RSD (%)
Intra-Day				
R trimipramine	50	49.32±1.57	1.4	3.2
	150	152.17±1.96	1.5	1.3
	250	247.63±4.08	0.9	1.6
S trimipramine	50	48.90±2.47	2.2	5.1
	150	151.27±2.92	0.9	1.9
	250	253.15±3.68	1.3	1.5
Inter-Day				
R trimipramine	50	48.45±2.16	3.1	4.5
	150	151.85±2.07	1.2	1.4
	250	252.19±1.85	0.9	0.7
S trimipramine	50	47.80±1.87	4.4	3.9
	150	148.67±2.35	0.9	1.6
	250	249.12±1.86	0.4	0.7

[a] Based on n=3 for intra-day assay and n=9 for inter-day assay.

Linear calibration curves were obtained in the 25-400ng/mL range for each trimipramine enantiomer. Standard curves were fitted to the linear regression equation y=ax+b, where y represents the ratio of drug/internal standard peak heights, a and b are constants, and x is the trimipramine concentration. Typical regression parameters of a (slope), b (y-intercept), and correlation coefficient were calculated to be 0.00151, 0.006525, and 0.99984, respectively, for R trimipramine and 0.00147, 0.01789, and 0.9994, respectively, for S trimipramine (n=3). The precision, calculated as %RSD and accuracy calculated as % error of the method, was ascertained using spiked samples at 50, 150, and 250ng/mL levels.

The intraday precision and accuracy as expressed by %RSD and %error were in the range 1.3-3.2% and 0.9-1.4% (n=3), respectively, for the R trimipramine and 1.5-5.1% and 0.9-2.2% (n=3), respectively, for the S enantiomer. The interday precision and accuracy were in the range 0.7-4.5% and 0.9-3.1% (n=9), respectively, for the R enantiomer and 0.7-3.9% and 0.4-4.4% (n=9), respectively, for the S enantiomer.

The detailed data is listed in Table 1. The minimum detectable concentration of each enantiomer was determined to be 10ng/mL (S/N=3). The limit of quantitation was found to be 25ng/mL for each enantiomer.

In conclusion, an HPLC method has been developed and validated for the assay of R and S trimipramine in human serum using β-CD as the mobile phase additive and a Prodigy ODS(3) column. The method uses an octadecylsilane solid phase extraction for sample clean-up and is applicable to the separation and quantitation of each trimipramine enantiomer in the 25-400ng/mL range. In addition, the method is free of interference from 2-hydroxy-desmethyl trimipramine, 2-hydroxy trimipramine, and desmethyl trimipramine, the three major metabolites of trimipramine.

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REFERENCES

1. R. N. Gupta, J. Chromatogr., **576**, 183-211 (1992).

2. C. S. S. Walter, Proc. R. Soc. Med., **64**, 282-290 (1971).

3. G. Gross, X. Xin, M. Gastpar, Neuropharmacol., **30**, 1159-1166 (1991).

4. H. R Olpe Hauser, R. S. G. Jones, Eur. J. Pharmacol., **111**, 23-30 (1985).

5. L. Julou, O. Lean, R. Ducrot, Comptes Rendus Biol., **155**, 307-312 (1961).

6. A. Abernathy, D. J. Greenblatt, R. I. Shader, Clin. Pharmacol. Ther., **35**, 348-352 (1984).

7. H. Hatori, E. Takashima, T. Yamada, O. Suzuki, J. Chromatogr., **529**, 189-193 (1990).

8. S. Hartler, B. Hermes, C. Hiemke, J. Liq. Chromatogr., **18**, 3495-3505 (1995).

9. G. Gulaid, G. A. Jahn, C. Maslen, J. Dennis, J. Chromatogr., **556**, 228-233 (1991).

10. M. Mazhar, S. R. Binder, J. Chromatogr., **497**, 201-212 (1989).

11. L. Wen-nuei, P. D. Frade, Ther. Drug Monit., **9**, 448-455 (1987).

12. E. Eap, L. Koeb, E. Holsboer-Trachsler, P. Baumann, Ther. Drug Monit., **14**, 380-385 (1992).

13. D. Haupt, C. Pettersson, D. Westerlund, Fresenius J. Anal. Chem., **352**, 705-711 (1995).

14. G. A. Ponder, S. L. Butram, A. G. Adams, C. S. Ramanathan, J. T. Stewart, J. Chromatogr. A-, **692**, 173-182 (1995).

15. D. T. Witte, J. Bosman, T. De-Boer, B. F. H. Drenth, K. Ensing, R. A. De-Zeeuw, J. Chromatogr., **553**, 365-372 (1991).

16. J. Hermansson, J. Chromatogr., **316**, 537-546 (1984).

17. M. L. Hilton, D. W. Armstrong in **New Trends in Cyclodextrins and Derivatives**, D. Duchene, ed., Editions de Santè, Paris, 1991, Ch.15 p.536.

18. E. Ameyibor, J. T. Stewart., J. Liq. Chromatogr. & Related Technol. in press, 1997.

19. J. C. Reepmeyer., Chirality, **8**, 11-17 (1996).

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AN IMPROVED METHOD FOR THE ISOLATION OF THE LIGNAN CONSTITUENTS OF *SAURURUS CERNUUS* BY REVERSE PHASE COLUMN CHROMATOGRAPHY

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ABSTRACT

The above-ground parts of the aquatic weed, *Saururus cernuus* contain many constituents with varying and significant biological acitivities. In the earlier method of isolation, the concentrated methanolic extract was fractionated by a 2-3 step solvent partition, and the appropriate fractions subjected to two or three steps of chromatography, as well as other manipulations, which together, made the isolation a somewhat elaborate and time-consuming operation, especially when a medium to large-scale extraction is to be carried out.

In an effort to simplify this process, the methanolic extract concentrate is now partitioned between benzene and water, and the organic extract subjected directly to reverse phase column chromatography. Most of the lignan constituents could be isolated either directly, or after one small additional column, where necessary.

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This method has some distinct advantages over the earlier scheme. Besides being simpler, and more readily applicable for large-scale work-up, the chlorophylls and other liipophilic components, which usually contaminate the lignan fractions in the normal phase silica column, remain on the column until almost all of the lignan constituents are eluted, thus aiding the purification.

The previously obtained lignan components, can all be isolated readily by the use of this simplified procedure.

INTRODUCTION

The aquatic weed, *Saururus cernuus* L (N.O. Sauraraceae) which grows mainly in the eastern United States, was known and used during the 19th century for its anti-inflammatory activity.[1,2] A systematic study was undertaken in our laboratory which yielded a number of novel lignan and other constituents.[3-6] The most important of these were the dineolignan type compounds named manassantins A and B (**1** and **2**), which showed potent neuroleptic acitivity.[7-9]

Because of the continued interest in this and other activities found in this plant, a reexamination of the isolation process used earlier was undertaken, with the objective of simplifying and streamlining the process, and for possible large scale applicability. Based on earlier, successful applications of a reverse phase column technique for the fractionation of the crude extracts of *Taxus brevifolia*, for the isolation of paclitaxel and several of its analogues,[10,11] use of this principle was studied with the extracts of *Saururus cernuus*, and the details of these studies are provided in this communication.

MATERIALS AND METHODS

Plant Material

The above-ground parts of the plant (which has been previously identified by the University of Florida Herbarium, where a voucher specimen was submitted) are collected locally, in and around Gainesville, FL during May-September, when the plant is found most commonly, dried in the sun and stored until needed for extraction.

Extraction and Partition

The dried plant material was ground to a coarse mesh (0.5-1 cm) and extracted in 25 lb quantities in a stainless steel tank using methanol. The extract was drained after 24 h and the extraction was repeated 3-4 more times, using absorbance at 275 nm as a guide, to indicate the concentration of extractables. Usually four extractions gave most of the desired constituents. The extracts were concentrated under reduced pressure to a thick green syrup, which was partitioned between water (5 gal) and benzene (5 gal). The organic layer was separated and the aqueous layer extracted a second time using benzene (3 gal). The aqueous layer was concentrated to a syrup and set aside for further study. The combined benzene extract which contained the bulk of the absorbance at 275 nm, representing the lignan constituents was concentrated to a dark green semi-solid (5% of the weight of the dried plant), referred to as the "extract solids" and stored until use.

Chromatography

A column was set up using C_{18} bonded silica gel (approximately 800g, 15-35 micron size, Phase Separations, Inc., Norwalk, CT) using methanol, in a threaded glass column of the Mitchell-Miller type (2.5 x 24", Ace Glass Co. Vineland, NJ), suitable for low pressure liquid chromatography). The column was equilibrated with 40% methanol in water, which made it ready for use.

The extract solids (150 g) was dissolved in methanol (450mL) by warming if necessary, and to this solution was added approximately a 150g equivalent of the equilibrated silica gel (about 15-20% of the silica from the above column) with stirring. While the stirring is being continued, 400 mL of the 40% methanol in water was added, followed by water (600mL), to make an approximate total volume of 1500 mL. The mixture was warmed in a water bath to approximately 50°C and the stirring continued until there was no visible green precipitate or oily material, and an aliquot of the silica gel/sample slurry, taken in a test tube, settled readily to give a relatively clear supernatant. The slurry was then filtered using light suction and the solid, re-slurried using part of the filtrate, and the thick slurry added to the column.

The clear filtrate was then pumped on to the top of the column using a metering pump (Eldex-Fisher Scientific Co.). From time to time, the column feed was checked to see that it remained clear, and if not, to either warm briefly or add minimal amounts of methanol to it, until it became clear, so as to prevent blockage of the pump.

Following the addition of the sample, the column was eluted with a step gradient of methanol-water (50, 55, 60, 65, 75, and 85% methanol). Fractions (200mL) were collected and monitored by uv absorbance (275 nm) and TLC. The change to the next concentration of the solvent was determined by the results of the monitoring of the fractions. For example, when the absorbance values rose as a result of the previous change, the solvent was continued until a definite trend to lower values was seen. Similarly, when the TLC showed the trend towards decreasing intensity of the major spot, and no new spot has shown a tendency to increase, the solvent is changed to the next level. In general, 2-4 multiples of the hold-up volumes of the column were used. After the 85% methanol-water was completed, the solvent was changed to 100% methanol, which was later changed to a mixture of methanol, ethyl acetate and ligroin (2:1:1). Most of the chlorophylls and other lipid-soluble components which were held up on the column during the run, started eluting when the 100% methanol was started, and the three-solvent mixture hastened their elution. After the column has been thus completely stripped of the green color, it was washed with methanol, equilibrated with 40% methanol in water, and is made ready for another chromatographic run.

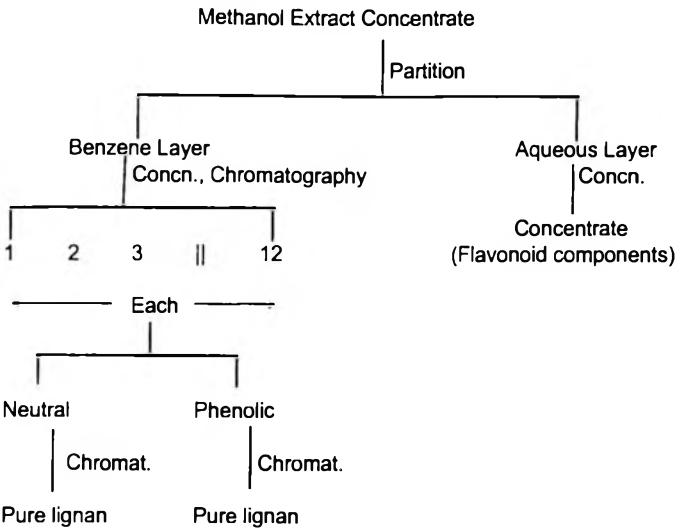
Based on the monitoring data, the fractions were combined into small groups (3-5 fractions) and concentrated to an oil and set aside for further work. The concentrates were examined by TLC to study the number, relative proportions, and nature of the components, eg. neutral, phenolic, lignan, non-lignan, etc. Some of the major lignan constituents isolated earlier[3-6] from this plant, such as austrobailignan-5 (most lipophilic), saucernetin, saucerneol (phenolic), manassantin B, and manassantin A (most hydrophilic), were used as markers to orient the others on the TLC.

The appropriate concentrates from above, were each partitioned counter-currently, using two separatory funnels, and the solvent system: methanol, 0.2 N aqueous sodium hydroxide, benzene and ligroin (1:1:1:1), to separate the phenolic and the neutral components. The aqueous methanolic layers containing the phenolic fractions were partially concentrated, neutralized, and extracted with benzene. The neutral and phenolic fractions thus separated, were each subjected to a brief column on normal phase silica for final separation and purification.

EXPERIMENTAL

Analytical HPLC was performed using two different units. For routine use, a combination of Waters 501 pump, with a U6K injector, a 486 tunable absorbance detector, and a Goertz Servogor 120 recorder was used. For determination of purity and quantitative information on composition etc., a setup containing a Waters 600 E

Scheme for the Fractionation of the *Saururus* Extract



pump with gradient control system, a 996 photodiode array detector, a 717 autosampler coupled with an NEC-386 computer and printer was used. Waters Millenium 2.1 program was used with the instrument. Standard columns (4.6 mm x 25 cm, Whatman, Partisil) packed with C_8-bonded silica (5 micron diameter) were used with either of the solvents: 60% acetonitrile-water or 60% acetonitrile, 10% methanol and water. The flow rate for both columns was 0.5 mL per min. The detector was set at 275 nm.

Thin-layer chromatography was carried out using silica gel HF-60, 254+366 (EM Science/Fisher) coated on microscope slides, by pouring as a slurry and drying the slides in an oven. The solvent systems consisted of 10-20% acetone/benzene, 5-10% methanol/dichloromethane, or 20-40% ethyl acetate/ligroin. Visualization was by a uv lamp, and by charring with 1 N H_2SO_4 in which most of the lignan constituents gave a scarlet red color, whereas those lignans containing methylenedioxy groups gave a violet brown color. Quantitative TLC was run using 2x3" slides on which the sample was applied as a band and developed. The bands were each scraped out, eluted with methanol, and the absorbance and the uv spectrum run on each, directly and after addition of a base to determine shifts due to phenolic compoumds. Column chromatography was carried out using silica gel (Fisher 100-200 mesh or 235-425 mesh, with a solvent sequence of ligroin → benzene → benzene with acetone (5-10%) and benzene with methanol (5-10%).

Alternatively, mixtures of ligroin and ethyl acetate were also used. Melting points were determined on Fisher-Johns apparatus and were uncorrected. The following instrumentation was used to record the spectra described here: uv, Perkin-Elmer Lambda 3B; ir, Perkin-Elmer PE-1420; and nmr, Varian VXR-300, Varian Gemini-300 and General Electric QE-300 spectrometers. The nmr spectra were recorded in δ values using TMS as an internal standard. The assignments were made with the help of COSY, HETCOR and APT spectra. Mass spectra (FAB) were obtained on a Finnegan Mat 95Q spectrometer using a cesium gun operated at 15 Kev of energy.

RESULTS

Before discussing the elution sequence for the components from the reverse phase column, it may be noted that a total of nearly 11 lignan components were obtained pure from this column to illustrate the applicability of the procedure. There are several other minor components which can also be isolated. An HPLC trace of these standards along with that of the extract solids is shown in Fig.1. The elution profile as seen by the absorbance values at 275 nm is shown in Fig. 2.

The elution of the lignans started, as the 50% methanol/water was started, and continued until the 85% methanol/water was completed. The elution sequence and the respective yields of the various lignans are shown in Table 1. The major peak in the elution profile based on the uv absorbance (Fig. 2) was found to be due to a non-lignan compound which is not investigated here further. Of the various lignans that were obtained and characterized (the bold numbers refer to the structures which are shown in Fig. 3), austrobailignan-5 **11** appears to be the major lignan component of the extract.

Characterization of the Major Lignans from *Saururus*

Bis-demethyl saucernetin **3**: Fractions 11-20 were combined, concentrated to an oil (1.5 g) and subjected to partition in the solvent system described under Materials and Methods. The phenolic fraction (0.6 g) was purified by a small silica column (25 g) in benzene/ligroin (1:1). Elution with 2% acetone in benzene gave the major component, obtained as a colorless powder, yield. 0.15 g, ^{1}H nmr, (δ): 0.68, d, J=6.6 Hz, 6H, H-9,9`; 2.24, m, 2H, H-8,8`; 3.87, s, 6H, 2xOMe; 5.42, d, J=6 Hz,2H, H-7,7`; 6.75-6.90, m, 6H, H-Ar.

The above compound was characterized by methylation carried out in acetone by refluxing with dimethyl sulfate and potassium carbonate to yield the dimethyl ether which was crystallized and found to be identical with saucernetin[3].

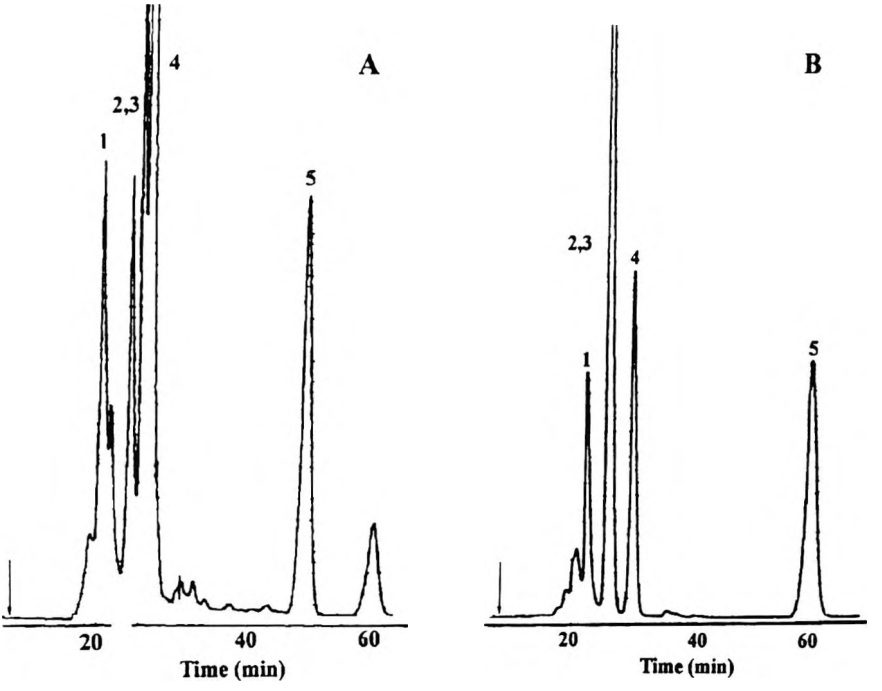


Figure 1. LC Trace of A) Extract of Saururus, B) Mixture of Sc-1, SC-2, SC-6, SC-7 and SC-8. 1: Manassantin A (**1**); 2: Saucerneol (**7**); 3: Manassantin B (**2**);4: Saucernetin (**5**), 5: Austrobailignan-5 (**11**).

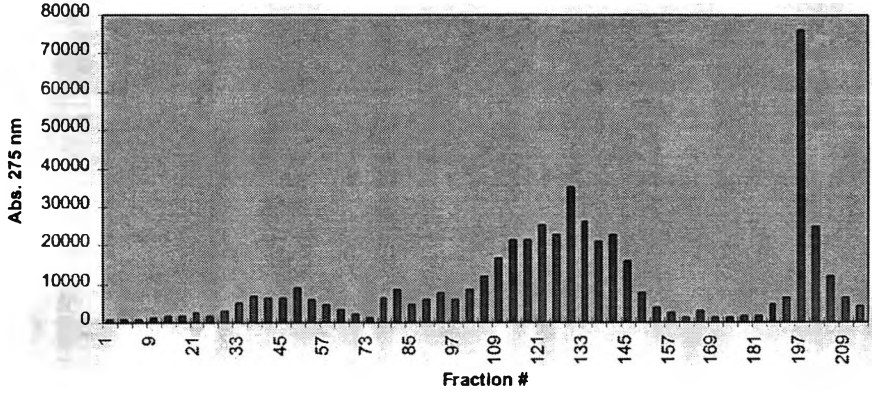


Figure 2. Elution Profile of the Extract of *Saururus*.

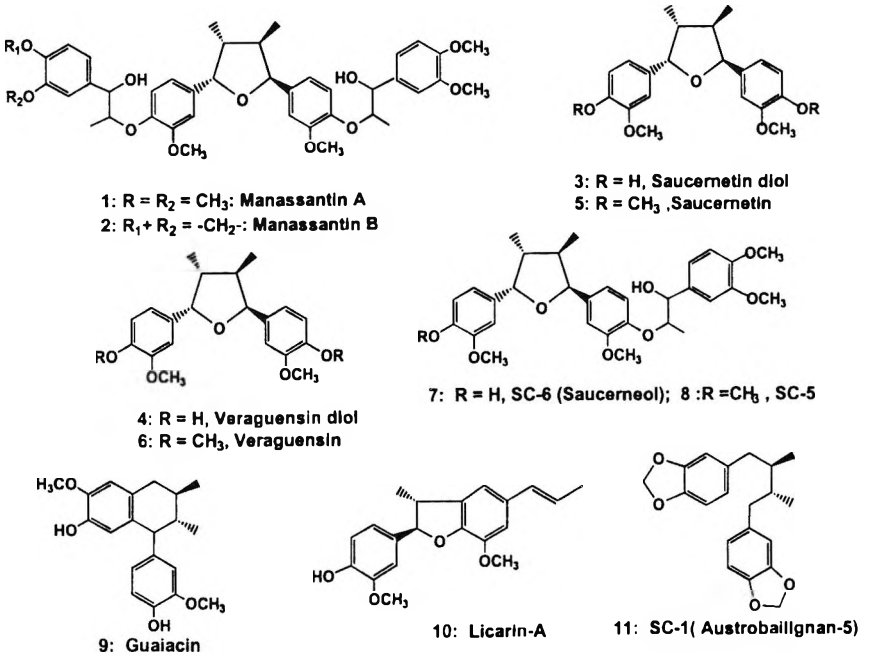


Figure 3. Structures of some *Saururus* lignans.

Bis-demethyl veraguensin **4**: Fractions 21-30 processed by the method given under 3. The major fraction from the column yielded **4** as a colorless glassy solid, yield, 0.1 g. Its nmr spectral characteristics were identical with those reported.[12,13] Saucernetin **5**: Fractions 36-50 were combined and concentrated to an oil (6 g) which was subjected to the phenolic partition. The neutral fraction (2.5 g) was chromatographed on a normal phase silica column in benzene/ligroin (1:1). Elution with benzene gave **5**, which was obtained as a colorless crystalline solid, yield, 1.5 g. Its physical and spectral properties were identical with those of saucernetin described earlier.

The proton nmr spectrum was reported earlier[3], but not the ^{13}C spectrum, which is given here: (δ) 14.73, C-9,9'; 44.02, C-8,8'; 55.88, 2xOMe, 83.51, C-7,7'; 109.6, 110.8, 118.4, 134.0, 147.9, 148.6, C-Ar. In the HETCOR spectrum, the following interactions were observed: a) the ^{13}C signal at 14.7 and ^{1}H peak at 0.69, b) the ^{13}C peak at 44.0 and the ^{1}H signal at 2.27, c) ^{13}C peak at 55.9 and the methoxyl signal at 3.9, and d) ^{13}C peaks at 109.6, 110.8 and 118.4 with the aromatic proton signals at 6.9.

Table 1

Elution Sequence of the Saururus Lignans

	Compound	Yield
1	SC-2 diol **3**	0.15 g
2	SC-3 diol 4	0.1 g
3	SC-2 **5**	1.5 g
4	SC-3 **6**	0.1g
5	SC-6 **7**	0.9 g
6	SC-5 **8**	0.3 g
7	SC-8 **1**	3.1 g
8	Guaiacin **9**	0.25 g
9	Licarin **10**	0.2 g
10	SC-7 **2**	0.3 g
11	SC-1 **11**	15 g

Veraguensin **6**: Elution of the above column with 2% acetone in benzene gave the minor component **6**, as a colorless crystalline solid whose physical and spectral properties were identical with those reported earlier for veraguensin[3]. Saucerneol 7: The phenolic fraction (3 g) obtained from fractions 36-50 as given under **5** was purified by chromatography on a normal phase silica column using benzene. Elution with 5% acetone/benzene gave **7**, obtained as a colorless powder, yield, 1.6 g. Its physical and ^{1}H nmr spectral properties were identical with those reported earlier.[4] The ^{13}C nmr spectrum has not been reported before and is given here: δ 14.8, C-9,9'; 16.9, C-9''; 44.1, C-8,8'; 55.9, OMe; 78.4, C-7''; 83.5, C-7,7'; 84.0, C-7''; 108.9, 110.1, 110.9, 113.9, 118.7, 119.0, 119.9, 132.5, 133.8, 136.6, 144.5, 146.2, 146.4, 149.0, 150.5, C-Ar.

Saucerneol methyl ether (SC-5, **8**): Fractions 66-75 were combined and concentrated (2 g). It was freed from the phenolic components by base partition. The neutral fraction (1 g) was purified by chromatography on silica gel in benzene.

The major component **8** was obtained by elution with 2% acetone in benzene, as a colorless powder, yield, 0.4 g), ^{1}H nmr spectrum: 0.70, d, J=6 Hz, 3H, H-9; 0.72, d, J=6 Hz, 3H, H-9'; 1.17, d, J=6Hz, 3H, H-9''; 2.25, d, 2H, H-8,8'; 3.90, 3.93, s, 15H, 5xOMe; 4.13, m, 1H, H-8''; 4.65, d, J=8.1 Hz, 1H, H-7''; 5.46, d, J=6 Hz, 2H, H-7,7'; 6.8-7.0, m, 9H, H-Ar; ^{13}C nmr spectrum, 14.8, C-9,9'; 16.9, C-9''; 44.0, C-8,8'; 55.9, OMe; 78.3, C-7''; 83.3, C-7,7'; 109.6, 110.0, 110.1, 110.8, 118.7, 119.9, 132.2, 132.6, 136.6, 146.6, 147.9, 148.6, 148.98, 148.9, 150.5, C-Ar.

The spectral data indicated that the compound **8** was the methyl ether of saucerneol. This was confirmed by methylating saucerneol in acetone with dimethyl sulfate and potassium carbonate and showing that the product was identical with **8**.

Manassantin A **1**: Fractions 76-95 were combined, concentrated (12 g), and partitioned to free it from any phenolic components. The neutral fraction (8 g) was chromatographed on silica gel (175 g) using benzene. Elution with 5% acetone in benzene gave 1 as a colorless powder, yield, 4.5 g. Its ^{1}H and ^{13}C nmr spectral properties were identical with those described.[4]

Licarin **10**: The phenolic components (2.7 g) from fractions 76-95 were purified by chromatography on silica using benzene. Elution with benzene gave **10** as a colorless crystalline solid. Its physical and spectral properties were identical with those described earlier.[14]

Guaiacin **9**: From the column described under **10**, elution with 2% acetone in benzene gave **9**, obtained as a colorless crystalline solid, the physical and spectral properties of which were identical with those described earlier.[13]

Manassantin B **2**: Fractions 96-104 were combined, concentrated and freed from phenolic components. The neutral fraction (1.5 g) was purified by chromatography on silica gel using benzene. Elution with 5% acetone in benzene gave **2** as a colorless powder, yield, 0.6 g; ^{1}H nmr spectrum, 0.72, d, 6 Hz, 6H, H-9,9'; 1.15, 1.17, d, J=6 Hz, H-9'', 9'''; 2.29, m, 2H, H-8,8'; 3.87, 3.89, 3.92, 3.93, s, 4xOMe; 4.11, m, 2H, 8'', 8'''; 4.62, 4.64, d, J=9 Hz, 2H, 7'',7'''; 5.46, d, J=6 Hz, 2H, H-7,7'; 5.94, s, 2H, O-CH_2-O; 6.78-7.00, m, 12 H, H-Ar; ^{13}C nmr, 14.8, 14.8, C-9, 9'; 16.8, 17.0, C-9'',9'''; 44.1, 44.1, C-8,8'; 55.8, O-Me; 78.3, 78.3, C-7'', 7'''; 83.3, 83.3, C-7, 7'; 83.8, 83.9, C-8'', 8'''; 100.9, OCH_2O; 108.0, 107.5, C-5, 5'; 110.1, 110.1, 110.1, 110.9, C-2, 2' 2'', 2'''; 118.6, 118.6, C-6,6'; 119.9, 120.9, C-6'', 6'''; 118.6, 118.8, C-5'', C-5'''; 136.4, 136.5, 132.6, 134.0, C-1,1', 1'', 1'''; 146.2, 146.4, 147.3, 147.7, C-3, 3', 3'', 3'''; 150.2, 150.5, 148.8, 149.0, C-4, 4', 4'', 4'''.

Austrobailignan 5 **11**: Fractions 191-205 were combined, concentrated (30 g) and subjected to chromatography on silica using 1:1 benzene/ligroin. The major component was obtained by elution with 3:1 benzene/ligroin, and it was obtained as a colorless crystalline solid, whose physical and ^{1}H spectral properties agreed with those described earlier.[16,3] Its ^{13}C nmr spectrum was not described in the past and it is given here: (δ), 13.8, C-9,9'; 38.2, C-8,8'; 41.1, C-7,7'; 100.7, O-CH_2-O; 107.9, C-5,5'; 109.8, C-2,2'; 121.7, C-6,6'; 133.4, C-1,1'; 145.4 C-4,4'; 147.4 C-3,3'.

DISCUSSION

The order of elution of the compounds from the reverse phase column (Table 1) deserves some comment. The order does not seem to correlate entirely with either the polarity, molecular weight, or the mobility on TLC on normal phase silica. For example, SC-1 (**11**) (austrobailignan-5) has the highest R_f (1.0, most lipophilic) of the lignans of *Saururus* as seen in TLC (10-20% acetone in benzene), followed by those of intermediate R_f (0.6-0.7): SC2 (**5**) (saucernetin), SC-3 (**6**) (veraguensin) and guaiacin (**9**). Significantly slower than these are the sesqui and dilignans such as SC-5 (**8**), SC-6 (**7**), SC-7 (**2**) and SC-8 (**1**), which appear in that order in the TLC (R_f 0.1-0.2). In the reverse phase column, as expected, SC-1 (**11**) was the last to be eluted. However, SC-2 (**5**) and 3 (**6**) (with higher R_f values than SC-6 (7), -7(**2**) and -8 (**1**) emerge from the column before SC-8, 6 and 7. Also, compounds that show the same R_f in TLC are separated significantly in the reverse phase column, which is seen often.

The procedure described here for the processing of the extract of *Saururus* using the reverse phase column, clearly has some advantages over the one based on the use of a normal phase silica columns. Among the advantages, the following are important to note. First, it involves fewer steps than were used before, because the two to three stage solvent partitions can be replaced by one partition step (water/benzene), which separates the water-soluble flavonoid glycosides from the lipophilic lignans and other constituents. By applying the concentrated benzene extract directly on the reverse phase column, the need for handling the lignan constituents in two or three subgroups is eliminated. In spite of the more complex mixture that is being applied to the C_{18} bonded silica column, the resolution was quite satisfactory. Secondly, in the normal phase silica column, the chlorphylls and carotenoid components were partially eluted as the eluting solvent changed each time, such that, most fractions when concentrated were greenish yellow, whereas, in the reverse phase column this leaching of the pigments was to a much smaller extent, and whatever amounts of these pigments present in these samples could be readily removed when they are subjected to the second (silica) column. Thus, the noncrystalline lignans such as SC-5, -6, -7 and -8 could be readily obtained as homogeneous, colorless solids with much less effort.

One of the difficulties in the normal phase silica column used earlier process was the co-elution of some of the sesqui and dineolignans (SC-5, -6, -7 and -8). Thus, even after the removal of SC-6 by the phenolic partition, the elution pattern of SC-5, 7 and 8 still gave mixtures such as SC-5 with decreasing amounts of SC-7, and SC-7 with decreasing amounts of SC-8. To obtain the last two in pure form, it was necessary to acetylate the mixture, separate the acetates, and regenerate the original compounds by saponification. In the current procedure, this was not

necessary. SC-8 and SC-7 separated well from each other, actually requiring a different solvent mixture for the elution of each. Most of the fractions showed single spots, while those which may contain mixtures can be purified readily in the subsequent "small" column. Similarly, many of the fractions obtained earlier by the normal phase silica column, which were assumed to be SC-7, were found by nmr spectra (only the latter having the methylenedioxy signal at δ 5.9) to be mixtures of SC-6 and SC-7. These mixtures could be readily separated by the reverse phase column, with SC-6 eluting first and SC-7, later, after a change of the solvent (see figs). The important overall result was that even though relatively large quantities (100's of pounds) of the plant were extracted before, only a portion of the earlier fractions could be taken up to homogeneity, the rest being left as mixtures. In this current procedure, these mixtures, as well as new extractions, could be processed readily, such that some of these components could be obtained for the first time, in 10-50 g quantities in a relatively short time.

The fractions from the initial reverse phase column, are combined based on the TLC and HPLC data, separated into the neutral and phenolic components. This type of neutral/phenolic separation is best done at this stage rather than at the original total extract stage. The phenolic lignans do not partition into aqueous base from solvents such as dichloromethane or even benzene. In order to separate most of the phenolics from the neutral components, it is necessary to use a solvent system listed under Materials and Methods, in which the benzene is diluted with ligroin (1:1) to lower the solubility of the ionized phenols, and methanol is added to the aqueous layer to increase the solubility of the ionized phenols. The partition also works best when one is using dilute solutions and thus, to do this at the original crude extract stage will require rather large volumes, and it is not convenient. Even when carried out under optimal conditions, some phenolic lignans still partially remain in the organic layer, eg. licarin and SC-6.

After the phenolic partition, the samples are subjected to the second column. The column used is usually a much smaller column, and can be either a reverse phase, or a normal phase column. At this point, other reverse phase column packings such as the CN, phenyl, etc. bonded silica column can offer specific advantages for resolution and thus can be used. Such information may be obtained during the HPLC analyses using columns containing these packings. Similar choices may be made for the normal phase column between various types of silica, florisil etc.

One of the most important advantages of the new procedure is its adaptability to larger scale operations. Based on the earlier experience with the processing of the extracts of *Taxus brevifolia*, the laboratory size columns on 150-200 g of the crude

extract could be scaled up to 2.3-2.7 kg of the extract using a 6"x6' column. Judging from the ease of preparing the sample and applying to the column in the present case, similar scale-up can be carried out on the extracts of *Saururus* if such need arises. The solvents used such as methanol/water mixtures, as well as the fact that the column can be used again and again, offer an economic advantage. Lastly, unlike the case with a normal phase silica or florisil columns, on a reverse phase column, none of the components of the extract can be "lost" due to irreversible adsorption on the silica.

The most important part of the process is the preparation of the sample for the reverse phase column. In most applications of preparative reverse phase chromatography reported in the literature, where this step is either the last or next to the last step, the sample is already of high degree of purity. In the current process, the reverse phase column is the first step in the purification scheme, and hence the sample is the crude extract itself. Because of its highly lipid soluble nature, the sample has little or no solubility in the solvent to be used, 40% methanol in water. Thus, it has to be applied as a suspension, which may suggest many problems because of the nature of lipophilic impurities such as chlrophylls, carotenoids, waxes, and such, normally present in the plant extracts. It is, thus, difficult to believe that such a column can perform satisfactorily. However, the C-8, or the C_{18} bound silica seems to absorb (or even dissolve) this lipophilic material so that no free oily or waxy material is left after the sample preparation as described, and the slurry is easily applied. The column performs as though a soluble sample has been used.

REFERENCES

1. D. L. Phares, Am. J. Pharm., **39**, 468, (1867).

2. F. P. Porchier, **Resources of the Southern Fields and Forests, Medical, Economical and Agricultural**; Walker Evans, and Cogswell, Charleston, (1869).

3. K. V. Rao, F. M. Alvarez, J. Nat. Prod., **45**, 393-397 (1982).

4. K. V. Rao, F. M. Alvarez, Tetrahedron Lett., 4947-4950 (1984).

5. K. V. Rao, N. S. Prakasa Rao, J. Nat. Prod., **53**, 212-215 (1990).

6. K. V. Rao, G. S. Reddy, J. Nat. Prod., **53**, 309-312 (1990).

7. K. V. Rao, V. N. Puri, P. K. Diwan, F. M. Alvarez, Pharmacol. Res. Commun., 19, 629-638 (1987).

8. K. V. Rao, V. N. Puri, Life Sci., **42**, 2717-2720 (1988).

9. K. V. Rao, V. N. Puri, H. S. El-Sawaf, Europ. J. Pharmacol., **179**, 367-376 (1990).

10. K. V. Rao, J. B. Hanuman, C. Alvarez, M. Stoy, J. Juchum, R. M. Davies, R. Baxley, Pharm. Res., **12**, 1003-1010 (1995).

