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VOLUME 19 NUMBER 15 1996

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

September 1996

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CODEN: JLCTFC 19(15) i–iv, 2385–2558 (1996)
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Volume 19, 20 Numbers, 1996: $1595.00
ISSN: 1082—6076 CODEN: JLCTFC

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Effective with Volume 6, Number 11, this journal is printed on acid-free paper.

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2385–2394 (1996)

MODIFIED LIQUID CHROMATOGRAPHIC ASSAY FOR DILTIAZEM AND METABOLITES IN HUMAN PLASMA

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ABSTRACT

Diltiazem (DTZ) is an anti-hypertensive and anti-anginal agent which is also used clinically as a metabolic inhibitor to reduce cyclosporin-A metabolism. The present communication describes an HPLC/UV method for measuring DTZ and 3 of its major metabolites. This method has a LOQ below 2.5 µg/L and within run CV's ranging from 11.3 to 1.9% at concentrations of 2.5 and 100 µg/L, and between-run CV's ranging from 10.5 to 4.9% at concentrations of 75 and 350 µg/L, respectively. The method has been applied successfully to 11 renal transplant recipients taking a variety of other drugs.

INTRODUCTION

Diltiazem (DTZ) is calcium channel blocking drug used pharmacologically in the treatment of hypertension and angina, and has also been used for an economic purpose by inhibiting the metabolism of the expensive immunosuppressant drug, cyclosporin-A (CsA).[1,2,3]

Diltiazem has been shown to be extensively metabolised to form 3 major metabolites N-monodesmethyl-diltiazem (MA), desacetyl-diltiazem (M1) and N-monodesmethyldesacetyl-diltiazem (M2). It has more than a 20-fold inter-subject variability in metabolite concentrations[4] and inter-species variability in its metabolic profile has been noted.[5] The primary route of DTZ metabolism is by the P450IIIA4 iso-enzyme system.[6] This isoenzyme is present in significant concentrations in the wall of the upper small intestine,[7,8,9,10] in addition to the liver, and has been demonstrated in animal studies to be a site of significant DTZ metabolism.[11,12] Its is perhaps not surprising, therefore, that DTZ suffers from a high "first pass" effect as the oral dose is absorbed from the gastric lumen.

There are also a number of commercially available DTZ formulations, including various "extended release" products. Whilst there are pharmacokinetic studies comparing two or three of these formulations,[13,14,15,16,17,18,19] there is very limited data in the full range of formulations and applications of DTZ, particularly metabolic interactions with other substrates for the P450IIIA4 isoenzyme, such as the clinically important "cyclosporin-A-sparing effect".[1,3,20,21,22,23]

Previous methods for analysis of DTZ (and its metabolites) have been reported, but may involve complicated equipment which may not be readily available in most laboratories,[24,25] or may have confined themselves to parent DTZ.[26,17] One early approach, which required minimal sample purification, appeared to limit column life and may have plasma interferences from patient samples, which could cause problems[27] or were less sensitive than the method described.[28,29]

The present method was developed from that kindly provided by LP Hackett and coworkers (personal communication, 1993), and describes a liquid chromatographic/UV method for the simultaneous determination of DTZ and the 3 metabolites in human plasma samples, applicable to pharmacokinetic studies.

MATERIALS AND METHODS

Stock Solutions

Separate stock solutions (100 mg base /L) of the pure substances, DTZ, MA (fumarate salt), M1 and M2 (kindly provided by Marion Merrell Dow Research Institute, Cincinnati, Ohio), were prepared in glass-distilled deionised water and these solutions serially diluted into single mixture of 10 mg/L and 1

mg/L of each compound. Desipramine HCl (Ciba-Geigy Australia Ltd, Sydney), was adopted as the internal standard and a 5 mg/L solution prepared. These solutions were held at -20°C between assays. All solvents were HPLC grade (BDH Laboratory Supplies, Poole, England) and phosphate buffer was Univar grade (Ajax Chemicals, Auburn, New South Wales, Australia).

Plasma Extraction

Calibration standards were prepared in 1.0 mL of human plasma (previously shown to be devoid of interferences in the chromatographic system described) at concentrations of 10, 50 100, 250, 500 and 750 µg/L of DTZ and each of the DTZ-metabolites. Patient heparinised plasma samples (1.0 mL) and quality control samples, were aliquoted into 15 mL screw-capped extraction tubes in parallel with this calibration curve. Each tube was spiked with the internal standard (50 µL), basified with 100µL Na_2HPO_4 / K_2HPO_4 (50 mM, pH=7.5) and vortex-mixed briefly. Diethyl ether (5 mL) was added before capping and shaking horizontally for 20 min at 100 rpm. Phases were separated by centrifugation (10 min at 3000 rpm) followed by snap-freezing in a dry-ice/ethanol bath. The organic phase was decanted into a conical tube containing 100µL of 50 mM HCl. Tubes were vortex-mixed for a further 60 sec and the HCl phase snap-frozen and ether phase decanted to waste. The acid extract was transferred to auto-sampler tubes and 50 µL injected into the HPLC system described.

Chromatography

The reverse phase chromatography was performed isocratically using a mobile phase comprising acetonitrile: Na_2HPO_4 (40mM, pH=5.5) (25:75) pumped at 1.0 mL/min (Spectra Physics, model P4000) through a 5µm reverse phase column (Lichrocart, RP-SelectB, 10 cm x 4 mm id, part #50829, E. Merck, Darmstadt, Germany), maintained at 40°C and the eluted substances detected by UV absorption at 215 nm (Spectra Physics, model AS2000). Aliquots (50µL) of extracted samples were injected using an autosampler (SpectraPhysics, model AS3000) and quantitation provided by a soft-ware data system (SpectraSystem version 1.2, Spectra Physics).

Statistical Considerations and Patient Studies

Assay performance (precision and accuracy) was assessed by extracting replicates of plasma solutions containing DTZ and each of the 3 metabolites at 2.5, 10 and 100 mg/L within a single run (n = 6), and at 75 and 350 mg/L

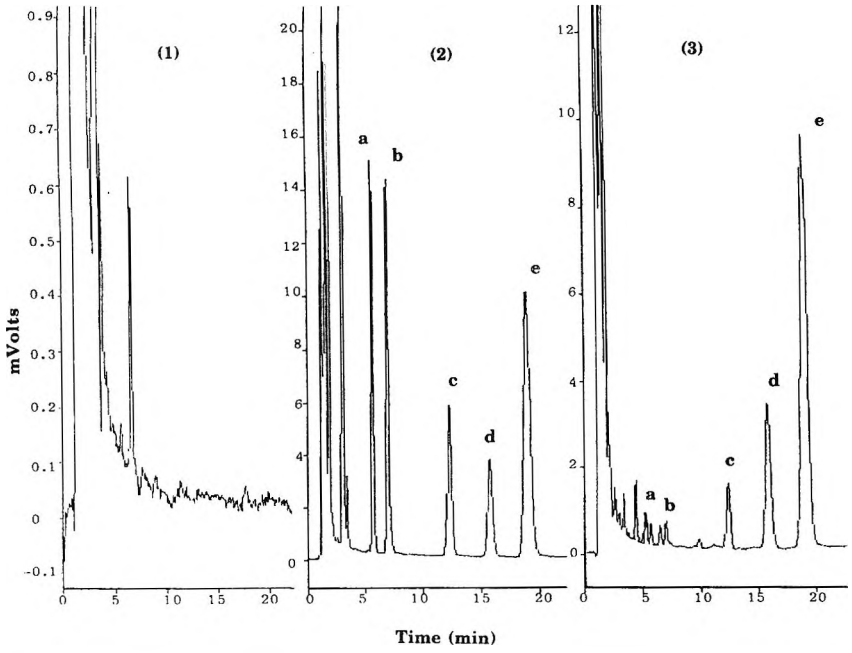

Figure 1. Sample chromatograms showing; panel (1) a "blank" plasma extracted from a patient not taking DTZ, panel (2) a plasma extracted from a sample spiked with 100 µg/L of M2 (a), M1 (b), MA (c), DTZ (d) and internal standard (e), and panel (3) a plasma sample from a renal transplant patient prescribed 60 mg DTZ tds. Note that the vertical attenuation varies in the 3 panels.

between runs (n=6). The robustness of the assay was assessed by application to samples from 11 unselected renal transplant recipients taking DTZ, plus a variety of other medications (including aspirin, atenolol, azathioprine, cephalexin, cyclosporin-A, frusemide, insulin, nifedipine, prazosin, prednisolone, ranitidine, sorbide nitrate), as this was the intended population for the initial application of the method. These patient samples included, were received by this laboratory for routine therapeutic drug monitoring assays of other drugs and, where DTZ was indicated, as part of "other drug therapy" for the patient on the assay request form.

RESULTS

Examples of the 3 chromatograms obtained using the method described, are shown in Figure 1. This shows samples of; (a) a "blank" sample (drug-free

Table 1

Precision and Accuracy Study Results for Parent Diltiazem (DTZ) and the 3 DTZ Metabolites*

Compound	Concentration added (μg/L)	Measured Concentration (mean ± SD) (μg/L)	CV%
Within run (n = 6):			
DTZ	2.5	3.36 ± 0.38	11.2
	10	9.67 ± 0.78	8.1
	100	91.8 ± 1.75	1.9
MA	2.5	3.46 ± 0.39	11.2
	10	10.34 ± 0.43	4.2
	100	91.9 ± 0.82	0.9
M1	2.5	3.57 ± 0.33	9.1
	10	10.31 ± 0.50	4.9
	100	95.0 ± 2.97	3.1
M2	2.5	3.76 ± 0.43	11.3
	10	10.41 ± 0.40	3.8
	100	91.6 ± 1.11	1.2
Between run (n = 6):			
DTZ	75	81.5 ± 0.40	4.9
	350	401.4 ± 41.2	10.3
MA	75	68.0 ± 7.2	10.5
	350	386.5 ± 28.8	7.5
M1	75	72.6 ± 7.2	9.9
	350	389.9 ± 35.7	9.2
M2	75	67.2 ± 5.7	8.4
	350	350.8 ± 30.8	8.8

* MA=N-monodesmethyl-DTZ, M1=desacetyl-DTZ, M2=N-monodesmethyl-desacetyl-DTZ.

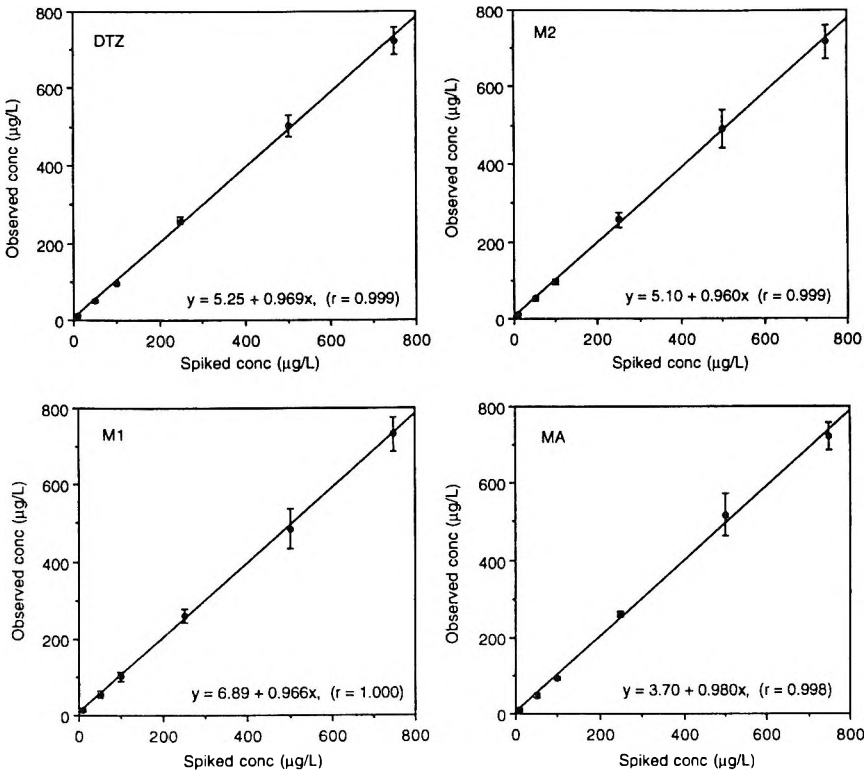

Figure 2. Calibration stability data for parent DTZ and each of the 3 metabolites from 6 analytical runs of the method described. Data shown are means ± sd's. The regression line and correlation coefficient are also presented.

plasma extracted), (b) a sample spiked with 100 µg/L of the 4 DTZ compounds (DTZ, MA, M1 and M2) and the internal standard, and (c) a sample from a patient taking 60 mg DTZ tds at steady-state, showing the presence of the parent DTZ and the 3 metabolites. The retention times for these 5 compounds were DLZ = 15.8 min, MA = 12.2 min, M1 = 6.9 min, M2 = 5.6 min and the internal standard = 18.9 min.

Whilst there were other peaks observed in some patient samples, presumably representing other medication (and/or metabolites) or endogenous substances, none were found to co-chromatograph with the 4 DTZ compounds of interest or internal standard.

The results of the precision and accuracy studies are presented in Table 1, which shows acceptable data both within and between runs. The CV at the lowest concentration studied (2.5 μg/L) was around 11%.

Figure 2 shows the stability of the calibration curves for DTZ and each of the 3 metabolites presented as means ± standard deviations (n=6). The regression line and correlation coefficient (r) is presented on the relevant figure for each compound.

DISCUSSION

The assay of DTZ and its metabolites in a single assay has proved a challenge to many centres. The clear discrimination of 5 peaks (ie., 4 DTZ-related compounds plus the internal standard) in a single isocratic run from a plasma extract is seldom easy, particularly, where patient samples will contain other drugs and their metabolites, as well as, endogenous substances. Furthermore, the patients studied were from a renal transplant population where a spectrum of other drugs (including cyclosporin-A and metabolites) present would be expected. This patient population had biochemical indices of renal function at or above the upper limit of normal, which might result in an increased likelihood of interference from a variety of endogenous substances.

The concentration of 2.5 μg/L, which was adopted as the limit of quantification (LOQ) for practical purposes, could be viewed as conservative. One could go to lower concentrations if one applied the method acceptance criteria recommendations of adopting a 20% CV at the LOQ (precision and accuracy, within and between runs).[30]

Peak resolution was found to be sensitive to the pH of the mobile phase of the method described. A particular feature of DTZ and metabolites are their stability, as they have been reported to break-down on storage.[31] In the present study, it was found that storage of both stock aqueous solutions or patient samples at -20°C, was not associated with detectable deterioration over a period of at least 1 month, when compared with freshly prepared solutions.

The HPLC/UV method described for the assay of DTZ and 3 of its metabolites in human heparinised plasma, has acceptable precision and accuracy, as well as sensitivity and specificity suitable for clinical and/or pharmacokinetic applications. The sample preparation is no more complex than many other drug analyses and should not pose a significant problem for laboratories with experience in this field.

ACKNOWLEDGEMENTS

This work was presented, in part, to the Australasian Society for Clinical and Experimental Pharmacologists and Toxicologists (1995) and published in abstract form in the proceedings.

REFERENCES

1. J. M. Pochet. Y. Pirson, Lancet, **1**, 979 (1986).

2. H. H. Neumayer, K. Wagner, Lancet, **2**, 523 (1986).

3. D. M. Leibbrandt, R. O. Day, Med. J. Aust., **157**, 296-297 (1992).

4. P. K. F. Yeung, C. Prescott, C. Haddad, T. J. Montague, C. McGregor, M. A. Quilliam, M. Xei, R. Li, P. Farmer, Eur. J. Drug Metab. Pharmacokinet., **18**, 199-206 (1993).

5. P. K. F. Yeung, S. J. Mosher, M. A. Quilliam, T. J. Montague, Drug Metab. Dispos., **18**, 1055-1059 (1990).

6. L. Pichard, G. Gillet, I. Fabre, I. Dalet-Beluche, C. Bonfils, J.- P. Thenot, P. Maurel, Drug Metab. Dispos., **18**, 711-719 (1990).

7. W. H. Peters. P. G. Kremer, Biochem. Pharmacol., **38**, 1535-8 (1989).

8. J. C. Kolars, R. Schmiedlin, J. D. Schuetz, C. Fang, P. B. Watkins, J. Clin. Invest., **90**, 1871-1878 (1992).

9. D. R. Krishna, U. Klotz, Clin. Pharmacokinet., **26**, 144-160 (1994).

10. P. B. Watkins, Gastrointest. Pharmacol., **21**, 511-525 (1992).

11. W. Hornsy , M. Lefebvre, G. Caille, P. du Souich, Pharm. Res., **12**, 609-614 (1995).

12. A. H. Hikal, C. Y. Anderson, Arzneimittelforschung, **42**, 101-102 (1992).

13. S. Guimont, H. Landriault, K. Klischer, M. Grace, C. Lambert, G. Caille, D. Gossard, A. Russell, A. Raymond, E. Hutchings, et al., Biopharm. Drug Dispos., **14**, 767-778 (1993).

14. V. Hutt, F. Janik, J. Kapper, G. Pabst, V. Ravelli, M. Maccari, H. Jaeger, Arzneimittelforschung, **43**, 737-743 (1993).

15. S. V. Dange, D. H. Nandal, V. S. Gokhale, K. U. Shah, D. B. Kadam, Indian J. Physiol. Pharmacol., **36**, 205-208 (1992).

16. E. De Bernardis, P. Candido, R. Lorefics, M. Picari, V. Rizza, Arzneimittelforschung, **42**, 25-27 (1992).

17. P. Du Souich, N. Lery, L. Lery, F. Varin, S. Boucher, M. Vezina, D. Pilon, J. Spenard, G. Caille, Biopharm. Drug Dispos., **11**, 137-147 (1990).

18. K. Murata, H. Yamahara, K. Noda, Pharm. Res., **10**, 1165-1168 (1993).

19. L. Brorson, A. Arvill, P. Lofdahl, E. Jorgensen, T. Fraser, H. Larsson, A.-M. Olsson, S. -O. R. Olsson, Eur. J. Clin. Pharmacol., **47**, 75-79 (1994).

20. J. Brockmoller, H-H. Neumayer, K. Wagner, W. Weber, G. Heinimeyer, H. Kewitz, I. Roots, Eur. J. Clin. Pharmacol., **38**, 237-242 (1990).

21. A. Chrysostomou, R. G. Walker, G. R. Russ, A. J. F. D'Apice, P. Kincaid-Smith, T. H. Mathew, Transplantation, **55**, 300-304 (1993).

22. A. McLachlan, S. Tett, Ther. Drug Monit., **17**,400 (1995).

23. K. L. Tortorice, K. L. Herm-Duthoy, W. M. Awani, K. V. Rao, B. L. Kasiske, etal., Ther. Drug Monit., **12**, 321-328 (1990).

24. P. Hubert, P. Chiap, J. Crommen, J. Pharm. Biomed. Anal., **9**, 883-887 (1991).

25. V. Ascalone, M. Locatelli, B. Malavasi, J. Chromatogr. Biomed. Appl., **657**, 133-140 (1994).

26. B. H. Jensen, C. Larsen, Acta Pharm. Nord., **3**, 179-180 (1991).

27. V Ascalone, L. Flaminio, J. Chromatogr., **495**, 358-360 (1989).

28. M. Delwar Hussain, Y. K. Tam, B. A. Finegan, R. T. Coutts, J. Chromatogr., **582**, 203-209 (1992).

29. P. K. F. Yeung, T. J. Montague, B. Tsui. C. McGregor, J. Pharm. Sci., **78**, 592-597 (1989).

30. V. P. Shah, K. K. Midha, S. Dighe, I. J. McGilveray, J. P. Skelly, A. Yacobi, T. Layoff, C. T. Viswanathan, C. E. Cook, R. D. McDowall, K. A. Pittman, S. Spector, Pharmaceut. Res., **9**, 588-592 (1992).