11. K.V. Rao, R. S. Bhakuni, J. Juchum, R. M. Davies, J. Liq. Chrom. & Rel. Technol., **19**, 427-447 (1996).

12. G. Schmedia-Hirschmann, F. Tsichritzis, J. Jakupovic, Phytochemistry, **31**, 1731-1735 (1992).

13. P. K. Agrawal, A. K. Pathak, Magnetic Resonance in Chemistry, **32** 753-773, (1994).

14. C. J. Aiba, R. G. C. Correa, O. R. Gottleib, Phytochemistry, **12**, 1163-1166 (1973).

15. P. L. Majumdar, A. Chatterjee, G. C. Sengupta, Phytochemistry, **11**, 811-813 (1972).

16. S. T. Murphy, E. Ritchie, W. C. Taylor, Australian J. Chemistry, **28**, 81-84 (1975).

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EVALUATION OF TAXOIDS FROM *TAXUS SP.* CRUDE EXTRACTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The extraction procedures and analysis methods by high-performance liquid chromatography (HPLC) for taxine B (**1**), isotaxine B (**2**) and 10-deacetylbaccatin III (**3**), expected or already known "precursors" of paclitaxel or active analogues, were developed for in series analyses of *Taxus* needles.

INTRODUCTION

Paclitaxel (Taxol®) and docetaxel (Taxotere®) are two new chemotherapeutic agents which are used for the treatment of cancer. These two drugs are currently prepared from 10-deacetylbaccatin III (10-DAB),[1] a neutral taxoid extracted from the leaves of the yew tree (*Taxus sp.*).[2,3] Taxine B and isotaxine B are basic taxoids, also isolated from the leaves of *Taxus sp.*[4-6] Taxine B, isotaxine B (further noted taxines B) and 10-DAB can be used to synthesize other taxane analogues of biological interest.

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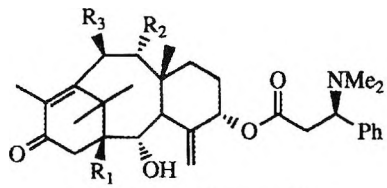
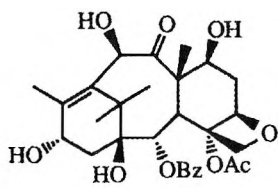
In order to select the highest taxoid plants, convenient methods for in series analyses were needed : a number of methods have already been described for paclitaxel content determination, some for 10-DAB evaluation,[7-12] very few for taxines B.[6] As part of our studies on the analysis of taxoids, we wish to report herein the analytical methods that we developed, involving extraction procedures and final quantitation by HPLC of taxines B and 10-DAB.



1 $R_1 = R_2 = OH$, $R_3 = OAc$
2 $R_1 = R_3 = OH$, $R_2 = OAc$
4 $R_1 = H$, $R_2 = OH$, $R_3 = OAc$
5 $R_1 = H$, $R_2 = OAc$, $R_3 = OH$



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MATERIALS

Plant Material

The yew stems were air-dried at room temperature. Dry needles were then removed from the stems and ground. Taxines B and 10-DAB standards were prepared from this plant material (*vide infra*).

Apparatus

The HPLC system consisted of a Waters 616 pump, Waters 717 plus autoinjector, and Waters 996 photodiode array detector (PDA) with a NEC Image 466es computer (Millennium software system) for controlling the analytical system and data processing.

HPLC columns

All the colums were Waters : Analytical columns : Nova-Pak® Silica, 4 µm, 3.9 x 150 mm ; Nova-Pak® C_{18}, 4 µm, 3.9 x 150 mm ; Symmetry™ C_{18}, 5 µm, 4.6 x 250 mm. Semi-preparative columns: Prep. Nova-Pak® HR Silica, 6 µm, 25 x 100 mm.

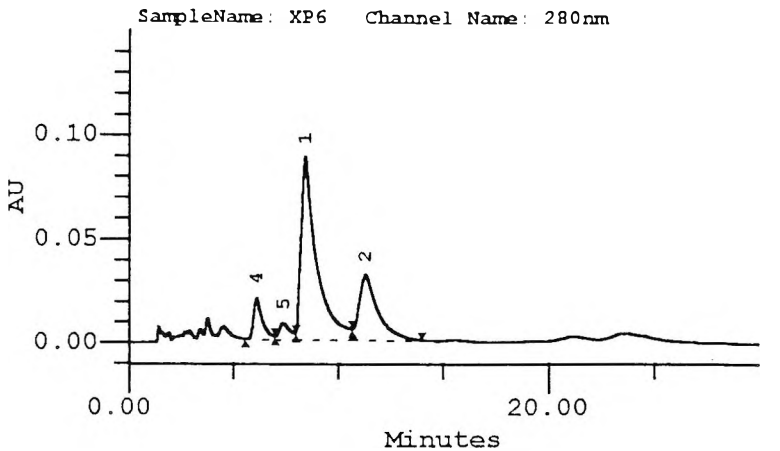

Figure 1. Crude alkaloidal mixture : isocratic, normal phase, separation of "Taxines B" on Nova-Pak Si 4 mm, 3.9 mm x 150 mm column. Mobile phase : $CHCl_3$/MeOH/Et_3N (99.4/0.5/0.1, v/v), 1 mL/min. flow rate **1** = taxine B ; **2** = isotaxine B ; **4** = 1-deoxytaxine B ; **5** = 1-deoxyisotaxine B.

METHODS

Extraction

Procedure for basic taxoids

The ground dry needles (50 g) were moistened with 25% NH_4OH and extracted with CH_2Cl_2 (5 x 200 mL) at room temperature for 7 days. The concentrated CH_2Cl_2 solution was extracted with 2% aqueous HCl until Mayer's test was negative. The combined aqueous layers were basified to pH 9 with 25% NH_4OH and extracted with CH_2Cl_2 (until negative Mayer's test). The CH_2Cl_2 solution was successively washed with H_2O, dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo* to provide a crude alkaloidal mixture (TA = total alkaloids).

Procedure for neutral taxoids[2,3]

The ground dry needles (10 g) were extracted with ethanol 95% (60 mL) at room temperature for 1 H. This was repeated 6 times: the resulting organic extracts were evaporated *in vacuo* to give the "ethanolic extract". This

"ethanolic extract" was partitioned between CH_2Cl_2 and H_2O, and the aqueous fraction extracted with CH_2Cl_2. The combined organic layers were finally dried over anhydrous Na_2SO_4, filtered and evaporated to dryness (35°, under reduced pressure) and provided the "CH_2Cl_2 extract".

Standards

Taxine B and isotaxine B were separated and purified by preparative HPLC from the crude alkaloidal mixture of *Taxus baccata*. 10-DAB was also isolated from the leaves of *Taxus baccata*, by classical chromatography, from the crude extract of neutral taxoids.[2,3] Taxines B and 10-DAB were identified in our laboratory from their spectral characteristics.

HPLC Analysis

Taxines B determination

Two methods were developed. Samples for injection were prepared in $CHCl_3$ for method 1, in DMF for method 2 (concentration : 5 mg/mL). The injection volumes were 10 µL at once for standards and samples.

The data were collected over 200-400 nm range of the absorption spectrum and all the chromatograms were plotted at 280 nm.

Method 1

The HPLC column was a Nova-Pak® silica (4 µm, 3.9 x 150 mm). The eluent was isocratic, consisting of $CHCl_3$/MeOH/Et_3N (99.4/0.5/0.1, v/v) at a flow rate of 1 mL/min., in 35 min. The t_R value (retention time) for taxine B and isotaxine B were 8.9 min and 11.2 min (after 35 min all the polar compounds were eluted) (Figure 1). Standards were purified by using a semi-preparative column : Prep Nova-Pak® HR Silica (6µm, 25 x 100 mm). The eluent was isocratic, consisting of heptane/isopropanol/Et_3N (90/10/0.1, v/v) at a flow rate of 8 mL/min. in 50 min. The t_R value for taxine B and isotaxine B were 20.0 min and 25.5 min.

Method 2

The HPLC column was either a Nova-Pak® C_{18} (4 µm, 3.9 x 150 mm) or a Symmetry™ C_{18} (5 µm, 4.6 x 250 mm).

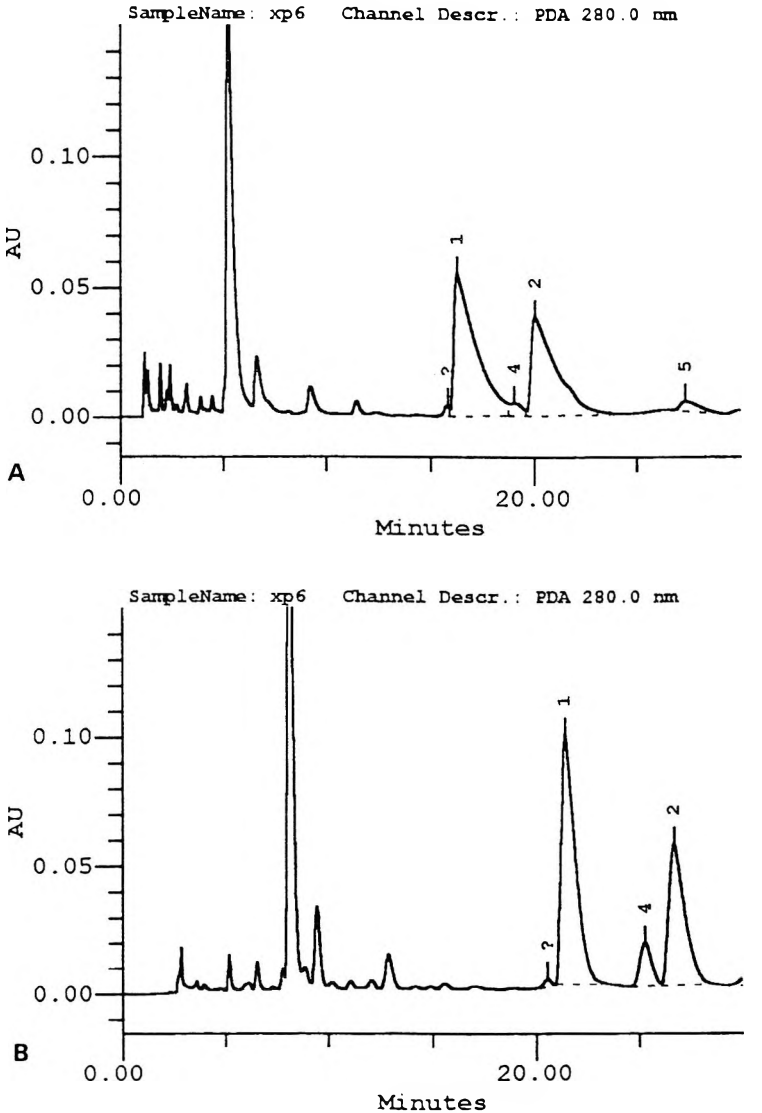

Figure 2. Crude alkaloidal mixture : isocratic, reversed phase, separation of "Taxines B". **2A**. Separation on Nova-Pak C18, 4µm, 3.9 x 150 mm column. **2B**. Separation on Symmetry, 5 µm, 4.6 x 250 mm column. Mobile phase for both columns was CH_3CN/H_2O/TFA (23/77/0.05, v/v),1 mL/min flow. **1** = taxine B ; **2** = isotaxine B ; **4** = 1-deoxytaxine B ; **5** = 1-deoxyisotaxine B.

The eluent was isocratic consisting of $CH_3CN/H_2O/TFA$ (23/77/0.05, v/v) at a flow rate of 1 mL/min in 35 min; then, a linear gradient to $CH_3CN/H_2O/TFA$ (50/50/0.05, v/v) over 10 min. The taxine B and isotaxine B were clearly resolved respectively at 21.4 and 26.7 min (Figure 2).

10-Deacetylbaccatin III determination

Samples for injection were prepared in DMF and kept at 10°C (20 mg/mL). The injection volumes were 10 μL at once for standards and samples. The data were collected over 200-350 nm range of the absorption spectrum and all the chromatograms were plotted at 240 nm. The HPLC column was a Nova-Pak® C_{18} (4 μm, 3.9 x 150 mm).

The eluent was isocratic consisting of 100% solvent A (MeOH/H_2O/AcOH, 39/61/0.1, v/v) in 14 min, then linear gradient to 100% solvent B (0.1% AcOH in MeOH) in 10 min and isocratic with solvent B (100%) in 10 min. The flow rate was 1 mL/min. The t_R value for 10-DAB was 10.7 min (Figure 3).

RESULTS AND DISCUSSION

As part of an ongoing project on the selection of the "best" plants from which a biotechnological production of active taxoids or "precursors" of them will be developed, as well as silviculture in open field conditions and new semisyntheses of paclitaxel (= Taxol®) analogues, we needed to develop convenient methods for in series taxines B and 10-DAB content evaluation.

For Taxines B

From total alkaloids two HPLC methods were developed to separate the taxines B. The first one consisted in using a silica column; two elution systems were tested with analytical and preparative objectives :

A. $CHCl_3$/MeOH/Et_3N (99.4/0.5/0.1, v/v)

B. Heptane/isopropanol/Et_3N (90/10/0.1 v/v)

From the crude basic taxoid extract the elution order of the constituents differs with the elution system. Purified taxine B and isotaxine B were isolated by using HPLC semi-preparative column. When eluent (A) is used with preparative objective a solvatation is suspected between Et_3N and taxines B and

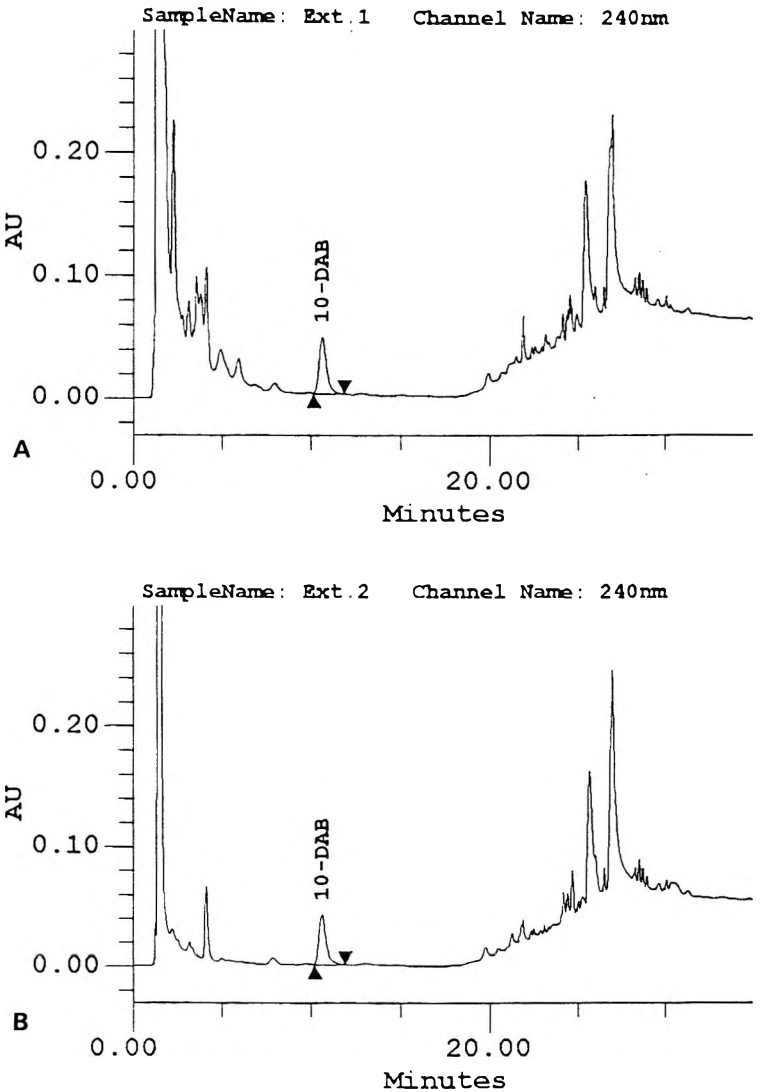

Figure 3. Crude neutral extracts : Separation on Nova-Pak C_{18}, 4 µm, 3.9 x 150 mm column. Mobile phase : MeOH/H_2O/AcOH (39/61/0.1, v/v) during 14 min. then a linear gradient to MeOH/AcOH (100/0.1, v/v) in 10 min., 1 mL/min. flow rate. **3A**. EtOH extract ; **3B** : CH_2Cl_2 extract ; 10 DAB = 10-deacetylbaccatin III.

Table 1

Characteristics of Columns

	Symmetry		Nova-Pak (Silica)		Nova-Pak C_{18}
	Taxine B (1)	**Isotaxine B (2)**	**Taxine B (1)**	**Isotaxine B(2)**	**10DAB (3)**
r^2	0.9999	0.9999	0.9995	0.9997	0.999
lowest RSD	0.01%	0.2%	0.3%	0.1%	0 02
highest RSD	2.6%	2.5%	1 5%	6.2%	2.1

a final partition CH_2Cl_2/H_2O is needed, with only 50% yield; with eluent (B) the higher boiling temperatures of heptane and isopropanol help along the triethylamine evaporation. In both eluents, the expected transesterification is observed, mainly in the way isotaxine B → taxine B; in the conditions of HPLC this transesterification is very fast, as much as it is when isotaxine B is kept in methanolic solution. A ratio 40/60 (isotaxine B/taxine B) is fastly obtained. Chromatograms were interpreted by co-injecting total alkaloids with each standard and recording the UV spectra.

A second method was carried out to try to separate, more efficiently, taxine B and isotaxine B from total alkaloids; it consisted in using reversed phase HPLC.

Two different columns were tested: Nova-Pak® C_{18} and Symmetry C_{18} (the last one was more recently available); the Symmetry column is especially convenient for basic molecules like taxines B: the chromatograms show peaks with very good symmetry.

The best solvent system was CH_3CN/H_2O/TFA (23/77/0.05, v/v). In this mobile phase, transacetylation appeared to be very slight: when taxine B was collected, its solution evaporated and the residue injected again, the measured isomerisation was 0.68 %; in the same conditions, isotaxine B showed 3% isomerisation.

Calibration curves were fixed up for both used methods. The linearity of the detector response between the peak area and the concentration was determined by injecting twice a series of five standard solutions ranging in concentration from 0.2 to 3.2 mg/mL: the relative standard deviation (R.S.D.) was calculated for each concentration (Table 1).

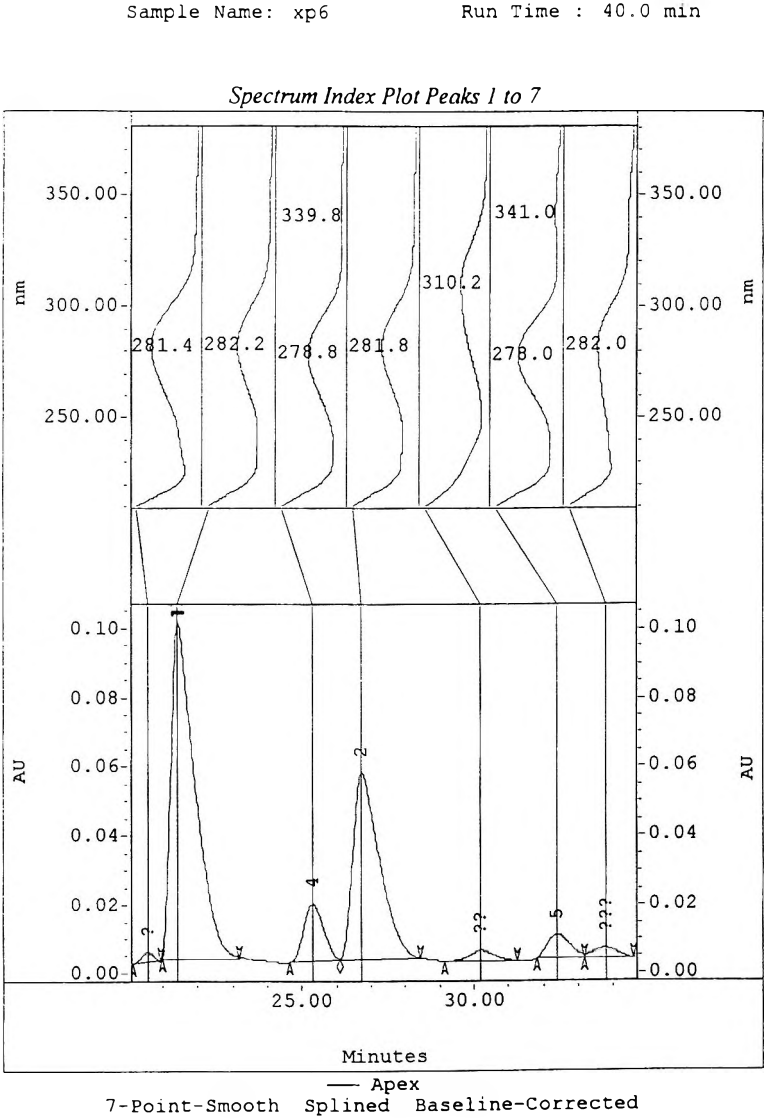

Figure 4. Diode array scans of "Taxines B" over 200 to 400 nm range of the absorption spectrum. **1** = taxine B ; **2** = isotaxine B ; **4** = 1-deoxytaxine B; **5** = 1-deoxyisotaxine B.

So, reversed phase HPLC of total basic taxoid extract provides a good manner to evaluate separately taxine B and isotaxine B contents but HPLC analysis on silica column is sufficient to evaluate globally taxines B. This method allowed to analyse easily more than 100 samples of various *Taxus sp.* needles; compared with the already published method,[6] it presents the advantage of avoiding the use of a buffer (the elimination of which is often tedious and the obtaining of pure compounds difficult).

These analyses showed significant variations in taxines B content : 0.13-7.53 g/kg ; they could represent 8.7-91% of the crude basic taxoid extract. Two other compounds were relatively abundant in some extracts; by semi-preparative HPLC, they have been separated, characterized and identified to 1-deoxytaxine B (**4**) and 1-deoxyisotaxine B (**5**).[6] (Figure 4).

For 10-Deacetylbaccatin III

After evaluation of 10-DAB in both extracts, ethanol and CH_2Cl_2, it was seen that the results were similar, 10-DAB being well "isolated" in both chromatograms; so, for in series evaluation, the EtOH extract, carried out faster, was preferred.

The separation of 10-DAB from the crude "ethanolic extract" by HPLC on a classical C_{18} column in previously described conditions was satisfactory. The 10-DAB peak appears sufficiently free of co-eluting material to allow the content determination.

The linearity of the detector response was determined by injection of standard solutions ranging in concentration from 15 to 200 µg/mL. A linear relationship was observed between the peak areas and the concentration of 10-DAB in the studied range; relative standard deviation (R.S.D.) was calculated for each concentration (Table 1).

To their credit, these new conditions of HPLC analysis provide, from crude ethanolic extracts of *Taxus* needles, clear chromatograms allowing an easy 10-DAB content determination. The analyses also showed important variations for 10-DAB content 3 - 959 mg/kg.

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REFERENCES

1. D. Guénard, F. Guéritte-Voegelein, P. Potier, Acc. Chem. Res., **26**, 160-167 (1993).

2. G. Chauvière, D. Guénard, F. Picot, V. Sénilh, P. Potier, Compt. Rend. Acad. Sci., Paris, série II, **293**, 501-503 (1981).

3. V. Sénilh, Thèse de Doctorat ès-sciences, Université Paris-Sud (Orsay), 3.02.1984.

4. E. Graf, S. Weinandy, B. Koch, E. Breitmaier, Liebigs Ann. Chem., 1147-1151 (1986).

5. L. Ettouati, A. Ahond, C. Poupat, P. Potier, J. Nat. Prod., **54**, 1455-1458 (1991).

6. L. H. D. Jenniskens, E. L. M. van Rozendaal, T. A. van Beek, P. H. G. Wiegerinck, H. W. Scheeren, J. Nat. Prod., **59**, 117-123 (1996).

7. K. M. Witherup, S. A. Look, M. W. Stasko, T. G. McCloud, H. J. Issaq, G. M. Muschik, J. Liq. Chromatog., **12**, 2117-2132 (1989).

8. K. M. Witherup, S. A. Look, M. W. Stasko, T. J. Ghiorzi, G. M. Muschik, J. Nat. Prod., **53**, 1249-1255 (1990).

9. H. N. El Sohly, E. M. Croom Jr., W. J. Kopycki, A. S. Joshi, M. A. El Sohly, J. D. McChesney, Phytochem. Anal., **6**, 149-156 (1995).

10. K. V. Rao, Pharm. Res., **10**, 521-524 (1993).

11. S.-S. Kwak, M.-S. Choi, Y.-G. Park, J.-S. Yoo, J.-R. Liu, Phytochem., **40**, 29-32 (1995).

12. G.-M. Liu, W.-S. Fang, X.-X. Zhu, Fitoterapia, 7, 149-151 (1996).

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TAXOL AND RELATED TAXANES FROM *TAXUS* CALLUS CULTURES

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ABSTRACT

This study presents a protocol for analyzing taxol and five related taxanes from tissue culture samples of *Taxus* spp. by high performance liquid chromatography (HPLC) using a reverse phase C_{18} column. Sep-Pak C_{18} cartridges were used for semi-purification of the crude extracts of the samples prior to analyses. Taxol, cephalomannine, 10-deacetyltaxol, 10-deacetylcephalomannine, baccatin III and 10-deacetylbaccatin III were well separated by the mobile phase of methanol : acetonitrile : water (25:35:45) at a flow rate of 1.0mL/min and detected at 227nm.

INTRODUCTION

Taxol[I], a cytotoxic diterpenoid natural product initially isolated from the stem bark of *Taxus brevifolia* Nutt. by Wani et al.,[1] is of great interest for its unique structure and excellent activity against ovarian cancer, breast cancer

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and other types of cancer.[1,2] Presently, the commercial source of taxol is the bark of *Taxus brevifolia*, which grows slowly and yields relatively low amounts of taxol. Although others species of *Taxus* also contain taxol,[1] and semi-synthesis from natural taxoids,[3] as well as total synthesis of taxol have been successful,[4] cell culture of *Taxus* spp., which provides rich stable supply of the antineoplastic agent taxol and related taxanes, may be viewed as a potential alternative to plant extraction currently in short supply.

The closely related taxanes of taxol[**I**], cephalomannine[**II**], 10-deacetyl-taxol[**III**] and 10-deacetylcephalomannine[**IV**] have shown less anticancer activity than taxol; baccatin III[**V**] and 10-deacetylbaccatin III[**VI**] can be converted to taxol through a semi-synthetic route. The analysis of these compounds[**I~VI**] from cultures of *Taxus* spp. can be used to select particular cell lines with high and stable yield of taxol/taxanes.

It is rather difficult to completely separate taxol[**I**] from cephalomannine[**II**] and 10-deacetyltaxol[**III**] from 10-deacetylcephalo-mannine[**IV**] by a common HPLC method, so several reported methods[1,2,5] utilized gradient elution technique or/and selected phenyl, cyano, and other special columns to modify the separation of taxol and the closely related taxanes. Although, it was reported, that a common HPLC method using a C_{18} column eluted in the isocratic mode for the determination of four taxanes[**I~IV**],[6] the determination of baccatin III[**V**] and 10-deacetylbaccatin III[**VI**] was not mentioned.

On the basis of reported methods,[1,2,6] we developed a HPLC method using a C_{18} column, eluted in isocratic mode, for analyzing taxol[**I**], cephalomannine[**II**], 10-deacetyltaxol[**III**], 10-deacetylcephalomannine[**IV**], baccatin III[**V**], and 10-deacetyl-baccatin III[**VI**] (Figure 1) from cultures of *Taxus* spp.

MATERIALS

A Spherisorb C_{18}, 5μm (250mm × 4.6mm) column was used for analysis. Sep-Pak C_{18} cartridges (Waters/Millipore Co., Milford, MA, USA) were used for pretreatment of the crude extracts of culture samples before HPLC analyses.

Acetonitrile (HPLC grade, Zhejiang Huangyan Experimental Chemical Factory, Zhejiang, China), methanol (G. R. grade, Beijing Chemical Factory, Beijing, China) and water (ultrapure) were filtered through G_5 filters prior to

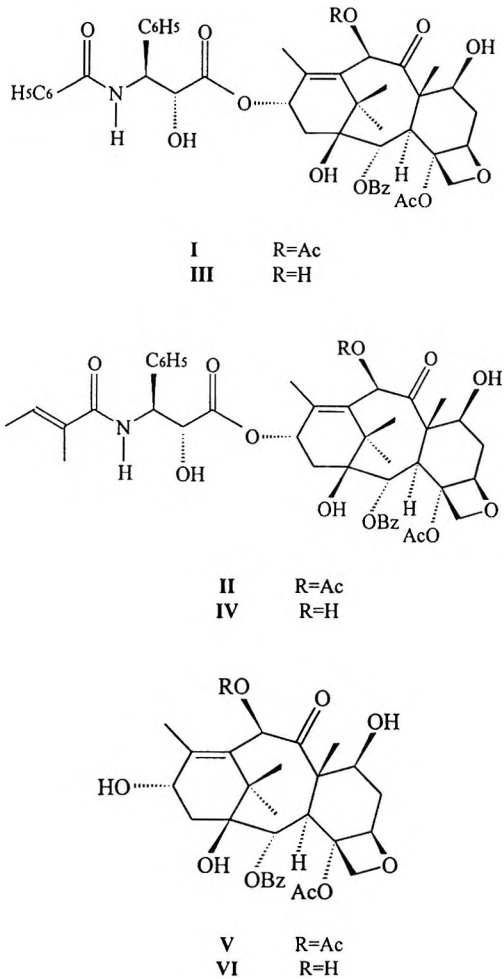

Figure 1. Structures of taxol and related taxanes.

use. Methanol and methylene chloride (both A. R. grade, Beijing Chemical Factory, Beijing, China) were used for the extraction of samples. Callus cultures were established from *Taxus yunnanensis, T. cuspidata*, and etc. Callus samples were harvested after 21~25 days of growth and dried at 55°C. Authentic samples of standards **I~VI** were generously provided by Dr. Weishuo Fang of our institute.

Instrumentation consisted of a LC-6A pump (Shimadzu), a Rheodyne 7125 Manual injector (Cotat, California, USA) and SPD-6A ultraviolet detector (Shimadzu) with a C-R3A integrator (Shimadzu).

METHODS

Dried callus cultures were pulverized and passed through 40-mesh sieves and extracted ultrasonically with a mixture of methanol and methylene chloride (10:1) for 30 min. The crude extract was evaporated to dryness and the residue was dissolved in methanol. A portion of the sample solution was loaded on a Sep-Pak C_{18} cartridge and eluated with water, 30%, and 85% methanol, respectively. The final eluate was evaporated to dryness and redissolved in a minimum amount of methanol.

HPLC analysis of 10μL fractions of the preparation was made with a mobile phase of methanol : acetonitrile : water (25:35:45) at a flow rate of 1.0mL/min with UV detection at 227nm.

All presumptive taxanes[**I~VI**] in calli of different species of *Taxus* cell lines were identified by comparison of the retention times of the chromatographic peaks with those of a standard mixture containing **I**, **II**, **III**, **IV**, **V**, and **VI** chromatographed in the same conditions. The co-injection of the sample and the standard mixture was done to confirm the compound existing in the sample. Each sample was injected duplicately and the average of the peak areas was used to quantify **I~VI** against external standard curves of the standards.

To inspect the recovery, the spiked samples containing definite amounts of the six standards[**I~VI**] were analyzed by the same procedure. By comparing different peak areas of the six compounds[**I~VI**] in samples and spiked samples, the recovery rates were obtained.

RESULTS AND DISCUSSION

The extraction solvent used by Wickremesinhe et al.[1] was methanol, which could effectively reduce the extraction of the highly hydrophobic components, such as oils, waxes, etc. in the plant tissue cultures. In our cultures, not only non-polar components, but also highly polar impurities were found. Therefore, methanol : methylene chloride (10:1) was used as the extraction solvent which could give satisfactory results for the analysis.

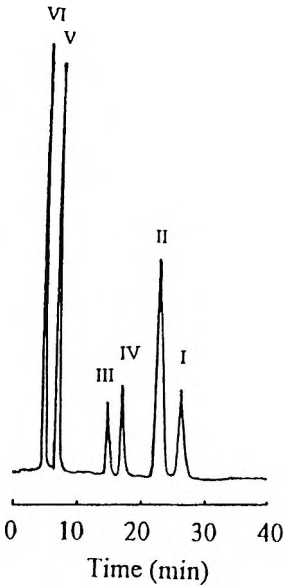

Figure 2. Separation of taxane standards. Peaks: I= taxol; II=cephalomannine; III= 10-deacetyltaxol; IV= 10-deacetylcephalomannine; V= baccatin III; VI= 10-deacetyl-baccatin III.

Sep-Pak C_{18} cartridges played an important role in the semi-purification step as Wickremesinhe et al.[1] reported. Neither non-polar components nor highly polar impurities were seen in the semi-purified fraction collected from the cartridge compared with the crude extract. Nevertheless, the following fraction eluted by pure methanol did not show any peaks of **I~VI**.

The mobile phase consisting of methanol, acetonitrile, and water was used on a phenyl, cyano, and any other special column except a C_{18} column to analyze taxanes[1,2] and on a μBondapak C_{18} preparative column to prepare taxol.[1] A mobile phase of water : acetonitrile : tetrahydrofuran (55:35:10) was used on a C_{18} column[6] but this took too much time to equilibrate the column before analysis, and tetrahydrofuran (THF) must be reevaporated in order to avoid baseline drift. Comparing these two kinds of mobile phases, we found that the mobile phase consisting of methanol, acetonitrile, and water was better and methanol : acetonitrile : water (20:35:45) which used to prepare taxol on a C_{18} preparative column[1] could be adopted for our analysis on a C_{18} analytic column. Equilibration time is relatively short and baseline is stable under this kind of mobile phase.

Table 1

The Linearity of Determination of Taxol and Related Taxanes

Compound	Range of Linearity (ng)	Equation of Linear Regression	Correlation Coefficient
Taxol	11.5 - 92	Y=328.1X - 565.8	0.9968
Cephalomannine	16.9 - 135	Y=98.51X + 1178	0.9994
10-Deacetyltaxol	9.6 - 76.8	Y=370.1X - 5.857	0.09996
10-Deacetylcephalo-mannine	14.5 - 116	Y=218.7X + 379.8	0.9998
Baccatin III	11.6 - 92.8	Y=165.0X - 86.30	0.9996
10-Deacetylbaccatin III	11.8 - 94.4	Y=135.6X - 80.61	0.9983

Table 2

Recovery Rate of Taxol and Related Compounds in Callus Cultures of Taxus spp.

Sample	I(%)	II(%)	III(%)	IV(%)	V(%)	VII(%)
TY-37	103.7	98.3	97.4	96.0	N.D.	N.D.
TY(2)2500-5	N.D.	N.D.	N.D.	N.D.	101.9	96.8
TN-20	N.D.	102.2	N.D.	N.D.	98.7	N.D.

I: taxol
II: cephalomannine
III: 10-deacetyltaxol
IV: 10-deacetylcephalomannine
V: baccatin III
VI: 10-decetyllbaccatin III
N.D.: not detected

Tests of different proportions of this kind of mobile phase showed that the proportion of methanol has an apparent effect on the separation and the retention times, but slight effect on the shape of the chromatographic peak. *I*~*VI* were well separated by the mobile phase of methanol : acetonitrile : water (25:35:45) at a flow rate of 1.0mL/min and detected at 227nm (Figure 2).

Table 3

The Contents of Taxol and Related Taxanes in Callus Cultures from Taxus spp.

Number of Cell Line	Kind of Medium	I(%)	II(%)	III(%)	IV(%)	V(%)	VI(%)
TY-37	D	0.052	0.13	0.018	0.029	ND	ND
TY-55	D	0.021	0.011	0.016	ND	ND	ND
TY(2)-500-5	D	0.0043	0.0044	0.0043	ND	ND	ND
TY(2)-500-6	D	0.0055	0.035	trace	ND	ND	0.042
TY(2)-500-8	D	0.011	0.010	0.0084	ND	ND	0.019
TN-20	C	ND	0.013	ND	ND	0.11	ND
TY(2)-1000-5	C	ND	ND	ND	ND	0.011	0.0081
TY(3)-7	B	ND	ND	ND	ND	0.0086	0.0057
TY(2)-2500-2	B	ND	ND	ND	ND	0.041	0.094
TY(2)-2500-4	B	ND	ND	ND	ND	0.012	0.0044
TY(2)-2500-5	B	ND	ND	ND	ND	0.076	0.031

I: taxol
II: cephalomannine
III: 10-deacetyltaxol
IV: 10-deacetylcephalommanine
V: baccatin III
VI: 10-deacetylbaccatin III
ND: Not detected

The isocratic analytical HPLC method showed good linearity for all six compounds **I~VI**. The mass of each taxoid was determined by comparison to its external standard curve over the range of approximately 10ng to 100ng (Table 1). Recovery rates of taxol and related taxanes in callus cultures of *Taxus* spp. were from 96.0% to 103.7% (Table 2).