31. J-L. Bonnefous, R. Boulieu, C. Lahet, J. Pharm. Sci., **81**, 341-344 (1992).

Received February 14, 1996
Accepted February 29, 1996
Manuscript 4083

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2395–2407 (1996)

DETERMINATION OF OCHRATOXIN A IN CEREALS AND FEED BY SAX-SPE CLEAN UP AND LC FLUORIMETRIC DETECTION

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ABSTRACT

We have developed a sensitive, reliable and highly specific method for the determination of ochratoxin A (OA) in cereals and animal feed. The samples were extracted in acidified acetonitrile and, they were then thoroughly purified by a new procedure combining a pH-controlled liquid-liquid partition with a strong anionic exchange solid-phase extraction. Both RP-TLC and RP-HPLC methods for semi-quantitative and quantitative determination have been described respectively, together with a RP-HPLC confirmation procedure, via conversion to OA-methyl ester. The TLC and HPLC determination limits were 8.0 ng/g and 0.10 ng/g respectively. The mean recovery from spiked samples was 95%.

INTRODUCTION

Mycotoxins are secondary fungal metabolites, i.e. end-products of fungal cellular metabolism and acting either in fungal cell differentiation or as antibiotics against microbial competitors and predators.

A large number of cereals and other crops are often contaminated with mycotoxins, produced by different fungi, either in the field or during storage. The ochratoxin A (OA), chemically (R)-N- [(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl) carbonyl]-L-phenylalanine, is produced by Penicillium and Aspergillus genera (i.e. A. ochraceus, P. viridicatum).[1] OA is one of the most important reasons accounting for mycotoxic nephropathy, mainly in swine species.[2] This mycotoxin has been found to contaminate a variety of agricultural commodities, particularly cereals, different kinds of beans (soya, cocoa, coffee), red and black peppers, cottonseed, citrus fruits, and peanuts and tobacco.[3] OA has carcinogenic, nephrotoxic, teratogenic and immunotoxic properties for a wide number of animals.[4-5] For example, it causes a proteic synthesis inhibition at cellular level and, in the kidney, it induces periglomerular and interstitial fibrosis leading to glomerular atrophy. Furthermore OA, as a dihydro-hydroxycumarin derivative, acts as an anti-vitamin K xenobiotic, leading to the haemorrhage syndrome. Swine are the most sensitive species to nephrotoxic effect. The minimal amount associated with swine nephropathy seems to range from 100 to 200 ppb.[6-7]

Many analytical methods have been developed,[8] including thin layer chromatography (TLC),[9-13] high performance liquid chromatography (HPLC),[13-19] gas chromatography (GC)[20] and enzyme-linked immunosorbent assay (ELISA).[21-22] Often, the procedures of extraction and clean up of the samples are too time-consuming, and the final extract is frequently matrix-dependent in regard to interfering substances. This results in unsatisfactory and insufficient clean up levels, thus strongly affecting its detection limit. Some published clean up methods mainly employ a pH-controlled liquid-liquid partition, frequently combined with a solid-phase extraction on silica or C_{18} sorbents.[13,16,18,19,23,24] This widespread approach, though easily applicable, is not so specific as to provide high recoveries, together with free interferences extracts. A GPC method has been used,[25] which needs special requirements, such as a dedicated GPC system, when most laboratories do not have such systems. More recently, immunoaffinity columns (IAC) have been successfully used,[26] but unfortunately, they are too expensive for routine use.

The aim of our work was to develop a highly sensitive and readily reliable TLC/HPLC method for the determination of low OA concentrations indipendent of the characteristics of an analyzed matrix. Several kinds of cereals and mixed feed were analyzed by the procedure described. We thus stressed the clean up procedure. For the first time, we have described a very effective, and yet rapid, clean up method based upon a combination of a liquid-liquid partition and a solid-phase extraction. Our method has given us successful and high recovery rates, as well as very well clean extracts. These extracts were then suitable, either for TLC screening, HPLC analysis or HPLC confirmation as methyl ester, without any additional purification.

EXPERIMENTAL

Reagents

Ochratoxin A, in a crystalline form, was purchased from Sigma. A 500 ng/μL stock solution in acetonitrile was then prepared. For sample spiking and TLC/HPLC analysis, 1 ng/μL standard solution was prepared in the HPLC mobile phase, acetonitrile-0.2 % orthophosphoric acid solution (55:45, v/v). Stock and standard solutions were protected from light in screw-cap amber tubes, and were stored frozen at -20° C. Methyl ester of OA was prepared by esterification of corresponding OA standards. All chemicals and solvents were purchased from Analyticals Carlo Erba (RPE reagent). The solvents employed for standards and mobile phase preparation were of HPLC grade (Lichrosolv Merck). All water used was bi-distilled and de-ionized by a Milli-Q Water System. A typical 100 mL tris.HCl buffer solution (pH=7.20), was prepared by mixing 25 mL of 0.2 M tris solution with 4.5 mL of 1 M HCl, and diluting it to 100 mL with de-ionized bi-distilled water. Isolute XL-SAX columns (500 mg sorbent, 10 mL volume), were supplied by IST-StepBio. The KC 18 RP-TLC 10x10 cm plates were purchased from Whatman.

Apparatus

The flask shaker was universal table shaker (mod. 709) from Chimica Omnia. The centrifuge was a Beckman J6-MC model. The SPE columns were connected to an SPE vacuum manifold apparatus, supplied from Supelco. The HPLC equipment was obtained from Perkin-Elmer and consisted of a series 200 LC quaternary pump, equipped with a Rheodyne injector (50 μl loop). The detector was an LC-240 fluorescence spectrophotofluorimeter. The chromato-integrator was a PE Nelson 1022 LC plus model. Separations were made at room temperature, on a 5 μm Supelcosil LC-18 250x4.6 mm column (Supelco).

Extraction of Cereals and Mixed Feeds

20 g of representative finely ground commodities were mixed with 80 mL of 3% acetic acid in acetonitrile in a 250 mL jar. It was closed and automatically shaken for 30 min. The raw extract was clarified by filtration through a folded filter. A 60 mL aliquot of the filtered mixture was transferred into a 100 mL round bottomed flask and evaporated until dry at 45° C. The residue was then dried under a helium flow for 1 min, in order to remove any acetic acid traces. The residue was quantitatively and accurately transferred to a 40 mL capped Nalgene centrifuge tube by using two 2.5 mL portions of n-

hexane, alternating with another two 5 mL aliquots of tris.HCl buffer (pH=7.20). A pH control of the aqueous phase was made and, if necessary, was corrected by adding 10 µl of 45 % NaOH solution. Afterwards, the bi-phase system was vigorously shaken on a vortex for 5 min, and then centriguged for 20 min at 4000 rpm, at 20° C. The upper organic layer was discharged off. Whenever a gelification of the aqueous phase occurred, further n-hexane washing was thoroughly made without affecting the recovery. A 5 mL aliquot of the aqueous buffered phase was collected and submitted to SAX-SPE clean-up.

SAX Column Clean-Up

The buffered aqueous extract was subjected to a SPE-SAX clean up procedure. The SAX column, connected to a vacuum manifold, was rinsed with 5 mL of methanol and conditioned with 5 mL of tris.HCl buffer solution (pH=7.20), at a flow rate of 1 drop/sec. It was recommended that the column was not allowed to run dry. The aqueous extract (5 mL) was passed through the SAX column at a flow rate of ca 0.5 mL/min to be purified. The column was then washed with 5 mL of buffer solution, in sequence, followed by 5 mL of deionized water, 5 mL of methanol and finally 4 mL of 1 % acetic acid in methanol.

The flow rate was not important at these washing stages. Ochratoxin A was then eluted off the SAX column by 7 mL of 5% acetic acid in methanol, at a flow rate of 1 mL/min. The eluate, collected in a 10 mL test tube, was then dried under a gentle stream of nitrogen at 45° C, reconstituted in 250 µl of HPLC mobile phase, and stored at - 20 ° C until analyzed by TLC and/or HPLC.

TLC Screening

A 10x10 cm RP-TLC plate was marked (maximum 6 mm diameter) with 20 µl aliquots of sample and spiked extract, together with 2, 5, 10, 15, 20 µl of OA standard solution (1 ng/µl). The spots were completely dried before they were developed. The plate was developed in a equilibrated tank with the mobile phase, acetonitrile-0.5 M sodium chloride in 0.2 % orthophosphoric acid solution (55:45, v/v). The solvent was kept to a minimum. It should wet no more than the bottom 3 mm of the plate, once it is placed in the tank.

After about 15 min developing time, the plate was air-dried and observed under long wave (365 nm) UV light. The OA appears as a blue-green spot with a retention factor of 0.5. Sample spots were compared with spike and standards.

HPLC Analysis

High performance liquid chromatograph, with fluorescence detector, was employed for the quantification of ochratoxin A and, then, its presence was confirmed as a methyl ester, after proper derivatization. Acetonitrile-0.2 % orthophosphoric acid (55:45, v/v) was used as a mobile phase, at a flow rate of 1 mL/min. A typical injection volume was 50 µl. The excitation and emission wavelenghts were set at 340 nm and 460 nm respectively. The concentration of OA was calculated by means of a linear, six concentration level, calibration graph in a range of 0.15-25 ng injected, based upon the peak area.

HPLC Confirmation by OA Derivatization to Methyl Ester

The eluate from the SAX column was placed in an amber screw-cap tube and dried under a gentle nitrogen stream at 45° C. Accordingly to the Zimmerli's method,[26] the residue was dissolved in 2.5 mL of methanol and 100 µl of 37 % HCl solution. The tube was closed and kept at room temperature for at least 20 h, protected from the light. The reaction mixture was evaporated until dry, under a nitrogen flow at 45° C. The residue was finally reconstituted in 250 µl of HPLC mobile phase, 50 µl of it were then analyzed for OA methyl ester content by HPLC.

RESULTS AND DISCUSSION

Acidified organic solutions turned out to be effective extraction media for ochratoxin A from cereals and mixed feed (13,19,27). OA is endowed of a common carboxylic moiety, whose estimated pKa value is 3.5 (on the basis of phenylalanine-containing dipeptides). It, therefore, appeared that an acidic extraction solvent ensures not only the complete conversion of the ionized to the non-ionized form and, consequently, the disrupting of strong ionic interactions with positively charged functional groups of the matrix components, but also, the analyte transfer to the bulk solution. In our study, the choice of acetic acid arose because it is readily soluble in organic solvents and yet sufficiently volatile to be easily removed from the medium. In our opinion, the use of any other inorganic acid was not advisable. Indeed, a neutralization step is needed before subjecting the sample to SAX clean up. This could provide a remarkable ionic strenght increase and, consequently, a reduced isolate retention on the anionic exchanger, by establishing competitive equilibria. Moreover, the extraction organic solvent, used in this work (acetonitrile), played a fundamental role in promoting the uptake of the ochratoxin A. This was either by denaturating the proteic matrix, responsible for *in vivo* interacting with this mycotoxin, or by acting as a hydrogen bond acceptor towards ochratoxin A.[28]

For the clean up of raw extracts, a new combined procedure of liquid-liquid partition and solid-phase extraction was used. A typical pH-controlled liquid-liquid partition, as reported elsewhere,[18,24] is based upon an unselective distribution between a non-polar organic solvent, such as chloroform, and a strongly alkaline aqueous solution, such as sodium hydroxide. This is incapable of distinguishing the analyte from many other acid interferences, and thus, uptaking all of them without any discrimination of their relative acidities. We greatly reduced the pH of the aqueous receiving phase at a value just enough for complete ionization of OA and a few other acid compounds, which mainly had pKa values lower than the ochratoxin A. We applied the pH-controlled liquid-liquid partition between a very apolar solvent such as n-hexane and a buffer solution of tris.HCl, with appropriate pH (7.2) and ionic strenght (0.045 M). This allowed not only an effective defatting of the raw extract, but also a quantitative partition of the analyte in the neutral aqueous phase. Indeed, the pH value of 7.20 was sufficient for a complete conversion of the ochratoxin A to water-soluble ionized form. It was fortunately unable to ionize other acidic interferences weaker than OA, hence partitioning on behalf of the organic phase. In this way, the OA transfer into the neutral aqueous solution was more specific and, above all, more selective. Nevertheless, the choice of tris.HCl buffer solution, rather than of other inorganic buffers, greatly contributed to this recognition selectivity. Such an organic buffer could favourably interact as quaternary ammonium salt, with the ochratoxin A, by a reasonable ion pair mechanism,[29] in order to competitively displace it from the interactions with some matrix components, thus, stabilizing it in the aqueous medium. Furthermore, a remarkable share in this stabilization was brought by the hydroxymethyl groups of the buffer molecule, acting as hydrogen bond donors towards OA. Indeed, we recovered quantitatively the ochratoxin A, together with more polar interferences by HPLC analysis of such buffered solution.

A subsequent step of solid-phase extraction was necessary not only to remove these interfering substances but also mainly for selectively preconcentrating the analyte in an organic phase. Instead of the usual and aspecific silica or C_{18} SPE,[18,24] we successfully exploited, for the first time, the carboxylic moiety. This makes ochratoxin A suitable for specifically and selectively interacting with the trimethylaminopropyl group of the strong anionic exchanger sorbent.

Figure 1 (right). Chromatogram of (a) blank maize sample containing no OA, (b) the same sample spiked to contain 5 ng/g OA and (c) a maize flour sample naturally contaminated with 1.70 ng/g OA.Conditions: mobile phase, acetonitrile-0.2 % orthophosphoric acid (55:45, v/v), column Supelcosil LC-18 (250x4.6 mm), flow rate 1 mL/min, excitation at 340 nm, emission at 460 nm.

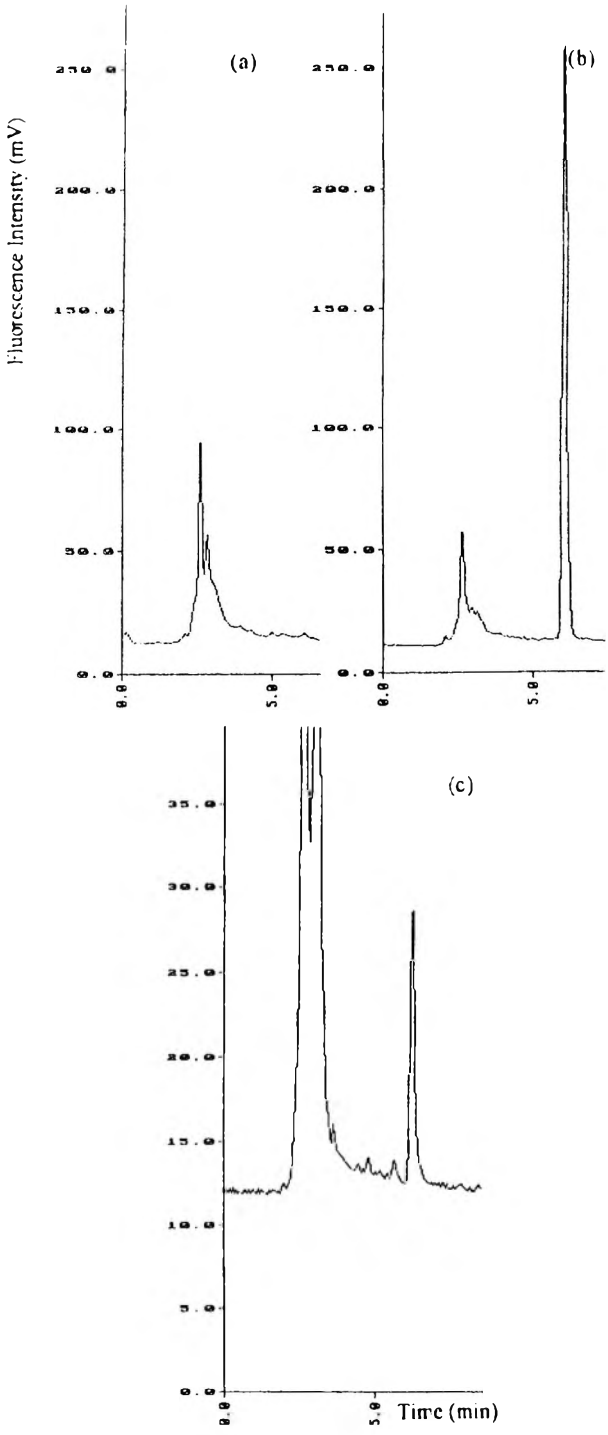
Fluorescence Intensity (mV)
(a)
(b)
(c)
Time (min)

Table 1

Recovery of Ochratoxin from Spiked Samples of Blank Control Maize

Concentration Range of the Sample (ng/g)	Number of Samples	Mean Recovery (%)	CV (%)
1 - 5	5	94	7
10 - 50	6	96	7
100	4	95	4

Conditioning the ion exchange sorbent, before applying sample solutions, with 5 mL of tris.HCl buffer (pH=7.20; I=0.045 M), resulted in a pre-equilibration step with a low selectivity chloride counter-ion. This gave the OA anionic form the best chance of displacing the counter-ion and remaining on the sorbent. Nevertheless, the low ionic strenght (0.045 M) of the sample provided the strongest isolate retention. Indeed, no loss in the OA amount during the loading step occurred, as confirmed by HPLC. Likewise, all three subsequent washing steps allowed the removal of most interferences, without any loss of the mycotoxin. In particular, washing with methanol eluted off a number of matrix interfering substances, presumably polar non-ionic molecules, retained even on SAX by non-functionalized silanolic group fractions.

We further improved the cleaning by modulating the acidity percentage in methanol. Acidity strongly affects the OA retention behavior on the SAX sorbent. Indeed, we observed that a 1% amount of acetic acid in methanol eluted nearly all remaining interferents, together with a variable amount of OA, depending upon volume used. Washing with 6 mL of 1% acetic acid in methanol yielded a loss in OA content from 1 to 7%. Evidently, the elution volume of OA with this low acidity solvent is far higher. Thus, a reduction to 4 mL provided no loss of OA and, meanwhile, there was a complete or almost complete elimination of interferences. OA recovery was finally achieved by increasing the acetic acid from 1 to 5%, 7 mL of this elution mixture were sufficient. In Figure 1, typical chromatograms of (a) a blank maize sample, (b) a sample spiked containing 5 ng/g ochratoxin A and (c) a naturally contamined maize flour sample with a concentration of 1.70 ng/g are shown, all showing the high cleaning level achieved.

Using OA standard solution, the absolute detection limit was 40 pg, at a signal-to-noise ratio of 3:1. We evaluated the limit of determination in maize sample at 0.10 ng/g. At this spiking level, the mean recovery of five triplicates was 97% with a CV of 12%. In Table 1, are listed the recovery and the CV value of maize samples spiked in the range 1-100 ng/g.

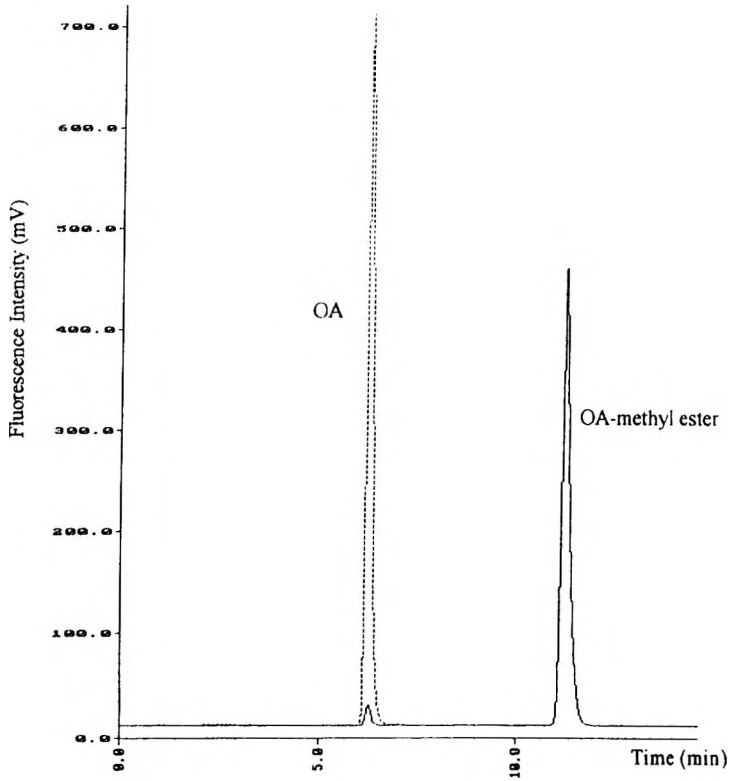

Figure 2. Overlapped chromatograms of OA (20 ng) and its methyl ester derivative.Conditions: mobile phase, acetonitrile-0,2 % orthophosphoric acid (55:45, v/v), column Supelcosil LC-18 (250x4.6 mm), flow rate 1 mL/min, excitation at 340 nm, emission at 460 nm.

Low variation coefficients, indicating a narrow data dispersion, confirmed that recovery rates were independent of spiking level, as well as the good repeatibility of the method. In the range 1-100 ng/g, the overall recovery percentage was 95% with CV 6%.

Wherever high HPLC sensitivity is not important, a reverse phase, thin-layer chromatography procedure was developed, in order to recognize rapidly an OA contamination in analyzed samples. The spotted extracts were the same, alternatively submitted to HPLC for quantitative determination and confirmation. The almost complete absence of interferences allowed an unambigous identification of the ochratoxin A spot (Rf=0.5).

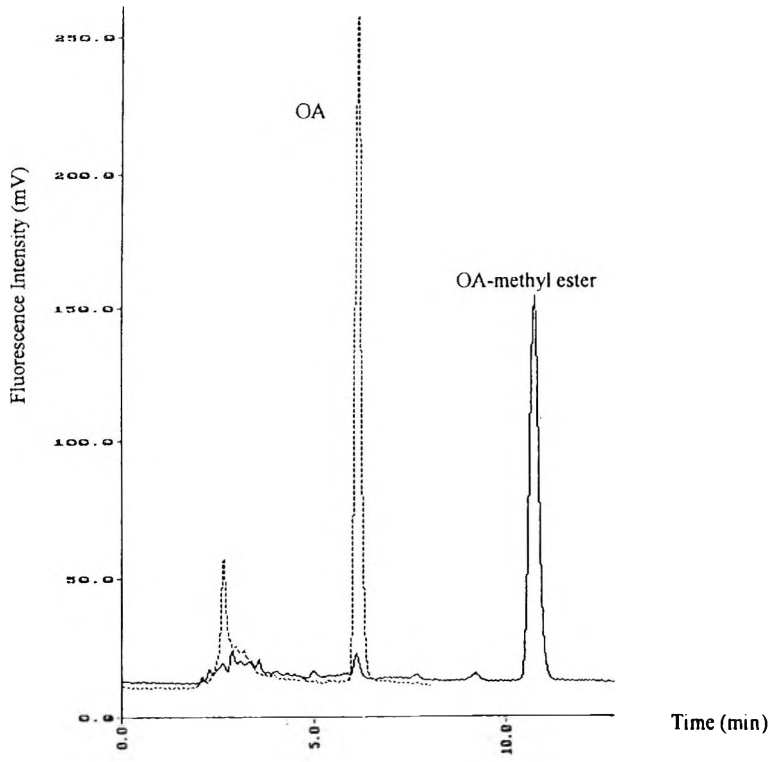

Figure 3. Overlapped chromatograms of OA and its methyl ester in extracts of 10 ng/g spiked maize sample. Conditions: mobile phase, acetonitrile-0,2 % orthophosphoric acid (55:45, v/v), column Supelcosil LC-18 (250x4.6 mm), flow rate 1 mL/min, excitation at 340 nm, emission at 460 nm.

Moreover, we observed a remarkable increase in the OA fluorescence intensity on reverse phase plates rather than direct phase.[9-13] This was presumably due to a reduction of the quenching process.

The combination of such factors provided a detectable limit of 8.0 ng/g, which was very low from a TLC point of view. The mobile phase used for plates development was the same as for HPLC analysis, except for the presence of 0.5 M NaCl in order to avoid the disruption of binding properties of the layer. This ensured the stability of the stationary phase without affecting the separation.

Besides TLC monitoring and HPLC quantitative determination, we exploited a confirmation method by conversion to methyl ester of ochratoxin A and sequent HPLC analysis. Following the derivatization protocol of Zimmerli,[26] OA was converted into the methyl ester. By lengthening the reaction time to at least 20 hours, there was an improvement of mean yield to 97%, which was calculated on unreacted ochratoxin A. Figure 2 displays the overlapped chromatograms with reference to 20 ng of OA, before and after derivatization. The ochratoxin A peak decreased and a new peak appeared five minutes afterwards, due to a less polar molecule than OA, almost definately the corresponding OA-methyl ester. Figure 3, shows the typical overlapping of OA and OA-methyl ester chromatograms, relatively to a 10 ng/g spiked maize sample. We are currently applying this matrix-independent method on other types of animal feed, which has also confirmed good reproducibility.

ACKNOWLEDGEMENT

We wish to thank Dr. Robert Coates (Centro Linguistico, University of Brescia) for manuscript correction.

REFERENCES

1. R. R. Marquardt, A. A. Frohlich, D. Abramson, Can. J. Physiol. Pharmacol., **68**, 991-999 (1990).

2. P. Krogh, Acta Pathol. Microbiol. Scand. Sect. A, Suppl., **269**, 1-28 (1978).

3. C. F. Jelinek, A. E. Pohland, G. E. Wood, J. Assoc. Off. Anal. Chem., **72**, 223-230 (1989).

4. R. R. Marquardt, A. A. Frohlich, J. Animal Sci., **70**, 3968-3988 (1992).

5. R. Roschenthaler, E. E. Creppy, G. Dirheimer, J. Toxicol. Toxin Rev., **3**, 53-86 (1984).

6. P. Krogh, in E. Eaker and T. Wadstrom, **Natural Toxins**, Pergamon Press, Oxford, 1980, pp. 673-680.

7. P. Krogh, N. H. Axelsen, F. Elling, N. Gyrd-Hansen, B. Hald, J. Hyldgaard-Jensen, A. E. Larsen, A. Madsen, H. P. Mortensen, T. Moller, O. K. Petersen, V. Ravnsko, M. Rostgaard, O. Aalund, Acta Pathol. Microbiol. Scand. Sect. A. Suppl., **246**, 1-21 (1974).

8. V. Betina, Journal of Chromatography Library- volume 54, **Chromatography of Mycotoxins, Techniques and Applications**, Elsevier, Amsterdam, 1993.

9. D. M. Wilson, W. H. Tabor, M. W. Trucksess, J. Assoc. Off. Anal. Chem., **59**, 125-127 (1976).

10. S. Swanson, R. Corley, D. White, W. Buck, J. Assoc. Off. Anal. Chem., **67**. 580-582 (1984).

11. B. A. Robert, M. E. Glancy, S. P. D. Patterson, J. Assoc. Off. Anal. Chem., **64**, 961-963 (1981).

12. G. B. Osborne, J. Sci. Food Agric., **30**, 1065-1070 (1979).

13. M. W. Howell, P. W. Taylor, J. Assoc. Off. Anal. Chem., **64**, 1356-1363 (1981).

14. G. M. Ware, C. W. Thorpe, J. Assoc. Off. Anal. Chem., **61**, 1058-1062 (1978).

15. P. Lepom, J. Chromatogr., **355**, 335-339 (1986).

16. H. Cohen, M. Lapointe, J. Assoc. Off. Anal. Chem., **69**, 957-959 (1986).

17. M. Sharman, S. McDonald, J. Gilbert, J. Chromatogr., **603**, 285-289 (1992).

18. V. Seidel, E. Poglits, K. Schiller, W. Lindner, J. Chromatogr., **635**, 227-235 (1993).

19. W. Langseth, Y. Ellingsen, V. Nymoen, E. M. Okland, J. Chromatogr, **478**, 269-274 (1989).

20. Y. Jiao, W. Blaas, C. Ruhl, R. Weber, J. Chromatogr., **595**, 364-367 (1992).

21. O. Kawamura, S. Sato, H. Kajii, S. Nagayama, K. Ohtani, J. Chiba, Y. Ueno, Toxicon, **27**, 887-897 (1989).

22. J. R. Clarke, R. R. Marquardt, A. Oosterveld, A. A. Frohlich, F. J. Madrid, M. Dawood, J. Agric. Food. Chem., **41**, 1784-1789 (1993).

23. H. Terada, H. Tsubouchi, K. Yamamoto, W. Hisada, Y. Sakabe, J. Assoc. Off. Anal. Chem., **69**, 960-964 (1986).

24. B. Hald, G. M. Wood, A. Boenke, B. Schurer, P. Finglas, Food Addit. Contam., **10**, 185-207 (1993).

25. C. Dunne, M. Meaney, M. Smyth, L. G. M. Th. Tuinstra, J. Chromatogr., **629**, 229-235 (1993).

26. B. Zimmerli, R. Dick, J. Chromatogr. B, **666**, 85-99 (1995).

27. E. Josefsson, T. Moller, J. Assoc. Off. Anal. Chem., **62**, 1165-1168 (1979).

28. E. Rajakyla, K. Laasasenaho, P. J. D. Sakkers, J. Chromatogr., **384,** 391-402 (1987).

29. A. Breitholtz-Emanuelsson, M. Olsen, A. Oskarsson, I. Palminger, K. Hult, J. Assoc. Off. Anal. Chem., **76**, 842-846 (1993).

Received November 24, 1995
Accepted February 13, 1996
Manuscript 4035

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2409-2417 (1996)

DETERMINATION OF CLOZAPINE AND ITS TWO MAJOR METABOLITES IN HUMAN SERUM BY LIQUID CHROMATOGRAPHY USING ULTRAVIOLET DETECTION

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ABSTRACT

A new, simple, reverse phase, and highly reproducible HPLC-UV method that has all the comprehensive features of good column, mobile phase, internal standard and extraction has been developed for the assay of clozapine (CLZ), norclozapine (NCLZ) and clozapine-N-oxide (CLZNO) in human serum. The method is very easy to adapt, overcomes the problems of earlier methods and is very economical. Amoxapine is the internal standard. All three analytes are extracted from alkaline serum using ethyl acetate and the absolute extraction efficiency is ~95%. The UV detector is set at 230 nm. The mobile phase is a mixture of phosphate buffer (0.05M, pH 2.7), acetonitrile and methanol (62:20:18 v/v) and contains 2.5 mL triethylamine per liter of solution. The detection limit of the method is 2 ng/mL for CLZ and NCLZ and 4 ng/mL for CLZNO. The mean CV for the method is 5%.

INTRODUCTION

A typical antipsychotic agent, clozapine (CLZ, Clozaril) is commonly given to schizophrenic patients refractory to conventional neuroleptic agents. The drug has minimal extrapyramidal side effects, but can cause agranulocytosis and dose dependent seizures.[1] Norclozapine (NCLZ), and clozapine-N-oxide (CLZNO) are the two major metabolites of clozapine in humans. The two compounds NCLZ and CLZNO have minor antipsychotic activity. A strong correlation between serum concentrations of analytes and CLZ dose has been shown, but the correlation between serum CLZ concentration and its antipsychotic or side effects are not as yet clearly defined.[1-3]

Although there are many methods for the analysis of CLZ alone, or both CLZ and NCLZ simultaneously, there are only four HPLC methods for the simultaneous analysis of all three compounds.[4-7] These methods however have one or more of the following defects: difficult to adapt, cost ineffective, require column heating, poor choices of internal standard, analytical column, and the UV detector wavelength setting, long assay times and poor extraction efficiencies of all compounds. We have developed an isocratic, reverse phase, and ambient temperature HPLC method that overcomes most of the above problems. We have validated our method, by determining accuracy and precision data using quality control samples, and the steady state concentrations of CLZ, NCLZ, and CLZNO, in 80 samples from 43 schizophrenic patients.

MATERIALS AND METHODS

Analytical grade clozapine, norclozapine and clozapine-N-oxide, were gifts from Sandoz Pharmaceutical Corporation, E. Hanover, NJ. Amoxapine, was obtained from Research Biochemicals International, Natick, MA.

The mobile phase is a mixture of aqueous buffer (0.05 M sodium monobasic phosphate, contains 2.5 mL triethylamine per liter of solution and adjusted to pH 2.7 using phosphoric acid), acetonitrile and methanol in the ratio 62:20:18 v/v. The mobile phase flow rate was 1.3 mL/min.

Working standards (CLZ : NCLZ : CLZNO), of the following concentration (µg/L) triplets, were prepared in drug free serum: 25/25/12.5, 150/150/75, 300/300/150, 500/500/250, 800/800/400 and 1000/1000/500. The standard solutions were aliquoted in 2 mL polypropylene tubes and kept frozen at -20°C. The frozen standard solutions were quite stable for at least 6 months. The working internal standard solution contained 200 ng of amoxapine per 50 µL of 0.1 M hydrochloric acid solution.

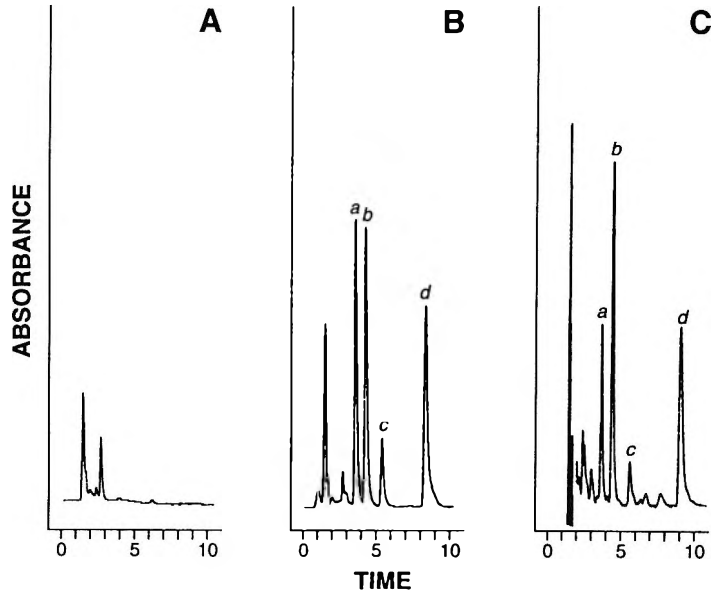

Figure 1. Chromatograms of human serum extracts. A - Blank drug free serum. B - Serum standard containing 150 µg/L each of NCLZ(a), and CLZ(b), and 75 µgL of CLZNO(c). C - Patient sample containing 212 µg/L of NCLZ(a), 290µg/L of CLZ(b), and 52 µg/L of CLZNO(c). Peak (d) represents 200 ng of amoxapine

Equipment

The HPLC system consisted of a pump (Waters, Model 510, Milford, MA), an autosampler (Waters, Model 717), a self packed C_8 guard column (Upchurch, Oak harbor, WA, Model C130-B, containing Perisorb RP-8), an analytical column (Supelco C_8-DB, 3 µm, 100 X 4.6 mm; Supelco Inc., Bellefonte, PA), a variable wavelength ultraviolet detector (Waters, Model 480, with a 1 cm pathlength flow cell) and an integrator (Hitachi, San Jose, CA, Model D-2500). The ultraviolet detector was set at 230 nm.

Extraction

0.5 mL of standard, quality control or patient's serum, 50 µL (200 ng) of internal standard, 400 µL of 1M sodium hydroxide and 6 mL of ethyl acetate were all pipetted into a 10 mL Teflon tube. The tubes were screw capped and shaken for 15 min in an Eberbach shaker, and centrifuged at 4000 X g for 10

min. The top organic layer was transferred to another Teflon tube and the analytes back extracted with 3 mL of 0.05 M hydrochloric acid. In the last step, the top organic layer was transferred to another tube and the ethyl acetate was evaporated under nitrogen in a water bath at 45°C. The residue was dissolved in 120 μL of mobile phase and 30 μL injected into the HPLC system.

RESULTS AND DISCUSSION

Figure1 illustrates typical chromatograms of extracts of human serum: A) a blank - drug free serum, B) drug free serum standard of clozapine and its metabolites, C) a patient sample in this study. There are no interfering peaks in the blank. The peaks are baseline resolved, amoxapine elutes well away from all the clozapine metabolites peaks, and the run time per sample is about 9 min. Serial standardization was performed on each analysis day. The good reproducibility (CV 5%) of the response factors, in about 30 assays so far in this study, clearly points to the robustness of the assay.

Table 1 summarizes the precision, recovery, and accuracy data for the assay. Two somewhat different solid phase extraction methods for CLZ, NCLZ, and CLZNO from serum have been reported,[4,7] but the absolute extraction efficiency of the metabolites by both methods is low. In our experience, our liquid-liquid extraction method has uniform and very high absolute extraction efficiency (~90%, n=6) for all three compounds (Table 1).

The choice of an internal standard for this HPLC assay has been a difficult one for many workers. Volpicelli,[5] "after extensive research," chose triprolidine as her internal standard. Schulz,[6] who had the highest extraction efficiency and sensitivity of all published methods, had an assay run time of 25 min per sample perhaps due to their internal standard ----- imipramine. Fadiran[7] chose n-methylspiperone as the internal standard, so that all the analytes will have uniform extraction efficiency by their solid phase extraction method. Numerous different compounds have been used as internal standards in the assay. We chose amoxapine as the internal standard, and it eluted at about 9 min and away from clozapine and all its metabolites. Amoxapine is very stable in solution and extracted very well (95 ± 3% absolute recovery; n=6). The good reproducibility and accuracy of our method (Table 1) is, at least, partially due to our choice of amoxapine as the internal standard.

A variety of columns (C_6, C_8, C_{18}, phenyl, cyano, cation exchange and silica) have been used in the assay. The nature and extent of reports on the normal phase and cation exchange columns, do not indicate that these methods are simple and did not meet our objective to develop a simple and easy to adapt method. C_8 columns are the choice lately for separating basic drugs.

Table 1

Precision and Recovery Data for all CLZ, NCLZ and CLZNO in Drug Free Human Serum

Spiked Concn*	Measured Concentration* CLZ	NCLZ	CLZNO*
		Mean, CV%, Recovery %	
		Inter-assay (n = 6)	
40	42, 7.0, 105	36, 4.3, 90	18, 5.3, 90
80	74, 6.0, 93	78, 5.1, 98	38, 4.5, 95
400	375, 3.6, 94	379, 2.8, 95	179, 4.1, 90
800	745, 1.9, 93	761, 1.5, 95	378, 2.5, 95
		Intra-assay (n = 6)	
40	44, 5.4, 110	37, 1.0, 92	21, 4.4, 105
80	74, 4.1, 92	80, 2.6, 100	38, 8.0, 95
400	388, 2.5, 97	380, 1.6, 95	182, 3.8, 91
800	744, 1.5, 93	761, 1.0, 95	382, 4.3, 96

* concn. μg/L,, CLZNO concns. in all control samples are half of that for CLZ.

The Supelco C_8-DB, 3 μm (100 X 0.46 cm) has been quite reliable for our assays of ketamine, midazolam, thiothixene, etc. The mobile phase (refer experimental section for composition) for the work, was quickly optimized and it contains the least amount of acetonitrile (20%, which is the most expensive of the solvents) compared to other works. The mobile phase flow rate was 1.3 mL per min and, as such, the method is one of the most cost effective. Ion-pairing agents, like heptanesuphonic acid increased the retention times of the compounds at the low pH mobile phase. Addition of triethylamine decreased the retention times of peaks.

Different workers have chosen different ultraviolet wavelengths (215, 230, and 254 nm) for detection. Greater assay sensitivity results when the detector is set at 215 nm.[8] But, the extraction method, the nature of the analytical column used, and the composition of the mobile phase, influence the selection of detector wavelength. Detectors set at 254 nm[7] clearly have poor detection limits.