The contents of **I~VI** in callus cultures from various species of *Taxus* were determined. A compound could be assumed to be present in the sample, if the peak of that compound remained single when co-injected with an authentic standard. The results showed that the contents of **I~VI** varied in different cell lines established in various kinds of medium (Table 3), where medium A was 6, 7-V basic medium contained 0.1mg/L KT, 1.0mg/L IAA and 1.0mg/L 2, 4-D, medium B was medium A which contained 20mg/L phenylalanine(Phe), medium C was medium A which contained 20mg/L Phe and 2.5g/L soybean

powder (SP), and medium D was medium C which contained 50mg/L vanadyl sulphate. The cell line TY-37 has the highest contents of **I** and **II**, and TY(2)2500-5 the highest content of **V**. Details of the work on *Taxus* callus culture will be reported elsewhere.

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REFERENCES

1. E. R. M. Wickremesinhe, R. N. Arteca, J. Liq. Chromatogr., **16(15)**, 3263 (1993).

2. K. M. Witherup, S. A. Look, M. W. Stasko, T. G. McCloud, H. J. Issaq, G. M. Muschik, J. Liq. Chromatogr., **12(11)**, 2117 (1989).

3. G. M. Cragg, S. A. Schepartz, M. Suffness, M. R. Grever, J. Nat. Prod., **56(10)**, 1657 (1993).

4. R. A. Holton, C. Somoza, H. B. Kim, F. Liang, R. J. Biediger, P. D. Boatman, M. Shindo, C. C. Smith, S. Kim, H. Nadizadeh, Y. Suzuki, C. Tao, P. Vn, S. Tang, P. Zhang, K. K. Murthi, L. N. Gentile, J. H. Liu, J. Am. Chem. Soc., **116**, 1597 (1994).

5. R. E. B. Ketchum, D. M. Gibson, J. Liq. Chromatogr., **18(6)**, 1093 (1995).

6. W. Fang, Y. Wu, J. Zhou, W. Chen, and Q. Fang, Phytochem. Anal., **4**, 115 (1993).

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A SIMPLE CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF PYRIMIDINES AND THEIR DIHYDROGENATED METABOLITES

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ABSTRACT

Fluorouracil (5-FU) is a pyrimidine analog widely used in the treatment of numerous malignancies. One major metabolic pathway is the reduction of its double bond by dihydropyrimidine dehydrogenase (DYPD), whose activity is a strong determinant of 5-FU plasma levels. Recent findings show that deficiencies in DYPD are less rare than generally assumed and play a major role in 5-FU toxic effects. We developped a simple, sensitive and accurate liquid chromatographic method that allows the simultaneous determination of uracil, 5-fluorouracil and their dihydrogenated metabolites in plasma. This method offers a useful tool for the detection of defects in pyrimidine degradation. HPLC was carried out by using Spherisorb ODS1 (10 cm) and ODS2 (25 cm) columns serially mounted, and 10 mM phosphate buffer, pH 3.0, as the mobile phase with UV detection at 205 nm.

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Many parameters, such as mobile phase pH, ionic strength, column temperature, were found to have a marked influence on the results. We calculated the ratio dihydrouracil/uracil and could describe a Gaussian distribution of this ratio in a population of 78 healthy volunteers.

INTRODUCTION

Fluorouracil (5-FU) is widely used in the treatment of a large range of tumors and according to various schedules. Recently, the concept of dose-intensity has been applied to 5-FU and high doses of this drug are currently administered, mostly by continuous infusion, over 5 to 21 days.[1] The use of high doses by continuous infusion, and the enlargement of the indications have generated a new profile of toxicity. Several studies have reported a high individual variability of 5-FU metabolism and a close link between its toxicity and its individual pharmacokinetic parameters.[1,2] Moreover, some authors have reported a relationship between 5-FU plasma levels and the response to treatment.[1,2]

5-FU metabolism is predominantly linked to the activity of dihydropyrimidine dehydrogenase (DYPD), a key enzyme of endogenous pyrimidine metabolism, which is submitted to a genetic polymorphism.[1,3-5] Complete deficiencies have been previously reported,[4-6] in which extremely high plasma levels of 5-FU were maintained for a long time after a low dose of 5-FU, with a severe, sometimes fatal, subsequent toxicity.[7,8] There is a genetic polymorphism of 5-FU metabolism, and a large range of DYPD activity, with a Gaussian distribution, has been shown among a large population of patients.[3,5] Moreover, a relationship between the DYPD activity in lymphocytes and 5-FU plasma levels has been reported in several studies.[3-5,9]

These results raise the question of the detection of DYPD deficiencies. A method using a test-dose of 5-FU appears difficult to set in current practice, since the plasma kinetics of 5-FU is complex. Moreover, this test leads to a risk of lethal toxicity in case of complete DYPD deficiency. On the other hand, the determination of DYPD activity in lymphocytes by a radioenzymatic technique, as described by Harris et al.,[4] would be an elegant solution. However, this method is long, tedious, and needs a large quantity of blood for Ficoll separation, as well as radioactive substrate. It cannot be easily and widely used in current practice. Finally, the coefficient of correlation between DYPD activity and 5-FU plasma levels is only 0.34 and it is not known whether the enzyme activity level in lymphocytes is a reliable reflect of the enzyme activity in organs, such as liver, lung, and kidney, where this enzyme is largely

widespread. Some authors have measured uracil, the endogenous substrate, in plasma, but its levels are influenced by several parameters and it does not provide a good detection of DYPD disorders.[10,11]

We have developed a new HPLC method which enables us to measure simultaneously, in plasma, DYPD substrates and their dihydrogenated metabolites. Thus, on a single chromatogram, one can measure both uracil (U) and its reduced metabolite, dihydrouracil (UH_2), and 5-FU and its metabolite, dihydrofluorouracil (FUH_2). This technique is simple, selective, very sensitive, and allows one to determine the ratio UH_2/U, which can be considered as representative of DYPD activity.

MATERIALS AND METHODS

Chemicals

5-FU, U and UH_2 were purchased from Sigma (Saint-Quentin-Fallavier, France). FUH_2 was obtained from Hoffmann-La Roche AG (Basel, Switzerland). Ammonium sulfate, potassium dihydrogen phosphate, phosphoric acid, and all other chemicals used to prepare buffers, as well as isopropanol, chloroform, and ethyl acetate, were of HPLC grade (Cofralab, Gradignan, France). The water used was of Milli-Q grade (Millipore) and was degassed with helium before use.

Extraction

To 500 µl plasma samples, 2.5 µg of 5-bromouracil (internal standard), dissolved in 25 µL water, were first added, followed by 200 µL of 10 mM phosphate buffer, pH 3.0, and 100 µL of chloroform, and the solution was vortex-mixed. Plasma proteins were precipitated with 1,800 mg ammonium sulfate. The tubes were then vortex-mixed for 1 min and 7 mL isopropanol in ethyl acetate (15/85), as extraction solvent, were added. The tubes were gently mixed for 15 min in a rotatory stirrer (45 turns per min) and centrifuged for 4 min at 4°C (max 8,000 *g*). The supernatant was transferred to a glass tube and the solution was evaporated at 56°C for 20 min under a stream of nitrogen. The dry extract was reconstituted with 200 µL of 10 mM phosphate buffer, pH 3.0, mixed for 1 min, and 50 µL of chloroform were added. The solution was centrifuged at 4°C for 3 min and the supernatant was recovered, vortex-mixed, and 40 µL of it were injected onto the column.

Chromatography

All reversed phase analyses were performed with a Kontron chromatograph (Kontron, Montigny-le-Bretonneux, France), equipped with an autosampler (model 465), a pump (model 422 S), a variable-wavelength UV detector (model 430), set at 260 or 205 nm. A thermostated oven was used (Peltier's effect, 5°C to 100°C, from B.A.E.I., Phase Sep, Pessac, France). Peak areas were determined by electronic integration (MT2, Kontron).

The following analytical reversed phase columns, purchased from Waters (Saint-Quentin-en-Yvelines, France), were evaluated for separation : Lichrocart C_{18} (250 x 25 mm, 5 µm particle size), Symmetry C_{18} (250 x 4.6 mm, 5 µm particle size), Spherisorb ODS1 (250 or 150 x 4.6 mm, 5 µm particle size), Spherisorb ODS1 (100 x 4.6 mm, 3 µm particle size), Spherisorb ODS2 (250 x 4.6 mm, 5 µm particle size), Spherisorb ODS2 (150 x 4.6 mm, 3 µm particle size).

The mobile phase consisted in 0.01 M potassium phosphate buffer adjusted at pH 3.0 with phosphoric acid and was used routinely at a flow rate of 0.6 mL/min. The total analysis time required in these conditions for each run was 90 minutes (Figure 1). Final results could be given within 2 hours after reception of the blood sample. At least 12 plasma samples could be extracted and analyzed per day.

Sample extraction was usually done in the morning and the samples were injected in the afternoon and overnight, so as to treat the data in the next morning.

Resolution of the Peaks on the Chromatograms

The resolution factor R was calculated by using equation :

$$R = 2\frac{T_2 - T_1}{W_1 + W_2}$$

T and W being the retention times and base widths of the peaks, respectively. Two peaks were regarded as reasonably well separated when $R \leq 1$, since at this value only 2 % of peak overlap occurs.[12] Larger values of R reflect better separation.

Method Validation

Linearity

5-FU, FUH_2, U, UH_2 and 5-bromouracil were dissolved in milli-Q water at a concentration of 1 mg in 10 mL and stored at - 20°C. Standard solutions were prepared by further dilution of the appropriate standard into milli-Q water. Plasma standards were prepared in a series of polypropylene mini-Eppendorf tubes, by the addition of 25 µL standard solution of the selected compounds, 25 µL of the internal standard and 475 µL of human plasma for a final total volume of 525 µL. The dilutions were 1/1600, 1/800, 1/400, 1/200, 1/100, 1/50, 1/20, 1/10, 1/5, 1/2, giving concentrations of 6.25, 12.5, 25, 50, 100, 200, 500, 1000, 2000, 5000 µg/L, respectively. Each of the compounds was injected directly onto the column and peak area data were recorded. 5-bromouracil solutions were prepared by dilution from a 1 mg/mL solution in milli-Q water with sonication until complete dissolution.

Calibration graphs were obtained using the least-squares method. Peak area ratios between each analyte and the internal standard were used to construct the least-squares regression curves. We determined the concentrations of selected compounds in unknown plasma samples by interpolation between known concentrations.

Precision

Repeated injections (n = 5) were performed on a single day to establish the within-day coefficient of variation (precision). The between-day coefficient of variation was determined similarly. Carry-over between injections was minimal. Before each run, the syringe was rinsed and the injector loop was back-flushed with mobile phase at a flow-rate of 1.3 mL/min. Samples were run in order of increasing concentration.

Accuracy

Repeated injections (n = 5) were performed on a single day to establish the mean accuracy. The accuracy was expressed as the ratio (x 100) of the concentration measured to the concentration added.

Mean analytical recovery

5-FU, FUH_2, U and UH_2 recoveries were evaluated using similarly prepared standards. Different concentrations were studied, whereas concentrations of 5-bromouracil were maintained constant. The peak areas

measured were then compared to those recorded without extraction. Recovery was calculated by reference to unextracted aqueous solutions to which identical quantities of internal standard had been added.

Detection limit

The limit of quantitation (LOQ) and the limit of detection (LOD) of the selected compounds in plasma were determined.

RESULTS

Wavelength Selection

The absorption spectra of 5-FU, U, and 5-bromouracil exhibited two absorbance maxima at 205 and 260 nm. The wavelength 260 nm does not permit to visualize some catabolites of 5-FU and U, especially the hydrogenated ones, UH_2 and FUH_2, which have an absorbance maxima at 205 nm. We selected this wavelength for the simultaneous determination of these analytes.

Influence of Various Parameters on the Extraction Yield

Ammonium sulfate

Sample pretreatment procedures involve a deproteination step that can be accomplished by using either a precipitating agent, a membrane ultrafiltration, or an ion-exchange column. We chose a precipitating agent to avoid the use of a preparative cartridge[13] and to perform at the same time that step and the liquid-liquid extraction. We selected ammonium sulfate because it did not affect recovery in plasma samples, and the quality of the chromatograms was excellent, according to the literature and our own experience.[14]

The protein precipitation usually performed with trifluoro- or trichloroacetic acid was unfavorable, because 5-FU coprecipitated with these chemicals. Ice-cold ethanol had no effect on recovery, but it made the use of an internal standard impossible, due to overlap with interferent peaks.[15]

We tested several amounts of ammonium sulfate, from 600 to 2,000 mg. The recovery was stable but 1,800 mg permitted a better separation of the aqueous and organic phases and, thus, provided an easier withdrawal of the aqueous phase.

Extraction Solvent

We compared different systems for extraction of the compounds from plasma. The recovery was calculated by reference to the unextracted aqueous solution to which identical quantities of internal standard had been added. We selected a solution of 15 % isopropanol in ethyl acetate that provided excellent results for 5-FU extraction in previous studies and in our own experience.[16-17] We tested different volumes of this solution, especially, the ratio of organic and aqueous phase and we found that the best combination was 7 mL isopropanol-ethyl acetate, 200 µL phosphate buffer (10 mM, pH 3.0) and 500 µL of plasma. This addition of 200 µL of mobile phase improved the recovery, compared to either nothing or 200 µL water, maybe because of the light acidification it produced. The addition of chloroform for extraction was used for eliminating interfering compounds and did not affect the extraction of the compounds.

We also tested an acidic extraction with sulfuric or phosphoric acid. They did not give good results, because they simultaneously increased the extraction of interfering compounds. The use of diethylether decreased the extraction yield and led to the occurrence of a compound which interfered with 5-FU.

Mixing appeared to be an essential step in the extraction. We tested the type of mixing by comparing vortex-mixing and gentle mixing in a rotative stirrer (45 turns per min). They provided equivalent extraction yield, but gentle mixing gave more reproducible results and was not operator-dependent.

Choice of the Internal Standard

Several compounds were tested : 5-fluorocytosine, 5-chlorouracil, 5-bromouracil, and 5-fluorouridine. We compared their extraction yield with isopropanol/ethyl acetate and the resolution of their peaks on the chromatogram. We selected 5-bromouracil that had an excellent recovery (90 %) and appeared isolated on the chromatogram. Unfortunately, its retention time is relatively long, 45 to 50 min.

Influence of Different Parameters on the Retention Times

Column type

Table 1 describes the columns used and shows the resolution factors for FUH_2 and UH_2 (R1), UH_2 and U (R*2*), U and 5-FU (R*3*), as an estimate of the performance of each system. The results with each column are described

Table 1

Influence of Column Characteristics on the Resolution Factor

Column	Length (mm)	Particle Size FUH_2 (μm)	Resolution Factor (R) UH_2 R1	U R2	5-FU R3
ODS1	100	3	3.3	0.34	0.64
ODS1	250	5	7.43	0.4	1.9
ODS2	150	3	<1	<1	1.5
ODS2	250	5	0.18	1.6	2.5
ODS1-ODS2	100+150	3+5	2.9	1	1
ODS1-ODS2	100+150	3+3	2.9	0.6	0.78
ODS1-ODS2	150+250	5+5	3.2	1.7	2
ODS1-ODS2	100+250	3+5	2.3	1.6	2
ODS1-ODS2	250+250	5+5	4.4	1.5	0.8
Symmetry	250	5	<1	<1	1.2
Lichrocart	250	5	0.38	0.93	1.33

below. ODS1 columns gave a good resolution of dihydrogenated compounds, but they were less efficient for separating U and 5-FU. On the other hand, ODS2 columns separated 5-FU and U well, but not dihydrogenated metabolites. Using both types of columns serially mounted, we could obtain a good separation of the 4 compounds. The best results were obtained with the combination of ODS1 (100 mm length, 3 μm particle size) and ODS2 (250 mm length, 5 μm particle size) columns.

Other columns, such as Symmetry and Lichrocart, have been tested but they did not provide a good resolution of FuH_2 and UH_2. A typical chromatogram obtained in the optimal conditions as described here is presented on Figure 1.

Figure 1 (right). A typical chromatogramme obtained with a plasma extract containing pyrimidines and dihydrogenated metabolites; *above*: blank chromatogram, retention times: UH_2 = 16.6 min, U = 18.4 min. There is no peak at 5-FU, 5-FUH_2 and 5-BU retention times; *below*: 5-FuH_2, 5Fu and 5-BU retention times are 14.59 min, 19.48 min and 53.20 min, respectively. Conditions as follows : 2 columns serially mounted, ODS1 (150, 5μm) and ODS2 (250, 5 μm); flow rate = 0.6 mL/min; column temperature = 12°C.

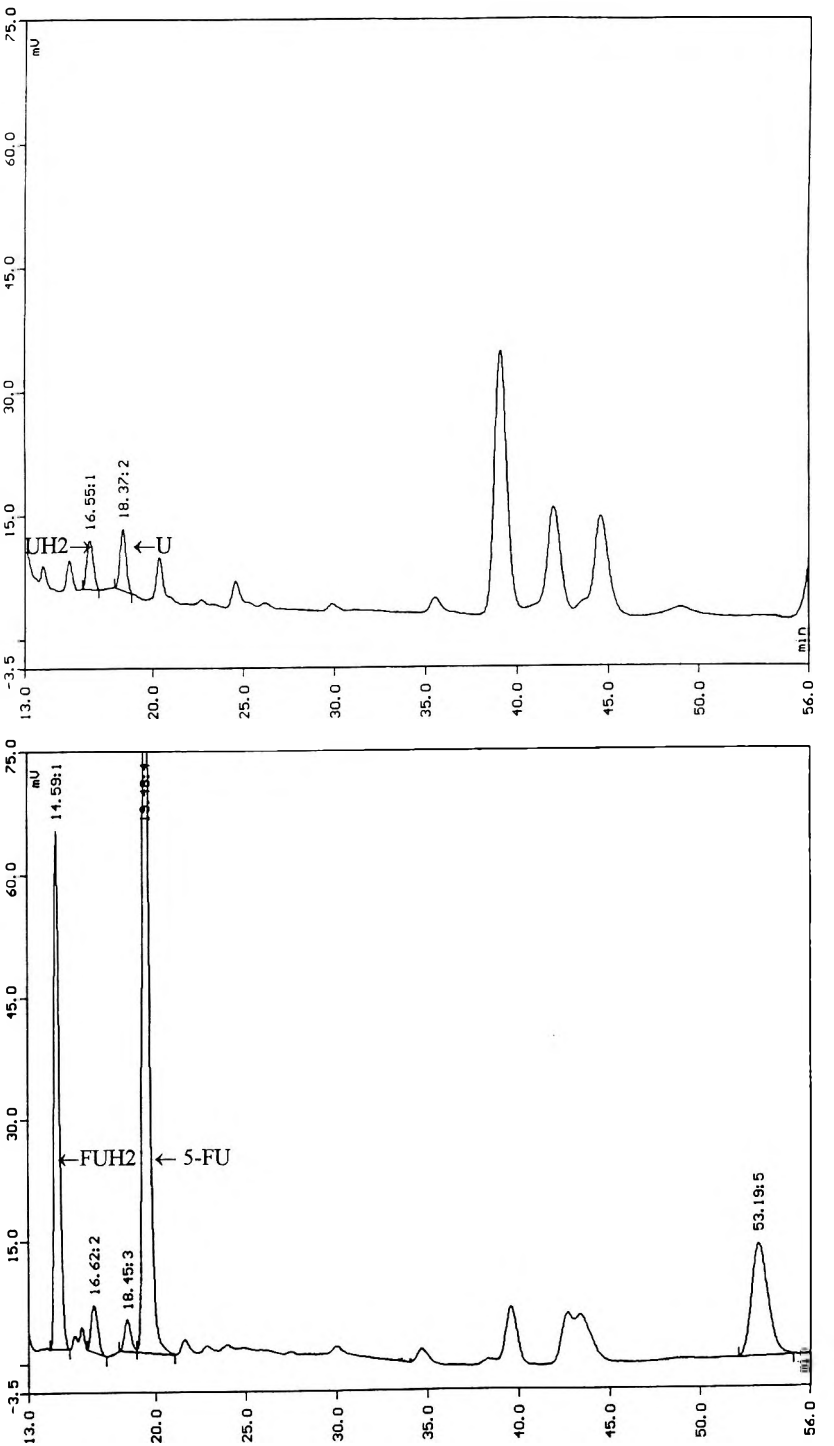
mU
75.0
60.0
45.0
30.0
15.0
-3.5
16.55:1
18.37:2
UH2
U
min
13.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
56.0
mU
14.59:1
FUH2
5-FU
16.62:2
18.45:3
53.19:5

Table 2

Influence of Flow Rate on the Resolution Factor

	Resolution Factor (R)			
Flow Rate (mL/min)	**FUH_2**	**UH_2**	**U**	**5-FU**
	R1	**R2**	**R3**	
0.6	2.7	1.94	3.03	
0.7	2.5	1.86	3.06	
0.9	2.5	1.85	2.9	

Table 3

Influence of Column Temperature on the Resolution Factor

	Resolution Factor (R)			
Temperature	**FUH_2**	**UH_2**	**U**	**5-FU**
	R1	**R2**	**R3**	
24°C	2.7	1.94	3.03	
15°C	3.44	2.23	1.77	
12°C	3.73	2.53	1.58	
8°C	4.07	2.85	1.3	

Flow-rate

We tested several flow rates (Table 2) and selected 0.6 mL/min because it provided the better resolution, and avoided too high column pressure (> 170 bars).

Temperature

The column temperature influences greatly the retention time of each analyte in different ways. Table 3 shows the resolution factors at three different temperatures. We selected 12°C which provided the best results for the separation of the dihydrogenated metabolites and pyrimidines.

Method Validation

Linearity

Quantitations of 5-FU, U, FUH_2 and UH_2 were obtained from calibration curves in which the peak area ratio drug/internal standard was plotted against the drug concentration. There was a linear relationship between the peak area ratios of the selected compounds over the concentration range 12.5-5,000 µg/L. The correlation coefficients for the calibration curves were all > 0.9999 (n = 5) for each compound (Figure 2).

We tested different concentrations of 5-FU and FUH_2 on one hand, and U and UH_2 on the other. New schedules use very high doses of 5-FU, according to the concept of dose-intensity, and high FUH_2 concentrations in plasma can be expected, whereas the plasma concentrations of the natural substrates are much lower, rarely over 200 µg/L.

Precision

The data for the validation of the within-day and between-day precisions are presented in Table 4. The results show very low coefficients of variation, even for low plasma levels.

For all compounds, the within-day and the between-day reproducibilities were always lower than 2% and 4%, respectively.

Accuracy

The accuracy, expressed as the ratio of compound added to that measured, is also presented in Table 4. It remains in the range 2-4% for low concentrations and was generally around 1% for higher concentrations.

Detection limits

The limit of quantitation (LOQ) of U, 5-FU, UH2, and FUH2 was 6 µg/L.

Mean analytical recovery

Table 5 shows that the recoveries ranged between 75% for FUH_2 and 90% 5-bromouracil.

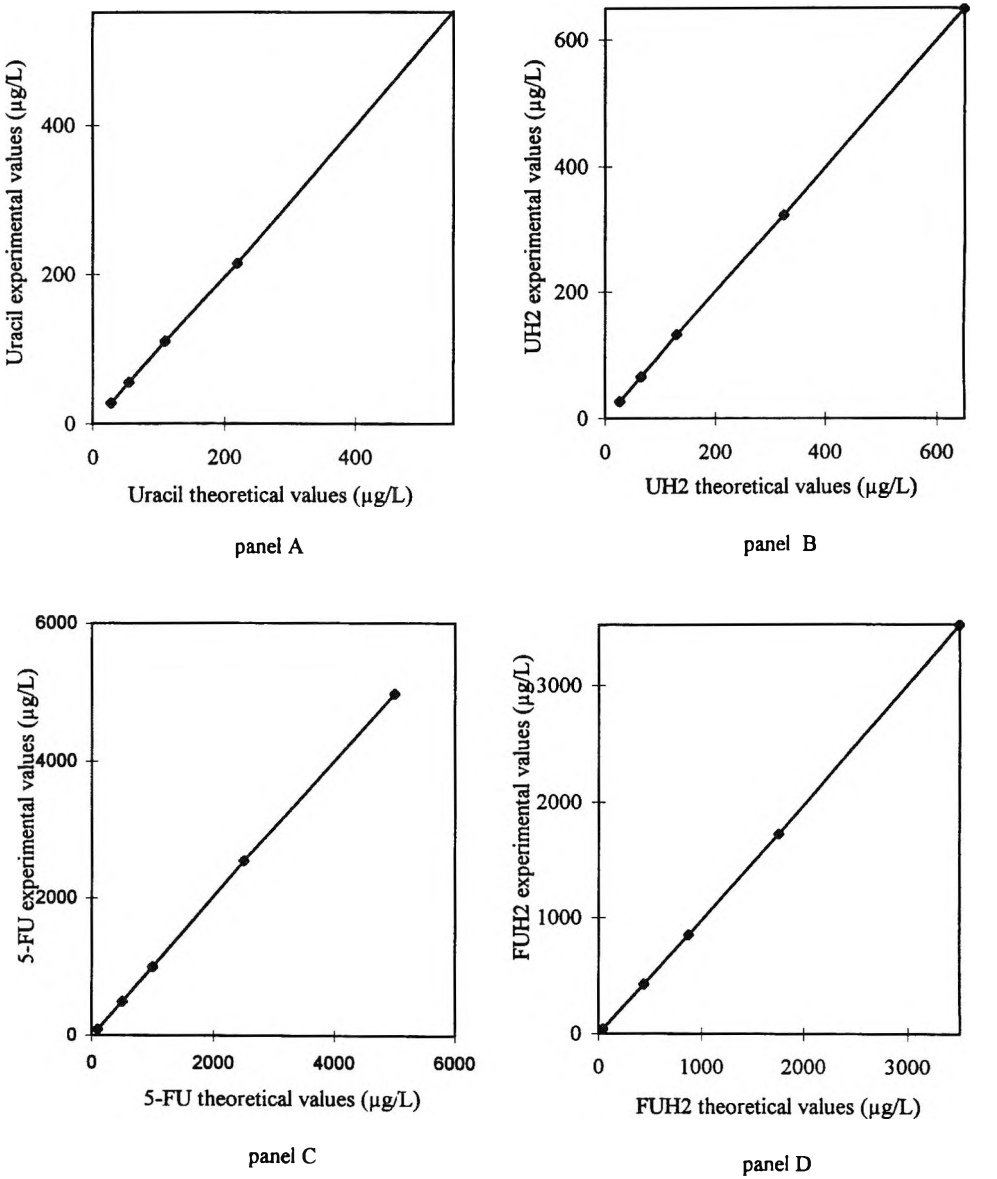

Figure 2. Study of the linearity of pyrimidines and their metabolites extractions. Regression slopes and coefficients of correlation were as follows : U (panel A) : $y=0.9999x$, $R^2=0.9998$; UH_2 (panel B) : $y=x$; $R^2=0.999$; 5-FU (panel C) : $y=0.9999x$, $R^2=0.9998$; FUH_2 (panel D) :$y=0.9999x$, $R^2=0.9997$.

Table 4

Reproducibility and Accuracy of the Determinations of U, UH_2, 5-FU, and FUH_2 in Plasma

Dihydrofluorouracil

	Within-Day (n=5)			Between-Day (n=5)		
Conc μg/L	**Mean±SD**	**CV%**	**Accuracy**	**Mean±SD**	**CV%**	**Accuracy%**
43.75	42.44±0.8	1.8	3	45.58±9.1	2	4.1
437.5	427.1±5.5	1.3	2.4	435.14±11	2.6	0.6
875	860.4±1.5	1.75	1.67	871.3±12	1.4	0.43
1750	1744±8.4	0.5	0.35	1732.8±26	1.5	1
3500	3496±23.5	0.7	0.12	3495±37.4	1	0.13

Dihydrouracil

	Wthin-Day (n=5)			Between-Day (n=5)		
Conc μg/L	**Mean±SD**	**CV%**	**Accuracy**	**Mean±SD**	**CV%**	**Accuracy%**
26	25.86±0.2	0.2	0.54	25.9±0.34	0.85	0.47
65	66.53±0.6	0.95	2.35	66.9±1	1.53	2.8
130	132.7±1.5	1	2.08	133.1±1.6	1.19	2.4
325	324.7±1.5	0.46	0.01	324.5±1.4	0.43	0.15
650	652.7±7.5	1.6	0.41	653.7±8.9	1.4	0.56

Uracil

	Wthin-Day (n=5)			Between-Day (n=5)		
Conc μg/L	**Mean±SD**	**CV%**	**Accuracy**	**Mean±SD**	**CV%**	**Accuracy%**
27.5	26.9±0.5	1.85	2.22	27±1	3.8	1.86
55	55.95±0.7	1.16	1.7	55.45±1	1.9	0.8
110	108.2±1.2	1.13	1.7	108.8±2.3	2.1	1.14
220	220.5±2.3	1.02	0.22	220±1.8	0.8	0.04
550	550.7±6.5	1.17	0.12	550.8±4.9	0.89	0.15

5-Fluorouracil

	Wthin-Day (n=5)			Between-Day (n=5)		
Conc μg/L	**Mean±SD**	**CV%**	**Accuracy**	**Mean±SD**	**CV%**	**Accuracy%**
290	286.9±2.5	0.87	1.06	286.3±3.3	1.14	1.25
540	530.45±11	2.07	1.77	533.6±13	2.41	1.19
1040	1034±16.7	1.61	0.56	1034±15.3	1.48	0.56
2040	2056±23	1.11	0.78	2052±18	0.88	0.6
4040	4017.4±50	1.25	0.57	4030±43	1.07	0.22

Table 5

Mean Analytical Recovery (Mean ± SD) of the Four Compounds

n=8	FUH_2	UH_2	U	5-FU	5-BU
Mean Recovery	85±1.66	75.6±1.7	79.25±2.6	84.75±2.33	90.75± 2.33

Study of Dihydrouracil/Uracil Ratio in Healthy Volunteers

We measured plasma concentrations of U and UH_2 in a population of 47 healthy volunteers. Blood samples were collected in heparinized tubes and centrifuged within the next hour for 5 min at 8,000 *g*. The resulting plasma was then treated in a way identical to that described as final method, or immediately stored at -20°C and later transferred to a -70°C deep freezer until analysis. The values of the ratio of the concentrations of UH_2 to those of U are presented in Figure 3.

DISCUSSION

Pyrimidine metabolism disorders have recently been the focus of considerable attention. DYPD activity presents a large dispersion among individuals. Partial deficiencies may be rendered responsible for the toxic events encountered in the population of patients treated with 5-FU, which are related precisely to the 5-FU steady-state plasma levels.[6-8]

Complete deficiencies seem to be rather exceptional, but are responsible for the occurrence of an extremely severe toxicity of 5-FU, frequently lethal in patients as early as the first course of treatment.[6-8,10,11] Such patients generally present no signs other than this extreme sensitivity to 5-FU, but may also present clinical symptoms such as mental retardation or neurologic disorders.[10,11]

Several authors have developed techniques for the determination of DYPD activity in lymphocytes using a radiolabelled substrate; this method is accurate, but difficulties arise when dealing with large populations, owing to the complex procedures used for measuring enzyme activity.[3-5,9] In addition, the DYPD activity in lymphocytes may not relect the enzyme activity present in the organs, which are in charge of the major part of the metabolic transformation of 5-FU, especially the liver.

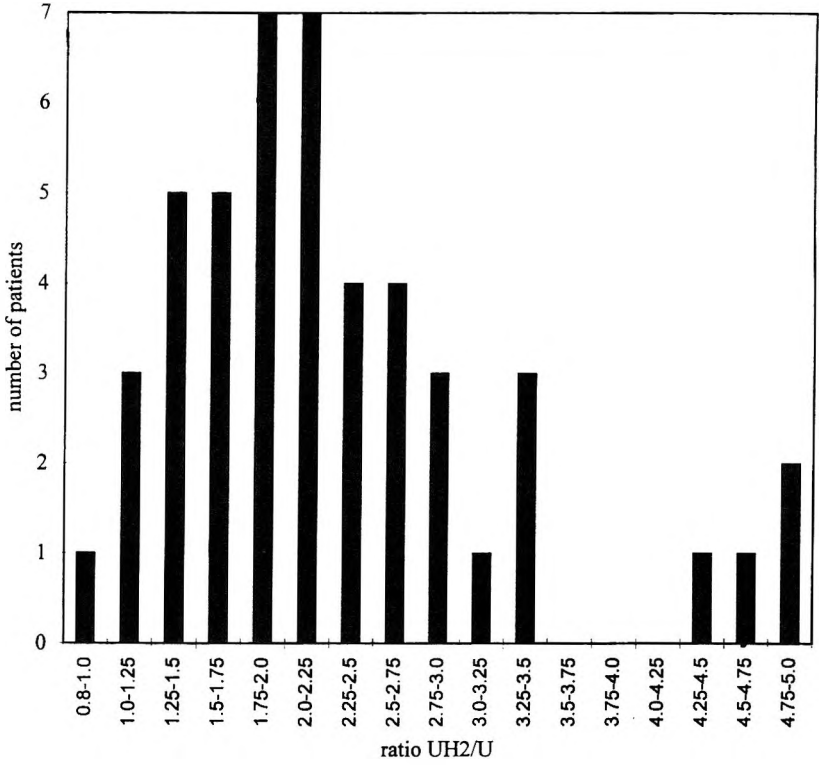

Figure 3. Distribution of UH_2/U ratios in a population of 47 healthy volunteers. This distribution is Gaussian.

It could be possible to approach this problem by the evaluation of the circulating levels of the dihydro derivatives, either of a naturally occurring pyrimidine, uracil, or of 5-FU itself. Methods have been previously reported for the measure of uracil, thymine, and their metabolites in urine.[10,18,19]

However, none of them reported the normal values of excretion of thymine and uracil in healthy populations, and no reference values for the ratios of dihydrometabolites to original pyrimidines in urine.

The method we propose allows the determination of both U and UH_2/U ratio in plasma. More than the single measurement of U concentration in plasma, it gives a reflect of the endogenous DYPD substrate and of its metabolite. It appeared to be sensitive, linear, and therefore, very suitable for

the routine studies. A further advantage of the method over previously HPLC published methods is the simplicity of extraction.[16,20,21] Sample preparation is rapid, but the time required for chromatography is rather long, since the total analysis time required for each run is 90 minutes.

Final results can be given within two hours after blood sampling. Therefore, about 12 samples can be handled daily, enabling injections and calculations to be done overnight, making this technique quite adapted to routine applications.

In a population of 47 healthy volunteers, the UH_2/U ratio followed a Gaussian curve that can be compared to that of DYPD activity, as reported previously by Etienne et al.[3] We are now currently exploring the relationship between this ratio and the plasma levels of 5-FU in patients treated with this drug, in order to evaluate the predictibility of this parameter on 5-FU toxicity.

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REFERENCES

1. G. Milano, P. Roman, P. Khater, M. Frenay, N. Renée, M. Namer, Int. J. Cancer, **41**, 537-541 (1988).

2. E. Gamelin, E. Dorval, Y. Dumesnil, P. Maillart, P. Burtin, M. J. Goudier, P. Gesta, F. Larra, Cancer, 77, 441-451 (1996).

3. M. C. Etienne, J. L. Lagrange, O. Dassonville, R. Fleming, A. Thyss, N. Renée, M. Schneider, F. Demard, G. Milano, J. Clin. Oncol., **12**, 2248-2253 (1994).

4. B. E. Harris, R. Song, S. J. Soong, R. B. Diasio, Cancer Res., **50**, 197-201 (1990).

5. Z. Lu, R. Zhang, R. B. Diasio, Cancer Res., **53**, 5433-5438 (1993).

6. R. B. Diasio, T. L. Beavers, J. T. Carpenter, J. Clin. Invest., **81**, 47-51 (1988).

7. M. Tuchman, J. S. Stoeckler, D. T. Kiang, R. F. O'Dea, M. L. Ramnaraine, B. L. Mirkine, N. Eng. J. Med., **313**, 245-249 (1985).

8. C. H. Takimoto, Z. H. Lu, R. Zhang, M. D. Liang, L.V. Larson, L. R. Cantilena, J. L. Grem, C. J. Allegra, R. B. Diasio, E. Chu, Clin. Cancer Res., **2**, 477-481 (1996).

9. R. A. Fleming, G. Milano, A. Thyss, M. C. Etienne, N. Renée, M. Schneider, F. Demard, Cancer Res., **52**, 2899-2902 (1992).

10. J. A. J. M. Bakkeren, R.A. De Abreu, R. C. A. Sengers, F. J. M. Gabreëls, J. M. Maas, W. O. Renier, Clin. Chim. Acta, **140**, 247-256 (1984).