Table 2

Daily Dose Levels and Plasma Concentrations of CLZ, NCLZ and CLZNO Obtained by the Method

Patient ID	Dose mg/Day	Concentrations in μg/L CLZ	NCLZ	CLZNO
1	600	486	344	71
2	600	891	474	207
3	400	392	312	152
4	500	388	254	176
5	500	468	574	152
6	125	284	215	48
7	300	398	338	31
8	600	550	489	140
9	250	145	85	73
10	150	140	78	57

In this work, ethyl acetate extraction did not affect the detection at 230 nm. While there are distinct advantages in using photodiode array detector, particularly in eliminating interferences, good interference studies can still be cost effectively done using an UV detector. According to Beer's law, higher pathlength flow cell increases the sensitivity of the method. The Waters (Model 480) UV detector with a 1 cm pathlength flow cell, enabled us to achieve very good sensitivities (2 ng/mL for CLZ and NCLZ and 4 ng/mL for CLZNO). The noise to signal ratio was 1 to 5 during these sensitivity studies.

Patient Results

Table 2 gives dosage and plasma concentrations of all the three analytes, CLZ, NCLZ, and CLZNO, for a few patients included in the study. As noted in earlier studies,[4-8] there is a lot of interpatient variability. In a few instances, metabolite concentrations are greater than those of the parent drug.

In the following discussions, the results of six patients who were taking the drugs fluoxetine, or dilantin or valproic acid, which are known to affect the metabolism of clozapine,[7] were excluded. The mean daily dose for the remaining 37 patients was 463 ± 165 mg/day (range 125-900). The mean serum concentrations of CLZ, NCLZ, and CLZNO were 517 ± 317, 304 ± 199 and 122 ± 72 μg/L respectively.

Table 3

Drugs Checked for Possible Interference and their Relative (to Norclozapine) Retention Times

Drug	Relative Retention Times
Atenolol	0.50
Norlclozapine	1.00 (5.5 min)
Bupropion	1.13
Clozapine	1.19
Paroxetine	1.49
Clozapine-N-oxide	1.50
Trazadone	1.50
Sertraline	1.85
Inderal	2.10
Dilantin	2.26
Doxepin	2.29
Medazepam	2.35
Amoxapine	2.60
Carbamazepine	2.70
Desmethylsertraline	2.80
Haloperidol	3.10
Loxapine	3.11
Verapamil	3.70
Imipramine	3.89
Oxazepam	3.91
Thiothixene	3.96
Valproic acid	4.55
Desipramine	4.90
Cogentin	4.98
Nortryptiline	6.00
Amitryptiline	6.10
Diazepam	6.94
Trifluoperazine	8.50
Fluoxetine	8.60

The correlations between daily dose and plasma concentrations of CLZ, NCLZ, and CLZNO were $r = 0.6244$, $r = 0.6482$, and $r = 0.6995$ respectively. The mean ratio in individuals NCLZ/CLZ = 0.662 ± 0.202, and CLZNO/CLZ = 0.2779 ± 0.151. These results are very similar to values reported by Volpicelli[5] and McCarthy.[8]

Interference Studies

The specificity of the assay was determined by injecting the drugs that our patient population were taking along with clozapine. Table 3 gives the retention times of these drugs checked in our method. About 200 ng of the drug was injected into the HPLC system. The drug bupropion could interfere with clozapine; paroxetine and trazodone interfere with clozapine-N-oxide; carbamazepine and desmethylsertraline coelute with amoxapine. We noted, that the patients in this study were not given carbamazepine, but either Dilantin or valproic acid. Although, Dilantin itself does not interfere in the assay, its metabolites were found to coelute with CLZ and NCLZ. Similar other drug metabolites could interfere in the assay and caution should be exercised in interpreting the results.

CONCLUSIONS

Our HPLC method for the simultaneous assay of CLZ, NCLZ, and CLZNO, offers many distinct advantages over the existing four similar methods.[4-7] It has nearly all the comprehensive salient features for a successful HPLC method. These include the analytical column, mobile phase, internal standard, extraction procedure and the UV detector with a long path length flow cell and set at the sensitive wavelength of 230 nm. Optimizing these features, make any HPLC assay simple, sensitive, easy to adapt, reliable and cost effective. Briefly, in our view, the analytical columns tried in earlier methods[4-8] have been a handicap, requiring a higher percentage of acetonitrile in the mobile phase, column heating, and perhaps precluded the choice of a good internal standard.

ACKNOWLEDGMENTS

We most sincerely thank Drs. Sally Guthrie, John DeQuardo, Stephan Taylor, and nurse JoAnn Goodson for their very valuable assistance with paperwork obtaining the IRB forms, outpatient clinic directors' permission to obtain patient samples, and other efforts making the project a success.

REFERENCES

1. R. J. Baldessarini, F. R. Frankenburg, N. Engl. J. Med., **324**, 746-754 (1991).

2. M. W. Jann, Pharmacotherapy, **11**, 179-195 (1991).

3. M. W. Jann, S. R. Grimsley, E. C. Gray, W. H. Chang, Clin. Pharmacokinet., **24,** 161-176 (1993).

4. H. Weigman, C. Heimke, J. Chromatogr., **582,** 209-216 (1992).

5. S. A. Volpicelli, F. Centorrino, P. R. Puopolo, J. Knado, F. R. Frankenburg, R. J. Baldessarini, J. G. Flood, Clin. Chem., **39**, 1656-1659 (1993).

6. E. Shultz, C. Fleishhaker, H. Remschmidt, Pharmacopsychiat., **28**, 20-25 (1995).

7. E. O. Fadiran, J. Leslie, M. Fossler, D. Young, J. Pharm. and Biomed. Anal., **13**, 185-190 (1995).

8. P. T. McCarthy, S. Hughes, C. Paton, Biomed. Chromatogr., **9**, 36-41 (1995).

Received December 20, 1995
Accepted January 15, 1996
Manuscript 4053

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2419–2422 (1996)

MECHANICALLY BLENDED SILICA-BASED BONDED PHASES. APPLICATION OF THE SIMPLE MODULATION OF THE POLARITY OF THE STATIONARY PHASE TO T. L. C.

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ABSTRACT

A mixture of polycyclic aromatic hydrocarbons and aza-arenes was used to test C_{18}/cyano mixed phases prepared by mechanical mixing of silica-based bonded phases for one-dimensional T.L.C. It was shown that for a normal phase polarity mechanism, the modulation in polarity of the stationary phase, obtained by simple variation of the percentage of the cyano phase, contributed to an improvement in the resolution of the mixture.

INTRODUCTION

In order to achieve an improvement in resolution, R, on a T.L.C. plate, a solvent is introduced or exchanged in the mobile phase based on Snyder's[1] triangle of selectivity, α ; the retention factor, R_f may also be optimized.[2] If double behavior phases are used such as the cyanopropyl phase,[3] then by changing the mechanism α and thus R can then be strongly modified. However, a certain rigidity limits this type of phase since on the one hand the ratio [cyano group] / [methylene group] is fixed, and on the other hand the total quantity of cyano group is approximately constant depending on the reproducibilities of the phase and layer preparations.

When these constraints are removed by use of mechanically blended mixed-mode phases,[4,5] we have shown that an improvement in R can be obtained by introducing a new parameter. The variable retention force of mixed C_{18}/cyano stationary phases has thus been successfully tested on a mixture of polycyclic aromatic hydrocarbons (P.A.H.) and aza-arenes.

MATERIALS

The P.A.H. and aza-arenes used were pyrene (Fluka), benzo[b]fluoranthene (Aldrich), acridine (Sigma), benzo[h]quinoline (Fluka) and phenanthridine (Fluka) of purity : > 97 %, 99 %, > 95 %, > 99 %, and >98 % respectively.

The characteristics of the pure commercial phases used : C_{18}-bonded silica and CN-bonded silica were as described previously.[4]

METHODS

The preparations of the blended phases and the plates, together with the development and detection techniques, have been described previously.[4] All the results were confirmed three times.

RESULTS AND DISCUSSION

The mixture tested contained two P.A.H. not resolvable on the unmixed C_{18} phase[6] : pyrene and benzo[b]fluoranthene, both hydrophobic and polarizable, and three isomeric aza-arenes : benzo[h]quinoline, acridine and phenanthridine, all hydrophobic, basic and dipolar.

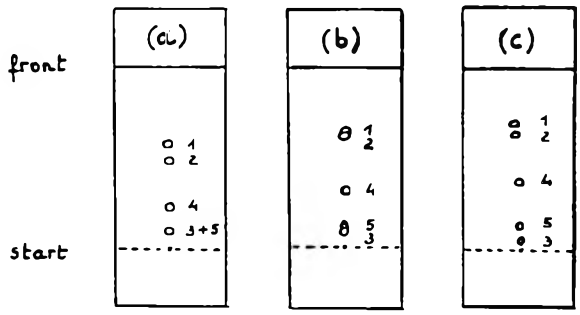

Figure 1. Effects of variations of the polarities of the mobile phase and the stationary phase on the resolution. Compounds : 1 = pyrene, 2 = benzo[b]fluoranthene, 3 = acridine, 4 = benzo[h]quinoline, 5 = phenanthridine. Stationary phases : (a) C_{18}/cyano 50/50, (b) C_{18}/cyano 50/50, (c) C_{18}/cyano 30/70,. Mobile phases : (a) hexane, (b) hexane / chloroform 85/15, (c) hexane / chloroform 80/20.

According to Smith and Cooper's triangle of selectivity, relating to bonded hydrophilic phases, the cyanopropyl phase is essentially characterized by the dipole orientation.[7] Combinations of such a phase with a C_{18} phase should thus be complementary with respect to the mixture being studied. Although a 50/50 mass percent C_{18}/cyano combination, combined with an eluent such as hexane, has proved to be satisfactory for the separation of certain P.A.H.,[4] when it was applied to aza-arenes it caused the co-elution of acridine and phenanthridine (Fig. 1a), which are strongly retained and in addition have the same basicity (pK_a = 5.60). In order to move these two compounds into an R_f zone more favorable to R,[2] the polarity of the eluent was increased. By addition of chloroform, essentially a proton donor[1] with respect to the basic aza-arenes, or by addition of nitroethane, essentially a creator of dipole-dipole interactions[1] with respect to the P.A.H. and the aza-arenes, it was only possible to achieve a weak increase in R for the two aza-arenes. This was at the expense of the P.A.H. whatever the percentage of modifier in the eluent. The best result obtained is shown (Fig. 1b).

On the other hand, an increase of the dipole-dipole type stationary phase-solute interaction forces by accentuation of the cyano phase percentage, combined with a slight increase in the eluent polarity, led to resolution of the mixture (Fig. 1c). We have shown with the C_{18}/cyano combinations that in the 50 - 100 % cyano range, the mechanism is normal phase polarity, as long as the polarity P' of the eluent does not reach the value 3.5.[4] With an invariant mechanism, the modulation of the polar phase percentage in a mixed phase is

thus easy to achieve and very flexible, constituting an additional variable which would seem to be of some interest. Studies are continuing with a view to defining this and to suggest predictive models.

REFERENCES

1. E. Heilweil, " A Systematic Approach to Mobile Phase Design and Optimation for Normal and Reversed - Phase Chromatography ," in **Techniques and Applications of Thin-Layer Chromatography**, J. Touchstone and J. Sherma (eds), Wiley, N.Y., 1985, pp. 37-49.

2. G. Guiochon, F. Bressolle, A. Siouffi, J. Chromatogr. Sci., **17**, 368-386 (1979).

3. W. Jost, H. Hauck, in **Instrumental High Performance Thin Layer Chromatography**, Proc. 4th Intern. Symp., Selvino, R.E. Kaiser (ed.), Inst. for Chromatography, Bad Duerkheim, 1987, pp. 241-253.

4. Z. Hajouj, J. Thomas, A. M. Siouffi, Analusis, **22**, 404-407 (1994).

5. Z. Hajouj, J. Thomas, A. M. Siouffi, J. Liquid Chromatogr., **18**, 887-894 (1995).

6. H. T. Butler, M. E. Coddens, S. Khatib, C. F. Poole, J. Chromatogr. Sci., **23**, 200-207 (1985).

7. P. L. Smith, W. T. Cooper, Chromatographia, **25**, 55-60 (1988).

Received June 13, 1995
Accepted June 23, 1995
Manuscript 3898

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2423–2441 (1996)

SEPARATION AND PURIFICATION OF INSULINS ON COATED SILICA SUPPORT FUNCTIONALIZED WITH SIALIC ACID BY AFFINITY CHROMATOGRAPHY

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ABSTRACT

High performance liquid affinity chromatography (HPLAC) is a powerful method for purification and analysis of biological compounds. It combines the speed and the efficiency of high performance chromatography with the selectivity of affinity chromatography methods. The selectivity depends, first, on the nature of the ligand used; this latter must exhibit a specific binding affinity towards the product to purify; second, it depends on adsorption and desorption processus that improve, or not, the protein affinity for the ligand.

The presence of N-acetyl neuraminic acid surrounding the insulin receptor structure shows that this acid may develop specific interactions with insulin.

We performed the grafting of sialic acid on coated silica supports. The performances of these supports towards insulin were studied by HPLAC. The ligand specificity was tested in presence of two forms of insulin, porcine and bovine insulins, which, differ from each other by only two amino acids in their structure. The support carrying sialic acid exhibits both affinity and specificity for insulin in solution, since it allows the separation of the two forms of insulin. This result is confirmed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) assay. RP-HPLC is a widely used method for the separation of several compounds that exhibit structural homologies, such as, for instance and in particular, the different forms of insulin.

In order to confirm the specificity and the affinity of the support bearing sialic acid for insulin, the elution of a pancreatic extract, consisting of numerous proteins including insulin, is performed. The support allows the insulin purification from a pancreatic extract with high purification yields.

INTRODUCTION

In high performance liquid affinity chromatography (HPLAC), a ligand is a component that may interact with the product to separate in a specific and reversible way. The original concept of ligand-product interaction in HPLC implies the notion of specificity. One ligand is specific for one given protein. The notion of strickness regarding ligand specificity has progressively evolved from very conservative, to a much wider concept, with the appearance of very broad specificity ligands. These latter, are called general or pseudo-specific ligands such as, for instance, enzyme cofactors, calmoduline[1] or lectines[2] or non biologic molecules as dye[3] and metallic ions.[4] Because of their broad specificity, a same affinity support can be used to purify a series of proteins.

The ligand used in the present study, N-acetyl neuraminic acid (NANA) belongs to the family of "general ligands". It binds IgG[5] just as well as sialidases[6] and it develops an affinity for insulin too.[7,8] At first sight, one can consider that there is no specificity; nevertheless, the particular and selective conditions of adsorption and desorption of each of these proteins create the specificity. Indeed, the conditions that allow the binding of IgG or sialidase on the support do not permit the fixation of insulin on immibilized sialic acid. Moreover, the conditions used to adsorb insulin on the support (water 5% methanol)[7] are little selective. Therefore, we felt the need to find another method to confirm, or not, the specificity of our support for insulin.

Table 1

Interspecies Variations of Insulin Amino Acid Sequence (18)

Species	A-8	A-9	A-10	B-30
Human	Thr	Ser	Ile	Thr
Bovine	Ala	Ser	Val	Ala
Porcine	Thr	Ser	Ile	Ala
Sheep	Ala	Gly	Val	Ala
Horse	Thr	Gly	Ile	Ala
Sperm Whale	Thr	Ser	Ile	Ser

In recent years, a large number of reports of studies of the reversed phase chromatography of insulin have been published. In most of them, insulin has been used as one among several polypeptides or proteins in order to caracterize the separation capacity of the system.[9-16] The separation of insulin and its derivatives represents a typical challenge to the separation capacity of reverse phase supports.

Insulin is a 6000 g/mol polypeptide constituted of 51 amino acids contained within two peptide chains: an A chain with 21 amino acids and a B chain with 30 amino acids.[17] The chains are connected by two disulfide bridges. In addition, there is an interchain disulfide bridge that links positions 6 and 11 in the A chain. The cleavage of disulfide bond implies a loss of insulin activity. This dimeric structure is conserved in the animal kingdom but slight variations are observed according to the species. Porcine insulin differs from human by only one amino acid, alanine instead of threonine at the carboxyl terminus of the B chain (position B30), and from bovine by two amino acids A^8 and A.[10] (see Table 1).[18]

The performance of our support was tested towards both bovine and porcine insulin, in order, to evidence the specificity of our ligand for insulin. Such work could also contribute to a better understanding of interaction mechanisms between insulin in solution and the ligand immobilized in HPLAC. The support was used, then, to attempt the purification of insulin from a pancreatic extract.

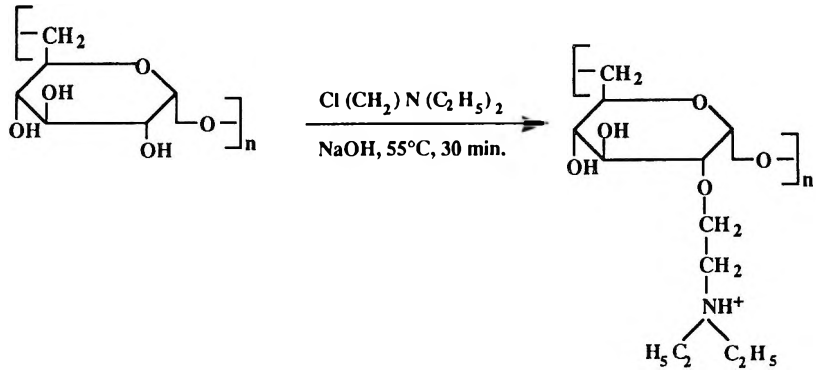


Figure 1. Reaction of substitution of dextran by 2-chloro-N,N-diethylaminoethan.

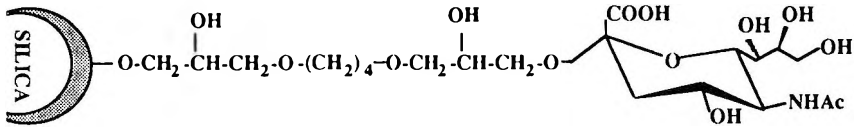


Figure 2. Structure of affinity support (SID-NANA).

MATERIALS AND METHODS

Synthesis of the Affinity Support

The synthesis of coated silica supports functionalized by N-acetyl neuraminic acid was carried out as reported previously.[7] The preparation of the affinity support is performed in two steps. First, silica beads are coated with dextran substituted by a calculated amount of diethyl-aminoethyl (DEAE) functions to hide negative charges at its surface. Second, ligands are immobilized using a coupling agent. The substitution of dextran T70 (68 000 g/mol) (Pharmacia, Bois d'Arcy, France) by 2-Chloro-N,N-diethyl-aminoethane (Janssen Chemica, Noisy Le Grand, France) is performed in a very alkaline medium at 55°C for 30 min. (Fig. 1).

The substitution rate of dextran with DEAE is determined by elemental analysis of nitrogen. The conditions for dextran modifications for an optimal passivation were previously determined[19,20] to obtain a proportion of dextran units carrying DEAE groups (Dx-DEAE) varying from 4 to 13%.

Silica beads (particle size 15-25 µm, porosity 1000 Å), kindly provided by Biosepra (Villeneuve la Garenne, France), are impregnated with a modified dextran solution (8 g of Dx-DEAE in 100-mL) adjusted to pH 11. Dextan coated silica is cross-linked with 1,4-butanedioldiglycidyl ether (BDGE) (Sigma, La Verpillière, France). The amount of Dx-DEAE covering the silica (SID) beads is determined by spectrophotometric assay of the sugar units after acid hydrolysis and by elemental analysis of carbon. Prior to the ligand coupling, the quality of the Dx-DEAE coverage of the silica support is evaluated by testing the elution of standard proteins on the support under high performance size-exclusion chromatographic (HPSEC) conditions.