11. R. Berger, S. A. Stoker-de Vries, S. K. Wadman, M. Duran, F. A. Beemer, P. K. de Bree, J. J. Weits-Binnerts, T. J. Penders, J. K. van der Woude, Clin. Chim. Acta, **141**, 227-234 (1984).

12. A. A. Miller, J. A. Benvenuto, T. L. Loo, J. Chromatogr., **228**, 165-176 (1982).

13. M. Barberi-Heyob, J. L. Merlin, B. Weber, J. Chromatogr., **581**, 281-286 (1992).

14. P. L. Stetson, U. A. Shukla, W. D. Ensminger, J. Chromatogr., **344**, 385-390 (1985).

15. W. Jäger, M. J. Czejka, J. Chromatogr., **532**, 411-417 (1990).

16. M. J. Del Nozal, J. L. Bernal, A. Pampliega, P. Marinero, M. Pozuelo, J. Chromatogr., **656** 397-405 (1994).

17. L. S. F. Hsu, T. C. Marrs, Ann. Clin. Biochem., **11**, 272-276(1980).

18. S. Sumi, K. Kidouchi, S. Ohba, Y. Wada, J. Chromatogr. B, **672**, 233-239 (1995).

19. A. H. Van Gennip, S. Busch, L. Elzinga, A. E. M. Stroomer, A. van Cruchten, E. G. Scholten, N. G. G. M. Abeling, Clin. Chem., **39**, 380-385 (1993).

20. A. R. Buckpitt, M. R. Boyd, Anal. Biochem., **106**, 432- 437 (1980).

21. W. Voelter, J. Chromatogr., **199**, 345- 354 (1980).

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ON-LINE SAMPLE PRETREATMENT AND DETERMINATION OF LANTHANIDES IN COMPLEX MATRICES BY CHELATION ION CHROMATOGRAPHY

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ABSTRACT

The rapid determination of lanthanides in complex matrices has been developed by an on-line sample pretreatment of chelation ion chromatography. In the present system, selective chelating resin and sulfonated cation exchanger concentrated lanthanides while eliminating bulk quantities of alkali, alkaline earth and transition metals from sample matrices. The concentrated lanthanides which were separated on a mixed-bed ion exchange column were eluted by a concentration gradient of

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oxalic acid (Ox) and diglycolic acid (DGA), coupled with post-column spectrophotometric detection with 4-(2-pyridylazo) resorcinol (PAR) at 520 nm. The overall analysis time after sample digestion was less than 55 min. The method detection limits (signal-to-noise ratio 3:1) from heavy lanthanides to light lanthanides were in the range of 0.6 -5.5 ng mL^{-1} and the R.S.Ds were within 1.8%-5.5%. This method was applied to soil, ore, nodular cast iron, plant, grain, and rare-earth fertilizer samples. The results of those real sample analyses were satisfactory.

INTRODUCTION

Lanthanides played important roles in many current technological fields. They could improve the performance of iron and the yield of crops.[1,2] They were also important catalysts in the petroleum industry and as fluorescent labels for biological molecules.[3] The increasing utilization of lanthanides has enhanced the need for rapid and sensitive methods for their determination.[4]

Numerous analytical techniques have been employed in the determination of trace lanthanides. Ion chromatography was one of the most effective and simple methods which were capable of separating individual lanthanides.[5] But, the high concentration of certain metals such as alkali, alkaline earth, iron, aluminum, and other transition metals usually interfered with the separation and determination of trace lanthanides in the complex matrices. Heberling et al.[6] has successfully separated and determined the transition and lanthanide metals in synthetic solution by utilizing a conventional, mixed-bed ion exchange column and selective complexing eluent. However, this method could only be used in the samples which had same magnitude level of the lanthanides and interferences.[7] It could not be used to the real complex samples.

Some off-line techniques have been used to eliminate matrix, to produce a lanthanides fraction free of alkali, alkaline earth, and transition metals.[8,9] Due to high selectivities of iminodiacetate-based resins towards transition metals, and property of the complexes to be kinetically labile, they had been widely used for enrichment of trace lanthanides from the complex matrices.[10-13] They have been used to graphite furnace atomic absorption spectrophotometry,[14] inductively coupled plasma mass spectrophotometry,[15] X-ray fluorescence spectrophotometry and neutron activation analysis.[16-18] But the off-line sample handling steps were too tedious and the reproducibility of the method relied heavily on the operator skill.

In order to eliminate the remaining interferences of complex matrices, chelation ion chromatography was the new technique which combined the on-line analyte concentration and matrix elimination.[19-23] Unlike conventional ion-exchange concentration methods, which were typically not selective for ions of the same valency,[10] chelation concentration was a selective concentration method. It could concentrate all the lanthanides while eliminating the high concentrations of alkali, alkaline earth metals, iron, aluminum, and other transition metals prior to ion chromatography separation. This paper described the method development and application of chelation ion chromatography for determination of lanthanides in soil, plant, grain, nodular cast iron, ore, and rare-earth fertilizer samples. The detection limit was greatly improved and the total analysis time was greatly shortened to less than 55 min after sample digestion.

EXPERIMENTAL

Instrumentation

Chromatographic analyses were performed on a metal-free Dionex DX-300 ion chromatography (Dionex Corp., Sunnyvale, CA, U.S.A), equipped with two advanced gradient pumps (AGP), a MetPac CC-1 chelation column (50 mm × 4 mm, I.D. packed with a styrene-based macroporous 12% cross-linked iminodicacetate-functionalized chelating resin, the particle was 20 μm and the capacity of resin was about 0.9 mequiv.), a TMC-1 concentrator column (25 mm × 3 mm, I.D. containing fully sulfonated PS-DVB cation-exchange resin with high capacity 2.2 mequiv.), an IonPac CG5 Guard column, and an IonPac CS5 analytical column (250 mm × 4.6 mm, I.D.,13 μm bead diameter polystyrene divinylbenzene functionalized with both quaternary ammonium and sulfonate functional groups), a 3.66 mL injection loop and a Dionex variable wavelength detector with a post-column reactor. The MetPac CC-1 and TMC-1 columns were used for sample pretreatment. The lanthanides separation was performed on the IonPac CS5 column.

All measurements were made at room temperature and all samples were filtered through a 0.45-μm filter prior to injection. Data collection and operation of all components in the system were controlled by Dionex AI-450 chromatographic software interfaced via an ACI-2 advanced computer interface to an AST Power Premium 3/33 computer.

Reagents and Standards

Ammonia solution, glacial acetic acid, hydrochloric acid, nitric acid, ammonium acetate, ethanol, lithium chloride, and lithium hydroxide monohydrate were of analytical-reagent grade reagents (Peking Chemical Works, Peking, China), perchloric acid, hydrofluoric acid, oxalic acid dehydrate, and 4-(2-pyridylazo) resorcinol (PAR) were of guaranteed-reagent grade reagents (Peking Chemical Works, Peking, China), pyridine-2,6-dicarboxylic acid (PDCA) was of chromatographic grade reagent (Aldrich, U.S.A), diglycolic acid (DGA) was of chromatographic grade reagent (Fluka).

Working standard solutions were prepared daily by standards (1000 $\mu g\ mL^{-1}$) which were obtained from National Research Centre for Certified Reference (China). Solutions were prepared with pretreated water, which was purified with a ML-Q system (>18MΩ, Millipore, Waters chromatography division, Oslo, Norway).

Eluents and Post-Column Reagent Solution

All reagents were prepared with the 18MΩ de-ionized water. Eluents: E_1: 4.0 M HCl- 65% EtOH; E_2: 2.0 M NH_4OAc, PH 5.5; E_3: 1.0 M HNO_3, 0.1 M Ox; E_4: 0.1 M NH_4NO_3, PH 3.4; E_5: 18MΩ DI water; E_6: 0.1 M Ox, 0.19 M LiOH ; E_7: 0.1 M DGA, 0.19 M LiOH; E_8: 0.006 M PDCA , 0.01 M LiOH and 0.05 M LiCl; "carrier" acid: 0.1M HNO_3

Post-column reagent: 0.8 mM PAR, 1.0 M glacial acetic acid and 3.0 M ammonia solution. The eluted lanthanides were detected after post-column reaction with PAR at 520 nm. The flow-rate was 0.7 $mL\ min^{-1}$.

Samples and Sample Preparation

Samples used in this study were Baotou ore and Baotou east ore (Beijing General Research Institute of Mining & Metallurgy), GBW 07401, nodular cast iron (National Research Centre for Certified Reference, China), soil, the leaf and grain of corn (Research Center for Eco-Environmental Sciences of Academia Sinica), "changle" rare-earth fertilizer 1 and 2 (Chinese Science and Technology University), among which GBW 07401 was certified reference soil sample. Lanthanides in GBW 07401 were well-distributed, stable, and had accurate content. It was used to evaluate the accuracy of method.

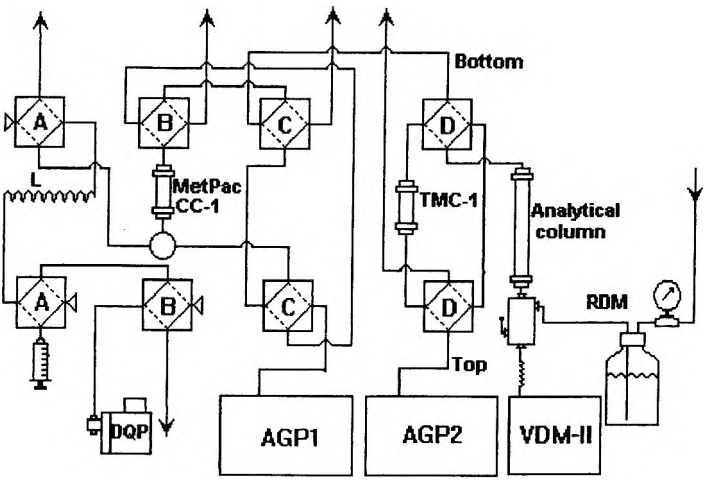

Figure 1. Schematic of the chelation ion chromatography system.

The first seven samples: 250 mg of powdered sample was weighted respectively. The sample was added into a closed polytetrafluoroethyene (PTFE) beaker and wetted with a small amount of water, 5 mL of concentrated nitric acid was added and heated nearly to dryness. Then 20 mL of 50% (w/w) hydrofluoric acid was added and heated to dryness. Furthermore, 5 mL of 60% (w/w) perchloric acid was added to the residue and heated until white fumes of perchloric acid appeared. After allowing to cool, 2 mL of concentrated nitric acid was added and evaporated to dryness again. Finally, the residue was dissolved to 25 mL with 0.1 M HNO_3.

The two rare-earth fertilizer samples were diluted directly by 0.1 M HNO_3 respectively. Before injection, each solution needed to be further diluted to a proper concentration within its linear range and filtered through a 0.45-μm filter.

Experimental Procedure

The detailed schematic of the chelation ion chromatography system was shown in Figure 1. Valve A&B were controlled by V5 of AGP1, valve C was controlled by V6 of AGP1, valve D was controlled by V5 of AGP2.

Table 1

Chelation Concentration and Matrix Elimination Operating Conditions for Analysis of the Lanthanides (AGP-1 Program)

Time (min)	E_1 (%)	E_2 (%)	E_3 (%)	E_4 (%)	Valve A# Valve B#	Valve C#	Flow Rate (mL min^{-1})	Simple Pump
0.0	0	100	0	0	off	on	1.0	on
2.0	0	100	0	0	off	on	2.0	off
4.0	0	100	0	0	off	on	2.0	off
4.1	0	0	0	100	off	on	2.0	off
5.0	0	0	0	100	off	on	2.0	off
5.1	100	0	0	0	on	on	1.5	off
9.5	100	0	0	0	on	on	1.5	off
9.6	0	0	100	0	off	off	1.0	off
13.5	0	0	100	0	off	off	1.0	off
13.6	0	0	0	100	off	off	2.0	off
17.0*	0	0	0	100	off	off	1.0	off
17.1	0	0	100	0	on	off	2.0	off
18.5	0	0	100	0	on	off	2.0	off
18.6	0	100	0	0	on	off	3.0	off
21.0	0	100	0	0	on	off	0.0	off
55.0	0	100	0	0	off	off	2.0	off
60.0	0	100	0	0	on	off	0.0	off

* Begin sample analysis

off: real line connected, on: dotted line connected

Sample pump flow rate: 2.0 mL min^{-1}

The AGP1 pretreatment program shown in Table 1 was entered into the method file in AI-450 operating software and subsequently down loaded onto the AGP1. The AGP2 concentration gradient program listed in Table 2 was entered through the AGP2 front panel. These elution containers were pressurized with N_2 to 5 psi. When the operation started, sample was flushed by acid carrier to the mixing tee where it was buffered with ammonium acetate solution and entered the MetPac CC-1 column. All anions and monovalent cations could not be retained. Following, the alkaline earth metals were selectively removed by acetate buffer solution. Then, the other metals were transferred to the TMC-1 column by HCl-EtOH solution, and there rinsed by Ox-HNO_3 solution further. The lanthanides were quantitatively retained while iron, aluminum and other transition metals were eluted to the waste. Finally,

Table 2

Gradient Separation Program for Analysis of the Lanthanides (AGP-2 Program)

Time (min)	E_5 (%)	E_6 (%)	E_7 (%)	E_8 (%)	Valve D#	Flow Rate (mL min^{-1})
0.0	0	0	0	100	off	1.0
17.0*	0	0	0	100	on	1.0
18.0	0	0	0	100	on	1.0
18.1	100	0	0	0	on	1.0
21.0	100	0	0	0	on	1.0
21.1	40	60	0	0	on	1.0
26.0	30	70	0	0	on	1.0
35.0	59	25	16	0	on	1.0
45.0	54	25	21	0	on	1.0
50.0	54	20	26	0	on	1.0

* Begin sample analysis

off: real line connected, on: dotted line connected

after the AGP1 delivered ammonium nitrate to convert the TMC-1 column from hydrogen form to ammonium form, the AGP2 delivered PDCA to elute lanthanides from TMC-1 column to CS5 column. Following separation, the lanthanides were detected photometrically using PAR postcolumn reagent at 520 nm.

RESULTS AND DISCUSSION

Matrix Elimination and Concentration

The first matrix elimination step was performed on a MetPac CC-1 column to remove alkali and alkaline earth metals from sample matrix. The MetPac CC-1 column contained macroporous iminodiacetate chelating resin which had a very high affinity for transition and lanthanide metals compared to the alkali and alkaline earth metals. The higher valency of the metal ion, the stronger bound the metal ion was to the resin. Before the sample stream passed through the MetPac CC-1 column, the sample previously loaded into the sample loop was flushed by acid carrier and buffered on-line with 2.0 M

ammonium acetate eluent in the PH range of 5 to 6. All anions and monovalent cations were not retained. By using the ammonium acetate eluent further, alkaline earth metals could be selectively eluted, while most of the transition and all lanthanide metals remained quantitatively bound to the resin. 10 mg of Ca could be eluted completely within 5 mL eluent. The other alkaline earth metals had the same results. Changes of the flow-rate from 1 to 3 mL min^{-1} did not influence the recoveries of the retained metals. Then, the remaining ammonium acetate was removed by ammonium nitrate, or else the high concentration of ammonium acetate would crystallize in the MetPac CC-1 column.

The selective elimination process proceeded further to remove the bulk quantities of iron, aluminum and other transition metals on high capacity TMC-1 concentrator column. The concentrator column contained fully sulfonated cation exchange resin which had high affinity for multivalent cations. The selective matrix removal was based upon the stable metal chloride complexes in a mixture of 4.0 M hydrochloric acid - 65% ethanol eluent. The mixture not only promoted the formation of relatively stable metal chloride complexes, it also decreased the distribution coefficient of the metal complexes on the cation exchange resin.[19] It was the effect of high concentration of ethanol that was a reduction of water molecules around the metals, a decrease in the forces binding the coordinated hydrated shell, and a decrease in the size of the outer hydration cloud. As a result, the transition metal chloride complexes formed in the mixture were more stable than in the aqueous system.

On the other hand, the lanthanides formed less stable metal chloride complexes. Thus, the relatively stable metal chloride complexes of transition metals were selectively removed from the TMC-1 column while lanthanides were quantitatively retained and concentrated. 10 mg of transition metals could be eluted within 6 mL eluent. But there were still partial iron and aluminum on the column. Some of them were from the matrix that could not be eluted completely and the others were introduced by the eluents, so, they were eluted further by 1.0 M Ox- 0.1 M HNO3 eluent, which existed as $Fe(Ox)_3^{3-}$ and $Al(Ox)_3^{3-}$. The acidity of this eluent was very important, or else, the lanthanides would be eluted as $La(Ox)_3^{3-}$. Before the lanthanides were eluted to the CS5 column, the TMC-1 column must be converted from hydrogen form to ammonium form, otherwise the remaining matrices would interfere with the separation of the lanthanides. Sometimes, they could overlap with the peak of La. Table 1 showed the optimization of chelation concentration and matrix elimination operating conditions.

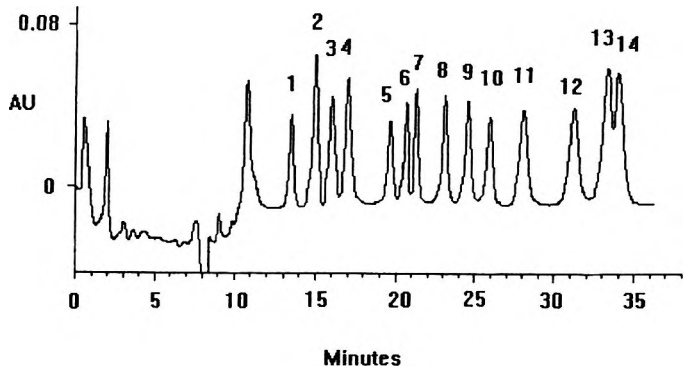

Figure 2. Chromatogram of lanthanides in synthetic solution. Chromatographic conditions as in Table 1 and 2 , Post-column reagent : 8×10^{-4} M PAR, 3 M NH_3,1 M HOAc , Flow-rate: 0.7 mL min^{-1} , Detection λ= 520 nm. Peaks: 1= La (240 ng mL^{-1}), 2 = Ce (200 ng mL^{-1}) , 3 = Pr (96 ng mL^{-1}), 4 = Nd (96 ng mL^{-1}), 5 =Sm (32 ng mL^{-1}), 6 =Eu (32 ng mL^{-1}), 7 =Gd (32 ng mL^{-1}), 8 =Tb(32 ng mL^{-1}), 9 =Dy (32 ng mL^{-1}), 10 = Ho (40 ng mL^{-1}), 11 = Er (32 ng mL^{-1}), 12 = Tm (53.3 ng mL^{-1}), 13 = Yb (64 ng mL^{-1}), 14 = Lu (80 ng mL^{-1}), A l (3.0 mg mL^{-1}) , Fe (3.0 mg mL^{-1}), Ca (6.0 mg mL^{-1}), Mg (6.0 mg mL^{-1}), Cu, Ni, Zn, Co, Mn (200 μg mL^{-1}) respectively.

Chromatographic Separation

The separation of lanthanides was accomplished by anion exchange of lanthanide-chelator complexes on the mixed-bed IonPac CS5 analysis column. In this separation, PDCA eluent removed iron and aluminum from the analysis column introduced by Ox and DGA eluents. The metal contaminants commonly found in these eluents, such as iron and aluminum, existed as $Fe(Ox)_3^{3-}$ and $Al(Ox)_3^{3-}$ were bound strongly by the anion exchange sites of the resin. On the other hand, when the TMC-1 column was placed in-line with the analysis column, PDCA eluted the concentrated lanthanides and trace transition metals from the TMC-1 column as Metal-PDCA complexes to the IonPac CS5 column. The lanthanides formed stable trivalent anionic complexes. The transition metals formed stable divalent anionic complexes which were eluted before La. But, the remaining PDCA on CS5 column would affect the separation and detection of lanthanides. Thus, it must be removed by DI water first. Then the lanthanides were separated by using the gradient Ox and DGA eluents. The separation was based on the stability of the lanthanide-chelator complexes, the smallest ions formed the strongest complexes and were least negatively changed. Therefore, when the lanthanides was separated by anion exchange with Ox and DGA eluents, the eluent order was from La to Lu.

Before starting the analysis, the gradient separation required column equilibration after analysis with the PDCA eluent for 17 min. The analytical system equilibrated while the sample pretreatment steps were being performed. A series of concentration gradient separating conditions had been tested. From those experiments, an optimum condition was chosen as shown in Table 2. A typical chromatogram of synthetic solution was illustrated in Figure 2. The individual lanthanides peaks were well separated. Although there were bulk quantities of alkali metals, alkaline earth metals, aluminum, iron, and other transition metals in the solution, they could not affect the separation and determination of lanthanides.

Accuracy and Detection Limit

In this work, an optimized program shown in Table 1 and 2 was selected. It produced much sharper peaks with good peak separation and excellent calibration curves. It was clear from Figure 2 that the method sensitivity was different among the individual lanthanides. The absorbance of light-lanthanides was less than the middle and heavy-lanthanides. Table 3 illustrated the linear range, correlation coefficients, relative standard deviations (R.S.D.), and detection limits.

It was shown that all lanthanides had good linearities whose correlation coefficients were greater than 0.998. The R.S.D. based on >10X detection limits concentration was found to be in the range of 1.8%- 5.5% and the detection limits (signal-to-noise ratio 3:1) of this method were lower than ever.

Analysis of Standard Reference Soil Sample

As a validation of the analytical technique, a standard reference soil sample GBW 07401was analysed. Table 4 showed the comparison. They were the averages of three totally independent analyses involving sample digestion and chelation ion chromatography procedures. They were obtained based on the system calibration with our standards. It was found that the IC values were in good agreement with the certified values. Most of them had good recoveries. But some elements such as Eu and Tm had bigger deviations. This was because their concentrations in their solutions were close to their detection limits. The concentration of Lu was very low and covered by Yb peak, so it could not be determined. If they had enough concentrations, they would also have good accuracies.

Table 3

The Linear Range, Correlation Coefficients, R.S.D., and Detection Limits of Lanthanides

Lanthanide	Concentration Range (ng mL^{-1})	Correlation Coefficient (r)[1]	R.S.D. (%)[2]	Detection Limit[3] (ng mL^{-1})
La	15-480	0.9990	5.5	6.7
Ce	12.5-400	0.9992	4.1	2.7
Pr	6-192	0.9999	5.3	1.7
Nd	6-192	0.9998	4.8	1.3
Sm	2-64	0.9997	4.1	0.7
Eu	2-64	0.9999	3.8	0.7
Gd	2-64	0.9998	4.9	0.6
Tb	2-64	0.9999	1.8	0.7
Dy	2-64	0.9999	4.2	0.6
Ho	2-64	0.9998	4.6	0.7
Er	2.5-80	0.9998	2.1	0.9
Tm	3.34-106.7	0.9998	4.7	1.3
Yb	4-128	0.9996	4.4	1.5
Lu	5-160	0.9988	4.9	1.9

[1] diluted 1:1, 1:2, 1:4, 1:8, 1:16, 1:32
[2] concentration > 10X detection limits (n=7)
[3] signal-to-noise ratio 3:1

From Table 4 it can be seen which sample was spiked. The spiked recoveries of all lanthanides were acceptable. Because of the appreciable overlap between the Ho and Y peaks , it was difficult to obtain the separated values.

Figure 3 showed the chromatogram of GBW07401. Table 5 showed the main matrix composition in GBW 07401. It could be seen that most of the interference had been eliminated during the sample pretreatment. They did not affect the separation and determination any more. System blanks were well below the detection limits for all the lanthanides, and there was no indication of any memory effect when a blank was run immediately after a sample with high lanthanides concentration.

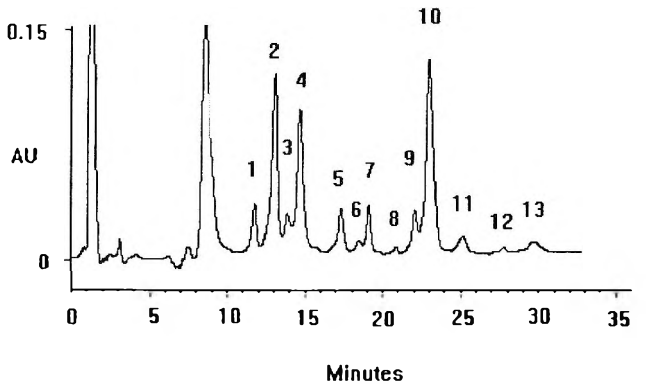

Figure 3. Chromatogram of lanthanides in GBW 07401. Similar chromatographic conditions to Fig 2.

Table 4

Lanthanide Analytical Results of GBW 07401 Soil Sample

Lanthanide	IC (μgg^{-1})	Certified (μgg^{-1})	Recovery (%)	Spiked (μgg^{-1})	Found (μgg^{-1})	Recovery (%)
La	36.0	34	105.9	19.2	54.8	97.9
Ce	64.7	70	92.4	16.0	80.3	97.5
Pr	7.0	7.5	93.3	7.68	14.6	99.0
Nd	29.2	28.0	104.3	7.68	36.4	93.8
Sm	5.25	5.2	101	2.56	7.8	99.6
Eu	1.21	1.0	121	2.56	3.86	103.5
Gd	4.64	4.6	100.9	2.56	7.24	101.6
Tb	0.69	0.75	92	2.56	3.36	104.3
Dy	4.44	4.6	96.5	2.56	7.12	104.7
Ho+Y	27.8	0.87+25	/	2.56	30.2	93.7
Er	2.87	2.6	110.4	3.2	6.12	104.4
Tm	0.50	0.42	119.0	4.4	5.0	102.3
Yb	2.32	2.66	87.2	5.12	7.68	104.7
Lu	N.D.	0.41	/	6.4	7.0	109.4

N.D.: not detected

Table 5

The Main Matrix Composition in GBW 07401

Composition	SiO_2	Al_2O_3	Fe_2O_3	MgO	CaO	MnO	ZnO	ZrO	Na_2O	K_2O
Content (%)	62.6	14.18	5.19	1.81	1.72	0.2	0.1	0.03	1.66	2.55

Table 6

Lanthanides Analytical Results of Nodular Cast Iron, Baotou Ore, Baotou East Ore, Soil, the Leaf and Grain of Corn Samples

	Nod Cast Iron		Baotou Ore		Baotou East Ore		Soil		Leaf		Grain	
	IC	ICP-MS	IC	ICP-MS	IC	ICP-MS	IC	ICP-MS	IC	ICP-MS	IC	ICP-MS
Lanthanide	($mg\ g^{-1}$)			($mg\ g^{-1}$)		($mg\ g^{-1}$)		($\mu g\ g^{-1}$)		($\mu g\ g^{-1}$)		($mg\ g^{-1}$)
La	4.59	4.50	4.52	4.72	5.33	5.55	20.4	32.8	18.2	18.51	N.D.	7.7
Ce	9.90	10.82	11.09	12.31	10.53	10.57	65.4	68.3	10.09	10.04	N.D.	192
Pr	0.89	0.79	2.23	2.05	1.39	1.27	7.2	7.3	3.92	3.45	N.D.	21
Nd	2.25	2.45	8.51	9.38	4.37	4.59	25.6	29.2	5.65	5.19	N.D.	88
Sm	0.187	0.192	0.83	0.92	0.41	0.42	5.2	5.4	0.57	0.51	N.D.	17
Eu	0.022	0.018	0.133	0.129	0.074	0.082	1.0	1.02	N.D.	0.045	N.D.	23
Gd	0.125	0.139	0.187	0.188	0.123	0.131	4.4	4.78	0.40	0.38	N.D.	12
Tb	N.D.	0.016	0.030	0.031	0.017	0.021	0.58	0.77	N.D.	0.045	N.D.	2.8
Dy	0.051	0.054	0.045	0.043	0.047	0.051	4.1	4.0	N.D.	0.013	N.D.	4.3
Ho	0.010	0.0088	0.018	0.016	0.006	0.0056	4.6	3.99	N.D.	0.0026	N.D.	1.9
Er	0.033	0.029	0.038	0.044	0.025	0.027	2.0	2.02	N.D.	0.022	N.D.	7.6
Tm	N.D.	0.005	N.D.	0.0014	N.D.	0.0005	0.59	0.32	N.D.	0.0007	N.D.	0.73
Yb	N.D.	0.027	N.D.	0.0057	N.D.	0.0018	2.4	1.9	N.D.	0.0015	N.D.	0.52
Lu	N.D.	0.009	N.D.	0.00075	N.D.	0.0009	N.D.	0.30	N.D.	0.0011	N.D.	0.27

N.D.: Not detected

These results indicated that the present chelation ion chromatography system was useful for the determination of lanthanides in samples with complex matrices. One of the most important advantage gained of applying this method was that the analytical media could be injected directly for analysis after sample digestion.

Analysis of Samples

Samples used in this study were nodular case iron, Baotou ore, Baotou east ore, soil, the leaf and grain of corn, whose lanthanides had prevously been

Table 7

Lanthanide Analytical Results of Rare-Earth Fertilizer 1 and 2 and Grains of Corn Samples

Lanthanide	Fertilizer 1				Fertilizer 2				Grain			
	IC	Spiked ($\mu g\ g^{-1}$)	Found	Recov. (%)	IC	Spiked ($\mu g\ g^{-1}$)	Found	Recov. (%)	IC	Spiked ($\mu g\ g^{-1}$)	Found	Recov. (%)
La	125	82.4	208.5	103.5	126	49.4	178	104.7	N.D.	9.6	9.25	96.4
Ce	38.6	68.7	104	95.3	41.2	41.4	81.6	98.4	N.D.	8.0	7.85	98.1
Pr	17.3	33	52	105.1	18.8	19.8	39.5	105	N.D.	3.84	4.09	106.5
Nd	41.2	33.0	74.3	100.4	47.9	19.8	67.6	100.1	N.D.	3.84	4.01	104.3
Sm	2.67	11.0	13.2	95.5	2.83	6.58	9.74	105	N.D.	1.28	1.31	102
Eu	0.37	11.0	11.2	98.4	0.32	6.58	7.1	102.9	N.D.	1.28	1.37	107
Gd	0.59	11.0	10.4	98	0.67	6.58	7.34	101.3	N.D.	1.28	1.27	99.1
Tb	N.D.	11.0	10.5	95.9	N.D.	6.58	6.34	96.2	N.D.	1.28	1.26	98.4
Dy	N.D.	11.0	10.9	99	N.D.	6.58	6.62	100.6	N.D.	1.28	1.36	106
Ho	N.D.	11.0	10.9	99	N.D.	6.58	6.38	97.1	N.D.	1.28	1.25	97.7
Er	N.D.	13.7	14.3	104	N.D.	8.23	8.33	101.2	N.D.	1.6	1.57	98.4
Tm	N.D.	18.9	18.5	97.9	N.D.	11.3	11.0	97	N.D.	2.2	2.3	104.5
Yb	N.D.	22.0	22.8	103.9	N.D.	13.2	12.9	97.6	N.D.	2.56	2.80	109
Lu	N.D.	27.5	28.7	104.4	N.D.	6.5	16.4	99.5	N.D.	3.2	3.15	98.5

N.D.: Not detected

determined by ICP-MS, and rare-earth fertilizer 1 and 2 whose lanthanides had not previously been determined. The chelation ion chromatography datum were presented in Table 6 and Table 7. It was seen from Table 6 that IC values also had a good agreement with the ICP-MS values. Thus, chelation ion chromatography had the similar accuracy with ICP-MS. It became one of the most effective method for separating and determining lanthanides.

The lanthanides concentration in grains of corn were too low to be determined. But its spiked recoveries were rather well. The lanthanides, in rare-earth fertilizer which were not determined by ICP-MS previously, had good spiked recoveries as shown in Table 7.

The properties of lanthanides were very similar. But, there were strong light lanthanides enrichment in samples (e.g. ore, nodular cast iron). All the lanthanides could not be determined in a single run. In order to maintain the lanthanides concentration in the analyte solution within the linear calibration range and above the detection limit of the technique, it was necessary to analyse in different concentrations for the same sample. But, some lanthanides in samples (e.g. grain, nodular cast iron, rare-earth fertilizer) were too low to be

determined. If the solutions were too thick, the concentration of the interference would be too high to be eliminated completely through the sample pretreatment and the high concentrated lanthanides would cover the low concentrated lanthanides. This needed to be studied further in our next work.

CONCLUSIONS

The on-line chelation IC technique performed accurate and precise analyses of lanthanides in complex matrices in a relatively short time and at low cost. The reagent and sample consumption were also relatively low compared to the off-line techniques. The instrument was extremely easy to operate and was not difficult to maintain. The sample preconcentration and matrix elimination covered a wide range of sample matrix concentration. It was an extremely versatile technique and the determination of lanthanide was easily adaptable to a wide variety of analytical problems in geological, agricultural and metallurgical fields.

REFERENCES

1. B. Zhao, E. W. Langer, Scandinavian Journal of Metallurgy, **6**, 287 (1982).

2. P. L. Liu, J. L. Tian, Y. Q. Li, T. J. Ju, Journal of Chinese Rare Earth Society, **13(2)**, 155 (1995).

3. M. Rizk, Y. El-Shabrawy, N. A.Zakhari, S. S. Toubar, L. A. Carreira, Talanta, **402(12)**, 1849 (1995).

4. B. T. Kilbourn, in R. G. Bantista, N. Jackson, eds., **Proceedings of an International Symposium held jointly by TMS and AusIMM during the TMS Annual Meeting,** San Diego, CA, March 1992, TMS (Mineral, Metals Materials), Warrendale, PA, 1991.p.209

5. M. Kumer, Analyst, **119**, 2013 (1994).

6. S. S.Heberling, J. M.Riviello, S. F.Mou, A. W. Ip, Res. Dev, **29(9)**, 74 (1987).

7. A. W. Al-shawi, R. Dahl, J. Chromatogr. A, **671**, 173 (1994).

8. A. P. leRoex, R. T. Watkins, Chem. Geol, **88**, 151 (1990).

9. R. M.Cassidy, Chem. Geol, **67**, 185 (1988).

10. J. P.Riley, D. Taylor, Anal. Chim. Acta, **40**, 479 (1968).

11. S. Hirata, K. Honda, T. Kuamany, Anal. Chim. Acta, **221**, 65 (1989).

12. R. R.Greenberg, H. M. Kingston, J. Radioanal. Chem., **71**, 147 (1982).

13. Y. Liu, J. D. Ingle, Jr., Anal. Chem, **61**, 520 (1989).

14. H. M.Kingston, I. L.Barnes, T. C.Rains, Anal. Chem, **50**, 2064 (1978).

15. E. M. Heithmar,T. A.Hinners, J. T.Rowan, J. M. Riviello, Anal. Chem, **62**, 857 (1990).

16. R. R.Greendery, H. M.Kingston, Anal. Chem, **55**, 1160 (1983).

17. H. M.Kingston, P. A. Pella, Anal. Chem, **52**, 2 (1981).

18. J. W.Jones, T. C. O'Haver, Spectrochim. Acta, **40B**, 263 (1985).

19. S. F. Mou, A. Sirirars, J. M.Riviello, Sepu., **12**,166 (1994).

20. A. Sirirars, H. M.Kingston, J. M. Riviello, Anal. Chem., **62**, 1185 (1990).

21. J. M.Riviello, A. Siriraks, R. M. Manake, R. Roehl, U. Alforque, LC.GC, **9**, 704 (1991).

22. A. Siriraks, J. M. Riviello, M. P. Harrold, **Pittsburg Conference Abstract**, 1992, p.1226.

23. Dionex, Technical Note 27.

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SEPARATION AND ISOLATION OF LIMONOIDS FROM *KHAYA SENEGALENSIS* BY DIRECT HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Isolation of various limonoids in pure form from the ethanol extract of the seeds of *Khaya senegalensis* (Desr.) A.Juss by Prep. HPLC procedure is described.

INTRODUCTION

The genus Khaya is a source of valuable timber, the African mahagony. Several species of this genus such as *K. grandifolia, K. anthotheca, K. ivorensis, K. senegalensis* etc. have been examined in great detail by Taylor and collaborators[1]. More than a dozen limonoids have been isolated from different species of this genus and from different parts of the species and structures assigned on the basis of spectral data and correlation studies.[2] All these compounds have been obtained by conventional column chromatography.

Experience in this laboratory has shown that preparative high performance liquid chromatography is exceedingly well suited for separation of tetranortriterpenoids as demonstrated in our studies on neem constituents.[3,4,5]

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Table 1

Isolation of Various Limonoids from the Seeds of *K. senegalensis*

Peak	RT (min.)	Compound	Amount Obtained
1	20.57	mixture	70 mg
2	23.24	6-hydroxy methyl angoensate	50 mg
3	27.37	2-hydroxy mexicanolide - New	150 mg
4	38.71	3-deacetyl-7-keto khivorin	120 mg
5	45.21	3β-hydroxy mexicanolide (Δ14,15 instead of 8,14)	35 mg
		3β-hydroxy mexicanolide (Δ8.30 instead of 8, 14) - New	10 mg
6	50.60	2,3-dihydroxy mexicanolide - New	100 mg
7	58.44	mixture	40 mg
8	64.55	mixture	20 mg
9	74.43	3β-hydroxy mexicanolide	945 mg
10	94.22	3-deacetyl khivorin	640 mg
11	103.37	3,7-dideacetyl khivorin	610 mg
12	MeOH wash	mixture	3000 mg

Khaya senegalensis (Desr.) A. Juss has been introduced into India in the state of Maharashtra as part of an afforestration programme and fruits are available in the month of February. We wished to test the various constituents of seeds of this species for their antifeedant and antifungal activities. Column chromatography of the extract from this source is difficult since there are a large number of compounds of closely similar structure. Direct preparative HPLC is fast, reproducible *ad nauseam* and does not involve the hassle of examining a large number of fractions by TLC. Herein, we report the isolation of various compounds from the ethanol extract of seeds of *K. senegalensis*. Nine compounds were obtained, six of which are known and three new (Table 1). Materials from peaks 1,7,8 & 12 proved to be mixtures, which will be examined in greater detail for separation and isolation of other constituents in pure state by further chromatography.

MATERIALS AND METHODS

Preparative High Performance Liquid Chromatography was carried out using a Shimadzu LC 8A HPLC system linked to CR4A data processor and the

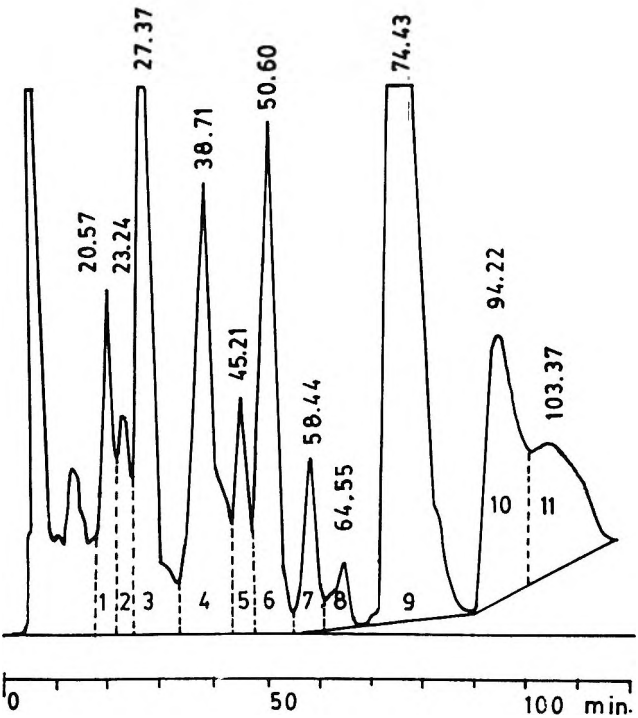

Figure 1. Preparative High Performance Liquid Chromatogram of the ethyl acetatefraction (VLC) from the seed extract of *Khaya senegalensis*.

peaks detected at 215 nm. Shimpack reverse phase (C_{18}) preparative column (25 cm x 20 mm i.d) was used for preparative runs and Phenomenex and Merck reverse phase columns (C_{18}) (25 cm x 4.6 mm) were used for analyses. Freshly powdered seeds (1.015 kgs) of *K.senegalensis* were extracted three times with n-hexane at room temperature and the defatted seed powder was extracted with ethanol.

The ethanol extract after removal of solvent (74.2 gm) was suspended in water and extracted with ethyl acetate. The residue (50.4 gm) was subjected to VLC using hexane and increasing quantities of ethyl acetate (10 - 50%) and then finally with ethyl acetate. The ethyl acetate fraction was concentrated to dryness in vacuo at 45° (10.5 gm). This was subjected to preparative HPLC for the isolation of the major limonoids.

RESULTS AND DISCUSSION

For each preparative run, 500 mg of the residue from ethyl acetate extract was dissolved in 2 mL of methanol, filtered through a Millipore filter (0.25 μm) and then injected into the preparative column (25 cm x 20 mm i.d.). The eluent flow rate was 10 mL / min. throughout the run. The individual peaks from peak 1 to 11 (Fig.1) were collected and evaporated. The purity of the compounds recovered from the peaks was checked by analytical HPLC. Identity of known compounds was established by spectral methods and comparison with literature data.

Peak 5 gave a mixture of two isomeric compounds which were separated by HPLC using ACN:MeOH:H_2O 35:30:35. In all three new compounds were obtained and their structures elucidated by spectral methods. Details will be published elsewhere.

REFERENCES

1. G. A. Adesida, E. K. Adesogan, D. A. Okorie, D. A. H. Taylor, B. T. Styles, Phytochemistry, **10**, 1845-1853 (1971).

2. E. K. Adesogan, D. A. H. Taylor, J. Chem. Soc. (C), 1974-1981 (1968).

3. T. R. Govindachari, G. Sandhya, S. P. Ganesh Raj, I. J. Chem., **31B**, 295-298 (1992).

4. T. R. Govindachari, G. Suresh, G. Gopalakrishnan, J. Liq. Chrom., **18**, 3465-3471 (1995).

5. T. R. Govindachari, G. Gopalakrishnan, G. Suresh, J. Liq. Chrom & Rel. Tech., **19(11)**, 1729-1733 (1996).

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HPLC DETERMINATION OF TRACE LEVELS OF BENZYLCHLORIDE, CHLOROBENZENE, NAPHTHALENE, AND BIPHENYL IN ENVIRONMENTAL SAMPLES

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ABSTRACT

A simple HPLC method with on-line preconcentration has been developed for the separation and the determination of trace levels of benzylchloride, chlorobenzene, naphthalene, and biphenyl in tap and surface water. The separation was achieved on the column 150 x 3.2 mm filled with Separon SGX C_{18}, d_p=5μm with the spectrophotometric detection at 220 and 250 nm. To enhance the selectivity of separation a water - methanol gradient was used. The method permits the measurement of benzylchloride, chlorobenzene, naphthalene, and biphenyl in environmental samples, with the detection limits of 10 ppt of naphthalene, 55 ppt of biphenyl, 90 ppt of benzylchloride, and 185 ppt of chlorobenzene (sample volume was 100 ml of water samples).

The assayed procedure has been applied for the quantitative determination of benzylchloride, chlorobenzene, naphthalene and biphenyl in tap water and river water.

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INTRODUCTION

In the past few years, there has been growing concern about the quality of our environment. Fortunately, at the same time, there have been great strides made for the trace level determination of a wide variety of organic pollutants in surface and drinking water. Water from polluted rivers is used to feed buffer reservoirs as a first step in the production of drinking water. As the quality of river water is not constant, continuous monitoring is necessary.

Benzylchloride, chlorobenzene, naphthalene, and biphenyl are important pollutants of the atmosphere being a product of many industrial processes (pharmaceutical industry, manufacturing of colours, organic synthesis, ...). In most cases, they can be emitted separately, depending on the technological processes. The emissions influence the quality of water. These substances represent a group of noxious organic compounds, therefore, legal requirements of many countries are increasing, making it necessary to determine them at very low level. Concern about environmental pollution and occupational hazards due to the presence of these toxic compounds in water lead to the development of an HPLC method after on-line preconcentration.

Numerous studies have been published about single determination of benzylchloride, chlorobenzene, naphthalene, and biphenyl in environmental samples including gas chromatography[1-7] and RP high pressure liquid chromatography.[8-10]

A more fundamental study on the determination of naphthalene and other PAH's in environmental samples by RP HPLC with on-line preconcentration, were presented in references.[9,11-12] A work of Rigly and al.[13] summarised the influence of different factors on the recovery of extraction of naphthalene on the membrane. The GC and HPLC methods were used for analysis of extracts. The HPLC method (C_{18} column, methanol/water or acetonitrile/water mobile phase) was used to study biphenyl in environmental water samples.[14-16] The method for the determination of chlorobenzene and other toxic compounds was described in reference.[17] The separation was made using Al_2O_3 column and UV detector was used for detection. Benzylchloride can be determined using a column containing macroporous polystyrene.[18]

In the present paper the method for separation and determination of benzylchloride, chlorobenzene, naphthalene, and biphenyl in surface and tap water is discussed. To improve the limits of determination, on-line preconcentration has been developed.

Table 1

Gradient of the Mobile Phase

t/min	% A	%B
0	35	65
18	15	85

EXPERIMENTAL

Chemicals

Standards of benzylchloride (Merck), chlorobenzene, naphthalene (Accu US Standard) and biphenyl (Supelco) were analytical grade. Methanol was supplied by Merck and was HPLC grade.

Prior the use, the water samples were filtered over a 0.45 μm membrane filter.

Apparatus

An HPLC system (Hewlett Packard, Series 1100) consisted of a quaternary pump, a injection valve Rheodyne, and a diode array detector.

A Separon SGX C_{18} column (150 x 3.2 mm I. D., 5 μm particle size) was used for the HPLC assay. A Separon SGX C_{18} precolumn (30 x 3.2 mm I. D., 5 μm particle size) was used for the on-line preconcentration. Before use, the precolumn was washed by methanol (to remove memory effect) and then by water. The volume of injected spiked water samples was 50 cm^3 in the case of river water and 100 cm^3 for redestilled and tap water. The desorption of compounds under study was by gradient of mobile phase according to Table 1 in back-flash mode.

Chromatographic Conditions

The gradient elution has been recommended for HPLC procedure. Eluent A was water. Methanol was used as eluent B. The linear gradient of the mobile phase is shown in Table 1.

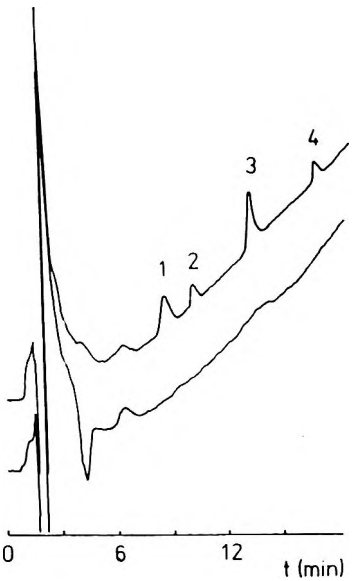

Figure 1. The chromatograms of the blank and the spiked redestilled water after on-line preconcentration (500 ppt of benzylchloride (1) and chlorobenzene (2), 100 ppt of naphthalene (3) and biphenyl(4)). Column: Separon SGX C_{18}, mobile phase: linear gradient of methanol in water from 65% to 85% during 18 min after injection of the sample, flow rate: 0.5 cm^3/min, detection: UV 220 nm. Precolumn: Separon SGX C_{18}, sample volume: 100 cm^3, flow rate: 2 cm^3/min.

The flow rate of the mobile phase was 0.5 cm^3/min and the flow rate of water samples into the precolumn was 2 cm^3/min. All experiments were done at the temperature of 25°C. A detection at 220 nm and 250 nm using the diode array detector was applied.

RESULTS AND DISCUSSION

In the experimental conditions described above, excellent and rapid separation has been achieved for all study compounds. Example of the HPLC separation of benzylchloride, chlorobenzene, naphthalene, and biphenyl using linear gradient elution is given in Figure 1. The advantage of the gradient elution was to not only decrease the time of analysis (great differences in the polarity of the quantified compounds), but also lowering of the limits of the detection (Table 2).

Table 2

The Limits of the Determination and Capacity Factors of Benzylchloride, Chlorobenzene, Naphthalene, and Biphenyl at Gradient and Isocratic Conditions

	Benzylchloride	Chlorobenzene	Naphthalene	Biphenyl
Gradient Elution				
LD	190 ppb	285 ppb	20 ppb	190 ppb
k	4.38	5.35	7.35	9.94
Isocratic Elution				
System A				
LD	1.3ppm	2.5 ppm	n.d.	n.d.
k	3.49	4.55	13.50	25.00
System B				
LD	n.d.*)	n.d.*)	0.1 ppm	0.65 ppm
k	2.65	3.25	4.01	6.13

LD = the limit of determination
system A = methanol/water (65/35 v/v)
system B = methanol/water (75/35 v/v)
n.d. = not determined
*) = low value of resolution between benzylchloride and chlorobenzene

When the isocratic elution is used the two chromatographic systems must be used for the separation. The first, for the separation of benzylchloride and chlorobenzene, includes the mixture of methanol in water 65 % / 35 % (v/v) as the mobile phase. The second system uses the mixture 75 % methanol in water (v/v) as mobile phase for the separation of naphthalene and biphenyl.

Whereas, all the study compounds can absorb the light of UV area the spectrophotometric detection is suitable. The wavelength 220 nm was used for the detection of benzylchloride, chlorobenzene, and naphthalene; and the wavelength 250 nm for the detection of biphenyl. At these wavelengths the maxima of absorption were founded.

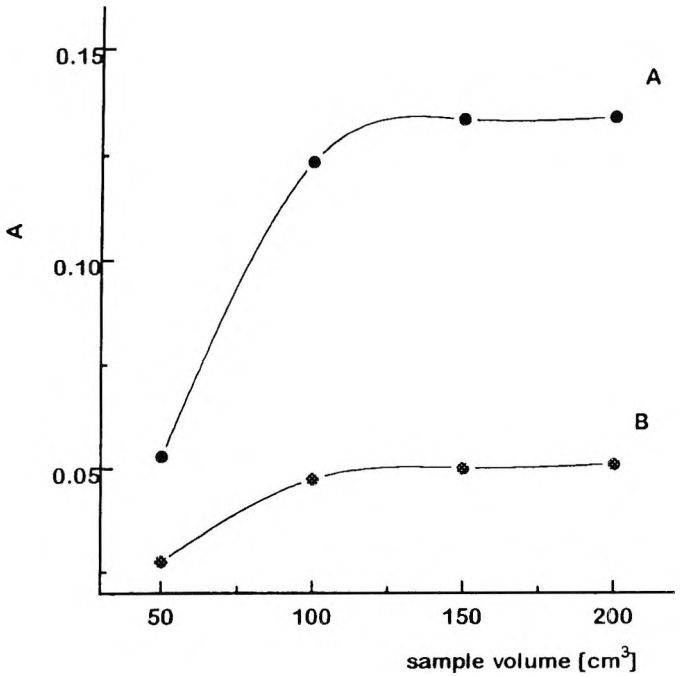

Figure 2. The breakthrough curves for benzylchloride (A) and chlorobenzene (B) at the concentration level 10 ppb. Precolumn: Separon SGX C_{18}, flow rate: 2 cm^3/min, A: area of the peak.

The most important criteria for on-line preconcentration is the breakthrough volume - the maximum sample volume that can be passed through the column without sample components breaking through. The properties of the packing material of the precolumn, physical and chemical parameters of the sample solution, flushing mode of mobile phase, the flow rate into the precolumn, and the temperature influence the retention of analytes. A reversed phase C_{18} packing material for the enrichment column was chosen to conform with the stationary phase used in the analytical column. A high capacity is required for the precolumn, whereas, selectivity and efficiency are important for the analytical column. The relationship of sample volume to the sorbent amount is determined by the substance with lowest retention.

The type and amount of packing material in the precolumn determines the maximum sample volume - the breakthrough volume. On-line trace experiment (and clean-up) by means of solid phase extraction, with short

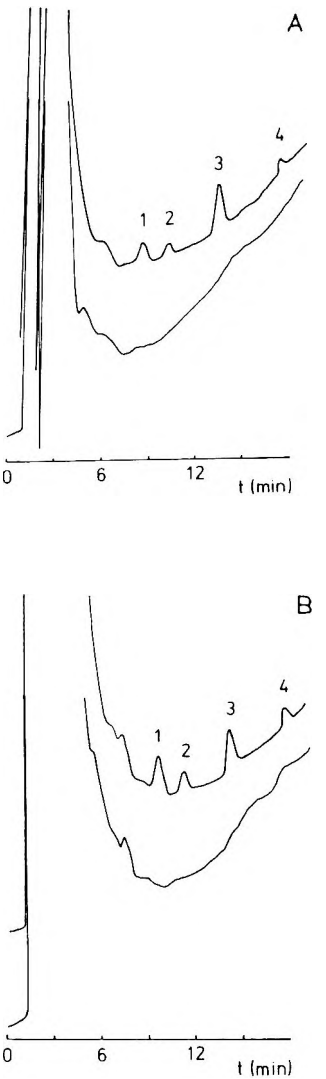

Figure 3. (A) The chromatograms of the blank and the spiked tap water after on-line preconcentration (500 ppt of benzylchloride (1) and chlorobenzene (2), 100 ppt of naphthalene (3) and biphenyl (4)). Sample volume: 100 cm^3. (B) The chromatograms of the blank and the spiked river (Danube) water after on-line preconcentration (1 ppb of benzylchloride (1) and chlorobenzene (2), 200 ppt of naphthalene (3) and biphenyl(4)). Sample volume: 50 cm^3. For the chromatographic conditions see Figure 1.

Table 3

The Limits of the Determination

Compounds	Redistilled Water/SV = 100 cm^3 With On-Line [ppt]	Redistilled Water/SV = 100 cm^3 Without On-Line [ppb]	Tap Water SV=100 cm^3 [ppt]	River Water SV=100 cm^3 [ppt]
Benzylchloride	90	190	145	145
Chlorobenzene	185	285	240	240
Naphthalene	10	20	15	30
Biphenyl	55	190	65	65

SV = sample volume

precolumns, is a popular column switching technique in liquid chromatography. In on-line experiments 3.2 mm diameter and 30 mm long precolumn was used. In this instance, the breakthrough studies were performed with the spiked redestilled water at the concentration level 10 ppb for all compounds. It was found that the capacity of the C_{18} precolumn was 105 cm^3 for benzylchloride and chlorobenzene, and in the case of naphthalene and biphenyl the breakthrough was more than 600 cm^3. The typical breakthrough curves for the spiked redestilled water (10 ppb of benzylchloride and chlorobenzene) with the breakthrough after 105 cm^3 are shown in Figure 2. The volume 100 cm^3 was used for the analysis of the redestilled and tap water. In the case of the surface (river) water, which contain many organic compounds, the sample volume containing 50 cm^3 was used for analysis.

As examples of real samples analyses, the chromatograms of the blank and the spiked tap water (500 ppt of benzylchloride and chlorobenzene, 100 ppt of naphthalene and biphenyl) are shown in Figure 3 (A) and chromatograms of the spiked river - Danube water (1 ppb of and chlorobenzene, 200 ppt of naphthalene and biphenyl) are shown in Figure 3 (B).

The determination of benzylchloride, chlorobenzene, naphthalene, and biphenyl was performed on the basis of measurement of absorbance at the wavelength of 220 nm (benzylchloride, chlorobenzene, naphthalene) and 250 nm (biphenyl) using calibration solutions containing known amounts of the compounds. In the range of concentrations from the limits of the determination (Table 3) to 10 ppb for all compounds the dependence of peak area on concentration was linear (correlation coefficients were over 0.999).

The limits of the determination afforded by HPLC method without preconcentration are in the range ppb and with on-line preconcentration in the range ppt. The lowest limits have been determined using a signal which gives 3 standard deviations above the mean blank signal. The results of the determination of the compounds under study in water are summarised in Table 3. In practice, it is obviously extremely difficult to eliminate all impurities in the reagent at levels corresponding to these very low limits of the determination.

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REFERENCES

1. W. A. Mc Clenny , Ch. R. Fortune, J. Environ. Sci. Heath A, **30(4)**, 901 (1995).

2. M. H. Hiatt, D. R. Youngman, J. R. Donnely, Anal. Chem., **66(6)**, 905 (1994).

3. M. Chai, C. L. Arthur, J. Pawliszyn, R. P. Belardi, K. F. Pratt, Analyst, **118(12)**, 1501 (1993).

4. S. F. Patil, S. T. Sonkar, J. Chromatogr., **684(1)**, 133 (1994).

5. K. Ventura, M. Dostal, J. Churáèek, J. Chromatogr., **642(1-2)**, 379 (1992).

6. F. Sun, Microchim. Acta, **113(1-2)**, 91 (1994).

7. I. Harrison, R. U. Leader, J. J. W. Higgo, J. C. Tjell, J. Chromatogr., **688(1-2)**, 181 (1994).

8. E. R. Brouvwer, A. N. J. Hermans, H. Lingeman, U. A. Th. Brinkman, J. Chromatogr., **669(1-2)**, 45 (1994).

9. S. Hatrik, J. Lehotay, Chem. Pap., **48(5)**, 334 (1994).

10. E. Bosch, P. Bou, M. Roses, Anal. Chim. Acta, **299(2)**, 219 (1994).

11. L. E. Vera-Avila, R. Covarrubias, Int. J. Environ. Anal. Chem., **56(1)**, 33 (1994).

12. F. Lai, L. While, J. Chromatogr., **692(1-2)**, 11 (1995).

13. L. J. Yoo, S. Shen, S. Fitzsimmons, M. Rigly, Proc. - Water Qual. Technol. Conf. PT., **1**, 745 (1993).

14. E. Bosch, P. Bou, M. Roses, Anal. Chim. Acta, **299(2)**, 219 (1994).

15. K. K. Verma, A. Verma, Anal. Lett., **15(11)**, 2083 (1992).

16. S. Butz, H. J. Stan, Anal. Chem., **67(3)**, 620 (1995).

17. G. De Vries, R. F. Rekker, J. Liq. Chromatogr., **16(2)**, 383 (1993).

18. Ch. Q. Wang, F. Svec, J. M. J. Frechet, J. Chromatogr., **669(1-2)**, 230 (1994).

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SIMULTANEOUS DETERMINATION OF NINE WATER AND FAT SOLUBLE VITAMINS AFTER SPE SEPARATION AND RP-HPLC ANALYSIS IN PHARMACEUTICAL PREPARATIONS AND BIOLOGICAL FLUIDS

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ABSTRACT

An automated reversed phase high performance liquid-chromatography (RP-HPLC) method is described, for the simultaneous analysis of water soluble [ascorbic acid (C), nicotinic acid, nicotinamide, folic acid, cyanocobalamine (B_{12}), and riboflavin (B_2)] and fat soluble (retinol, α-tocopherol, α-tocopherol acetate) vitamins. The compounds are separated after solid-phase extraction (SPE) on C_{18} cartridges, where water soluble vitamins pass unretained, while fat soluble vitamins are retained on the sorbent.

After isolation of the two fractions, water soluble vitamins are separated on a Lichrosorb RP-18 250x4.0 mm, 5 μm analytical column, using a gradient elution system consisting of CH_3OH-0.05 M CH_3COONH_4 (5:95 v/v) changing to (30:70 v/v) over a period of 20 min at a flow rate 1 mL/min. Fat soluble vitamins

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are separated on a Spherisorb RP-18 220x4.6 mm, 5 μm analytical column with an isocratic mobile phase consisting of CH_3OH-CH_3CN (30:70 v/v) at a flow rate of 1.5 mL/min.

A UV-vis detector operated at 270 nm and 290 nm for water soluble and fat soluble vitamins, respectively, is used for detection and quantitation of the analytes.

Xanthine is used as internal standard at a concentration of 4.2 ng/L for water soluble vitamins, while anthraquinone is used as internal standard at a concentration of 3.5 ng/mL for fat soluble vitamins.

Linearity is observed up to 10 ng/mL for ascorbic acid, folic acid, and riboflavin, up to 15 ng/mL for nicotinic acid, nicotinamide, and cyanocobalamine, while up to 20 ng/mL for all fat soluble vitamins. Limits of detection ranged from 2.5 - 5.0 ng for water soluble vitamins and 2.0 - 5.0 ng for fat soluble vitamins. Method's validation is achieved in terms of day-to-day and within-day reproducibility studies. Long term stability study is conducted during routine operation of the system over a period of ten consecutive days.

The developed method is applied to the analysis of pharmaceutical preparations: (tablets, injection solutions) and biological fluids: (blood serum, urine). SPE technique is used for the isolation of the vitamins from the matrix of human biological fluids: blood serum (40 μL) and urine (100 μL). High extraction recoveries are achieved using Merck Lichrolut RP-18 cartridges.

INTRODUCTION

Vitamins are vital to human life. The human metabolism is not able to synthesise these compounds, which have a catalytic function in anabolic and catabolic pathways. Therefore, a supplementation of these substances, most often only in μg or mg amounts per day, through food chain is necessary. Due to their function, vitamins are involved in developmental and fast reproducing processes like blood formation, maintenance of epithelial tissue, ossification of bone, eye functions, as well as, in the metabolic pathways of the central nervous system.

Malnutrition and metabolic diseases can lead to a vitamin deficiency, which shows very significant clinical symptoms, while excessive vitamin intake, particularly of fat-soluble vitamins, can result in different diseases.

Vitamins can be classified in two main groups: water-soluble and fat soluble vitamins. In the nomenclature of vitamins often abbreviations are used as common names, e.g., vitamin A, B, C, and so on. These abbreviations stand for the main active substance, but often compromise the biochemical effect of the vitamin itself plus its active metabolites.

The natural source of vitamins for human beings has always been food and drinks. Because artificial vitamin formulations, often complemented with minerals, can and will not replace the natural sources, our need for vitamins can be covered in the conventional way. Customer demands and marketing strategies have forced many food and drink producers to provide information on the vitamin content of their products in the product description.

Vitamins have been determined by a wide variety of techniques from which the most widely applied are the chromatographic ones: paper, thin-layer, column, gas-liquid, and high performance liquid chromatography (HPLC). The latter technique allows a rapid, simple, and selective determination of vitamins.

The analytical advantages of HPLC in comparison to the other analytical techniques are solvent economy, higher efficiency, mass sensitivity, easy coupling with other techniques, and finally small amounts of sample. This method requires relatively little clean-up and offers at once the possibility for the determination of several vitamins in the same run on the same column.[1,2]

Determination of vitamins can be done either by normal phase chromatography or by reversed phase chromatography which is the most common. Most of the analysts use very complex mobile phases like mixtures of three or four types of solvents[3-8] or phosphate buffers which are causing a lot of problems, if the analyst is not very cautious.[3,9-11] Many high performance liquid chromatographic (HPLC) methods for single or simultaneous multiple determinations of vitamins using either ultraviolet-visible absorbance (the most common techique for the detection of vitamins), electrochemical[12-16] or fluorimetric[13,17-21] detection have been published.

Most of the published methods involve the use of capillary columns,[22,23] gradient instead of isocratic elution,[8,24-26] two or three different detectors,[6,7,14,24,25,27,28] or changing the detection wavelength during the run.[26,29,30] Sometimes, diode-array detectors are used for the detection of

vitamins.[7,31] The complexity of these HPLC conditions, no doubt, effectively improves the selectivity and sensitivity of vitamin determinations, nevertheless, they are considered complicated and costly for routine analysis.

The first step in analysing vitamins is sample preparation. In most cases, they have to be extracted from the matrix, however, for the analysis of vitamins in additive raw material or soft drinks, a pre-treatment of the sample may not be necessary. It is important to verify that the chosen sample preparation method is suitable for the analysis of the vitamins of interest, because all vitamins are unstable during common sample preparation methods (boiling for deproteinisation, alkali- or acid-treatment).

For all vitamins it is recommended to use short-time and gentle extraction methods, which in some instances should be performed in a darkened place.

Determination of vitamins in pharmaceutical formulations (tablets, gelatine capsules, injections) is nearly always preceded by extraction with a solvent. Before the extraction can be carried out, these products have to be free from the tablet coating, capsule wall, or other shell vehicular. Sometimes the samples are saponificated.[3,10,32]

Separation of plasma-vitamins by HPLC is nearly always preceded by deproteination of the plasma with ACN, EtOH, MeOH and extraction with n-hexane or heptane.[7,9,14,17-18,23,27,33,34] Sometimes, sample preparation requires perchloric acid or trichloroacetic acid deproteinization of the plasma followed by centrifugation and filtration through a disposable filter.[9,11] To determine vitamin concentrations in tissues, homogenisation followed by the extraction with an organic solvent is the most widely used method.[5,18,19,31,35-38]

For the clean up procedure of biological samples, the technique of SPE extraction was applied in order to isolate the vitamins from the matrix. SPE is a rapid and solvent consuming technique, which leads to reduction in pollution and offers a wide sorbent selection.[25]

In the present paper, a simultaneous determination of water-soluble vitamins [ascorbic acid (C), nicotinic acid, nicotinamide, folic acid, cyanocobalamine (B_{12}), riboflavin (B_2], and fat soluble vitamins (retinol, α-tocoferol, α-tocoferol acetate) is proposed. Solid-phase extraction is used as a pre-treatment technique of biological samples and also as an isolation method of the two fractions of vitamins (water soluble and fat soluble vitamins), in case the two fractions of vitamins exist in the same sample.

EXPERIMENTAL

Instrumentation

The chromatographic system which is used for the analysis of vitamins consists of the commercial components: a Spectra Physics 8800 HPLC ternary pump (California USA), a Spectra Chrom 100 UV/VIS detector, operated at 270 nm for the analysis of water-soluble vitamins and 290 nm for the analysis of fat-soluble vitamins and a sensitivity setting of 0.002 absorbance units full scale (AUFS), a Rheodyne 7125 (California, USA) Injection valve with a 10 μL loop, and a Spectra Physics SP 4290 integrator.

The analytical columns which are used are: a Lichrosorb RP-18, 250x4 mm, 5 μm for the determination of water-soluble vitamins (MZ Analysentechnik, Mainz, Germany) and a Spherisorb RP-18, 220x4.6 mm, 5 μm for the determination of fat-soluble vitamins (Brown Lee, a part of Perkin-Elmer/ABI, Deerfield, USA).

A glass vacuum-filtration apparatus obtained from Alltech Associates, was employed for the filtration of the buffer solutions, using 0.2 μm membrane filters obtained from Schleicher and Schuell (Dassel, Germany).

A Glass-col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) are employed for the pre-treatment of biological samples.

The SPE study is performed on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International, a division of Varian (Harbor City, USA).

Lichrolut RP-18 SPE cartridges are supplied from Merck (Darmstadt, Germany).

All evaporations are performed with a 9-port Reacti-Vap evaporator (Pierce, Rockford, IL, USA).

UV spectra for selecting the working wavelength of detection are taken using a Varian DMS 100S UV/VIS double-beam spectrophotometer. All computations are achieved using a VIP 312 Computer.

Materials and reagents

Vitamins were purchased from Merck and were used without further purification.

Xanthine, used as internal standard for water-soluble vitamins, was purchased from Sigma (St. Louis, MO. USA). Anthraquinone, used as internal standard for fat-soluble vitamins, was purchased from Serva (Heidelberg, Germany).

HPLC gradient grade methanol was obtained from Lab-Scan (Dublin, Ireland) and acetonitrile was obtained from Merck.

Ammonium acetate pro analysi was purchased from Merck. All other reagents used were of analytical grade. Bis de-ionised water was used throughout analyses.

Chromatographic Conditions

Water-soluble vitamins

A reversed phase C_{18} Lichrosorb RP-18 250x4 mm, 5 μm, is used for the separation of water-soluble vitamins at ambient temperature. A variety of mobile phases was tested in order to find out the optimum chromatographic system for the analysis of vitamins. The mobile phases, were in principle, binary mixtures of an aqueous solution of ammonium acetate with methanol in several ratios. The final mobile phase is chosen in terms of peak shape, column efficiency, and chromatographic analysis time, selectivity, and resolution. The mobile phase consisted of:

Time (min)	MeOH (%)	CH_3COONH_4 0.05 M (%)
0	5	95
6	15	85
13	30	70
20	30	70

Flow rate 1 mL/min.

Fat-soluble vitamins

A reversed phase C_{18} Spherisorb RP-18 220x4.6 mm, 5 μm, was used for the separation of the fat-soluble vitamins at ambient temperature. The mobile phase consists of CH_3OH- CH_3CN (30:70 v/v) at a flow rate of 1.5 mL/min.

The mobile phases were selected among others for leading to optimal resolution of compounds, as well as to convenience regarding total time of analysis.

Xanthine (XA) was chosen to be used as internal standard for water-soluble vitamins, after an assay of a wide variety of organic compounds, (Bamifylline, 3-methyl XA, 7-methyl XA, 1-methyl XA, Caffeine) taking into consideration the sufficient resolution, as well as the spectra criteria. For the same reasons, Anthraquinone was chosen to be used as internal standard for fat-soluble vitamins, after an assay of a wide variety of organic compounds (Codeine, Tolfenamic acid, Flufenamic acid, Mefenamic acid, Paracetamol, Hyrdoxy-anthraquinone, Anthranilic acid).

RESULTS AND DISCUSSION

Resolution Factors

The separation between water-soluble vitamins, as shown in chromatogram presented in Figure 1 and the separation between fat-soluble vitamins, as shown in chromatogram presented in Figure 2, are sufficient as signified, also, from resolution factors Rs which are:2.45 for Ascorbic acid-Nicotinic acid, 2.80 for Antraquinone-Retinol, 2.03 for Nicotinic acid-Xanthine, 15.50 for Retinol-αTocoferol, 4.76 for Xanthine-Nicotinamide, 2.75 for αTocoferol - α Tocoferol acetate 3.10 for Nicotinamide-Folic acid,17.82 for Folic acid-Cyanocobalamine and 7.28 for cyanocobalamine-Riboflavin.

Calibration Curves

Water-soluble vitamins

Calibration of the method was performed by injection of mixed standard of water-soluble vitamins covering the entire working range. Twelve concentrations were used in the range 0.5-20 ng/μL.

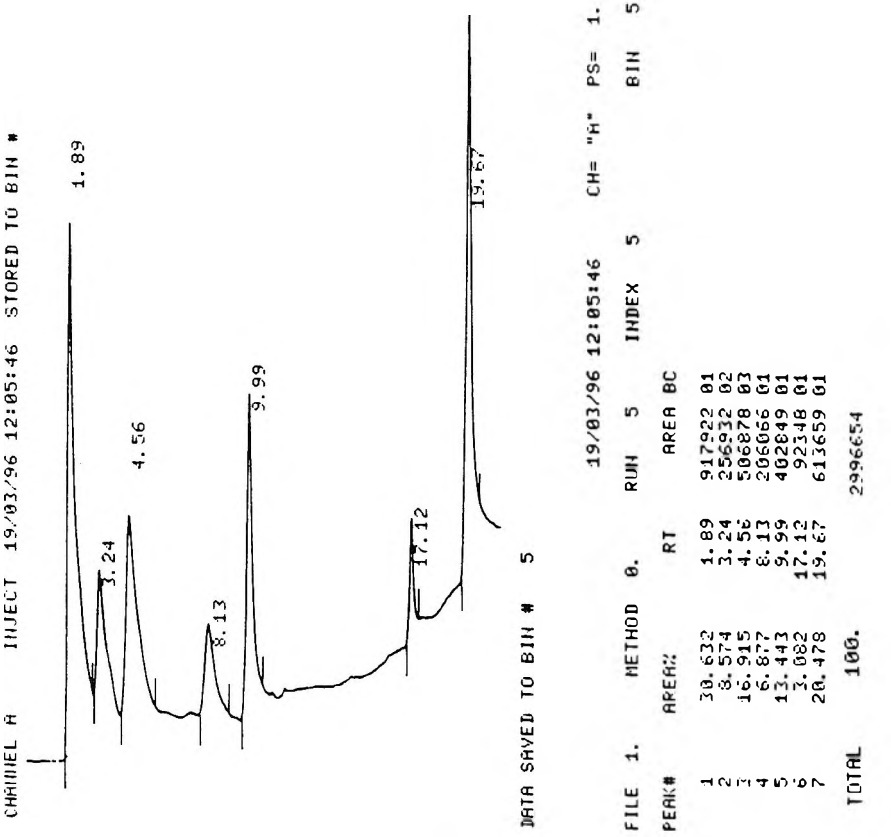

Figure 1. High performance liquid chromatogram of water soluble vitamins. Ascorbic acid (4.12ppm) 1.89min, Nicotinic acid (4.16ppm) 3.24min, Xanthine (4.20ppm) 4.56min, Nicotinamide (4.16ppm) 8.13min, Folic acid (3.12ppm) 9.99min, Cyanocobalamine (4.88ppm) 17.12min, Riboflavin (4.02ppm) 19.67min.

These working solutions were prepared and frozen at -18°C. The working solutions were stable about five to six days. The sensitivity setting of the UV/VIS detector was adjusted to give almost full scale deflection for the highest standard concentration. Each sample was injected five times.