The immobilisation of N-acetyl neuraminic acid on SID requires the use of a coupling agent to create covalent bonding between the ligand and the support (SID-NANA). In this study, NANA is coupled to the activated support by 1,4-butanediol diglycidyl ether (BDGE), a diepoxide agent, as previously described.[7]

Activation of dextran coated silica is carried out with BDGE in diethylether. NANA is coupled to the activated support in carbonate buffer for 48h. The amount of ligand fixed on the support is determined by spectrophotometric assay on the coupling solution supernatant using a periodate-resorcinol method.[21]

Elution of Insulin on SID-NANA in HPLAC

The HPLAC system consists of a pump (Spectra P100, Thermo Separation Products, Les Ulis, France) monitored by a programmer and equipped with an injection valve (Model 9126, Rheodyne, Merck, Nogent-Sur-Marne, France), connected to an UV-visible spectrophotometric detector (L-4000; Merck), an integrator (D-2520 GPC integrator; Merck) and a fraction collector (Model 203, Gilson, Sarcelles, France). The insulin used in HPLAC, is kindly provided by Diosynth S.A. (Akzo, Eragny-sur-Epte, France).

A 100 µL amount of insulin (porcine or bovine insulin or both porcine and bovine insulins), is injected on to the column (12.5 x 0.4 cm I.D.) containing the SID-BDGE-NANA support (the use of an injection vanne), at a flow-rate of 0.5 mL/min. The fractions corresponding to elution and desorption peaks are collected and analysed, first by SDS-PAGE electrophoresis (Phast system,

Pharmacia, Saint Quentin en Yvelines) and, second by reverse phase HPLC using a RP-18 column (5 μm) LiCrospher 100 (MERCK). The optimisation of the elution conditions used were determined previously.

RESULTS AND DISCUSSION

Synthesis of an Affinity Support

The first attempts to separate proteins in HPLC on unmodified mineral phases (silica or glass with a controlled porosity), have shown the presence of non specific interactions[22] then, mineral phases were modified by coating of hydrophilic polymers.[23,24] In our laboratory, we are currently using silica based supports which are coated with a calculated amount of diethylaminoethyl groups.

The dextran substitution is performed in order to confer a weak anion exchange capacity by DEAE groups into glycosidic units. The substitution rate of T70 dextran polymers by DEAE is 5% in our experimental conditions. The adsorption of Dx-DEAE on silica beads, results from interactions between anionic groups on the silica surface and DEAE groups carrying positive charges.

The coverage is further strengthened by cross-linking dextran chains with BDGE around the silica particles, that leads to the formation of ether-type bonds with polysaccharide hydroxyl groups. The polymeric coverage on the silica support, determined by elemental analysis, is 35 mg of Dx-DEAE per gram of silica.

In order to ascertain that the stationary phase used for coupling of the ligand will not undergo non-specific interactions with standard proteins, these proteins are eluted on the SID support under high performance size-exclusion chromatographic (HPSEC) conditions.[7]

The results show that no interaction occurs in the selected elution conditions. The SID support is neutral enough to be coupled to a biospecific ligand and to avoid non specific interactions with proteins in solution.

The BDGE activation is obtained by an hydroxyl function of the support with hydroxyl groups of the ligand (Fig. 2). The amount of fixed NANA on SID support is 10 mg/g silica and the coupling yield amounts 50%.

Table 2

Elution Conditions Used

	Ta	Tb	Gradient
a	Water 5% meethanol pH 8	0.05M phosphate buffer, 1M NaCl	0-10 min: 100% Ta 10-25 min: Ta→Tb 25-35 min: 100%Tb
b	Water 5% methanol pH 8	0.05M phosphate buffer, 1M NaCl	0-10min: 100% Ta 10-25 min: Ta→Tb 25-35 min: 100% Tb
c	Water 5% methanol pH 8	(0.05 M phosphate buffer, 0.2M NaCl): 90% ACN: 10%	0-10 min: 100% Ta 10-25 min: Ta→ Tb 25-35 min: 100% Tb
d	Water 5% methanol pH8	(0.05M phosphate buffer, 0.2M NaCl): 90% ACN: 10% Tc: distilled water	0-10 min: 100% Ta 10-25 min: Tc→ Tb 25-35 min: 100% Tb
e	Water 5% methanol pH 8	(0.05M phosphate buffer, 0.2M NaCl): 90% 48%Tc ACN: 10% Tc: distilled water	0-10 min: 100% Ta 10-18 min: Tc→52% Tb- 18-25 min: 52% Tb-48%Tc 25-35 min: 52%Tb-48%Tc →100%Tb
f	Water 10% methanol pH 8	0.05M phosphate buffer: 90% ACN: 10%	0-10 min: 100%Ta 10-16 min: Ta→50% Tb-50%Ta 16-25 min: 50%Tb-50%Ta 25-30 min: 50%Tb-50%Ta →100%Tb 30-35 min: 100%Tb

Ta: Adsorption buffer
Tb: Desorption buffer

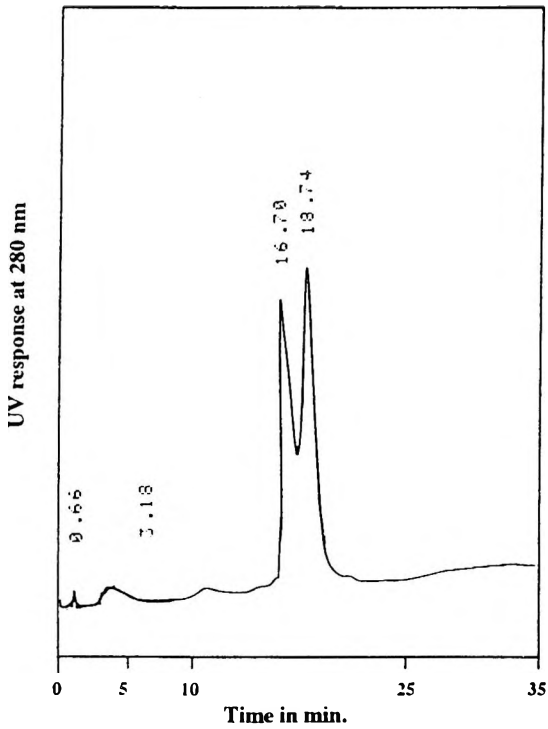


Figure 3. Elution of 100 µL of 50% porcine insulin with 50% bovine insulin mixture (1mg/mL) on SID-NANA. Column: 12.5 x 0.4 cm I.D.; Flow-rate: 0.5 mL/min; Eluents and elution program: See Table 2, f.
Note the fractions are called F1 and F2 (F1: Fraction of the first desorption peak; F2: Fraction of the second desorption peak).

Table 3

Purification Yield of Insulin on SID-NANA

Injection Quantity (µg)	F0 (µg)	F1 (µg)	Purification Yield (%)
85.3	11.2	74.1	87

Elution of Insulin on SID-NANA in HPLC

The elution profiles of a commercial mixture of insulin (porcine + 6% bovine) on the affinity support carrying sialic acid[7] show two desorption peaks. The Bradford assay[25] and SDS-PAGE electrophoresis show that the ratio between the two peaks corresponds to the mixture initially introduced on to the column. We, then, performed the elution of a 100µl of 50% porcine insulin with 50% bovine insulin mixture in the same conditions as described previously (Table2, a). A single peak of desorption is obtained; the two forms of insulin are not separated. We assumed that the second peak obtained from the elution of the mixture of insulin (porcine + 6% bovine) is due to the presence of desamidoinsulin in our sample.

Among the two main steps of HPLC i.e adsorption and desorption, the importance of the latter to increase the specificity throughout the elution step is well-known. We studied the influence of the desorption conditions on insulin elution, in order, to selectively unbind the two forms of insulin. These latter differ from each other by their hydrophobicity. For this purpose, we used mobile phases identical to those applied in RP-HPLC on C_{18} phase. Table 2 represents the different desorption conditions used in our study.

The use of a mixture, consisting of 70 volumes of 0.05M monosodic phosphate with 0.2M NaCl and about 30 volumes of acetonitrile (70:30, v/v), did not allow the separation of the two types of insulin (Table 2, b). Acetonitrile (ACN) high concentration could lead to a cleavage of interactions between both insulins and the chromatographic support. The ACN concentration is then decreased up to 10%; this did not affect the resolution of the two proteins (Table 2, c).

In order to optimize the conditions of desorption, we used a linear gradient of eluting buffer and TB (Table 2, d). Elution of bovine:porcine insulin mixture results were unsatisfactory, since a very poor second peak shape is obtained. To improve the two peaks resolution, we used a slightly concave gradient (Table 2, e) of same composition; two desorption peaks are obtained with similar retention times. The use of such a gradient seems to promise some improvement in the resolution of the different components of the sample.

The elution of the mixture is performed with some modifications of the mobile phase and the elution gradient. On one hand, NaCl, favouring hydrophobic interactions, is removed from the desorption buffer; on the other hand, the adsorption buffer is increased in methanol concentration to improve the binding yield.[7] The use of a broad concave gradient (Table 2, f), allows us to obtain a good separation of the two types of insulin with a difference of 2 min in retention times (Fig. 3). In order to identifly the two peaks, the elution of each

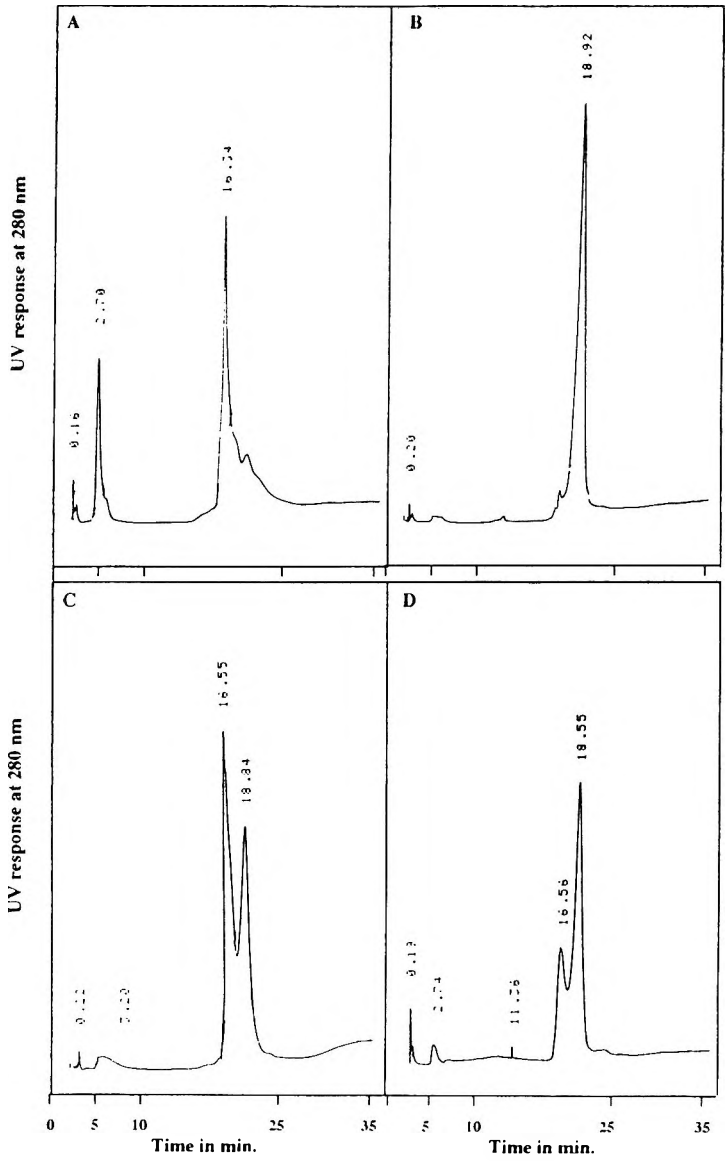


Figure 4. Elution of 100 µL amount of insulin (1 mg/mL) on SID-NANA (the same conditions as described in Figure 3). A: Porcine insulin; B: Bovin insulin: C: Porcine insulin with 10% bovine; D: Bovine insulin with 10% procine.

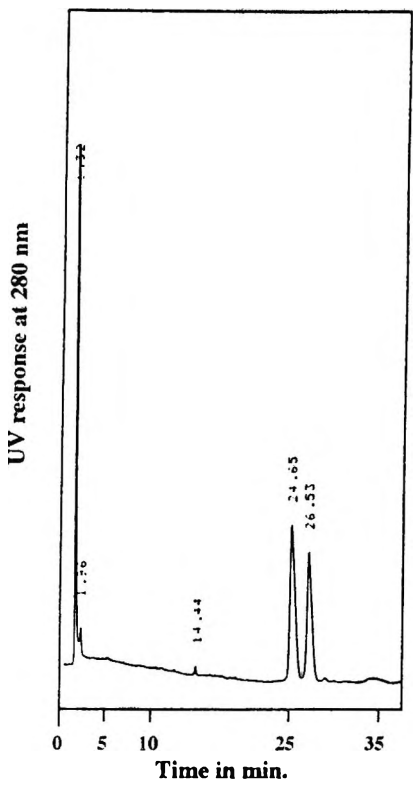


Figure 5. Elution of 100 µL of 50% procine insulin with 50% bovine insulin mixutre (1 mg/mL) on C_{18} (5 µm) LiChrospher 100. Column: 125 x 0.4 cm I.D.; Flow-rate: 1 mL/min; Eluants: Ta: Water 0.1 % TFA; Tb: Acetonitrile.

type of insulin, as well as, a mixture of porcine insulin with 10% bovine insulin and a mixture of bovine insulin with 10% porcine is performed. The elution profiles are presented in Figure 4. The individual elution of porcine insulin and bovine insulin is obtained; the size ratio of the peaks is consistent with the mixture initially introduced on the support. Moreover, bovine insulin develops a higher affinity for sialic acid than porcine insulin since it is eluted the last.

It is observed, that the same sample analyzed several times throughout the day, shows an increasing retention time, with a linear relationship existing between the retention times of the different components and the duration of the analysis due to the evaporation of the ACN. The fractions corresponding to the desorption peaks were collected, concentrated by means of micro-concentrator

of porosity 1000 and analyzed by SDS-PAGE electrophoresis. The presence of insulin is caracterized by a migration band of 6000 g/mol. Insulin is present in all fractions; insulin retained on the column is in a monomeric state since no migration band of 12000 g/mol occurs. The selective elution of porcine and bovine insulins on SID-NANA show evidence that this support develop distinct affinities for these two proteins. To confirm these results, collected fractions are analysed by RP-HPLC. RP-HPLC is a powerful method to detect small differences in the structure of different compounds and particularly in their hydrophobicity.

First of all, we undertook the optimization of elution conditions that will allow the separation of the two types of insulin with a significative resolution; then we analyzed the collected fractions from the two proteins elution in affinity chromatography on SID-NANA. We performed the elution of a mixture (50%/50%) of porcine and bovine insulins by RP-HPLC whose base principle is a very polar mobile phase and an hydrophobic stationary phase. Several modifications of the mobile phase are selected to achieve a satisfactory resolution.

First, the concentration of the organic component is fine tuned, checking how the percentages of ACN used could produce drastic alteration in the retention times. Then, a low concentration of trifluoroacetic acid (TFA) (0.1%, v/v) is added to improve the resolution by increasing the polarity of the mobile phase. A 100 μL amount of porcine or bovine insulin or a mixture of 50%-50% is injected on to column (C_{18}) using a concave gradient from water 0.1% TFA to 40% ACN. The composition of solvents and the elution program are presented in Figure 5. Elution profiles show that the two types of insulin are eluted selectively.

The analysis of collected fractions from the insulin samples elution on SID-NANA by RP-HPLC, reveal (Fig. 6) that the fractions corresponding to the first desorption peaks and the second desorption peaks are eluted at the same time as, respectively, porcine insulin and bovine insulin. This result confirms that porcine insulin is eluted before bovine insulin on the support carrying sialic acid and that the reverse order is obtained compared to their elution on C_{18} support.

Comparaison of Two Methods: HPLAC and RP-HPLC

In order to understand the interaction mechanisms that govern the separation of the two forms of insulin on the support functionalized with sialic acid, we performed the elution of insulins (porcine + bovine), first on the support carrying NANA under the elution conditions used in RP-HPLC and

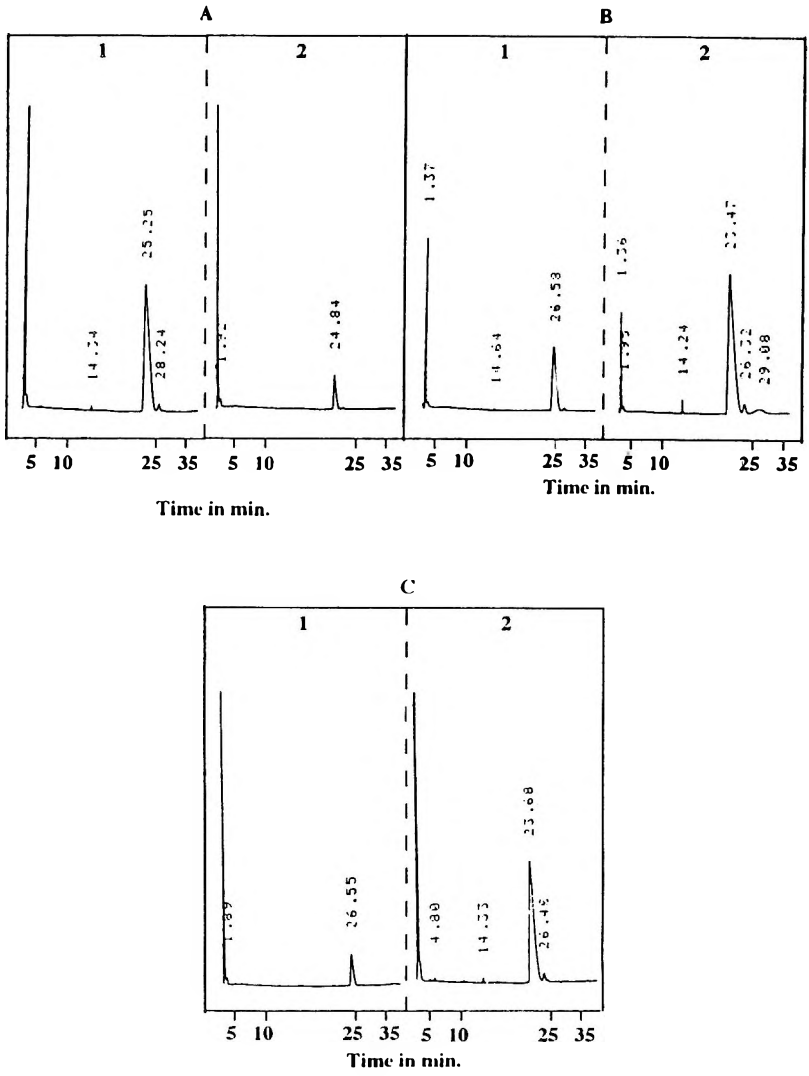
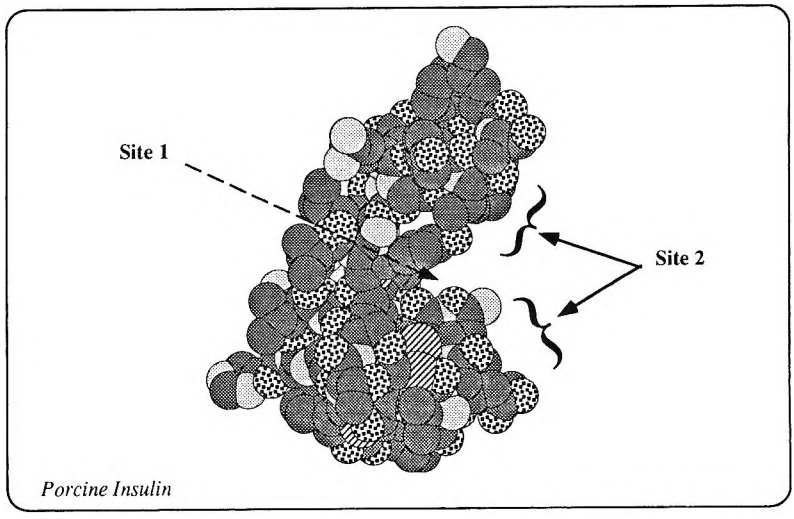


Figure 6. Elution of a 100 µL amount of collected fractions on C_{18} (5 µm) same conditions as described in Figure 5. A: 1-F1 of the insulins mixture 90% porcine + 10% bovine; 2-F2 of the insulins mixture 90% procine + 10% bovine; B: 1-F1 of the insulins mixture 50% porcine + 50% bovine; 2-F2 of the insulins mixture 10% procine + 90% bovine; C: 1-F1 of the insulins mixture 10% procine + 90% bovine; 2-F2 of the insulins mixture 10% porcine + 90% bovine.