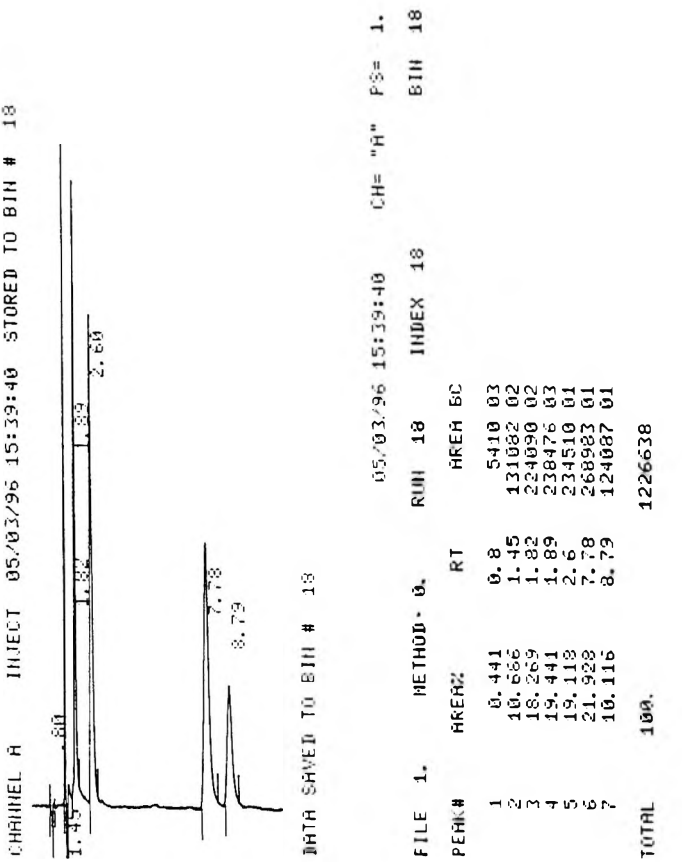

Figure 2. High performance liquid chromatogram of fat soluble vitamins. Anthraquinone(3.50 ppm) 1.89 min, Retinol (2.00 ppm) 2.60 min, α-Tocoferol (5.00 ppm) 7.78 min, α-Tocoferol acetate(4.50 ppm) 8.79min.

Linear correlation between absolute injected amount or concentration and peak area ratio was obtained for all water-soluble vitamins, using xanthine as internal standard, at a concentration of 4.20 ng/μL. The correlation coefficients of the calibration curves had a range of 0.99722-0.99931.

Fat-soluble vitamins

The same procedure described for the calibration of the method of water-soluble vitamins, was followed for fat-soluble vitamins as well. Stock standard and working solutions were prepared and frozen at -18°C. All solutions were

stable at that temperature, for at least one month. Linear correlation, between absolute injected amount or concentration and peak area ratio was obtained for all fat-soluble vitamins, using Antraquinone as internal standard, at a concentration of 3.50 ng/μL.

The correlation coefficients of the calibration curves had a range of 0.99926-0.99948.

Linear range

Linearity was observed up to 10.0 ng/μL for ascorbic acid, folic acid and riboflavin and up to 15.0 ng/μL for nicotinic acid, nicotinamide, cyanocobalamine.

In the case of fat-soluble vitamins linearity was observed up to 20.0 ng/μL.

Limits of detection

The detection limits were assessed in the presence of the internal standards. Those were considered to be the quantities which are producing a signal of peak height three times the size of background noise.

The detection limits are: 2.5ng for Ascorbic acid, 5.0 ng for Nicotinic acid, 5.0 ng for Nicotinamide 3.0 ng for Folic acid 5.0 ng for Cyanocobalamine, 2.5 ng for Riboflavin, 2.0 ng for Retinol, 5.0 ng for α-Tocoferol and 5.0 ng for α-Tocoferol acetate.

Precision and Accuracy

Method validation regarding reproducibility was achieved by replicate injections of standard solutions at low and high concentration levels where peak areas were measured in comparison to the peak area of the internal standard.

Statistical evaluation revealed relative standard deviations at different values for eight injections. Long term stability study was conducted during routine operation of the system over a period of ten consecutive days. Results are illustrated in Table 1.

Table 1

Precision and Accuracy for the Analysis of Vitamins

	Within-Day Precision and Accuracy for the Analysis of Vitamins (n=8)					Day-to-Day Precision and Accuracy for the Analysis of Vitamins (n=7)			
Analyte	**Added (ng)**	**Found (ng)**	**SD**	**RSD (%)**	**Recovery (%)**	**Found (ng)**	**SD**	**RSD (%)**	**Recovery (%)**
Water-Soluble Vitamins									
Ascorbic acid	41.2	42.2	0.6	1.4	102.4	41.0	0.2	0.5	99.5
	51.5	51.4	1.4	2.7	99.8	51.1	1.1	2.2	99.2
	61.8	60.1	1.0	1.7	97.2	61.2	1.6	2.6	99.0
Nicotinic acid	41.6	40.1	1.6	4.0	96.4	42.3	1.4	3.3	101.7
	52.0	51.3	1.7	3.3	98.6	50.9	2.8	5.5	97.9
	62.4	60.3	2.1	3.5	96.6	60.6	1.6	2.6	97.1
Nicotinamide	41.6	40.1	0.7	1.7	96.4	40.3	1.8	4.5	96.9
	52.0	51.2	2.8	5.5	98.5	51.9	3.1	6.0	99.8
	59.8	61.1	1.0	1.6	102.2	63.1	3.4	5.4	101.1
Folic acid	42.7	41.1	0.7	1.7	96.2	40.0	2.0	5.0	96.2
	48.8	50.0	1.4	2.8	102.5	50.6	1.0	2.0	97.3
	60.3	61.0	1.1	1.8	101.2	60.8	3.1	5.1	101.7
Cyanoco-balamine	40.2	39.9	0.9	2.2	99.2	42.8	1.2	2.8	100.2
	53.6	51.5	3.6	7.0	96.1	47.3	1.9	4.0	96.9
	61.0	60.4	2.9	4.8	99.0	60.8	2.6	4.3	100.8
Riboflavin	40.2	38.6	0.5	1.3	96.0	41.2	2.3	5.6	102.5
	53.6	53.6	0.8	1.5	100.0	53.2	3.6	6.8	99.2
	61.0	60.6	0.4	0.7	99.3	60.2	1.1	1.8	98.7
Fat-Soluble Vitamins									
Retinol	30.0	28.9	1.5	5.2	96.3	29.1	0.5	1.7	97.0
	40.0	38.2	1.0	2.6	95.5	39.6	1.1	2.8	99.0
	50.0	50.0	1.3	2.6	100.0	49.9	0.5	1.0	99.8
α-Tocoferol	34.2	32.2	1.0	3.1	94.2	33.1	0.6	1.8	96.8
	45.6	44.2	1.0	2.3	96.9	44.9	0.9	2.0	98.5
	57.0	55.6	0.5	0.9	97.5	55.2	0.8	1.4	96.8
α-Tocoferol acetate	31.2	29.4	0.4	1.4	94.2	30.1	1.1	3.6	96.5
	39.0	37.7	1.0	2.6	96.7	38.7	1.3	3.4	99.2
	54.6	54.0	1.9	3.5	98.9	54.2	0.5	0.9	99.3

Sample Pre-Treatment for Water Soluble Vitamins Analyses in Pharmaceutical Preparations

Pharmaceutical preparations: tablets and injection solutions, were treated as follows, in order to examine the applicability of the HPLC method of water-soluble vitamins determination:

Tablets

According to the label, a tablet contains 50 mg nicotinamide and 15 mg riboflavin.

After finely powdering of a tablet in a porcelain mortar, a portion of 0.1014 g was quantitatively transferred into a 100 mL volumetric flask and diluted to volume with water. The concentration of this solution was namely 98.1 ng/μL for nicotinamide and 29.4 ng/μL for riboflavin.

From this stock solution three working solutions were prepared by the proper dilution. Working solutions contained the internal standard xanthine, at a concentration of 4.2 ng/μL.

Aliquots of 10 μL were injected onto the HPLC analytical column.

High performance liquid chromatogram of water-soluble vitamins in tablet with xanthine as internal standard, is shown in Figure 3.

Injection solution 1

An ampoule of injection solution (volume 0.5 mL) contains 2 mg riboflavin and 100 mg nicotinamide, as stated on the label. Volume of 0.5 mL of this injection solution was quantitatively transferred into a 100 mL volumetric flask and diluted to volume with water. The concentration of this solution was namely 20 ng/μl for riboflavin and 100 ng/μl for nicotinamide.

From this stock solution three working solutions were prepared by the proper dilution in order to obtain concentrations: 1, 2 and 3 ng/μl. Working solutions contained the internal standard xanthine at a concentration of 4.2 ng/μl.

Aliquots of 10 μL were injected onto the HPLC analytical column. High performance liquid chromatogram of water-soluble vitamins in injection solution 1, with xanthine as internal standard, is shown in Figure 4.

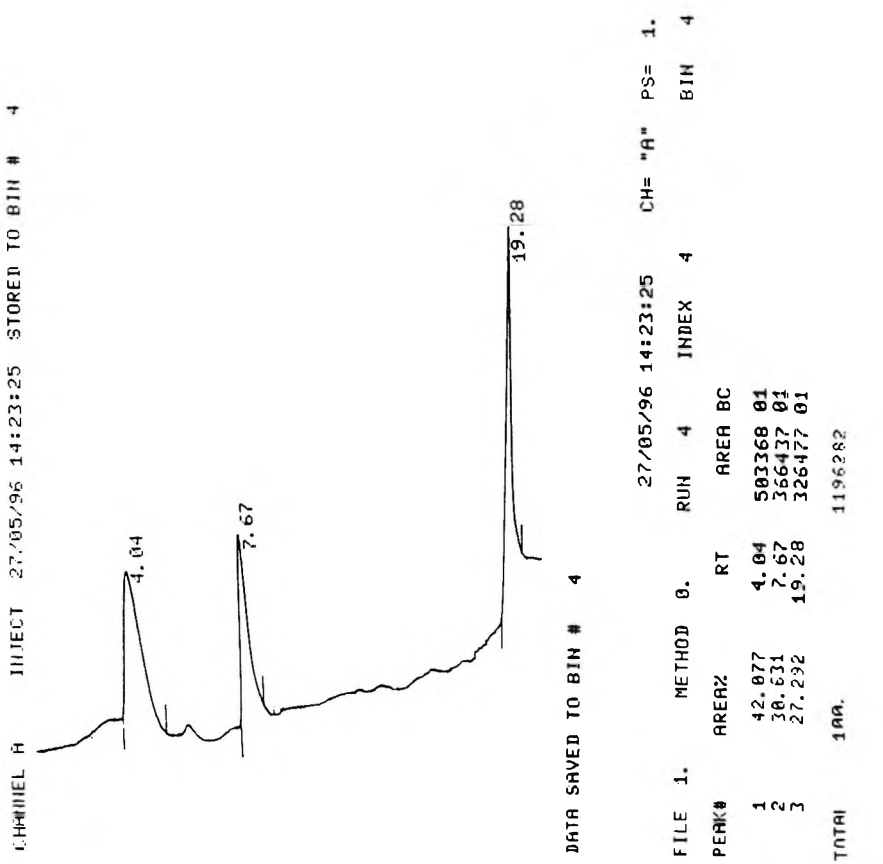

Figure 3. High performance liquid chromatogram of water soluble vitamins in tablet. Xanthine (4.2 ppm) 4.04 min., nicotinamide (7.85 ppm) 7.67 min. and riboflavin (2.35 ppm) 19.28 min.

Injection solution 2

According to the label, an ampoule of injection solution (volume 10 mL) contains 500 mg ascorbic acid and 50 mg nicotinamide. An aliquot of 2 mL was quantitatively transferred into an 100 mL volumetric flask and diluted to volume with water. The concentration of this solution was namely 100 ng/μL for ascorbic acid and 10 ng/μL for nicotinamide. From this stock solution three working solutions were prepared by the proper dilution, in order to obtain concentrations: 1, 2 and 3 ng/μL.

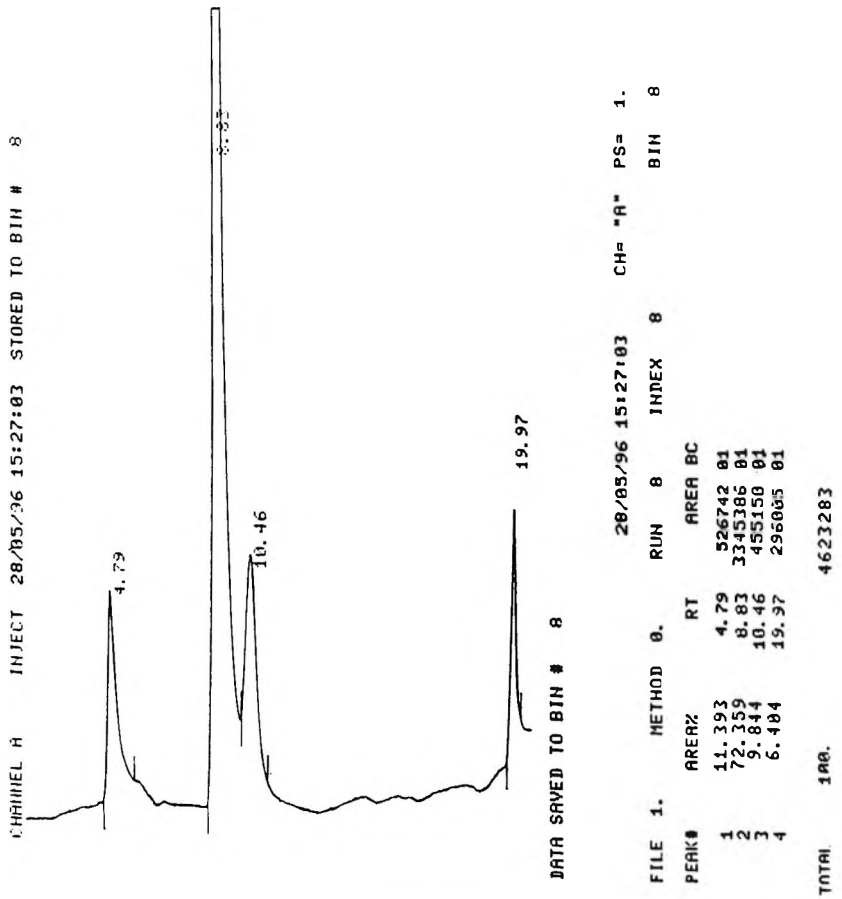

Figure 4. High performance liquid chromatograms of water soluble vitamins in injection solution 1. Xanthine(4.2 ppm) 4.79 min., Nicotinamide(80 ppm) 8.83 min., Riboflavin(1.6 ppm) 19.97 min., Unknown peak 10.46 min.

Working solutions contained the internal standard xanthine at a concentration of 4.2 ng/μL. Aliquots of 10 μL were injected onto the HPLC analytical column. High performance liquid chromatogram of water-soluble vitamins in injection solution 2, with Xanthine as internal standard, is shown in Figure 5. The experimental results from the analysis of water-soluble vitamins in pharmaceutical preparations are given in Table 2.

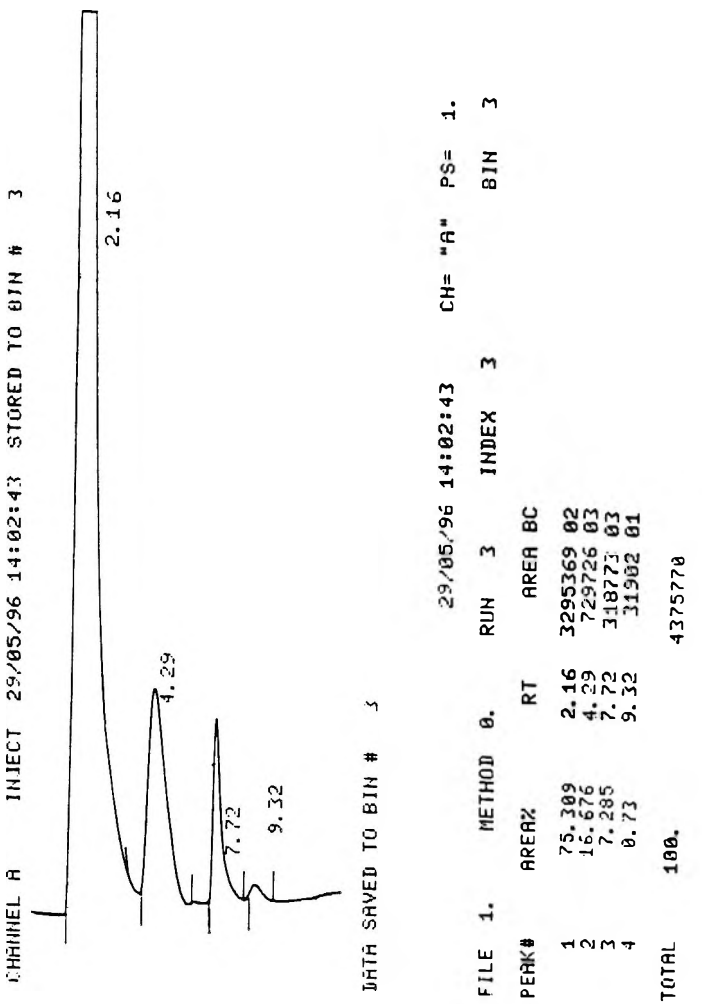

Figure 5. High performance liquid chromatogram of water soluble vitamins in injection solution 2. Ascorbic acid (45 ppm) 2.16 min., Xanthine (4.2 ppm) 4.29 min., Nicotinamide (4.5 ppm) 7.72 min., Unknown peak 9.32 min.

Solid-Phase Extraction

Solid-phase extraction cartridges (Lichrolut RP-18), fitted to the vacuum manifold, were conditioned by flushing with 3 mL methanol and 3 mL of water, prior to the addition of the sample. Then the sample (500 μL) was

Table 2

Experimental Results for the Analysis of Vitamins in Pharmaceutical Preparations by RP-HPLC with Xanthine as Internal Standard[a]

Sample	Analysed Quantity (ng)	Found (ng)±SD	RSD (%)	Labelled Amount (mg)	Found (mg)±SD	RSD (%)
Tablet						
Riboflavin	14.7	13.9±0.6	4.3	15.0	14.7±0.4	2.7
	23.5	23.1±0.9	3.9			
	35.3	35.4±1.0	2.8			
Nicotinamide	24.5	24.9±0.6	2.4	50.0	50.5±1.7	3.3
	49.0	47.7±1.0	2.1			
	78.5	77.9±2.7	3.5			
Injection Solution 1						
Riboflavin	10.0	9.5±0.5	5.3	2.0	1.9±1.1	4.7
	20.0	18.7±1.1	5.9			
	30.0	30.6±1.7	5.6			
Nicotinamide	10.0	10.1±0.4	4.0	100.0	98.0±2.6	2.7
	20.0	19.2±0.7	3.6			
	30.0	29.1±1.3	4.1			
Injection Solution 2						
Ascorbin acid	10.0	9.4±0.4	4.2	500.0	481.3±10.4	2.2
	20.0	19.7±0.6	3.0			
	45.0	44.6±1.9	4.3			
Nicotinamide	10.0	9.6±0.4	4.2	50.0	49.4±1.2	2.4
	20.0	20.2±0.7	3.5			
	30.0	30.1±1.2	4.0			

[a] (n=7)

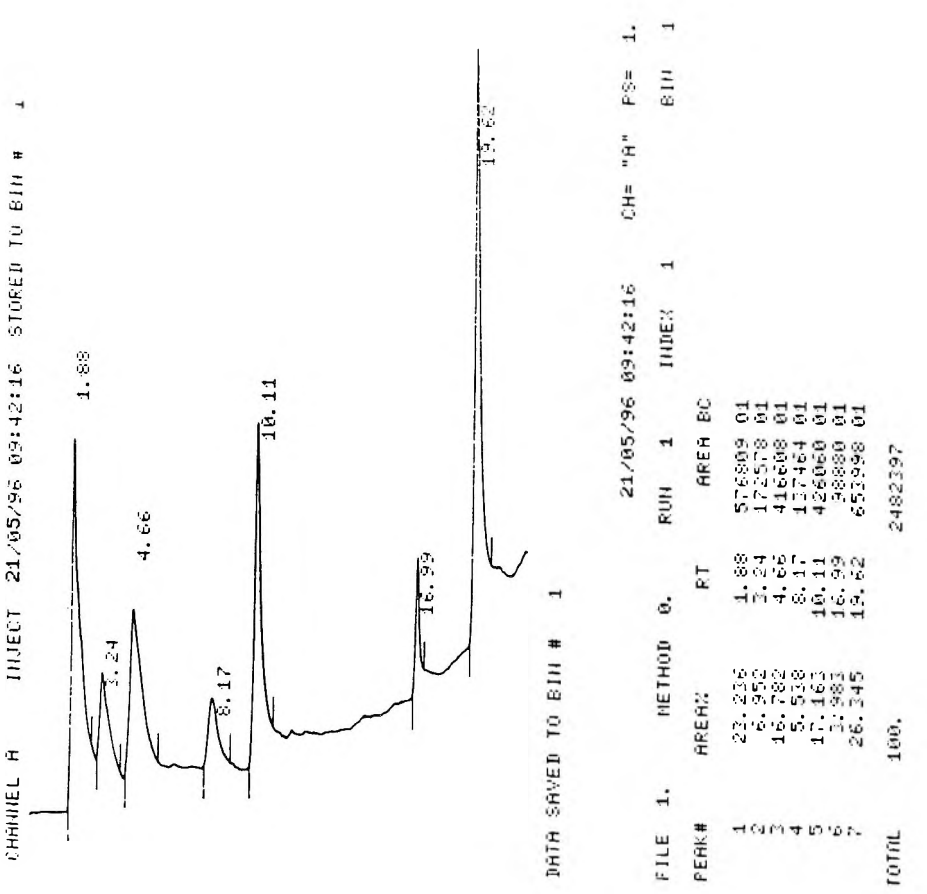

Figure 6. High performance liquid chromatogram of water soluble vitamins. Ascorbic acid (4 ppm) 1.88 min., Nicotinic acid (4 ppm) 3.24 min., Xanthine(4.2 ppm) 4.66 min., Nicotinamide (4 ppm) 8.17min., Folic acid (5 ppm) 10.11min., Cyanocobalamine min(5.5 ppm) 16.99 min., Riboflavin(5.05 ppm) 19.62 min.

applied to the cartridge. The fat-soluble vitamins were retained on the sorbent, while the water-soluble passed unretained. The water-soluble vitamins were collected into clean conical vials. Then fat-soluble vitamins were eluted by applying 3 mL of methanol and were collected into conical vials, as well. The solvent of the two selected fractions of vitamins was subsequently evaporated to dryness, at 45°C, under stream of nitrogen.

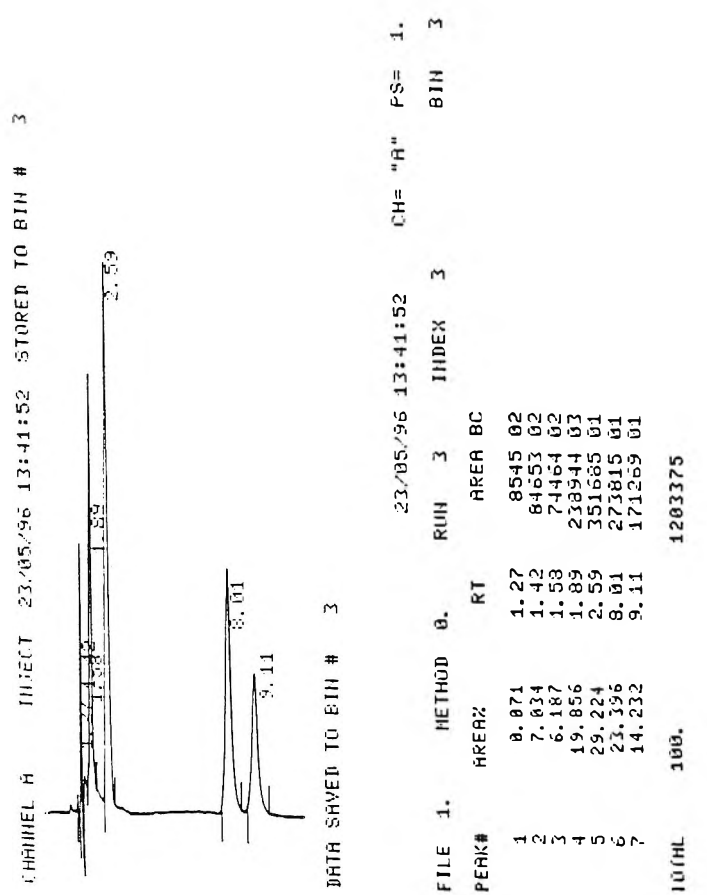

Figure 7. High performance liquid chromatogram of fat soluble vitamins. Anthraquinone(3.5 ppm) 1.89 min., Retinol (2.5 ppm) 2.59 min., α-Tocoferol (5 ppm) 8.01 min., α-Tocoferol acetate (6.5 ppm) 9.11 min.

The dry residue of water-soluble vitamins fraction was redissolved in 500 μL of methanolic solution of xathine (internal standard, 4.2 ng/μL), while the dry residue of fat-soluble vitamins fraction was redissolved to 500 μL of methanolic solution of Anthraquinone (internal standard, 3.5 ng/μL). Aliquots of 10 μL of each solution were injected onto the HPLC column.

Table 3

Recovery of Vitamins from Standard Solutions After SPE on C_{18} Cartridges Using Internal Standard

Analyte	Added (ng)	Found (ng)	SD	RSD (%)	Recovery (%)
Water-Soluble Vitamins					
Ascorbic acid	30.0	28.6	0.9	3.1	95.3
	50.0	49.1	1.7	3.5	98.2
Nicotinic acid	31.8	29.9	0.9	3.0	94.0
	53.0	52.0	1.9	3.6	98.1
Nicotinamide	29.4	28.0	1.3	4.6	95.2
	49.0	48.3	0.8	1.6	98.0
Folic acid	30.0	28.3	0.9	3.2	94.3
	50.0	48.3	1.0	2.1	96.8
Cyanocobalamine	30.0	28.3	0.6	2.2	94.3
	45.0	48.4	0.7	1.7	107.6
Riboflavin	30.3	26.7	1.7	6.4	88.1
	50.5	45.9	1.1	2.4	90.9
Fat-Soluble Vitamins					
Retinol	21.6	21.6	0.6	2.8	100.0
	32.4	31.8	1.3	4.1	98.2
α-Tocoferol	23.0	21.9	1.1	5.0	95.2
	34.5	31.4	1.2	4.1	91.0
α-Tocoferol acetate	25.3	23.2	0.8	3.5	91.7
	34.5	33.6	2.1	6.2	97.4

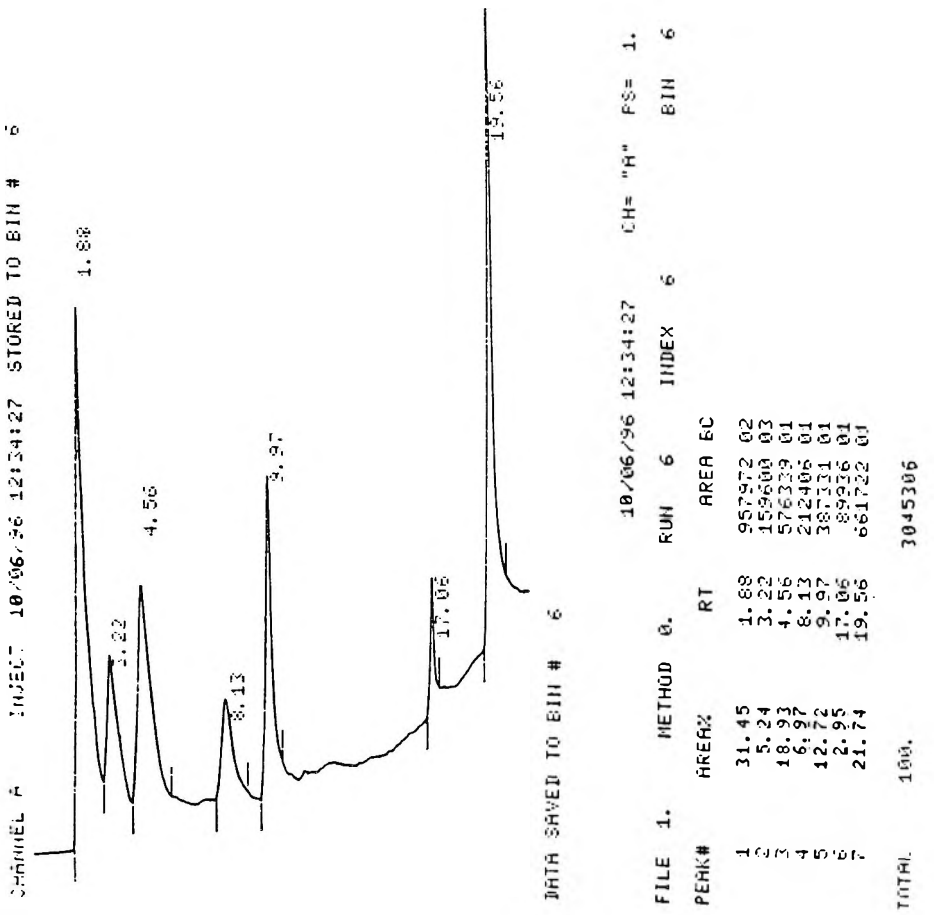

Figure 8. High performance liquid chromatogram of analysis of water soluble vitamins in spiked human blood samples. Ascorbic acid(4 ppm) 1.88 min., Nicotinic acid(3.19 ppm) 3.22 min., Xanthine(4.20 ppm) 4.56 min., Nicotinamide(4.40 ppm) 8.13 min., Folic acid(4.28 ppm) 9.97 min., Cyanocobalamine(4.50 ppm) 17.06 min. and Riboflavin(5.30 ppm) 19.56 min.

The correlation coefficients of the calibration curves had a range of 0.99917-0.99986 for water soluble vitamins and a range of 0.99904-0.99939 for fat-soluble vitamins. High performance liquid chromatograms of water-soluble and fat-soluble vitamins, after pre-treatment with SPE C_{18} cartridges are shown in Figures 6 and 7 respectively. The reproducibility and accuracy of solid phase extraction of vitamins were investigated. Results of recovery are shown in Table 3.

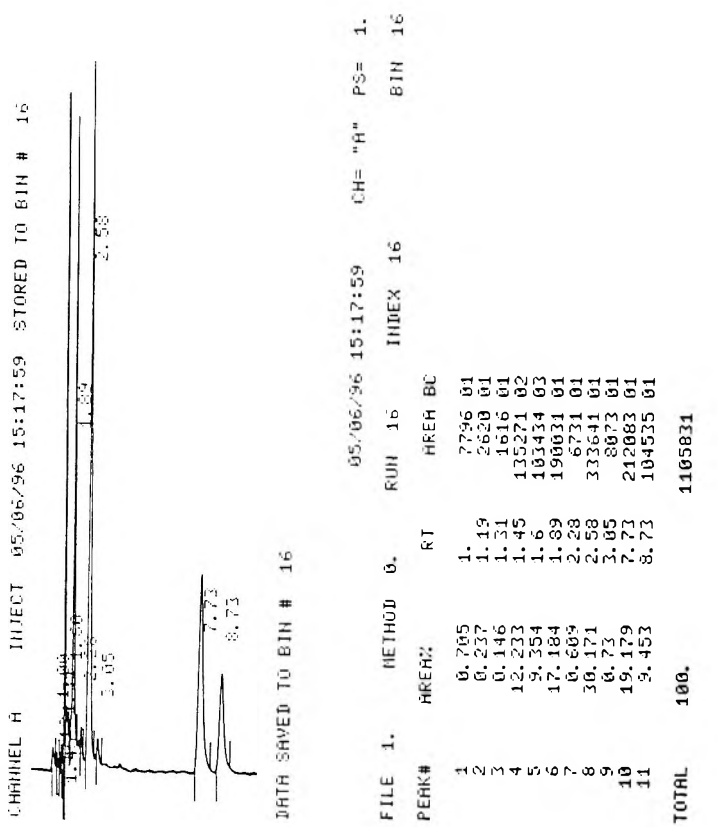

Figure 9. High performance liquid chromatogram of analysis of fat soluble vitamins in spiked human blood samples. Anthraquinone(3.50 ppm) 1.89 min., Retinol(3.41 ppm) 2.58 min., α-Tocoferol(5.10 ppm) 7.73 min. and α-Tocoferol acetate(4.90 ppm) 8.73 min.

Sample Preparation of Biological Fluids

Human blood plasma

Aliquots of 40 μL human blood plasma were treated with 80 μL of CH_3CN in order to precipitate proteins. After vortex mixing for two minutes, the sample was spiked with 100 μL of water soluble vitamins solutions, at concentration levels of 1, 3, 5, 7, 10 ng/μL.

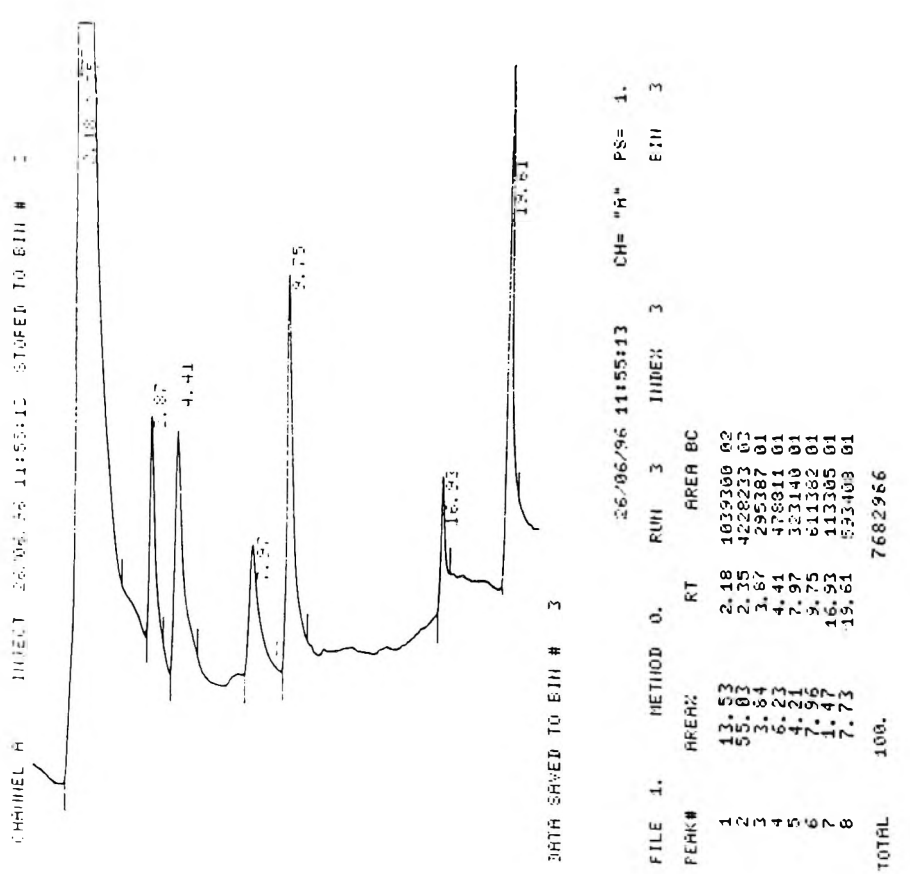

Figure 10. High performance liquid chromatogram of analysis of water soluble vitamins in urine samples. Nicotinic acid(6.24 ppm) 3.87 min., Xanthine(4.20 ppm) 4.47 min., Nicotinamide(6.60 ppm) 9.97 min., Folic acid(6.42 ppm) 9.75 min., Cyanocobalamine(6.0 ppm) 16.93 min. and Riboflavine(5.30 ppm) 19.61 min.

Then the sample was centrifuged at 3500 rpm for 15 min. and the supernatant was evaporated, at 45°C, under nitrogen stream, to remove organic solvents. Subsequently the sample was slowly applied to the solid-phase cartridge.

High performance liquid chromatograms of water-soluble and fat-soluble vitamins, extracted from human blood serum, are shown in Figures 8 and 9 respectively.

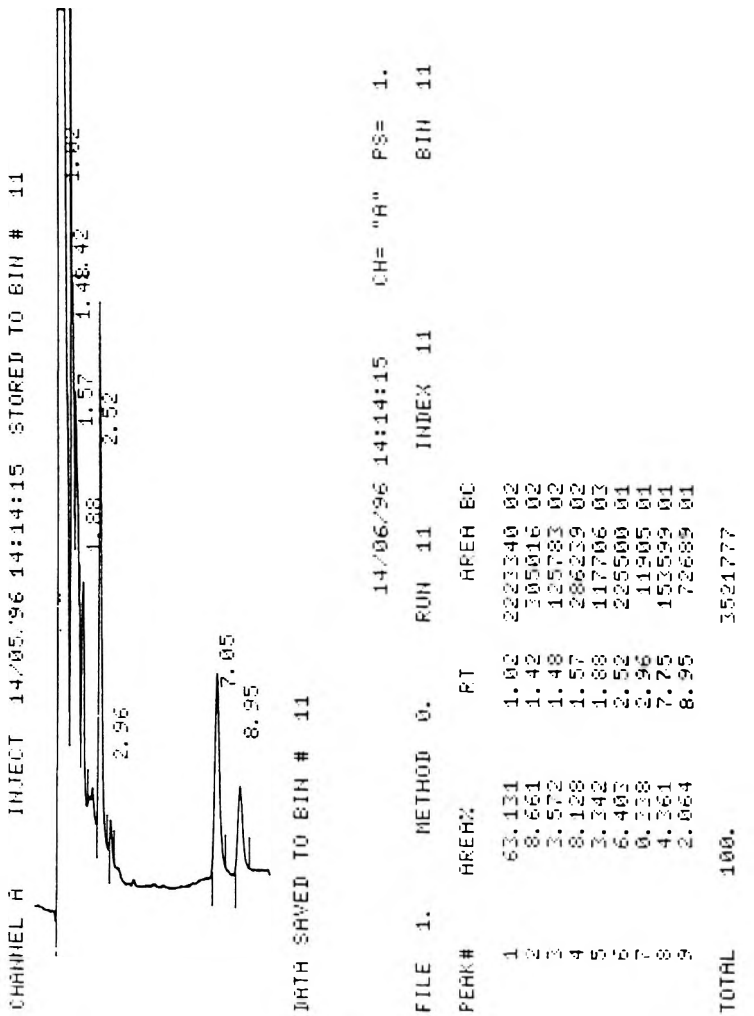

Figure 11. High performance liquid chromatogram of analysis of fat soluble vitamins in urine samples. Retinol(3.0 ppm) 2.52 min., α-Tocoferol(4.5 ppm) 7.75 min. and α-Tocoferol acetate(4.0 ppm) 8.95 min.

Urine

The same sample preparation method was followed for urine samples, after a small modification regarding sample volume. Thus in 100 μl of urine sample, 200 μl of acetonitrile were added and the procedure was followed as

described above. The serum and urine samples were pooled samples. No interference from endogenous compounds from sample matrix was observed in case of blood serum, in contrast to sample matrix of urine, in which some interferences observed. An interference in the fraction of water-soluble vitamin (retention time 2.48 min.) made the determination of vitamin C impossible. Also an interference in the fraction of fat-soluble vitamins (retention time 1.88 min) made the use of internal standard (Anthraquinone) impossible.

High performance liquid chromatograms of water-soluble and fat-soluble vitamins, extracted from human urine, are shown in Figures 10 and 11 respectively.

The correlation coefficients of the calibration curves for the analysis of vitamins in blood serum ranged between 0.99822 and 0.99982 for water soluble vitamins and between 0.99802 and 0.99968 for fat soluble vitamins. The correlation coefficients of the calibration curves for the analysis of vitamins in urine ranged between 0.99757 and 0.99943 for water soluble vitamins and between 0.99630 and 0.99954 for fat soluble vitamins. The precision and accuracy studies of SPE of vitamins from biological samples were conducted by spiking blood serum and urine samples, with known concentrations of the compounds and then by comparing obtained results, with those as calculated from regression equations. Results of recovery studies for serum and for urine samples are given in Table 4. Each value represents the mean of six measurements carried out.

CONCLUSIONS

Six water soluble vitamins (ascorbic acid, nicotinic acid, nicotinamide, folic acid, cyanocobalamine and riboflavin) and three fat soluble vitamins (retinol, α-tocopherol and α-tocopherol acetate) were separated into two fractions by means of solid-phase extraction and subsequently analysed by HPLC.

The developed method was further applied to multi-vitamin pharmaceutical preparations analysis and biological fluids analysis as well.

The binary eluent system used for water soluble vitamins and the isocratic eluent system used for fat soluble vitamins provide good separation, high selectivity and resolution within a minimum analysis time of 20 min for the fraction of water soluble vitamins and 9 min for the fraction of fat soluble vitamins.

Table 4a

Recovery of Vitamins from Human Blood Serum After SPE on C_{18} Cartridges[a]

Analyte	Added (ng)	Found (ng)	SD	RSD (%)	Recovery (%)
Water-Soluble Vitamins					
Ascorbic acid	10.0	8.3	0.6	7.2	83.1
	30.0	27.2	1.3	4.8	90.7
Nicotinic acid	10.4	8.8	5.2	5.8	84.6
	31.2	27.8	0.5	1.8	89.1
Nicotinamide	11.0	9.3	0.5	5.4	84.5
	33.0	31.8	0.4	1.2	96.4
Folic acid	10.7	10.7	0.7	6.5	100.0
	32.1	29.5	1.0	3.7	91.9
Cyanocobalamine	15.0	13.6	0.9	6.6	90.7
	30.0	29.2	0.9	3.1	97.3
Riboflavin	10.6	9.9	0.5	5.0	93.4
	31.8	29.6	1.0	3.4	93.1
Fat-Soluble Vitamins					
Retinol	11.2	10.2	0.3	2.9	91.1
	34.1	32.6	1.2	3.7	95.6
α-Tocoferol	12.6	11.1	0.6	5.4	88.1
	37.8	35.6	1.5	4.2	94.2
α-Tocoferol acetate	14.7	13.4	0.6	4.5	91.2
	34.1	33.6	1.1	3.3	98.5

[a] (n = 6)

Table 4b

Recovery of Vitamins from Human Urine Samples After Using Internal Standard[a]

Analyte	Added (ng)	Found (ng)	SD	RSD (%)	Recovery (%)
Water-Soluble Vitamins					
Nicotinic acid	10.4	9.4	0.5	5.3	90.4
	31.2	30.7	1.4	4.6	98.4
Nicotinamide	11.0	11.0	0.3	2.7	100.0
	33.0	32.4	1.6	4.9	98.2
Folic acid	10.7	10.2	0.2	2.0	95.3
	32.1	30.9	1.1	3.6	96.3
Cyanocobalamine	15.0	14.3	0.9	6.3	95.3
	30.0	29.1	1.7	5.8	97.0
Riboflavin	10.6	9.2	0.6	6.5	86.8
	31.8	28.6	1.9	6.5	89.3
Fat-Soluble Vitamins					
Retinol	11.2	10.3	0.6	5.8	92.0
	34.1	32.2	1.3	4.0	94.4
α-Tocoferol	12.6	11.4	0.5	4.4	90.5
	37.8	35.7	1.0	2.8	94.4
α-Tocoferol acetate	14.7	13.9	1.1	7.9	94.6
	34.1	34.1	1.8	5.3	100.0

[a] (n = 6)

Limits of detection are within 2-5 ng range for 10 μL injected sample volume. Day-to-day reproducibility was tested over ten consecutive days and repeatability (within day assay n=8) proved to be sufficient (RSD<7%).

Small volumes of biological fluids are required (40 μL of blood serum and 100 μL of urine).

REFERENCES

1. I. N. Papadoyannis, **HPLC in Clinical Chemistry,** Chromatographic Science Series, Volume 54, J. Cazes Ed., Marcel Dekker, New York, (1990).

2. A. Guillou, A. Choubert, J. D. Noue, Food Chem., **347(1)**, 93-99 (1993).

3. M. Amin, J. Reusch, The Analyst, **112**, 989-991 (1987).

4. J. Brown-Thomas, A. Moustafa, S. Wise, W.May, Anal. Chem., **60**, 1929-1933 (1988).

5. I. van Vliet, F. van Schaik, J. van Schoonhoven, J. Schiver, J. of Chromatogr., **553**, 179-186 (1991).

6. S. Seitz, R. Kock, H. Greiling, Fres. Z, Anal. Chem., **343**, 77-78 (1992).

7. A. Barma, D. Kostic, J. Olson, J. of Chromatogr., **617**, 257-264 (1993).

8. Z. Zaman, P. Fielden, P. Frost, Clin. Chem., **39(10)**, 2229-2234 (1993).

9. P. Edwards, P. Liu, G. Alan Rose, Clin. Chem., **35/2**, 241-245 (1989).

10. S. M. El-Gizawy, A. Ahmed, N. El-Rabbat, Anal. Lett., **24(7)**, 1173-1181 (1991).

11. T. Reynolds, A. Brain, J. of Chromatogr., **15(5)**, 897-914 (1992).

12. W. A. MacGrehan, E. Schoenberger, J. of Chromatogr., **417**, 65-78 (1987).

13. Y. Haroon, D. Bacon, J. Sadowski, J. of Chromatogr., **384**, 383-389 (1987).

14. M. Delgado Zammarrero, A. Sanchez Perez, C. Gomez Perez, J. Hernandez Mendez, J.of Chromatogr., **623**, 69-74 (1992).

15. H. Hasegawa, J. of Chromatogr., **605**, 215-220 (1992).

16. D. Zammarreno, M. M. Sanchez Perez, F. Moro, Analyst, **120(10)**, 2489-2492 (1995).

17. T. H. Hefferan, B. M. Chrisley, J. A. Driskell, J. of Chromatogr., **374**, 155-161 (1986).

18. Y. Usui, N. Nishimura, N. Kobayashi, T. Okanoue, M. Kimoto, K. Ozawa, J. of Chromatogr., **489**, 291-301 (1989).

19. S. Sharma, K. Dakshinamuri, J. of Chromatogr., **578**, 45-51 (1992).

20. W. A. MacCrehan, E. Schoenberger, J. Chromatogr. B., Biomed. Appl., **670(2)**, 209-217 (1995).

21. K. Sharpless, D. Duewer, Anal. Chem., **67**, 4416-4422 (1995).

22. D. Blanco, L. Sanchez, M. Cutierrez, J. Liquid Chromatogr., **17(7)**, 1525-1539 (1994).

23. D. Gomis, V. Arias, L. Alvarez, M. Alvarez, Anal. Chim. Acta, **315**, 177-181(1995).

24. K. Hirauchi, T. Sakano, S. Notsumoto, T. Nayaoka, A. Morimoto, K. Fujimoto, S. Masuda, Y. Suzuki, J. of Chromatogr., **497**, 131-137 (1989).

25. G. Chase, J. Akoh, W. O. Landen, J. of Liquid Chromatogr., **18(15)**, 3129-3138 (1995).

26. K. Epler, R. Ziegler, N. Craft, J. of Chromatogr., **619**, 37-48 (1993).

27. Mitsumasa Shino, Analyst, **113**, 393-397 (1988).

28. G. Li, J. Y. Li, Z. H. Hao, Y. Nie, Z. H. Meng, X. C. Li, Sepu., **13(6)**, 474-476 (1995).

29. B. Lee, S. Chua, H. Ong, C. Ong, J. of Chromatogr., **581**, 41-47 (1992).

30. P. Vinas, N. Campillo, I. Lopez Garcia, M. Hernandez Cordoba, Food Chem., **45**, 349-355 (1992).

31. S. Pikkarainen, M. Parviainen, J. of Chromatogr., **577**, 163-166 (1992).

32. K. Savolainen, K. Pynnoenen, S. Lapinjoki, M. Vidgren, J. of Pharm. Sciences, 77(**9**), 803- 805 (1988).

33. A. Clarke, C. Rowbury, Clin. Chem., **31(4)**,657-658 (1991).

34. S. D. Torrado, E. J. Caballero, R. Cadorniga, J. of Liquid Chromatogr., **18(6)**, 1251-1264 (1995)

34. J. C. Wallingford, B. A. Underwood, J. of Chromatogr., **381**, 158-163 (1986).

35. A. Deshuytere, H. Deelstra, Fresenius Z. Anal. Chem., **324**, 1-4 (1986).

36. H. E. Indyk, The Analyst, **113**, 1217-1221 (1988).

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DETERMINATION OF PROGESTERONE IN NANOCAPSULES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

In order to determine progesterone concentration in nanocapsules consisting of benzyl benzoate in a poly-ε-caprolactone shell, various methods were tested for preparation of samples. The opening of nanocapsules by a dissolution-dilution in acetonitrile was chosen. The method was validated. A reversed phase HPLC method using an acetonitrile-water mixture (75:25) as mobile phase, demonstrated that the entrapping of progesterone was almost total in the nanocapsules, and that, only traces of progesterone were present in the aqueous phase of the suspension. The lack of adsorption on the polymeric wall was also demonstrated.

INTRODUCTION

Colloidal drug carriers, nanoparticles, are used as drug delivery systems[1] and can enhance efficacy of drugs and reduce their toxicity.[2]

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These new drug carriers can modify the distribution of drugs and play a role in the pharmacokinetics : adsorption, metabolism and elimination of drugs.[3,4]

Progesterone, a steroid sex hormone, can be used in many pathologies.[5,6] By oral route, its hormonal activity is low.[7] Indeed, the bioavailability of progesterone is reduced by an intense intestinal and liver metabolism. So, it would be interesting to use a carrier system for progesterone administration. In order to study potential modification induced in progesterone metabolism, after its administration to rats under nanocapsules form, it was necessary to control the quality of these nanoparticle suspensions. To our knowledge, no method had been described for the determination of progesterone in nanoparticles.

For analytical purpose, the separation of nanocapsules of various xenobiotics from the aqueous supernatant was generally performed by ultracentrifugation.[8,9] and seldom by ultrafiltration centrifugation.[10] On another hand, the determination of progesterone in biological media was performed either by gas chromatography,[11] or both normal[12] and reversed phase[13,14] high performance liquid chromatography (HPLC). In the latter case, the mobile phase was methanol/water, acetonitrile/water or methanol/ acetonitrile/water.

In this paper, we describe a reversed phase HPLC method for the determination of progesterone in a nanocapsules suspension.

MATERIALS

Chemicals and Reagents

Progesterone was purchased from Upjohn, Fine Chemical Division (Michigan, USA). Acetonitrile, methylene chloride, ethyl acetate, and methanol were of liquid chromatographic grade and purchased from Prolabo (Paris, France). Distilled water, used for the mobile phase, was obtained from Fresenius (Malakoff, France).

Nanocapsules Suspension

Nanoprecipitation was the method chosen to prepare the nanoparticles.[15] Progesterone was dissolved in an oily solvent, benzyl benzoate. Poly-ε-caprolactone polymer was dissolved in acetone and added to the progesterone

solution to form solution A. Solution B was an hydroalcoholic solution containing surfactant (Synperonic®). After mixing solutions A and B, nanocapsules were formed. Evaporation of acetone, alcohol, and of a part of water, allowed us to obtain a concentration of 5 $mg.mL^{-1}$, which was in agreement with the requirements of administration to rats.

Apparatus

The HPLC system consisted of an isocratic solvent delivery pump (110A Beckman pump, San Ramon, CA, USA), a sample injector (Rheodyne Model 7125, Latek, Eppelheim, Germany) equipped with a 20 µL loop, a reversed phase column (Macherey Nagel C18 Nucleosil, 250 mm x 4 mm I.D., 5 µm particle size), and a variable-wavelength UV detector (Beckman Model 166). The data recording system consisted of an IBM personal computer PS/2 Model 8550.Z with Gold software system (version 5.1, Beckman). Ultracentrifugation was performed with a Beckman L8-55 Ultracentrifuge (Beckman Instruments), equipped with a 40TR rotor.

METHODS

Chromatography Conditions

Liquid chromatography was performed at room temperature. The mobile phase was an acetonitrile-water mixture (75 : 25 V/V) programmed to be delivered at a flow-rate of 1.0 $mL.min^{-1}$. The wavelength used for detection was 254 nm.

Standard Curves

A working solution of progesterone (0.055 $mg{\cdot}mL^{-1}$) was prepared in acetonitrile. This solution was diluted with acetonitrile to give the following final concentrations : 0.0055, 0.011, 0.022, 0.033, 0.044, 0.055 $mg{\cdot}mL^{-1}$ for the construction of standard curves. Stability of progesterone solutions in acetonitrile at various concentrations (0.0055 to 0.055 $mg.mL^{-1}$) was studied. These solutions were stored at 4°C and analysed periodically. Progesterone was eluted as a single peak, with a constant peak area, over a period of two weeks under these conditions, and no other peak of degradation was seen at λ = 210, 230 or 254 nm.

Preparations of Samples

Progesterone in the aqueous phase

Five mL of the nanocapsules suspension were centrifuged at 50,000 g for 45 min; it gave a clear supernatant liquid which was directly injected into the HPLC system. Concentrations were very low, and no dilution was necessary.

Progesterone in the nanocapsules suspension

The suspension of nanocapsules was treated according to either method A, B or C. For checking adsorption on the polymer surface, method D was used.

Method A

It consisted of an opening of nanocapsules by a dissolution-dilution in acetonitrile. 0.5 mL of nanocapsules suspension, previously kept under magnetic agitation, were diluted 1:200 with acetonitrile. A solution was obtained with formation of a residue of poly-ε-caprolactone insoluble in acetonitrile. This solution was filtered through a microfilter before being injected into the HPLC system.

Method B

Methylene chloride was used as solvent for nanocapsules opening. 5 mL of nanocapsules suspension were evaporated, and diluted with 150 mL of methylene chloride. The solution was dried using anhydrous sodium sulfate and then evaporated under reduced pressure. The residue was dissolved in 50 mL of methanol. This solution was diluted 1:20 with methanol and was filtered through a microfilter before being injected into the HPLC system.

Method C

Nanocapsules suspension was treated according to method B, using ethyl acetate instead of methylene chloride as solvent.

Method D

A progesterone solution in acetonitrile at a concentration of 5 $mg.mL^{-1}$ was diluted 1:200 with acetonitrile before being injected into the HPLC system. A progesterone and poly-ε-caprolactone solution in acetonitrile at concentrations of 5 and 12.5 $mg.mL^{-1}$ respectively, was diluted 1:200 with acetonitrile, filtered and injected into the HPLC system.

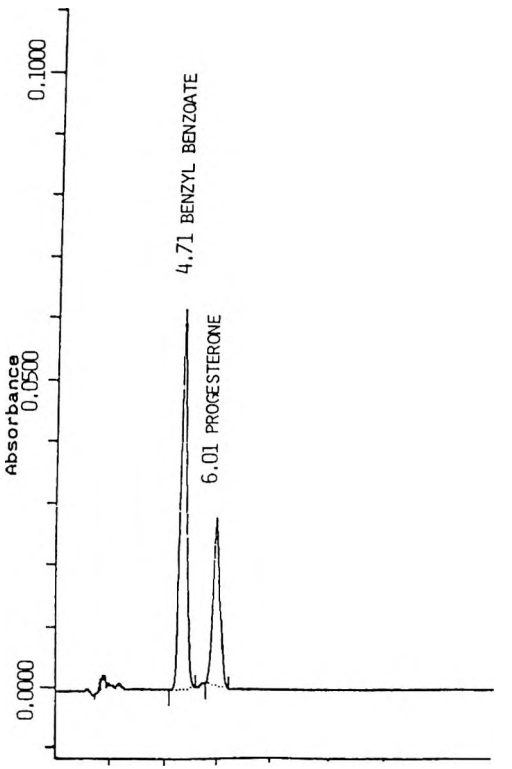

Figure 1. Chromatogram of nanocapsules suspension.

RESULTS AND DISCUSSION

Chromatograms

Typical chromatogram of progesterone and benzyl benzoate is reproduced in Figure 1.

Retention times for these compounds were 6 min and 4.7 min respectively; symmetrical peaks were obtained. Under these conditions, the synperonic® (component of nanocapsules) does not adsorb at 254 nm.

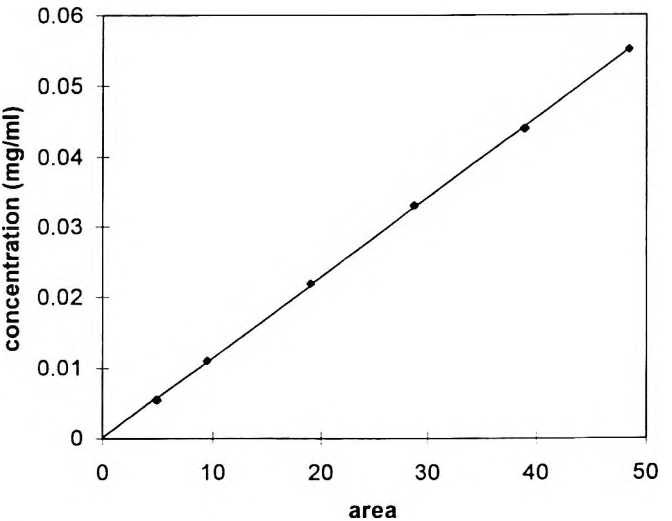

Figure 2. Linearity curve.

Validation

Linearity

Linearity was observed for concentrations of 0.0055 to 0.055 $mg.mL^{-1}$. The straight-line equation was $Y = 0.001132x + 0.000245$ (Figure 2). The correlation coefficient, r, was 0.9998. The validation results were established for two injections per concentration and for six concentrations.

Reproductibility

This study was performed on lots of nanocapsules suspension at a concentration of $5mg.mL^{-1}$, and using method A for determination of progesterone in nanocapsules suspension. For this purpose, the intra-assay and inter-assay (RSD) were established for six injections per determination on six lots (I,II,III,IV,V,VI), and the values are listed in Table 1.

The limit of detection was defined as the minimum drug concentration corresponding to twice the signal-to-noise ratio. It was evaluated as 0.5 $\mu g \cdot mL^{-1}$ in 0.5mL of nanocapsules suspension.

Table 1

Validation of the Method

Lots	Found Conc. ($mg \cdot mL^{-1}$)	Accuracy (%)	Intra-assay Variability RSD(%)	Inter-assay Variability RSD(%)
I	4.87	97.4	1.00	0.99
II	4.82	96.3	0.76	0.83
III	4.86	97.1	0.75	1.10
IV	4.92	98.4	0.26	1.00
V	4.87	97.3	1.03	0.72
VI	4.88	97.5	0.34	0.98

Table 2

Results of the Three Extraction Methods for Nanocapsules Suspension ($5mg \cdot mL^{-1}$)

	Concentration ($mg \cdot mL^{-1}$)	Precision (%)	Concentration (%)
Nanocapsules suspensions Method A (CH_3CN)	4.89	0.79	98
Nanocapsules suspensions Method B (CH_2Cl_2)	5.05	3.70	101
Nanocapsules suspensions Method C (AcOEt)	5.05	3.60	101

Comparison of Extraction Methods

In order to check the consistency of the values of total progesterone, measured. three parts of the same lot at the concentration of 5 $mg.mL^{-1}$ were treated separately according to method A. B and C. The values are compared in Table 2.

Table 3

Results of Encapsulation Checking

	Concentration ($mg \cdot mL^{-1}$)	Precision (%)	Concentration (%)
Aquesous phase free progesterone	0.0026	1.10	0.05
Nanocapsules suspension Method A(CH_3CN)	4.89	0.79	98

These results showed that the behaviour of these different solvents towards progesterone nanocapsules was identical. Acetonitrile, methylene chloride, ethyl acetate, identically extract the progesterone present in the nanocapsules suspension.

The precision obtained for the three methods showed that method A was more accurate. Moreover, this method was a simple dissolution-dilution in acetonitrile, so it was more reliable than the two other methods which were more complicated. Measures performed in the presence of an internal standard, diazepam, and without internal standard, gave identical results.

Checking the Quality of the Encapsulation

Progesterone determinations were performed simultaneously in the aqueous phase and in the whole nanocapsules suspension, on aliquots of the same lot of nanocapsules suspension at the concentration of 5 $mg \cdot mL^{-1}$. The experimental results listed in Table 3 are given both in $mg \cdot mL^{-1}$, and in percentage of the total amount of progesterone.

These results showed that progesterone was almost totally encapsulated, since the concentration of free progesterone (i.e. progesterone dissolved in the aqueous phase) was very low: 0.0026 $mg \cdot mL^{-1}$ (0.05 % of progesterone theoreticaly present). This result was expected because progesterone is a lipophilic drug which is not soluble in water ($s = 12\ \mu g.mL^{-1}$ at 37°C).[16]

A question remained. Was progesterone entirely dissolved in the oily solution entrapped in the nanocapsules, or was it partly adsorbed on the polymer surface? In order to solve this question, experiments were performed to clear up the ways of trapping progesterone in nanocapsules.

Lack of Adsorption of Progesterone on Poly-ε-caprolactone

The results obtained on solutions of progesterone in acetonitrile and of progesterone and poly-ε-caprolactone in acetonitrile, prepared according to method D, showed that the same values of progesterone concentration were found for the two solutions. This observation confirms that the same results were obtained when the extraction from nanocapsules suspension was performed, either by methylene chloride (method B) or by ethyl acetate (method C). So this lipophilic drug does not adsorb on the polymer. This leads us to conclude that progesterone is entrapped by simple inclusion inside the nanocapsules.

This lipophilic drug has a different behaviour than a hydrophilic compound studied in our laboratory, such as phenobarbitone,[17] which aside from being present in the aqueous phase of the suspension, was both entrapped in the oily solution and adsorbed on the nanocapsules wall. In that case, the results were different using method B or method C.

CONCLUSION

The HPLC method presented here, allows the evaluation of the total progesterone included in the nanocapsules suspension. This lipophilic drug is almost entirely entrapped in the nanocapsules without adsorption on the polymer constituting the nanocapsule wall. The method was validated and it can be used for the evaluation of the quality of the preparation of nanoparticule suspension.

REFERENCES

1. F. H. Roerdink, A. M. Kroon, eds., **Drug Carrier Systems**,John Wiley, New York (1989).

2. F. Puisieux , L. Roblot-Trempel, STP Pharma. Sci., **5**, 107-113 (1989).

3. L. Illum, N. W. Thomas , S. S. Davis, J. Pharm. Sci., **75**, 16-22 (1986).

4. N. Ammoury, H. Fessi, J. P. Devissaguet , F. Puisieux , S. Benita, J. Pharm. Sci., **79**, 763-767 (1990).

5. W. S. Maxson, J. T. Hargrove, Fert. Steril., **44**, 622-626 (1985).

6. S. Wright. **Physiologie Appliquée a la Médecine**, Flammarion Médecine Sciences, Paris (1980).

7. M. I. Whitehead, P. T. Townsend, D. K. Gill, W. P. Collins, S. Campbells, Brit. Med. J., **280**, 825-827 (1980).

8. L. Marchal-Heussler, D. Sirbat, M. Hoffman, P. Mainaut, Pharm. Res., **10(3)**, 386-390 (1993).

9. C. Michel, M. A. Prahamian, L. Defontaine, P. Couvreur, C. Damgé, J. Pharm. Pharmacol., **43**, 1-5 (1990).

10. S. -S. Guterres, H. Fessi, G. Barratt, J. -P. Devissaguet, F. Puisieux, Int. J. Pharm., **113**, 57-63 (1995).

11. E. Vanluchene, A. Hinting, M. Dhont, R. Serreyn , D. Vandekerckhove, J. Steroid Biochem., **35**, 83-89 (1990).

12. J. -T. Lin, E. Heftmann, I. R. Hunter, J. Chromatogr., **190**, 169-174 (1980).

13. R. B. Taylor, K. E. Kendle, R. G. Reid., J.Chromatogr., **385**, 383-392 (1987).

14. F. E.Francis, R. A. Kinsella Jr., J. Chromatogr., **336**, 361-367 (1984).

15. H. Fessi, J. P. Devissaguet, F. Puisieux , C. Thies, US Patent, 5, 118, 528 (1992).

16. R. Duclos. Thèse Doctorat Sciences Pharmaceutiques, Université de Rouen, 1989.

17. M. Berrabah, D. André, F. Prévot, A. M. Orecchioni , O. Lafont, J. Pharm. Biomed. Anal., **12**, 373-378 (1994).

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DETERMINATION OF THREE WATER-SOLUBLE ACTIVE INGREDIENTS IN QIANGLI YINGQIAO CONTAINING Vc TABLETS BY CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

A simple and rapid capillary zone electrophoretic (CZE) method was established for separation and determination of chlorphenamin males(CPM), paracetamolum(PAM), and vitamin C(Vc) within 3 min, using borate-phosphate buffer at pH 7.2. The influence of injection time on peak area, peak height and efficiency was studied in detail. The CZE method was successfully applied to quality control of CPM, PAM and Vc in Qiangli Yingqiao containing Vc tablets.

INTRODUCTION

Compared to Western medicine, the pharmacological actions of traditional Chinese medicine are slower, so taking this into consideration and considering their concerted actions, more and more combinations of traditional Chinese medicine and Western medicine have been adopted for pharmaceutical preparations in China.

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Qiangli Yingqiao containing Vc tablet is a typical example, consisting of great amounts of Yingqiao extract, which is a kind of water-insoluble traditional Chinese herbal preparation, and small amounts of three water-soluble ingredients--vitamin C(Vc), paracetamolum(PAM) and chlorphenamin males(CPM). Qiangli Yingqiao containing Vc tablet is a very common medicine which can resist influenza virus and treat influenza, cold, fever, cough, sore throat, and headaches due to colds.[1] However, the contents of these three water-soluble ingredients in the tablets aren't clearly indicated; it is, therefore, useful and necessary to develop a rapid and simple method for determination of Vc, PAM, and CPM in order to monitor the quality of the kind of Yinqiao tablet.

CE is being widely established within the pharmaceutical industry for the quality control and determination of drugs, and recently, used for analyses of different traditional Chinese medicines.[2-3] In previous papers,[4] we also developed a CZE method for identification and determination of aesculin and aesculetin in ash bark.

Although many papers have been published for analyses of CPM and PAM,[5-8] as well as Vc[9-10] in drugs, up to date no report appears on simultaneous separation and determination of the three substances. In this paper, we made attempts to enlarge the use of CZE in the pharmaceutical analysis, developing a rapid and simple CZE method for determination of Vc, CPM, and PAM in Qiangli Yinqiao tablet within 3 min. The effects of injection time on peak height, peak area, and peak width at half height were investigated in detail.

EXPERIMENTAL

Instrumentation

The CE system employed was a Waters Quanta 4000 (Waters Chromatography Division of Millipore, Milford, MA, USA), with a positive power supply. Waters AccuSep fused silica capillary (53cm × 75μm I.D.) were used throughout. A window for on-column detection, by removing the polyimide coating, was created 7.6cm from the end of the capillary (cathode). Direct UV detection was performed with a Hg lamp and a 254-nm optical filter. Samples were introduced from the anodic end of the capillary by hydrodynamic injections, where the sample vial was raised by 10cm. Data acquisition was carried out with a Maxima 820 Chromatography workstation

(Waters) with a system interface module connecting the CE system to the station. Data acquisition rate was 20 points s^{-1}. Collection of electropherographic data was initiated by a signal cable connection between the Quanta 4000 and the system interface module(SIM).

Chemicals

Authentic PAM and CPM were purchased from the National Institute for the Control of Pharmaceutic and Biological Products, P.R. of China. Vc is purchased in Shanghai Chemical plant, China (analytical grade). Two batches of Qiangli Yingqiao containing Vc tablets, from the same pharmaceutical factory, were purchased in a drug store and analyzed. Unless otherwise specified, all chemicals were of analytical reagent grade.

All solutions were prepared using filtered, degassed, and distilled water. Carrier electrolytes were filtered through 0.45μm membrane prior to use.

Sample Preparation

Ten tablets were accurately weighed and ground, an appropriate amount of the resultant powder was accurately weighed and 50mL water was added. After ultra-sonication for 5 min, the mixture was filtered and water was added to make the volume of the filtrate exactly 100mL. For the two batch tablets, the same preparations were used.

Procedure

The capillary was first purged with 0.5mol l^{-1} NaOH for 3 min, 3 min with water and 5 min with running buffer prior to each analysis. Overnight the capillary was stored in 0.5mol l^{-1} NaOH. At the beginning of each day, it was sufficient to rinse the capillary with water for about 10 min. All conditional experiments were completed using the test solution containing 0.22mg mL^{-1} of Vc, 0.27mg mL^{-1} of PAM, and 0.20mg mL^{-1} of CPM.

Experiments were performed to optimize the separation. Unless otherwise specified, the optimum conditions used were: running buffer prepared by mixing 20 mmol l^{-1} sodium tetraborate with 20 mmol l^{-1} sodium dihydrogen phosphate(pH=7.2), applied voltage of 25kV and hydrodynamic injections for 10s. All operations were at room temperature.

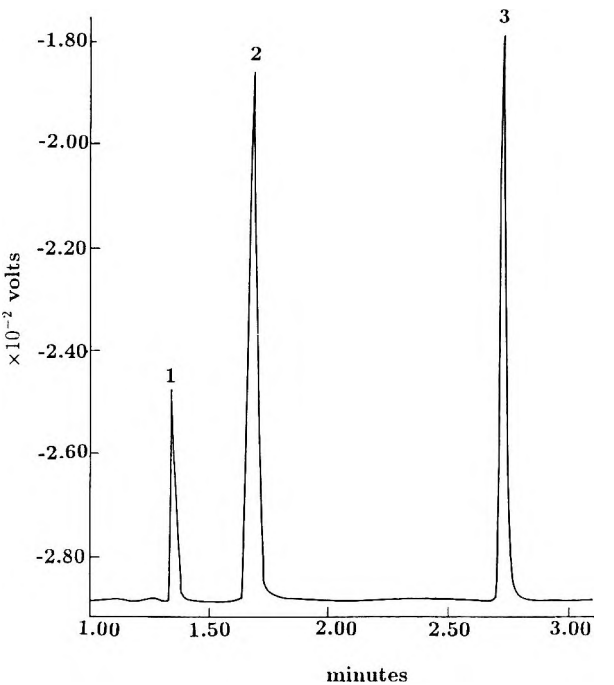

Figure 1. Typical electropherogram of standard mixture of CPM, PAM and Vc. Conditions: 20mmol l^{-1} borate--phosphate buffer at pH=7.2, voltage of 25kV, Hydrodynamic injection time for 10s, other conditions as experimental. Peak identifications are: (**1**) CPM, (**2**) PAM, (**3**) Vc.

RESULTS AND DISCUSSION

Selection of Buffer Solution

According to the complexation mechanism postulated by Lorand and Edwards,[11] borate can form anionic complexes with polyhydroxy compounds (i.e., polyols), thereby facilitating their separation by electrophoresis. The polyol-borate complex formation of catechols[12] and carbohydrates,[13] etc., has been exploited in CE. Here, we make best use of the advantage of the complex of borate with the ortho-dihydroxy in Vc. It is well known that, Vc is not stable and easy to reduce in solution, especially in basic solution, which gives rise to difficulty in determining its content. However, its complexation with borate

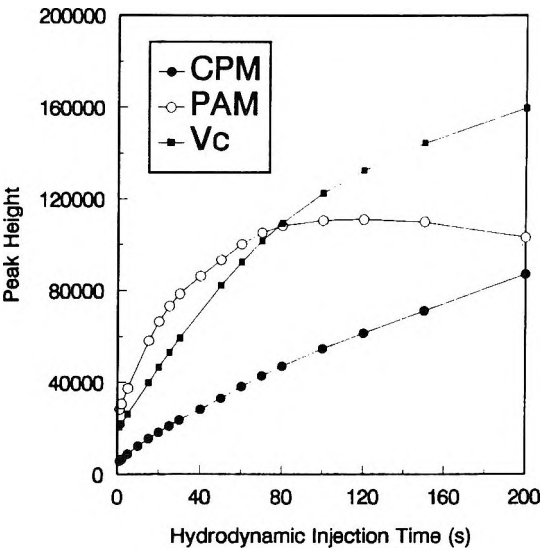

Figure 2. Dependence of peak height on injection time. Conditions as Figure 1 except injection time.

not only helped its separation from the other two analytes, but also prevented its reduction in the buffer solution. It was found out that a buffer solution of 20mmol l^{-1} borate mixing with 20 mmol l^{-1} phosphate in the pH range 7.0-7.5 could obtain better separation for Vc, CPM, and PAM, so pH 7.2 was selected in our experiments. Figure1 shows a typical CZE electropherogram of the standard mixture of Vc, CPM and PAM.

Effect of Injection Time

In the range investigated, it was found out that migration times and mobilities of the samples only change slightly with increasing injection time, so our emphasis was on effects of injection time on peak area, peak height, and the peak width at half-height. In order to observe the effects clearly, it is necessary to point out, emphatically, that for the three analytes, the concentration order is PAM > Vc > CPM and the sensitivity of CPM is also lower than that of PAM and Vc.