Site 1= A (Thr8-Ser9-Ile10) for Porcine Insulin
A (Ala8-Ser9-Val10) for Bovine Insulin

Site 2= A (Gly1-Val3-Glu4)
B (Arg22-Gly23-Phe24-Phe25)

Figure 7. Putative interaction sites between insulin and NANA grafted on SID.

second, on a column C_{18} by using the mobile phase applied in HPLAC. Most of insulin mixture eluted on SID-NANA under RP-HPLC conditions are not retained on the support. A low amount of insulin is adsorbed and desorbed from the column within a single peak. This result demonstrates that the two forms of insulin are eluted similarly. The conditions used in RP-HPLC allow the protein to develop hydrophobic interactions with the support. The hydrophobic interactions alone do not allow neither insulins retention on the support nor their separation.

DISCUSSION

We have previously demonstrated[7] that the interactions between insulin and the support carrying sialic acid, are complex and involve simultaneously hydrophilic, hydrophobic and ionic interactions. The cooperative effect of all these different interactions determines the affinity and the specificity.

The predominance of one type of interaction, depends on the nature of the elution solvent used. According to the solvent nature, different conformational changes of insulin occur so that the region of insulin structure implied in the insulin-ligand interaction is different. This interpretation infers the existence of different active sites on the insulin molecule.

In order to have an understanding of these interaction mechanisms, one must modelize the insulin molecule as an hydrophilic surface carrying negative charges and surrounding an hydrophobic pocket. A conformational modification occurs and regulates the expression of different apolar regions at the protein surface modifying the insulin-supports interactions. Porcine insulin differs from bovine insulin by only two amino acids A^8 and A^{10} (Table 1), the SID-NANA support allows the distinct resolution of each protein under well defined conditions. This performance suggests that residues A^8 and A^{10} are somehow involved in the interactions between the support and the peptide.

The conditions which allow the separation of the two different forms of insulin on the support, probably favour the appearance of a different active site for each protein at the protein surface. The difference in the affinity of the two types of insulin let us assume, that the active site (site 1) is constituted of the amino-acid sequences from A^8 to $A,^{10}$ Thr 8-Ser 9-Ile 10 for porcine insulin, and Ala 8-Ser 9-Val 10 for bovine insulin (Fig. 7). When the conditions used do not permit the separation of the two forms of insulin, it may suggest the occurence of a same binding site for both proteins at the surface. This site (site 2) is constituted of the amino-acid sequences from B 24 to B27 (Phe B24-Phe B25-Tyr B26-Thr B27) and from A1 to A4 (Gly 1-Val 3-Glu 4)[25] (Fig. 7). The interaction between insulin and the immobilized ligand occurs through the sequence (site 1) which provides the specificity to the complex and other regions (site 2) which ensure its stability.

Purification of Insulin from Pancreatic Extract

The present study evidences that the support bearing sialic acid allows a good resolution of the two types of insulin. The separation of both insulins was performed in purified medium.

In order to confirm the selectivity of the support for insulin, we proceeded, then, to the elution of a pancreatic extract consisting of numerous proteins including insulin. The pancreatic extract was kindly provided by J. OLIVIE from societe Diosynth S.A. (Akzo, Eragny-Sur-Epte, France).

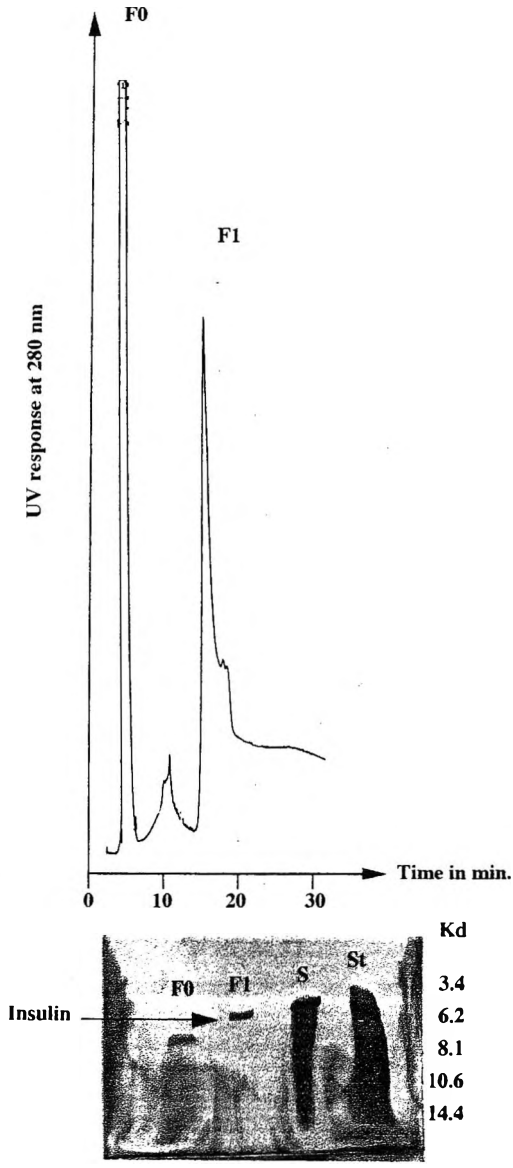


Figure 8. Elution profile of extract (100µL) on SID-NANA. For experimental details, see legend to Figure 3. SID-PAGE of pooled fractions F0 and F1 shown in chromatogram. unfractioned pancreatic extract (S) and standard proteins (St).

The chromatogram of the Figure 8 was obtained by injection of a 100 µl amount of a pancreatic extract, under the same conditions as those used for the resolution of the two types of insulin. The fractions corresponding to elution and desorption peaks are collected and analyzed by SDS-PAGE. The presence of insulin is revealed by a migration band of 6000 g/mol. The results described in figure 8 show that only insulin is retained and evidence the selectivity of the support towards insulin. Furthermore, the amount of insulin contained in the injected samples and the eluted fractions, is determined by RadioImmunoAssay (RIA, Cis biointernational, Gif Sur Yvette, France). The results presented in Table 3 show a purification yield of insulin of 87% from a pancreatic extract.

Finally, the silica based support coated with dextran-DEAE functionalized by sialic acid, allows a good resolution of the two types of insulin and the insulin purification from protein mixture. These results evidence the affinity and the specificity of sialic acid for insulin.

CONCLUSION

The present study demonstrates the influence of the mobile phase, especially the desorption phase on the performance of the affinity support functionalized with sialic acid residus. The interactions developed between insulin and the chromatographic support depend strongly on the nature of the eluting solution used. Insulin presents two binding sites, site 1 and site 2, which are not simultaneously expressed at the protein surface. In contrast to site 2, the expression of site 1, at insulin surface, allows the distinct resolution of each form of insulin. The expression of each site is regulated by reversible conformational changements that depend on the nature of the mobile phase used. The insulin-immobilized ligand interaction occurs through site 1, which ensures both specificity and selectivity of the complex and through site 2, which maintains the stability of the complex.

The support functionalized with sialic acid, exhibits an affinity for insulin with a high binding yield. It allows the separation of the two forms of insulin, i.e porcine and bovine insulins. The reverse elution order of the two forms of insulin obtained in HPLAC, in comparison with RP-HPLC, shows that the interaction mechanisms which permit the separation of the two insulins differ from those involved in RP-HPLC.

The support also allows the insulin purification from a pancreatic extract with high purification yields. The use of such a support could be broadened out to the separation of other forms of insulin, as well as, insulin analogues. These results may also contribute to a better understanding of interaction mechanisms between insulin with its receptor.

ACKNOWLEDGEMENTS

The authors would like to thank Mr J. Olivie for the samples of insulin and Mrs R. Siali-Lakhiari for revising and enhancing the quality of the manuscript.

REFERENCES

1. C. B. Klee, D. L. Newton, M. Krinks, in **Affinity Chromatography and Biological Recognition**, pp. 55, Academic Press, NY (1983).

2. M. Caron, D. Bladier, R. Joubert, Int. J. Biochem., **22**, 1379-1385 (1990).

3. C. R. Lowe, J. Pearson, Methods in Enzymology, **104**, 97-113 (1983).

4. A. M. Vijayalakshmi, in **Affinity Chromatography and Biological Recognition,** pp. 269-273, Academic Press, NY (1983).

5. A. Serres, E. Legendre, D. Muller, J. Jozefonvicz, J. Chromatogr., in press (1995).

6. J. Suzuki, K. Murakami,Y. Nishimura, J. Carbohydr. Chem., **12**, 201-108 (1993).

7. H. Lakhiari, E. Legendre, D. Muller, J. Jozefonvicz, J. Chromatogr. B., **664**, 163-173 (1995).

8. H. Lakhiari, D. Muller, J. Jozefonvicz, J. Chromatogr. A., **711**, 93-103 (1995).

9. B. V. Fisher, D. Smith, J. Pharm. Biomed. Anal., **4**, 377-387 (1986).

10. M. Ohta, H. Tokunaga, T. Kimura, Chem. Pharm. Bull., **31**, 3566-3570 (1983).

11. G. Szepesi, M. Gazdag, J. Chromatogr., **218**, 597-602 (1981).

12. D. J. Smith, R. M. Venable, J. Collins, J. Chromatogr. Sci., **23**, 81-88 (1985).

13. L. F. Lloyd, P. F. Corran, J. Chromatogr., **240**, 445-454 (1982).

14. A. N. McLeod, A. Manuer, S. P. Wood, J. Chromatogr., **502**, 325-336 (1990).

15. F. Vincent Warren, A. Brian Bidlingmeyer, J. Liq. Chromatogr., **8**, 619-634 (1985).

16. I. Ismail Salem, M. C. Bedmar, M. M. Medina, A. Cerezo, J. Liq. Chromatogr., **16(5)**, 1183-1194 (1993).

17. F. D. Steiner, Diabetes, **26(4)**, 322-344 (1977).

18. L. Hovgad, Thesis, University of Utah (1991).

19. X. Santarelli, D. Muller,J. Jozefonvicz, J. Chromatogr., **443**, 55-62 (1988).

20. F. L. Zhou, D. Muller, X. Santarelli, J. Jozefonvicz, J. Chromatogr., **476**, 195-203 (1989).

21. G. W. Jourdian, L. Dean, S. Roseman, J. Biol. Chem., **246**, 430-435 (1971).

22. S. Hjersten, J. Chromatogr., **87**, 325-331 (1973).

23. R. J. Blagrove, M. J. Frenkel, J. Chromatogr,. **132**, 399-404 (1977).

24. L. Letot, J. Lesec, C. Quivoron, J. Liq. Chromatogr., **4**, 1311-1322 (1982).

25. M. M. Bradford, Anal. Chem., **72**, 248-254 (1976).

26. N. McDonald, J. Murray-Rust, T. Blundell, Brit. Med. Bull., **45,** 2, 554-569 (1989).

Received January 23, 1996
Accepted February 25, 1996
Manuscript 4072

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2443–2456 (1996)

PREPARATIVE ISOLATION OF A SOLUBLE FORM OF BOVINE LUNG ANGIOTENSIN CONVERTING ENZYME BY AFFINITY AND SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

A high capacity process is described for the preparative purification of a soluble form of bovine lung angiotensin I-converting enzyme by affinity and size exclusion chromatography. The affinity purified enzyme was solubilized by tryptic attack for 1 h at 300C and separated by Sephacryl S-300 HR chromatography. A recovery of 68% was obtained. The purification procedure described here, enables one to obtain 27 mg of enzyme with a specific activity of 26 min^{-1} mg^{-1} from 1 kg of bovine lung. Molecular mass of native soluble ACE form was obtained by size-exclusion high performance liquid chromatography. Molecular mass of membrane-bound enzyme and the ACE form solubilized with trypsin, was found to be 170 kDa and 160 kDa, respectively, using disc gel electrophoresis in the presence of sodium dodecyl sulfate.

INTRODUCTION

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1), is a membrane-bound metallopeptidase that plays an important role in blood pressure regulation. Inhibition of ACE is a widely used approach in the treatment of hypertension.[1] ACE hydrolyzes angiotensin I to the potent vasoconstrictor angiotensin II, and converts the vasodilator bradykinin into an inactive peptide.[2,3]

ACE is a glycoprotein that consists of a single polypeptide chain of an apparent molecular mass, ranging from 130 kDa and 180 kDa[4,5] with two homologous domains, each bearing a potential catalytic site.[6] The difference in the molecular mass is probably due to variations in the glycosilation pattern of the protein from different species.

ACE has been identified in many tissues and is specially abundant in lung. This enzyme is an integral membrane protein anchored to the plasma membrane via its hydrophobic C-terminus.[5] A soluble form of the enzyme also exists in plasma.[7] For its purification, membrane-bound enzyme can be solubilized by detergents or, incubating the membranes with trypsin.[5]

The detergent-extracted, membrane-bound ACE produces aggregates when the detergent is eliminated, while ACE, from the trypsin-extracted samples, is present in non aggregated form even in absence of detergent.[8]

In this work, we describe the trypsin or subtilisin treatment of the purified ACE, to obtain a soluble form of bovine lung ACE in absence of detergent. Purified aggregate ACE was solubilized by tryptic attack for 1 h at 300C. The soluble form was separated by Sephacryl S-300 HR chromatography. A recovery of 68% was obtained. Our purification procedure enables us to carry out a large scale preparation of a soluble ACE form, and to obtain 27 mg of enzyme with a specific activity of 26 min^{-1} mg^{-1} from 1 kg of lung.

MATERIALS AND METHODS

Materials

Standard liquid chromatographic materials used were: Epoxy-activated Sepharose 6B, Sephacryl S-300 HR and Superdex 200HR 10/30 for FPLC, all from Pharmacia. Trypsin from bovine pancreas, subtilisin from Bacillus subtilis, and phenylmethanesulfonyl fluoride (PMSF) were obtained from Boehringer Mannheim. Lisinopril, furanacryloyl-L-phenylalanilglycylglicine, and N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonicacid) (Hepes) were purchased from

Sigma. Ethylenediaminetetraacetic acid (EDTA) was obtained from Merck; other chemicals were bought from either Sigma or Merck. Veal lungs were obtained from a local slaughterhouse immediately after the animals were killed.

Measurement of Protein Concentration

Protein concentration was measured according to biocinchoninic acid (BCA).[9] For pure lung ACE solutions, the concentration was determined by using rabbit-lung-enzymespecific absorbance at 280 nm of 225,000 M^{-1} cm^{-1}.[10]

Measurement of ACE Activity

ACE enzyme assay was performed by the spectrophotometric method of Holmquist et al.[11] at 25°C, with furanacryloyl-L-phenylalanilglycylglicine as substrate. Reaction mixtures contained 100 μM substrate in 50 mM Hepes, 300 mM NaCl, 10 μM zinc acetate and 0.7 μg/mL to 2 μg/mL of enzyme at pH 7.5. Absorbance measurements at 334 nm were carried out in a Beckman DU-70 spectrophotometer with the cells maintained at 25°C. One unit of activity produces Δ $-A_{334}$/min of 1.0. The specific activity of the purified protein was 24-26 min^{-1} mg^{-1}.

Purification of ACE

Step 1.- Preparation of the Homogenate. Up to 1 kg of fresh veal lung bought from a local slaughterhouse was taken immediately to our laboratory in crushed ice. From this moment on, the entire procedure was carried out at 4°C. Small portions of the lung were homogeneized in a blender with 10 mM Hepes, 400 mM NaCl, pH 7, at a 5:1 volume:weight ratio.

The homogenate was centrifuged at 9,000xg for 60 min in a Beckmann J2-HS centrifuge. The supernatant was separated and the precipitate was washed twice with the same buffer.

Step 2.- Extraction with Triton X-100. The precipitate was dispersed in 10 liters of a solution containing 10 mM Hepes, 400 mM NaCl, 100 μM $ZnCl_2$ at pH 7 (buffer A), which was made 0.5% in Triton X-100, stirred for 1 h, centrifuged at 9,000xg for 60 min, and the pellet was discarded. We have tested the stirring time of 2 h and 3 h and approximately the same ACE activity was obtained in the supernatant.

Step 3.- Ammonium Sulphate Precipitation. The supernatant was made 34% w/v NH_4SO_4 by addition of solid amonium sulphate, and stirred for 2 h and centrifuged at 9,000xg for 30 min. The supernatant was discarded, and the pellet was redissolved in 750 mL of buffer A and dialyzed against several changes of the same buffer. After dialysis, if any turbidity was still present, a final 30,000xg centrifugation for 30 min in a Beckmann XL-90 ultracentrifuge got rid of it.

Step 4.- Affinity Chromatography. The dialyzed solution was applied to a lisinopril/Sepharose 6B affinity column equilibrated in buffer A. Lisinopril was coupled to the epoxy-activated-Sepharose 6B as described by Bull et al.[12] Approximately 46 mL of the exchanger in a 1.6x23 cm column was used for a purification starting from 1 kg of lung. The flow rate, during chromatography, was 15 mL/h. ACE bound to the lisinopril/Epoxy-activatedSepharose 6B affinity column, the overall retention was 95%. The column was washed with 5 column volumes of buffer A; 3 column volumes of 0.5 M NaCl; 5 column volumes of buffer A and 2 column volumes of 10 mM sodium phosphate at pH 7. After 1.0 bed volume of 10 mM sodium phosphate, 5 mM EDTA at pH 7, at a flow rate of 15 mL/h has passed through the column, the flow was stopped during 24 h.

The enzyme was then eluted with the same buffer, at a flow rate of 50 mL/h. Fractions of 1 mL were collected over 5 mL of 20 mM Hepes, 0.1 mM NaCl, 50 TM $ZnCl_2$ at pH 7, monitoring the absorbance at 280 nm. A protein peak was detected and its fractions were pooled. Phosphate and EDTA was then eliminated by dialysis, against a solution 10 mM Hepes, 0.1 M NaCl, 10 μM $ZnCl_2$, at pH 7. The enzyme was concentrated with an Amicon cell using a XM30 membrane, to a protein concentration of approximately 1 mg/mL. Gel electrophoresis, under denaturating conditions of the purified enzyme, is shown in Fig. 1. Only one band is seen, which clearly demonstrates a high degree of purity for the obtained enzyme. The specific activity of the enzyme was 20 units/mg.

Step 5.- Trypsin treatment. The protein solution (1 mg/mL) was incubated for 2 h with bovine pancreatic trypsin (0.2 % w/w with respect to protein), in 10 mM Hepes, 0.1 M NaCl, 10 μM $ZnCl_2$, at pH 7 and 30°C. Proteolytic action was stopped by addition of PMSF (1 mM). The trypsin solution (1 mg/mL) was freshly prepared before the experiment by dissolving the enzyme in 0.1 mM HCl.

Step 6.- Sephacryl S-300 HR Chromatography. The ACE solution was finally chromatographed in a gel filtration column. Approximately 140 mL of Sephacryl S-300 HR in a 1.6x70 cm column, was used for a purification starting from 1 kg of lung. The solution that had been incubated with trypsin was applied at a flow rate of 30 mL per hour to the gel filtration column, equilibrated in 10 mM Hepes, 0.1 M NaCl, 10 μM $ZnCl_2$, at pH 7, and the enzyme was eluted with the same buffer.

The active fractions were collected, pooled and usually concentrated with an Amicon cell using a XM30 membrane, to a protein concentration of approximately 3 mg/mL. In this solution the enzyme retains its full activity for at least 6 months at 4°C.

Protein Electrophoresis Under Denaturing Conditions.

Polyacrilamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) of the enzyme, was performed on 7% or 6% gels according to Laemmli[13] using a Bio-Rad Protean II electrophoresis apparatus. Coomassie Brilliant Blue R-250 was used for staining the protein bands. Commercial protein size markers were used for calibration (Pharmacia).

Molecular Mass Determinations

Analytical high performance liquid chromatography (HPLC) was carried out on a Beckman apparatus. Effluents were monitored with a diode array detector (DU168). FPLC column was Superdex 200 HR 10/30 from Pharmacia.

The column was calibrated with tiroglobuline (669 kDa), ferritin (440kDa), alcohol deshidrogenase (150 kDa), bovine serum albumin (67 kDa), ribonuclease (13,7 kDa) and Blue Dextran (2000 kDa) from Sigma. The column was equilibrated with 10 mM Hepes, 0.1 M NaCl, 10 μM $ZnCl_2$ at pH 7.

Proteolysis

Membrane-bound converting enzyme that had been eluted from lisinopril/Epoxy-activatedSepharose 6B affinity column (1 mg/mL), was digested with bovine pancreatic trypsin or subtilisin (1/1000-1/10 w/w with respect to ACE) in 10 mM Hepes, 0.1 M NaCl, 10 μM $ZnCl_2$, pH 7, at 25°C. Proteolytic action was stopped by dilution in 50 mM HEPES, 300 mM NaCl, 10 μM zinc acetate (pH 7.5) and the activity was assayed inmediately.

Proteolysis of ACE was also stopped with PMSF (1 mM) and the peptides were separated by Superdex 200 HR 10/30 HPLC. For the SDS-PAGE experiments, the proteolysis was stopped by precipitation with ice-cold trichloroacetic acid (TCA) (5% final concentration).

Table 1

Purification of ACE Starting from 1 kg of Bovine Lung

Purification Step	Volume (mL)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Extraction with Triton X-100	9,600	19,560	920	0.04	1	100
Ammonium Sulphate Precipitation	960	4410	830	0.20	5	90
Lisinopril/Epoxy-Sepharose 6B	90	29	635	22	550	70
Trypsin treatment and Sephacryl S-300 HR Chromatography	8	27	700	26	650	68

RESULTS AND DISCUSSION

Purification of ACE

The main features of the ACE purification procedure described in the presvious section are shown in Table 1. As can be seen, 27 mg of ACE, with a specific acitivity of 26 units/mg was obtained from 1 kg of lung. Our procedure is based on the method of Bull et al.[12] for the purification of the ACE from rabbit lung. Before affinity chromatography, a precipitation with ammonium sulfate was performed according to Pantoliano et al.[14] This step reduced the volume to apply on the affinity column ten times. In this way, the fractionation step with 18% w/v ammonium sulphate made by Pantoliano et al.[14] was not necessary. The principal step of the purification is the affinity chromatography. The adsorbed protein was eluted with 10 mM sodium phosphate, 5 mM EDTA at pH 7, and the EDTA was removed by dialysis overnight. If the enzyme is eluted with lisinopril, it is necessary to dyalize the sample for several days to remove the lisinopril.[12,15] The main difference in our procedure is the trypsin treatment and the use of Sephacryl S-300 HR chromatography, as the last purification step. This trypsin treatment enables us to purify a soluble form of the enzyme. Figure 2A, shows the profile of the Superdex 200 HR 10/30 elution, which is similar to that reported by gel

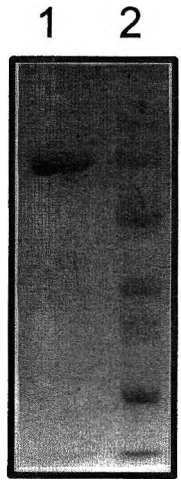

Figure 1. Purified bovine lung angiotensin converting enzyme (15 μg) separated by SDS-PAGE on 7% gels (lane 1). In lane 2 different molecular mass standards from Pharmacia were run, from top to bottom: myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), glutamic dehydrogenase (53 kDa).

filtration on Sephadex G-200 by Bull et al.[12] When ACE untreated with trypsin, was applied to the column, two protein peaks with ACE activity were obtained: a first major peak near the void volume, corresponding to aggregated angiotensin-converting enzyme with 21 units/mg, and a smaller peak, which traveled with apparent molecular mass around 310 kDa and had a higher specific activity of 26 units/mg. The area ratio was 9:1. Fractions of each peak were pooled and concentrated to approximately 0.4 mg/mL in a centricon 30 (Amicon) filter. The solutions were left one day at 4°C and were rechromatographed on the Superdex 200 HR 10/30 column. In each case, only one peak was obtained with the same elution time of the previous chromatography (Fig. 2, B and C).

Four samples of ACE (375-400 μg of protein) were incubated with increasing amounts of trypsin for 1h at 30°C in 10 mM Hepes, 0.1 M NaCl, 10μM $ZnCl_2$, pH 7, in a final volumen of 500 μL. After the reaction had been stopped with PMSF (1 mM), a fraction of each sample was applied to a column of Superdex 200 HR 10/30 on HPLC, which was equilibrated with the same buffer. Sample 1 (1/1000 w/w with respect to ACE), showed the same area ratio between peaks as that of samples untreated with protease (Fig. 2A).

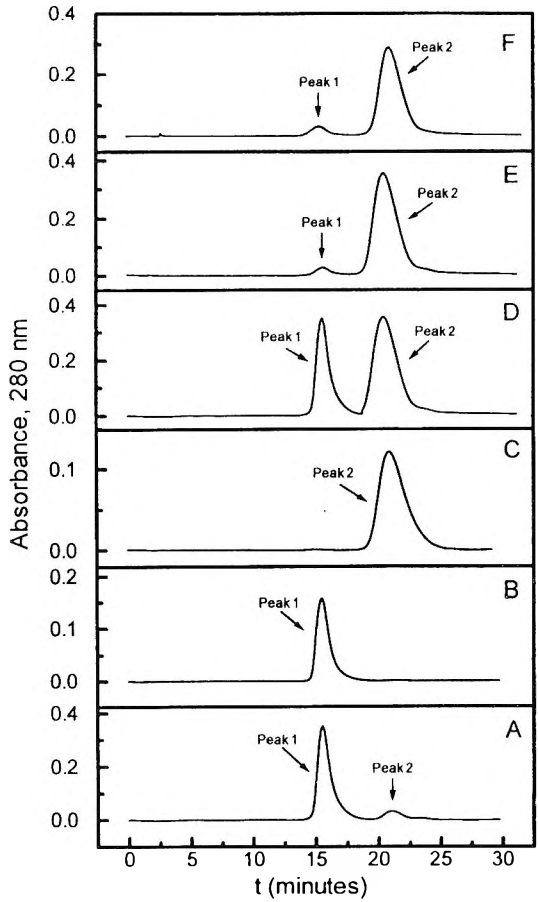

Figure 2. HPLC chromatograms of bovine lung angiotensin converting enzyme. The following samples were injected and absorbance monitored at 280 nm: (A) 500 µL ACE eluted on affinity column; (B) ACE from peak 1 fractions of chromatogram A; (C) ACE from peak 2 fraction in chromatogram A; ACE treated with trypsin: (D) 1 mg/ 500mg protein (E) and 1mg/200mg protein for 1 h at 300C; (F) ACE treated with subtilisin (1mg/200mg protein) for 1 h at 300C. The chromatography was performed on Superdex S-200 10/30 column equilibrated with 10 mM Hepes, 0.1M NaCl, 10 µM $ZnCl_2$ pH 7, at a flow rate of 0.5 ml./h at room temperature.

On increasing the amount of trypsin, the area of peak 2 was increased with respect to that of peak 1 (Fig. 2D). In the experiment to a 1/200 (w/w) trypsin/ACE ratio and higher, the 0.5 : 9.5 area ratio was obtained (Fig. 2E). The solubilization of

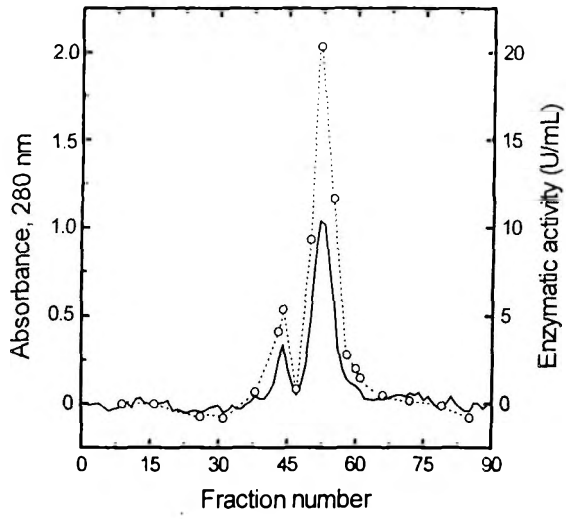

Figure 3. Elution profile in the Sephacryl S-300 HR chromatography; 29 mg of ACE incubated in the presence of trypsin and with a specific activity of 22 units/mg was applied on a 1.6x70 cm column and 1.3 mL fractions were collected. The enzymatic activity is shown with the open circles. The specific activity in the peak 2 pooled solution, containing 27 mg of protein, was 26 units/mg. The chromatography was performed at a flow rate of 30 mL/h at room temperature.

purified ACE was also carried out using subtilisin treatment. Figure 2F, shows an elution profile of a sample of ACE (0.75 mg/mL), incubated for 1 h at 30°C in the presence of subtilisin (1/200 w/w with respect to ACE) . The 1.5:8.5 area ratio was obtained. In all cases, the enzyme activity was 21 units/mg and 26 units/mg for the peak 1 and 2, respectively. The digestion with trypsin or subtilisin [1/200 (w/w) protease/ACE ratio], lets the enzyme to be solubilized. Thus, we introduced a trypsin treatment and the use of size exclusion chromatography as the last purification step. For 1 kg of bovine lung, we used a column of Sephacryl S-300 HR (1.6x70 cm). Fraction (1.3 mL) were collected at a flow rate of 30 mL per hour. As can be seen in Figure 3, the enzyme activity and amount of protein was obtained mostly closely parallel to that of peak 2 (0.5:9.50 area ratio). An elution profile similar to that shown in Fig. 3, was obtained when fractions of peak 1 were pooled, concentrated, incubated with trypsin in the same conditions indicated above and rechromatographed. Lanzillo et al.[8] had showed that the detergent-extracted membrane-bound converting enzyme produced aggregates, while the trypsin-extractedsamples were monomers.

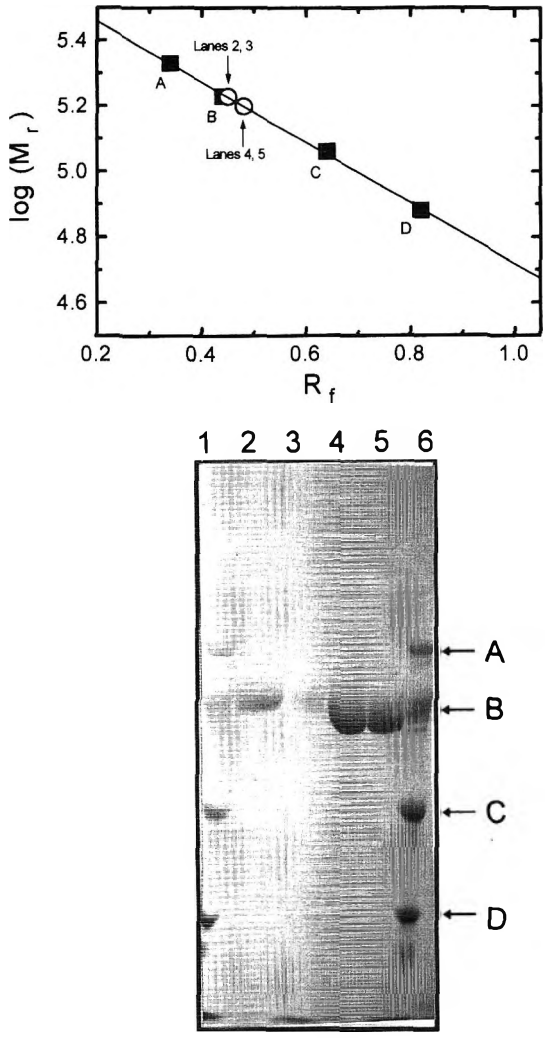

Figure 4. Slab gel electrophoresis of the samples obtained on the chromatographies in the Fig. 2. Trypsin-untreated ACE: lane 2 contained 12 μg of aggregate ACE from Fig. 2B; lane 4 contained 20 μg of soluble ACE form from Fig 2C. Trypsin-treated ACE: lane 3 contained 12 μg aggregate ACE from peak 1, Fig. 2E; lane 5 contained 20 μg of soluble ACE from peak 2 of Fig. 2E. Lanes 1 and 6 contained the following four protein molecular mass standards: A, myosin (212 kDa); B, α_2-macroglobulin (170 kDa); C, β-galactosidase (116 kDa); D, transferrin (76 kDa). Samples were electrophoresed in a 6% polyacrylamide gel under reducing conditions and were stained with Coomassie brilliant blue R-250.

Thus, the peak 1 in Figure 2, would correspond to ACE molecules with its membrane-bound sequence intact, which induces an aggregation among ACE molecules. Peaks 2 in Figure 2 might correspond to ACE molecules with the membrane-bound sequence deleted.

We have checked our purification procedure starting from lungs kept at -20°C for up to four months and have obtained the same results as above.

Molecular mass determination

Molecular mass determination was carried out by size exclusion chromatography on HPLC (SE-HPLC) and disc gel electrophoresis. The results of electrophoresis of chromatographed samples on Superdex 200 HR 10/30 column, under denaturing and reducing conditions, are shown in Fig. 4. Only one band is seen in all cases, the molecular mass of the fractions corresponding to the peak 2 is slightly smaller than those of the fractions of the peak 1.

Under these conditions, the subunit of aggregate species (peak 1, Fig. 2, B and E) were calculated to have a molecular mass of 170 kDa (lanes 2 and 3), which agrees with that reported previously.[3] Thus, the peak 1 in Fig. 2E should correspond to ACE molecules, which had not been cleavaged with trypsin. The molecular mass of peak 2 of the samples incubated in the absence (Fig. 2B), or the presence (Fig. 2F) of trypsin, were estimated to be approximately 160 kDa in SDS-PAGE (lanes 4 and 5).

These results seem to indicate, that the peak 2 (Fig. 2A) of samples untreated with trypsin, correspond to protein molecules that had lost a peptide of 10 kDa, probably from hydrophobic C-terminal part. These deletions might be carried out by a endogenous protease during the purification. The tryptic attack also produces the deletion of a peptide of approximately 10 kDa. Recently, Beldent et al.[16] have described that human plasma ACE is secreted from the membrane-bound enzyme, by the deletion of 140 amino acids (14 kDa) at the C-terminal part.

The calculated difference of 10 kDa, approximately in the apparent mass of the soluble ACE forms, separates by gel filtration without (Fig. 2A, peak 2) or with (Fig. 2D, peak 2) tryptic attack, is very close to the result described previously.[16] The discrepancy is probably due to the difficulty in obtaining precise high molecular mass by SDS-PAGE.

The results obtained by SE-HPLC, might indicate that the soluble ACE form obtained by treatment with trypsin, might be in its native state as homodimer. Although, it is necesary to consider that the linked oligosaccharides to polypeptide chain can interact with chromatography gel. This fact can modify its elution time.

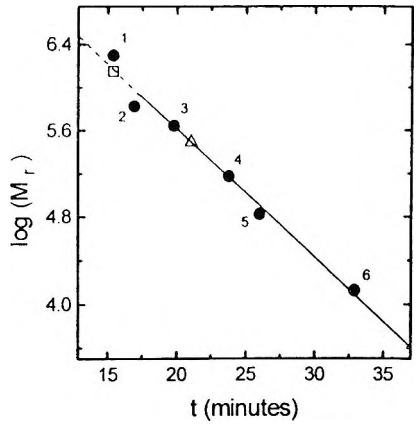

Figure 5. Molecular mass determination of the samples obtained on the showed chromatographies in the Fig. 2. Calibration curve for size exclusion-HPLC. A Superdex 200 HR 10/30 (10x300 mm) was equilibrated with 10mM Hepes, 0.1M NaCl, 10 μM $ZnCl_2$ (pH 7) and operated at 0.5 mL/h. At t=0 500 μL of the protein mixture (in the same buffer) were injected: Blue Dextram (2000 kDa), tiroglobuline (669 kDa), ferritin (440kDa), alcohol deshidrogenase (150 kDa), bovine serum albumin (67 kDa) and ribonuclease (13,7 kDa). Absorbance was monitored at 280 nm. The elution times are plotted vs log mol wt. The peak 1 fractions of the Fig 2B and 2E correspond to aggregates (□) while peak 2 fractions of the Fig 2C and 2E correspond to a soluble ACE form with a molecular mass of 310 kDa (Δ).

CONCLUSIONS

The described purification procedure enables us to obtain 27 mg of a soluble ACE form with a specific activity of 26 min^{-1} mg^{-1} , from 1 kg of bovine lung using affinity and size exclusion chromatography. A 650-fold purification was achieved with a 68% yield. A soluble angiotensin I-converting enzyme form was obtained from purified membrane-bound enzyme form, using trypsin or subtilisin treatment and size exclusion chromatography. The recovery of 93% was acheived. If peak 1 fractions (Fig. 3) were treated again with trypsin, a recovery of 96% can be obtained. The procedure described is more suitable than a tryptic digestion of the membranes, because of its higher yield and better purification. A molecular mass of 310 kDa was obtained for native soluble ACE form by size-exclusion HPLC. Molecular mass of membrane-bound enzyme and of the ACE form solubilized with trypsin, was found to be 170 kDa and 160 kDa, respectively, by SDS-PAGE. Thus, the trypsin solubilizes the protein by deletion of a fragment of 10 kDa, approximately.

ACKNOWLEDGEMENTS

This research was supported by grants PB93-0731 and PB93-1163 from DGICYT, Ministerio de Educacion y Ciencia, Spain. We are grateful for the excellent technical assistance of Dr. A. Cámara-Artigas.

REFERENCES

1. K. K. Griendling, T. J. Murphy, R. W. Alexander, Circulation, **87**, 1816-1828 (1993).

2. H. Y. T. Yang, E. G. Erdös, Y. Levin, Biochim. Biophys. Acta., **214**, 374-376 (1970).

3. M. R. Ehlers, J. F. Riordan, Biochemistry, **28**, 5311-5318 (1989).

4. R. L. Soffer, Annu. Rev. Biochem., **45**, 73-94 (1976).

5. N. M. Hooper, J. Keen, D. J. C. Pappin, A. J. Turner, Biochem J., **247**, 85-93 (1987).

6. L. Wei, F. Alhenc-Gelas, P. Corvol, E. J. Clauser, J. Biol. Chem., **266**, 9002-9008 (1991).

7. N. M. Hooper, Int. J. Biochem., **23**, 641-647 (1991).

8. J. J. Lanzillo, J. Stevens, Y. Dasarathy, H. Yotsumoto, B. L. Fanburg, J. Biol Chem., **260**, 14938-14944 (1985).

9. P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, D. C. Klenk, Anal. Biochem., **150**, 76-85 (1985).

10. R. Shapiro, J. F. Riordan, Biochemistry, **23**, 5225-5233 (1983).

11. B. Holmquist, P. Bünning, J. F. Riordan, Anal. Biochem., **95**, 540-548 (1979).

12. H. G. Bull, N. A. Thornberry, E. H. Cordes, J. Biol. Chem., **260**, 2963-2972. (1985).

13. U. K. Laemmli, Nature, **227**, 680-685 (1970).

14. M. W. Pantoliano, B. Holmquist, J. F. Riordan, Biochemistry, **23**, 1037-1042 (1984).

15. J. R. Schulleck, I. B. Wilson, Life Science, **45**, 685-690 (1989).

16. V. Beldent, A. Michaud, L. Wei, M. T. Chauvet, P. Corvol, J. Biol. Chem., **268**, 26428-26434 (1993).

Received February 28, 1996
Accepted March 18, 1996
Manuscript 4090

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2457–2476 (1996)

REDOX CHROMATOGRAPHY USING POLYPYRROLE AS A STATIONARY PHASE

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ABSTRACT

Chromatographic studies on the effect of exposure of polypyrrole coated stationary phases to redox reagents have been carried out. Polypyrrole containing chloride or dodecylsulfate as counterion was chemically synthesized and coated onto silica particles and packed into a chromatographic column. A series of standard compounds was used as the molecular probes. Changes in the chromatographic properties of polypyrrole as the result of the exposure to redox reagents were compared with those obtained by electrochemical treatment.