For the three analytes, as can be seen from Figure 2. the same tendencies are observed, that is, within the shorter injection time, the peak height increases with direct proportional to increasing injection time, whereas, when

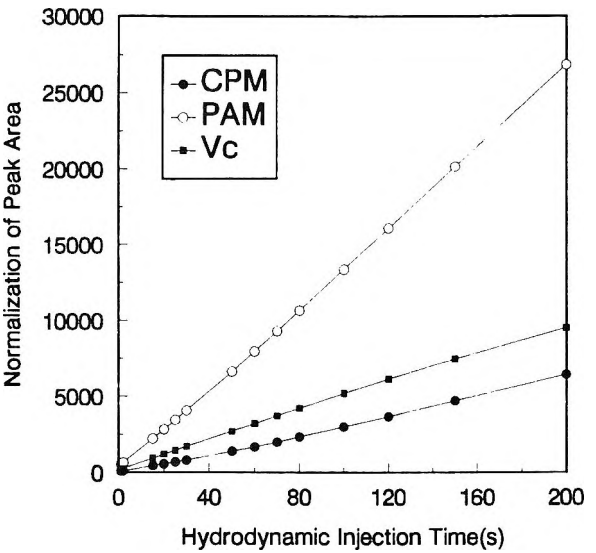

Figure 3. Dependence of peak area on injection time. Conditions as Figure 2.

injection time increases to a degree, the peak height no longer increases linearly with injection time. On the other hand, the greater the concentration, the worse the linear relationship between peak height and injection time. For peak area, however, as indicated in Figure 3, in the whole range studied, peak areas increase linearly with injection time. The reason is that, because the increased injection time results in higher sample concentrations, at higher sample concentration there is peak broadening or distortion which causes changes in peak height but not area. Therefore, for most analyses, peak area is preferred, especially, the normalization of CE peak areas (i.e. division of peak area by migration time) which is necessary to ensure correct quantitation when the solute concentration is high. Peak height can be used for quantitation at the lower concentration, especially, it may be much more desirable for trace analysis where a very small peak is superimposed on a noisy baseline.

As in HPLC, higher detection sensitivity can be obtained by longer injection time, whereas efficiency suffers significantly with injection time increasing, as observed in the experiment (illustrated in Fig 4), due to serious peak broadening, which leads to the reduced efficiency.[14] In other words, the effect of injection time on efficiency also suggested the dependence of efficiency on sample concentration, that is, higher sample concentration will result in a decrease in efficiency. So, a suitable injection time must be considered when selecting optimum separation conditions. Here, 10s injection time was selected.

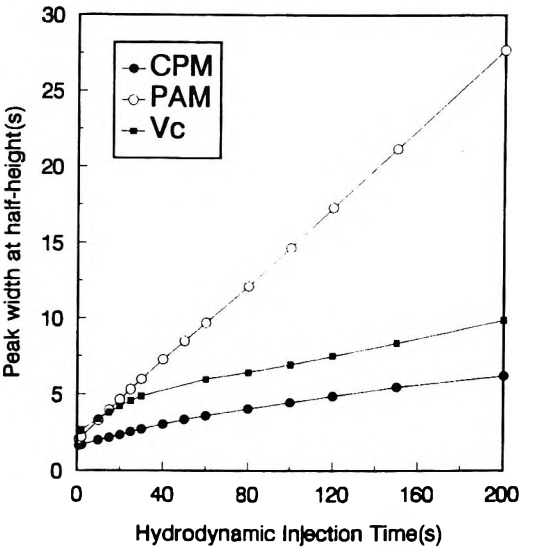

Figure 4. Effect of injection time on peak width at half-height. Conditions as Figure 2.

Effect of Applied Voltage

The influence of applied voltage was investigated. As observed from Fig. 5, increased voltage results in decrease in peak width at half-height, indicating that higher efficiency is obtained with increasing voltage. In addition, shorter analysis time is also observed, so 25 kV was selected.

Quantitative Analysis

Under the optimized conditions, the method showed good linear correlations based on the linear relationship between the normalization of CE peak area for PAM and Vc and peak height for CPM and sample concentration as shown in Table 1, where A is the normalization of CE peak area for Vc and PAM and the peak height for CPM, and C is concentration in $\mu g\ mL^{-1}$. Approximate detection limits were calculated at a signal-to-noise ratio of 2. The relative standard deviations (N=5) at 80 $\mu g\ ml^{-1}$ for all three analytes are 1.83%, 0.71%, and 2.10% for CPM, PAM, and Vc, respectively.

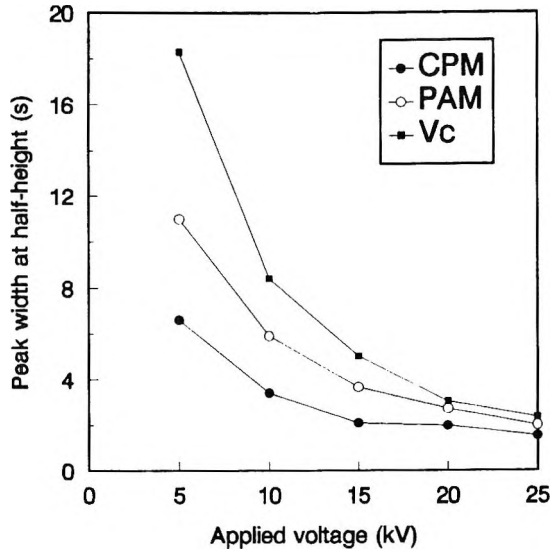

Figure 5. Effect of applied voltage on peak width at half-height. Conditions as Figure 1 except voltage.

Table 1

Linear Regression Analysis

Species	Linearity ($\mu g\ mL^{-1}$)	Detection Limit ($\mu g\ mL^{-1}$)	Linear Regression Equation	Correlation Coefficient
CPM	16.0 - 600.0	7.0	A = 0.97 + 0.27C	0.9998
PAM	6.0 - 2000.0	3.0	A = 7.82 + 1.61C	0.99993
Vc	3.0 - 1000.0	2.0	A = 3.2 + 1.42C	0.99991

Application

The CZE method established has been successfully applied to the separation and determination of three water-soluble ingredients in Qiangli Yinqiao containing Vc tablets. Table 2 gives the results of standard addition of recovery for these three ingredients in two batch tablets. Due to the content of CPM being very lower, it must be determined at the excess of PAM and Vc, consequently, two typical electropherograms for batch II are shown in Fig. 6.

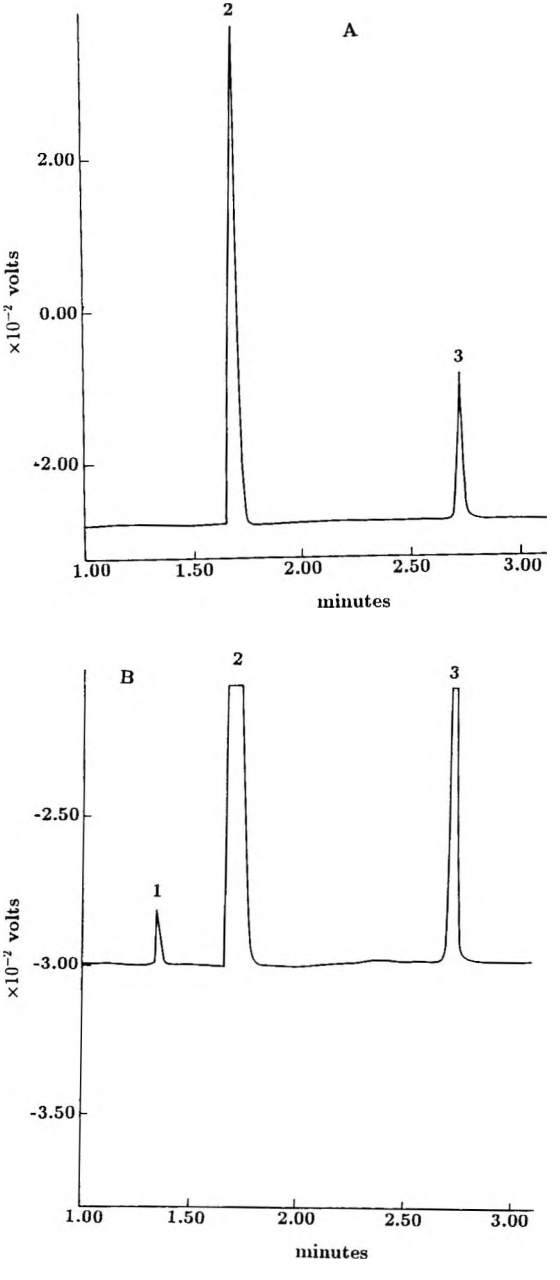

Figure 6. Electropherograms of CPM, PAM and Vc in Qiangli Yingqiao containing Vc tablet from Batch II. (A): for determination of PAM and Vc, (B): for CPM. Conditions as Fig. 1.

Table 2

Quantitative Analysis

	Species	Determined (mg tablet^{-1})	RSD (n= 9) (%)	Added (μg)	Recovery (%)	RSD (n=3) (%)
	CPM	0.203	3.17	120	98.6	0.65
				80	101.8	1.36
Batch I	PAM	60.57	1.53	120	99.0	0.23
				80	97.2	0.89
	Vc	12.15	2.78	50	96.5	2.6
				80	99.6	1.37
	CPM	0.214	2.84	80	102.2	1.16
Batch II	PAM	66.54	1.39	80	98.4	0.76
	Vc	15.42	2.47	80	98.7	1.43

CONCLUSION

The proposed CZE method was successfully used for separation and determination of CPM, PAM, and Vc in Qiangli Yinqiao containing Vc tablets within 3 min, indicating that the method can be promising in the quality control of such kinds of medicine containing these three ingredients. The relationships between injection time and peak area and peak height and peak width at half-height, suggest that normalization of CE peak area can ensure the correct quantitation either at lower or higher concentration, and that selection of injection time is important to CE separation.

The normalization of CE peak areas for quantitative analysis of Vc and PAM and peak height for CPM confirms that normalization of CE peak areas is preferred to peak height in quantitative analysis.

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REFERENCES

1. **New Edited Handbook of Chinese Medicine**, National Medicine and Science & Technology Press, Beijing. p.658(1988).

2. L. M. Liu, S. J. Sheu., J. Chromatogr., **639**, 323-328 (1993).

3. C. T. Chen, S. J. Sheu, J. Chromatogr., **710**, 323-329 (1995).

4. Z. Zhang, Z. Hu, G. Yang, Chromatographia, in press.

5. H. Nishi, T. Fukuyama, M. Matsuo, S. Terabe, J. Pharm. Sci., **79(6)**, 519-523 (1990).

6. H. Nishi, T. Fukuyama, M. Matsuo, S. Terabe, J. Chromatogr., **498**, 313-323 (1990).

7. M. E. Swartz, J. Liq. Chromatogr., **14(5)**, 923-938 (1991).

8. S. Fujiwera, S. Honda, Anal. Chem., **59**, 2773-2776 (1987).

9. S. Fujiwara, S. Iwase, S. Honda, J. Chromatogr., **447**, 133-140 (1988).

10. C. P. Ong, C. L. Ng, H. K. Lee, S. F. Y. Li, J. Chromatogr., **547**, 419-428 (1991).

11. J. P. Lorand, J. O. Edwards, J. Org. Chem., **24**, 69-774 (1959).

12. J. P. Landers, R. P. Oda, M. D. Schuchard, Anal. Chem., **64**, 2846-2851 (1992).

13. J. Lui, O. Shirota, M. Novotny, Anal. Chem., **63**, 413-417 (1991).

14. J. W. Jorgensen, K. D. Lukas, Anal. Chem., **53**, 1298-1302 (1981).

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OPTIMIZED AND VALIDATED HPLC METHODS FOR COMPENDIAL QUALITY ASSESSMENT. II. OPIUM ALKALOIDS

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ABSTRACT

The main opium alkaloids, such as morphine, codeine and papaverine have their own monographs in the modern pharmacopoeias. This paper includes reversed phase high performance liquid chromatographic procedures for the purity control of the mentioned opiates. The optimized and validated methods make possible the separation of morphine, codeine, thebaine, noscapine, papaverine and the detection of the four opiate impurities in morphine, codeine and papaverine. The limit of detection ranges from 0.01 up to 0.1%. Also, morphine content in codeine chloride can be quantitated with good accuracy.

INTRODUCTION

The most important opium-alkaloids, morphine, codeine, papaverine, are permanent members of the standard medical stock. Morphine itself, as an analgesic, is essential to endure severe pains. Codeine is one of the most often

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used antitussives even nowadays, and the same is valid for papaverine in the field of smooth musculature relaxation. Though, its therapeutical significance is less, noscapine also has its own monograph in the modern pharmacopoeias. Almost all of these monographs prescribe purity test(s) for the detection of "related" or "foreign" alkaloids (substances). Table 1 summarizes the prescribed tests of four pharmacopoeias (European Pharmacopoeia Ed.II., United States Pharmacopeia Ed.XXIII, Deutsches Arzneibuch Ed.10., Pharmacopoea Hungarica ed.VII). It may be seen that, only non-specific chemical reactions, dissolution tests (sulfuric acid) or TLC procedures under general addressing, as detection of "foreign" ("related") alkaloids, are prescribed. In conclusion, specific testing for an individual (i.e. named) opiate-impurity (e.g. codeine in morphine and vice versa) only exceptionally occurs. The general suitability of HPLC for compendial quality control was raised and illustrated in a previous paper[1] by the HPLC purity test of methylxanthine alkaloids . The present publication provides RP-HPLC methods for the quality testing of the three main natural opiates.

The HPLC separation of the major alkaloids in opium and in plant material has been the subject of several works.[2-5] Recently, capillary electrophoresis was used for the same purpose.[6] For the HPLC determination of the main opium alkaloid, morphine, in biological samples, numerous papers could be found. A few years ago Tagliaro et al.[7] gave an excellent overview of such separations and detection techniques. Similarly, HPLC separation of codeine[8,9] and also its metabolites[10] was the subject of publications. As morphine and codeine are common biological degradation products of each other, their separation and the HPLC separation of some of their other metabolites frequently emerge in the analysis of biological samples.[11-18] However, only a few works were found dealing with HPLC separation and determination of morphine,[19] codeine,[20,21] or papaverine in pharmaceutical preparations. Purity tests, by HPLC, suitable for compendial use, could not be found in the literature. In the present paper, optimized and validated RP-HPLC systems are suggested for the purity control of morphine, codeine, and papaverine. In each of these three substances the other two opiates, as well as noscapine and thebaine, can be selectively and sensitively detected.

EXPERIMENTAL

Chromatography

The HPLC apparatus is comprised in an ISCO pump Model 2350 (USA) combined with a Valco injector unit (10 μL loop). An ISCO variable

Table 1

Pharmacopoeial Methods for Testing Related Substances in Opium Alkaloids

Tested Alkaloid	Impurity Investigated (Method)			
	Eur. Ph. II	**USP XX III**	**DAB 10**	**Ph. Hg. VII**
Codeine	Foreign alk. (TLC) Morphine ($NaNO_2$)	Chromatographic purity (TLC) Morphine ($K_3Fe(CN)_6$ + $FeCl_3$)	Foreign alks. O,O-dimethyl-morphine (TLC) Morphine ($NaNO_2$)	Foreign alks., deg-radiation prods. (TLC) Morphine ($NaNO_2$)
Morphine	Related subs., Codeine (TLC)	Foreign alks. (isolation of, detn. by acidi-alkalimetry)	Related subs., Codeine (TLC)	Related alks. (as in USP) Organic impurities (TLC)
Noscapine	Related subs. (TLC)	Ordinary impts. (TLC) Morphine ($K_3Fe(Cn)_6$ + $FeCl_3$)	Related subs. (TLC)	Related alks., deg-radiation prods. (TLC) Morphine ($KH(IO_3)_2$
Papaverine	Foreign alks., Codeine (TLC)	Organic impts. (cryptopine, thebaine, etc.) (H_2SO_4 dissoln.)	Foreign alks. Codeine (TLC)	Related subs., degradation prods. (TLC)

absorbance detector (230-800 nm) was used. The equipment units, subsequent to the pump, was thermostatted (Column Heater-Chiller, Model 7955 Jones Chromatography Ltd., Wales) at 23° - 30° ± 1°C. 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) was packed in a

stainless steel column 250x4.0 mm I.D., BST, Budapest, Hungary. As mobile phases for optimization, sonically degassed and filtered mixtures of methanol and phosphate buffer solutions pH 3 and pH 8, methanol-water mixtures, occasionally containing pentanesulfonic acid or tetrabutylammonium bromide in different concentrations, were used.

The column void time was signalled by the injection of methanol. Each data of retention was calculated as an average of at least three parallel runs. The eluent flow rate for optimization was adjusted to 0.6-0.8-1.0-1.5 mL/min. The effluent was monitored at the wavelength of optimal detectability of the tested compound. After each experiment, the column was brought to the initial state by washing with 50 mL of methanol-water mixture, in which the amount of water corresponded to that of the buffer solution in the eluent.

A final purging of the column was performed with 50mL of methanol. In the case of the column being loaded by an eluent with pH 8, a prewash with 50 mL 10:90 mixture of methanol- phosphoric acid (pH 2.5) was made.

Materials

Tetrabutylammonium hydrogensulfate 97%, was from Aldrich. Pentanesulfonic acid natrium 98%, was also from Aldrich.

Buffer solutions at pH 3 and 8 were prepared by mixing the proper volumes of 0.067 M aqueous solution of potassium dihydrogen phosphate and disodium hydrogenphosphate (KH_2PO_4,$Na_2HPO_4.2H_2O$) anal. grade. Reanal. Budapest. The pH of the solutions was tested by potentiometry with an accuracy ± 0.02 unit.

Methanol RS for HPLC, Carlo Erba. Water, deionized, double distilled. Sodium chloride 99,99%, Aldrich.

Model Substances

Morphine chloride, Codeine chloride, Papaverine chloride, Noscapine chloride, met the requirements of the Hungarian Pharmacopoea ed. VII. Thebaine chloride was generously donated by Alkaloida (Tiszavasvári, Hungary) and was used without further purification.

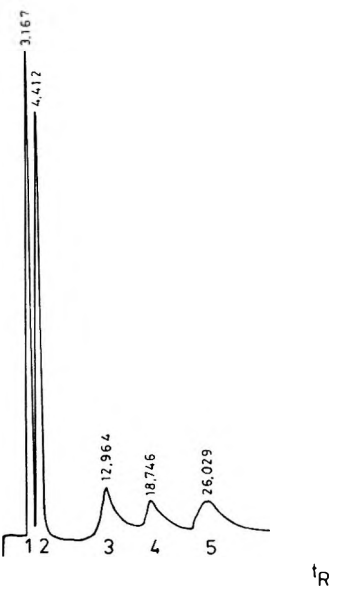

Figure 1. The detection of "related alkaloid" impurities in morphine. (Procedure see in the text under "Prescription."). (1) Morphine (extracted amount: 0.5 g). Impurities, added % (injected μg / 10 μL):(2) codeine 0.1 (0.5), (3) thebaine 0.1 (0.5) (4) noscapine 0.1 (0.5) (5) papaverine 0.02 (0.1).

RESULTS AND DISCUSSION

Morphine Chloride

The optimization of the procedure was performed by successive changing of the methanol content, pentanesulfonic acid concentration, and pH of the phosphate buffer in the eluent, as well as the flow rate and temperature.

None of the proven systems separated the peaks of morphine and codeine sharply enough for purity test purposes. The unique, amphoteric character of morphine, allowed its preseparation from the four related alkaloids by solubility difference, i.e.: from an appropriately alkalinized medium the non-phenolic related compounds can be extracted with chloroform, while morphine mostly remains, as phenolate, in the aqueous solution. Figure 1 shows the HPLC-separation of the four related alkaloids and morphine, when the latter was spiked with 0.1 % w/w of codeine, thebaine, noscapine, and 0.02 % w/w of

Table 2

Retention Time and Limit of Detection of Opium Alkaloids

Compound	Retention Time Min. Methanol Content, %*			Limit of Detn., μg**
	30	40	50	
Morphine	3.1	2.6	2.5	0.1
Codeine	4.4	3.3	2.7	0.1
Thebaine	21.7	8.1	4.3	0.25
Noscapine	41.3	10.9	4.9	0.4
Papaverine	54.3	13.9	5.3	0.05

* Eluent composition: Phosphate buffer, pH=3 - Methanol + 0.005M pentane-sulphonate Na

** At signal-to-noise ratio min. 3:1.

papaverine. It can be seen, that the peaks of codeine and morphine, as a consequence of the reduced amount of the latter, separate sharply, followed by the peaks of the other three substances moving with rather high retention. It was established, that chloroform extracts only about 0.5 % w/w (2.5 mg) of the examined morphine amount (500 mg).

Prescription

Test solution

0.5 g of the tested morphine chloride is dissolved in 10 mL of water and then, by the dropwise addition of 2M sodium hydroxide, the pH of the solution is adjusted to 11. This alkaline aqueous solution is extracted three times with ten mL portions of chloroform.

The combined chloroformic phase is filtered through a layer of anhydrous sodium sulfate and evaporated to dryness. The dry residue is dissolved in 10 mL of the eluent. 10 μL of this test solution is injected and chromatographed.

Mobile phase

The mobile phase was Phosphate buffer pH 3 - methanol (65:35) + 0.005M pentanesulfonic acid natrium.

Eluent flow rate

Eluent flow rate was 1 mL/min., the rather high retention time of thebaine, noscapine and papaverine (retention times see Table 2) is reduced by increasing the flow rate, after the complete elution of codeine, to 1.5 mL/min. (Also, gradient elution may be applied with the increasing of the methanol content of the eluent up to 40 % v/v after the first five minutes of development).

Evaluation

The appearance of a definite peak of codeine, thebaine, noscapine, and papaverine indicates the presence of as much, or more than, 0.1 μg (0.02%) 0.25 μg (0.05%) 0.37 μg (0.074%) 0.02 μg (0.004%) impurity respectively (see Table 2). It is to be noted, that codeine impurity, as much as 0.1 %, also can be quantitatively measured, using standard solutions.

Codeine Chloride

In case of the codeine a pretreatment, similar to that of morphine, to reduce the tested substance/impurity ratio, was not possible. Therefore, an unusually great amount of codeine, allowed by the loading capacity of the column and the suitable resolution from the adjacent peak (morphine) had to be chromatographed. The optimization of the chromatographic system was performed in the same manner as that of morphine. A chromatogram is shown by Figure 2.

It can be seen, though the complete elution of the large, tailing peak of 100 μg codeine takes about 10 minutes, the system is suitable for the detection of the four opiate impurities.

It is to be noted, that morphine can be detected and also quantitated with a good precision in the range from 0.2 up to 1.0 μg (Table 3). A higher amount of morphine may cause an overlapping with the peak of codeine.

Prescription

Test solution

0.1 g of codeine chloride is dissolved in 10 mL of the eluent. 10 μL of the solution is injected and chromatographed in the chromatographic system as that used for morphine.

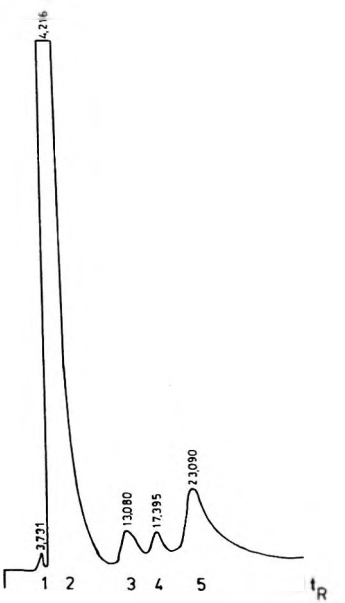

Figure 2. The detection of "related alkaloid" impurities in codeine (Procedure see in the text under "Prescription"). (2) Codeine (examined amount: 100 μg/10 μL). Impurities, added % = injected μg/10 μL: (1) morphine 0.1 (3) thebaine 0.25 (4) noscapine 0.25 (5) papaverine 0.1.

Table 3

Precision* (Repeatability) of the Morphine Content Determination in Codeine

Morphine in Codeine % (μg/100 μg)	S.D. %	Linearity (peak area/ concn.)
0.2	3.58	
0.4	3.60	
0.6	2.63	r = 0.9991
0.8	1.85	n = 5
1.0	2.28	

* It was determined by assaying 5 aliquots at each concentration.

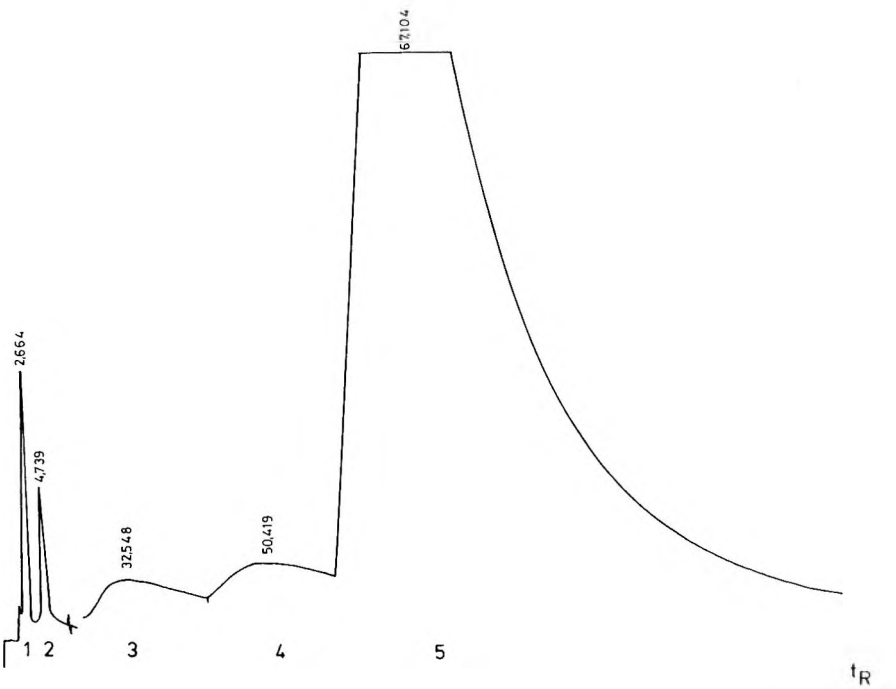

Figure 3. The detection of "related alkaloid" impurities in papaverine. (Procedure see in the text under "Prescription".) (5) Papaverine (extracted amount 0.5 g). Impurities: (1) morphine (2) codeine (3) thebaine (4) noscapine injected:1-1 μg.

Eluent flow rate

The eluent flow rate is 0.8 mL/min., and it changed to 1.5 mL/min. after the elution of codeine's peak.

Evaluation

The appearance of a definite peak of morphine, thebaine, noscapine and papaverine indicates the presence of the impurities as much or more than 0.1 μg (0.1%) 0.25μg (0.25%) 0.37 μg (0.37%) 0.004 μg (0.004%) respectively.

Papaverine Chloride

As the purity control of papaverine had to be considered at a reasonable wavelength of detection, 254 nm, papaverine exhibits an extreme sensitivity, i.e., with a large, tailing peak by which the small adjacent peak of noscapine impurity is overlapped. However, the different solubilities of papaverine and its potential impurities in ethanol offered a way to reduce the final concentration of papaverine. It was established that only 4.2% of the tested papaverine dissolves in the ethanol, while the impurities, being present in an amount 0.1-1%, are completely extracted into the ethanol. The chromatogram of a purity test, was made in the optimized chromatographic system, is shown by Fig. 3. The optimization was performed by the same manner as in case of morphine.

Prescription

Test solution

0.5 g of papaverine chloride is shaken in 3 mL of 96 % ethanol for 1 minute and filtered into a 50.0 mL volu-metric flask, diluted to volume with the eluent and mixed. 10 μL of the solution is injected and chromatographed.

Mobile phase

The mobile phase consists of phosphate buffer pH=3 - methanol 70:30 + 0.005 M pentanesulfonic acid natrium.

Eluent flow rate

The eluent flow rate is 1.5 mL/min. To enhance the elution of thebaine, noscapine, and papaverine, gradient elution up to 40% v/v of methanol content may be applied. the same as that of the testing of morphine.

Evaluation

The appearance of a definite peak indicates the presence of as much or more than 0.1 μg (0.1%) morphine and/or codeine, 0.25 μg (0.25%) of thebaine, 0.037 μg (0.037%) of noscapine.

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REFERENCES

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1. Zs. Budvári-Bárány, Gy. Szász, K. Gyimesi-Forrás, J. Liq. Chromatogr., **20**, 1233-1242 (1997).

2. H. W. Ziegler, T. H. Beasley, D. W. Smith, J. Assoc. Off. Anal. Chem. **58**, 888-897 (1975).

3. C. Y. Wu, J. J. Wittick, Anal.Chem., **49**, 359-363 (1977).

4. N. Evren, B. Sener, N. Noyanalpan, Gazi Universetesi Fakultesi Dergisi, **2**, 67-72 (1985).

5. A. Akhila, G. C. Uniyal, Indian J. Pharm. Sci., **45**, 236-238 (1983).

6. I. Bjornsdottir, S. H. Hansen, J. Pharm. Biomed. Anal., **13**, 687-693 (1995).

7. F. Tagliaro, D. Franchi, R. Dorizzi, M. Marigo, J. Chromatogr. Biomed. Appl., **488**, 215-228 (1989).

8. D. E. Easterling, R. K. Desiraju, W. R. Detorres, Pharm.Res., **3**, 45-47 (1986).

9. S. S. Mohammed, M. Butschkau, H. Derendorf, J. Liq. Chromatogr., **16**, 2325-2334 (1993).

10. K. Persson, B. Lindstrom, D. Spalding, A. Wahlstrom, A. Rane, J. Chromatogr. Biomed. Appl., **491**, 473-480 (1989).

11. I. N. Papadoyannis, B. Caddy, Microchem. J., **36**, 182-191 (1987).

12. J. Shah, W. D. Mason, Anal. Lett., **20**, 1493-1501 (1987).

13. S. C. Harris, M. A. Miller, J. E. Wallace, Ann. Clin. Lab. Sci., **18**, 297-305 (1988).

14. Z. R. Chen, F. Bochner, A. Somogyi, J. Chromatogr. Biomed. Appl., **491**, 367-378 (1989).

15. D. Wielbo, R. Bhat, G. Chari, D. Vidyasagar, I. R. Tebbett, A. Gulati, J. Chromatogr. Biomed. Appl., **615**, 164-168 (1983).

16. K. L. Crump, I. M. Mcintyre, O. H. Drummer, J. Anal. Toxicol., **18**, 208-212 (1994).

17. A. S. Low, R. B. Taylor, J. Chromatogr. Biomed. Appl., **663**, 225-233 (1995).

18. D. L. Lin, H. Liu., C. Y. Chen, J. Anal. Toxicol., **19**, 275-280 (1995).

19. M. Dolezalova, J. Pharm. Biomed. Anal., **10**, 507-514 (1992).

20. M. F. Powell, J. Pharm. Sci., **75**, 901-903 (1986).

21. G. A. Milovanovic, T. J. Janjic, L. Trifkovic, Mikrochim. Acta, **3**, 287-293 (1986).

22. B. W. Hadzija, A. M. Mattocks, J. Pharm. Sci., **76**, S27-S27 (1987).

23. E. Postaire, M. Hamon, B. Oulare, D. Pradeau, Ann. Pharm. Franc., **43**, 547-556 (1985).

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J. LIQ. CHROM. & REL. TECHNOL., 20(19), 3271–3277 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

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.

NOVEMBER 2 - 6: AAPS Annual Meeting & Expo, Boston, Mass. Contact: AAPS, 1650 King Street, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000.

NOVEMBER 2 - 6: 11th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Klyczynski, Electrosysthesis Co., 72 Ward Rd., Lancaster, NY 14086, USA.

NOVEMBER 6 - 8: 2nd North American Research Conference on Emulsion Polymers/Polymer Colloids, Hilton Head Is., SC. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561, USA.

NOVEMBER 7 - 8: 6th Conference on Current Trends in Computational Chemistry, Jackson, Miss. Contact: J. Leszcynski, Jackson State Univ., Chem. Dept., 1400 J. R. Lynch St., Jackson, MS 39217, USA. Tel: 601) 973-3482; Email: jersy@iris5.jusms.edu.

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: AIChE Annual Meeting, Westin Bonaventure/Omni Los Angeles, Los Angeles, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

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.

NOVEMBER 20 - 21: New Horizons in Chemistry Symposium, Los Angeles. Contact: J. May. Tel: (213) 740-5962; Email: jessy@methyl.usc.edu.

1998

FEBRUARY 26 - 28: Sample Handling and Analysis of Organic Pollutants, Archamps, France. Contact: ACTIVE Assoc. Office, c/o Pharmapeptides, CUR Business Park, 74166 Archamps, France.

FEBRUARY 28 - MARCH 1: 28th Annual Spring Prog. in Polymers: Degradation & Stabilization of Polymers, Hilton Head Is., South Carolina. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561. Email: ims@mhv.net.

MARCH 1 - 5: 28th International Symposium on Environmental Analytical Chemistry (ISEAC 28), University of Geneva, Switzerland. Contact: ACTIVE Assoc. Office, c/o Pharmapeptides, CUR Business Park, 74166 Archamps, France.

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 8 - 12: AIChE Spring National Meeting, Sheraton, New Orleans. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

MARCH 15 - 18: 14th Rocky Mountain Regional Meeting, Tucson, Arizona. Contact: R. Pott, Univ of Arizona, Tucson, AZ 85721.

MARCH 23 - 25: 5th Meeting on Supercritical Fluids: Materials and Natural Products Processing, Nice, France. Contact: F. Brionne, Secretariat, ISASF, E.N.S.I.C., 1 rue Grandville, B.P. 451, F-54001 Nancy Cedex, France. Email: brionne@ensic.u-nancy.fr.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

MAY 3 - 8: HPLC'98 – 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques, Regal Waterfront Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA

MAY 20 - 22: 32nd Middle Atlantic ACS Regional Meeting, Wilmington, Delaware. Contact: G. Trainor, DuPont Merck, P. O. Box 80353, Wilmington, DE 19880-0353. Email: reglmtgs@acs.org.

MAY 26 - 29: VIIIth International Symposium on Luminescence Spectrometry in Biomedical and Environmental Analysis: Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Las Palmas de Gran Canaria (Canary Islands), Spain. Contact: Dr. Jose Juan Dantana Rodriguez, University of Las Palmas of G. C., Chem. Dept., Faculty of Marine Sci., 35017 Las Palmas de G. C., Spain. Tel: 34 (9) 28 45.29.15 / 45.29.00; FAX: 34 (9) 28 45. 29.22; Email: josejuan.santana@quimica.ulpgc.es.

MAY 27 - 29: 30th Central Regional ACS Meeting, Ann Arbor, Michigan. Contact: D. W. Ewing, J. Carroll Univ., Chem. Dept., Cleveland, OH 44118. Tel: (216) 397-4241. Email: ewing@jevaxa.jeu.edu.

JUNE 1 - 3: 31st Great Lakes Regional ACS Meeting, Milwaukee, Wisconsin. Contact: A. Hill, Univ. of Wisc., Chem. Dept., Milwaukee, WI 53201, USA. Tel: (414) 229-4256; Email: reglmtgs@acs.org.

JUNE 2 - 5: Third International Symposium on Hormone and Veterinary Drug Residue Anbalysis, Congres Centre Oud Sint-Jan, Bruges, Belgium. Contact: C. Van Peteghem, Dept. of Food Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel: (32) 9/264.81.34;

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.

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.

JULY 12 - 16: SFC/SFE'98: 8th International Symposium & Exhibit on Supercritical Fluid Chromatography, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.
AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS, 1155 16th Street, NW, Washington, DC 20036, USA.

AUGUST 31 - SEPTEMBER 3: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Charleston Place, Charleston, South Carolina. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

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.

SEPTEMBER 13 - 18: 22nd International Symposium on Chromatography, ISC'98, Rome, Italy. Contact: F. Dondi, Chem. Dept., Universita di Ferrara, Via L. Borsari, 46, I-44100 Ferrara, Italy. Tel: 39 (532) 291154; FAX: 39 (532) 240707; Email: mo5@dns.Unife.it.

SEPTEMBER 23 - 25: International Symposium on Preparative and Industrial Chromatography and Allied Techniques (SPICA '98), Strasbourg, France. Contact: Mlle. Francoise Brionne, E.N.S.I.C., 1, rue Grandville, B.P. 451, F-54001 Nancy Cedex, France

SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania. Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

NOVEMBER 15 - 20: AIChE Annual Meeting, Fontainbleu/Eden Roc, Miami Beach, Florida. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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, TN 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 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036. Email: natlmtgs@acs.org.

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations, Seattle, Washington. Contact: Barr Enterprises, P.O.B. 279, Walkersville, MD 21793, USA.

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

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036. Email: natlmtgs@acs.org.

AUGUST 25 - 31: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

2002

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

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

2003

MARCH 23 - 28: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

SEPTEMBER 7 - 12: 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.

2004

MARCH 28 - APRIL 2: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC.

AUGUST 22 - 27: 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.

2005

MARCH 13 - 18: 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.

AUGUST 28 - SEPTEMBER 2: 230th 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.

2006

MARCH 26 - 31: 231st ACS National Meeting, Atlanta, GA. 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 10 - 15: 232nd ACS National Meeting, San Francisco, 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.

2007

MARCH 25 - 30: 233rd ACS National Meeting, Chicago, Illinois. 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 19 - 24: 234th ACS National Meeting, Boston, Massachusetts. 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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1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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