INTRODUCTION

Polypyrrole (Figure 1) is capable of a diverse array of molecular interactions.[1,2] Polymers with a great variety of properties can be produced by resorting to derivatives,[3,4] copolymers,[5,6] or incorporation of particular anions ($-A^-$)[7,8] during synthesis.

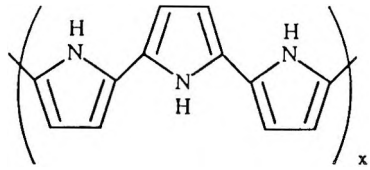


Figure 1. Polypyrrole.

Perhaps the most interesting property of conductive polymers is that their physicochemical properties can be modified *in-situ* by switching the oxidation state, either electrochemically or chemically. Several workers have made use of this property to design special liquid chromatographic columns,[9-11] with conductive packing materials. It has been demonstrated that the retention of analytes can be controlled by application of an electrical potential to the stationary phase.

The properties of conductive polymers can also be modified by exposure to chemical oxidants/reductants.[12-14] To date, however, the effect of chemical oxidation/reduction of conductive polymers on the chromatographic properties and, hence, molecular interaction capabilities, has not been reported. In this work, the effect of exposure to redox reagent solutions has been considered. Two polypyrrole-based conductive polymers, i.e., polypyrrole chloride and polypyrrole dodecylsulfate, were investigated. The redox reagents employed were ferric chloride and sodium sulfite.

For the purpose of the study, the polymers were chemically polymerised directly on the surface of silica particles which were then packed into chromatographic columns. A series of small molecules, polyaromatic hydrocarbons, basic drugs, and amino acids were used as the test compounds. The columns were chemically treated by injecting either ferric chloride or sodium sulfite solution.

EXPERIMENTAL

Reagents and Materials

All reagents were of analytical reagent (AR) grade unless otherwise stated. Pyrrole, LR grade (Fluka Chemika-BioChemika, Buchs, Switzerland) was distilled before use. HPLC grade methanol and acetonitrile were obtained from

Mallinckrodt Australia, Clayton, Victoria, Australia, while the water was purified using a Milli-Q water system from Millipore (Lane Cove, NSW, Australia). Benzene and toluene were purchased from Ajax (Auburn, NSW, Australia). Aniline and theophylline were commercially supplied by BDH (Poole, UK), while N,N-dimethylaniline (DMA) was from May and Baker (Dagenham, UK). Sodium sulfite, ferric chloride, sodium acetate (NaAc), and sodium chloride were from BDH, while sodium dodecylsulfate (SDS) and Tris-(hydroxymethyl amino methane) (Tris) was purchased from Sigma Chemical Co. (Castle Hill, NSW, Australia). Hydrochloric acid and glacial acetic acid (HAc) were obtained from Ajax. L-Tryptophan (Trp) and L-Tyrosine (Tyr) were also obtained from Sigma .

The acetate buffer solution, pH 3.8, was prepared from a mixture consisting of 440 mL 0.2 M HAc, 60 mL 0.2 M NaAc and 500 mL water. The buffer solution, pH 7.4, was prepared by mixing 420 mL 0.1 M HCl with 500 mL 0.1M Tris and 80 mL water. Packing materials were prepared as described previously.[9,10] Silica (Ultrasphere, Beckman Instruments, Gladesville, NSW, Australia) was used as received. The silica has particle size = 10 μm; surface area = 220 m^2/g; and pore size = 80 Å. Stainless steel columns (4.9 mm x 50 mm) were purchased from Alltech (North Strathfield, NSW, Australia). These columns were then packed with either polypyrrole chloride or polypyrrole dodecylsulfate-coated silica, using a column slurry packer, with methanol as the driving liquid. The test samples were dissolved in pure methanol or acetonitrile and diluted with the mobile phases if required. Trp and Tyr were dissolved in water, either individually or as a mixture.

Instrumentation

All chromatographic work was performed with an HPLC system which consisted of a Kortec K35D HPLC pump (ICI, Melbourne, Australia), a Rheodyne 7125 injector with 20 μL sample loop (Alltech), a variable wavelength UV-Vis detector (ICI) and a Kipp and Zonen BD41 strip chart recorder.

An electrochemical cell system, consisting of Pt disc (as working electrode), Ag/AgCl (as reference electrode), and a piece of RVC (as counter electrode) that was connected to a galvanostat (home-made), was used to prepare polypyrrole chloride-coated Pt electrode. A pH meter, Orion SA 520 (Linbrook, Thornleigh, MSW, Australia), was used for electrode potential measurement. Electrochemical redox manipulation was accomplished with a home-made potentiostat.

Chromatographic Measurements

Columns were flushed with water and methanol before use. The mobile phase flow rate was adjusted to 1 mL/min throughout the experiment. The eluent output was monitored at 254 nm. Retention times were recorded with a stopwatch and the dead time (t_0) was estimated from the retention of water. The mobile phase system used was a mixture of water-methanol or water-acetonitrile, the composition of which could be varied as required. The elution of amino acids was carried out with either buffer-methanol or buffer-acetonitrile eluent, depending on which column was being used.

The chromatographic properties of the column were manipulated by treating it with either 0.1 M Na_2SO_3 or 0.1 M $FeCl_3$. The reductant or the oxidant solution was injected into the column through the injector several times, while water was passing through the column at 0.3 mL/min. After each run of several injections, the column was flushed with 50-100 column volumes of water before the mobile phase to be used was employed.

Ferric chloride (E°= +0.771V) was chosen here as the oxidant for the following reasons. Firstly, it was used in the preparation of the stationary phase, thus avoiding any complication which could be brought in if using other chemicals. For example, if $Fe(NO_3)_3$ is used as the oxidant, NO_3^- would be incorporated into the polymer instead of Cl^- and this could change the properties of the film. Secondly, it has been reported that neutral polypyrrole films can be oxidised chemically by various metals such as Fe^{3+}.[15] The resulting oxidised films became more conductive.[16] Sodium sulfite is a common reductant (E^O = -0.93) and, because its reaction products are soluble in water, they are easily removed from the column.

RESULTS AND DISCUSSION

For the preliminary studies, the redox reagents were not included in the eluent, as this would complicate the interpretation of results. The inclusion of the oxidant/reductant in the eluent may also influence the chemical nature of the molecular probes being used and would certainly affect ion exchange processes occurring on the conducting polymer.

Conductive polymer films, such as polypyrrole, can be switched from conducting to non-conducting by reducing the polymer films, and they can be made conductive again by reoxidizing the film. The counterions are expelled during reduction and incorporated during oxidation according to:

$$(PPy)^{+}_{n}\,A^{-}_{(p)} \underset{-e}{\overset{+e}{\rightleftharpoons}} (PPy)^{o}_{n} + A^{-}_{(s)}$$

where $A^{-}_{(p)}$ is the counterion in the polymer and $A^{-}_{(s)}$ is the counterion in solution. This mechanism is applicable especially for small hydrophilic counterions. For large counterions, such as dodecylsulfate (DS^{-}), the situation could be different. According to Panero et al.[17] and Martinez et al.,[18] DS^{-} counterions are not easily released after the incorporation of the ions into the polymer matrix. This is not just because of their large size, but also because of the presence of the polar end and the long alkyl chain in the molecules, the former being compatible with the charged form of the polymer and the latter with the neutral polymer backbone. The counterion movement in the polymer containing such large counterions can be illustrated according to:

$$(PPy)^{+}_{n}\,A^{-}_{(p)} + C^{+}_{(s)} \underset{-e}{\overset{+e}{\rightleftharpoons}} (PPy)^{o}_{n}\,A^{-}_{(p)}\,C^{+}_{(p)}$$

where $C^{+}_{(s)}$ is the counter-cation in solution and $C^{+}_{(p)}$ is the counter-cation in the polymer. During polymer reduction, because the counter-anions are not easy to expel, the counter-cations are incorporated into the polymer to preserve the charge neutrality.

There have been some reports, however, that such large incorporated amphiphilic surfactant anions are released on the reduction of the polymer.[19-22] The loss could be up to approximately 50%.

In the study described hereafter $FeCl_3$ and Na_2SO_3 were used as redox reagents to manipulate the oxidation state of the stationary phases prepared from polypyrrole doped with Cl^{-} and DS^{-}. In oxidation-reduction reactions, the reagents work normally through electron transfer processes according to:

$$Fe^{3+} + e \rightleftharpoons Fe^{2+}$$

$$SO_4^{2-} + H_2O + 2e \rightleftharpoons SO_3^{2-} + 2OH^{-}$$

In the following discussion, a chromatographic examination of the effect of exposure of polypyrrole to the redox reagents is described.

Table 1

Elemental Composition of the Column Packings

Packings	Weight Percent				Mole Ratio		
	C	H	N	Cl	S	N/Cl	N/S
PPCl/Si	4.20	0.31	1.12	1.4	---	2.10	---
PPDS/Si	7.31	0.83	1.17	0.71	0.67	4.17	4.00

Table 2

Effect of Redox Treatment of PPCl/Si and PPDS/Si on Retention of Aniline and DMA*

	Redox Reagent Injection	PPCl		PPDS/Si
		Aniline k'	DMA k'	DMA k'
0.1M Na_2SO_3	0	1.8	1.7	4.7
	100	1.7	1.3	3.9
	200	1.0	0.6	3.5
	300	0.8	0.5	3.0
0.1M $FeCl_3$	40	2.7	2.2	3.4
	80	3.0	2.3	2.8
	120	---	---	2.4

* Mobile phase: 40% MeOH/H_2O for aniline and 60% MeOH/H_2O for DMA, at 1 mL/min.

Chemical Composition of Column Packings

The elemental composition of the polymer layer coated on the surface of silica particles for polypyrrole chloride (PPCl/Si) and polypyrrole dodecylsulfate (PPDS/Si) is presented in Table 1.

The mole ratio of N:Cl in PPCl/Si is 2.1 : 1.0, which suggests that one Cl^- counterion is associated with 2.1 monomer units. This result is lower than the expected value, which is normally in the range 3-4.[22] It has been reported, previously, that this probably is due to the fact that $[Fe(Cl)_4]^-$ was incorporated

as a counterion.[23,24] During PPyDS/Si preparation, both chloride and dodecylsulfate (DS^-) counterions were incorporated into the polymer matrix. The mole ratio of N : (Cl + DS) is 2.04 : 1.0, which indicates that every 2 pyrrole monomer units were associated with either one Cl^- or DS^- counterion.

Retention Behaviour of Benzene and Derivatives

The effect of chemical manipulation on PPCl/Si with the redox reagents upon the retention behaviour of benzene and toluene was investigated. No effect was found. Benzene and toluene are considered to be non-polar compounds. Their separation, in liquid chromatography, is therefore mainly determined by hydrophobic interaction.

In addition, benzene as an aromatic compound, is an electron donor through the π system. So also is toluene, which is even more hydrophobic due to the presence of the methyl group. Their retention on the column remained essentially unaffected by this redox manipulation. Similar results were obtained for PPDS.

Different results were obtained with aniline and DMA as test studies. The effect of redox reagent treatment of PPCl/Si on retention behaviour is presented in Table 2. The retention of these compounds decreased after the column was treated with the reductant and then increased even beyond the original retention values after the column was in contact with the oxidant. Aniline and DMA are amines which have a tendency to share their unpaired electrons; hence, they are electron donors through their lone-paired electrons.

The retention behaviour of aniline and DMA, after the column was treated with the reagents, indicated that such treatment changed the ability of the column to undergo electron donor-acceptor (EDA) interactions through the unshared paired electrons. The increased retention after reoxidation suggests that the polymer was then in a higher oxidation state than the rest potential that resulted in the initial retention values.

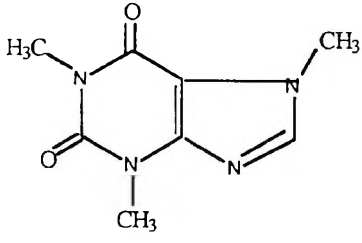
The possibility that DS^- counterions were expelled upon reduction and gradually leached out from the PPDS/Si column during the washing and contact with the mobile phase was indicated by the retention behaviour of aniline on this column. After the second run of column treatment with the reductant, the peak intensity of this analyte was lower and needed more injections to obtain a steady peak height. Although it eluted faster, it was clear that some part of the aniline was irreversibly adsorbed, which suggested that some reactive sites on the

surface of silica had became more accessible. This might also be due to irreversible changes in the polymer during treatment. Attempts were made to elute aniline after the column was reoxidised. After several injections, only very small peaks with irregular shapes were observed. This made it difficult to measure the retention times.

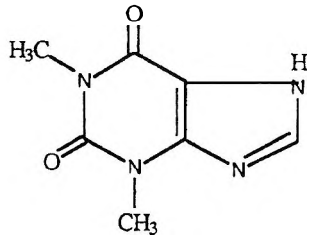
The retention behaviour of DMA on PPDS/Si is presented in Table 1. It has been observed previously that DS^- counterions intercalated in the polypyrrole stationary phase enhanced the capability of the column for hydrophobic interaction with DMA, as well as improving the selectivity.[24] As shown in Table 1, interaction reversibility, as observed on polypyrrole chloride column, is not shown here. Instead, the retention tended to decrease constantly as the column was subjected to reduction-oxidation treatments. This phenomenon seemed to suggest that, again, DS^- counterions were leaching out slowly during the course of chromatographic measurements. This process probably compensated or offset any effect from redox manipulation.

The effect of chemical manipulation on the chromatographic properties of the polymers was also tested using phenol as the test compound. Because phenol is a proton donor compound, the effect of the treatment on the proton-accepting ability of the polymers could be examined. It was found that the retention of phenol on both columns was essentially unaffected by the exposure of the coated polymers to the redox reagents, which indicated that its proton-accepting ability was not disturbed.

Retention Behaviour of Basic Drugs



caffeine



theophylline

The effectiveness of the chemical manipulation on PPCl/Si was also tested with the elution of theophylline and caffeine (Table 3). It is shown that this redox manipulation affected theopylline more than caffeine. These compounds have very similar molecular structures (see below), yet they have quite different basicities, which would reflect their capability of interacting through EDA interactions.

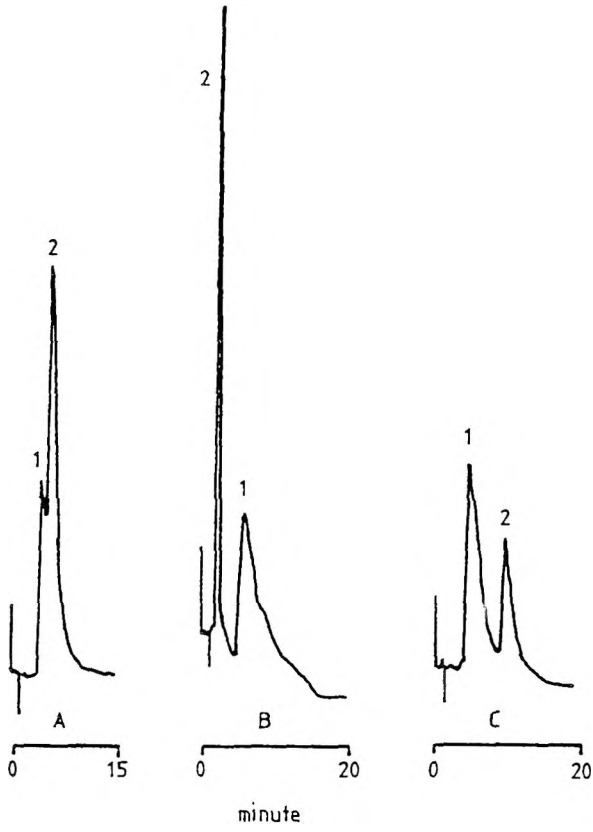


Figure 2. Effect of the injected redox reagents into PPCl/Si column on the chromatographic separation of theophylline (1) and caffeine (2).
Mobile phase : 65 % MeOH / H_2O at 1 ml / min. A: before the reagents were injected; B: after 1.0 mL 0.1 M Na_2SO_3 was injected; C: after 0.8 mL 0.1 M $FeCl_3$ was injected (following step B).

Theophylline is a stronger base (pK_a = 3.5) than is caffeine (pK_a = 0.6). This suggests that theophylline tends to donate its unshared paired electrons more readily, and is, hence, more sensitive to the changes in the EDA interactions capability of the column. If this argument holds true, it confirms, further, that the redox treatment did change the properties of the stationary phase to some degree. Upon reduction, the retention of theophylline decreased

and went to the original value upon reoxidation. The improvement in selectivity of the column, upon reduction, is more clearly demonstrated in the chromatograms (Figure 2).

The prediction that the presence of DS^- counterions would induce different kinds of interactions with the analytes was not obvious in experiments involving theophylline and caffeine. From the retention point of view, both analytes responded to redox manipulation in a parallel way; there was no essential improvement in selectivity. The retention behaviour of theophylline on the PPDS/Si column was different from that observed with the PPCl/Si column (Figure 3). As can be seen, column reoxidation did not restore the separation profile. All the results obtained from this column seemed to suggest that the use of redox manipulation to modify the properties of the stationary phase was not effective. Perhaps it changed to some degree, but then it was offset by the possible irreversible change in the polymer backbone on exposure to high pH. The possibility that Na^+ ions were incorporated, due to the treatment of the column with the reductant, should also be taken into account. The presence of these cations might induce different behaviour of the polymer when interacting with the test compounds.

Retention Behaviour of Amino Acids

In this study, two amino acids, i.e., L-tyrosine (Tyr) and L-tryptophan (Trp), were used as the probes for the following reasons:

1. Their isoelectric points (pI) are similar i.e., 5.67 and 5.88 for Tyr and Trp, respectively, which makes it easier to adjust the pH.

2. Both have aromatic rings, which makes it easy to detect by a UV detector without derivatisation.

3. Their hydrophobicities are quite different from each other, with Tyr being less hydrophobic than Trp,[25] which would result in different degrees of interaction with the polymeric phase.

Depending on the pH of the solution, the charges on the amino acids can be manipulated as illustrated below. In this work, the pH's of the buffer components in the eluent were 3.8 and 7.4, which were well apart from the pI values of the amino acids.

The effect of the injection of the Na_2SO_3 solution into the PPCl/Si column on the retention behaviour of Tyr and Trp is illustrated by the chromatograms in Figures 4 and 5a. At pH 3.8, the amino acids are positively charged. The side

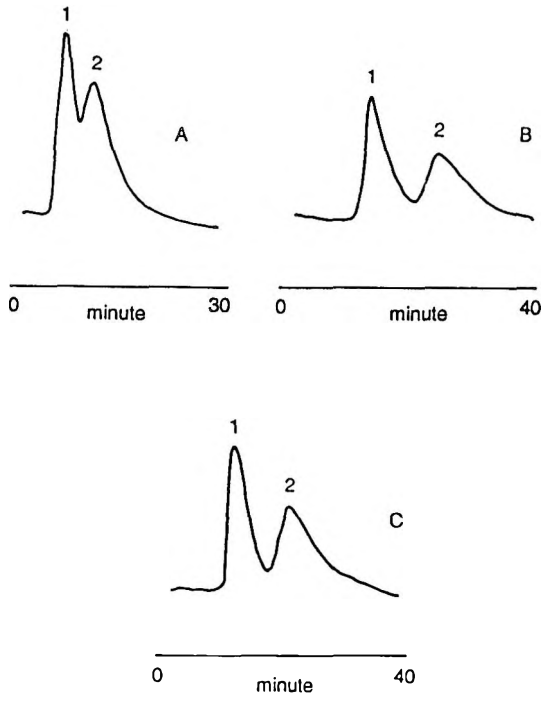
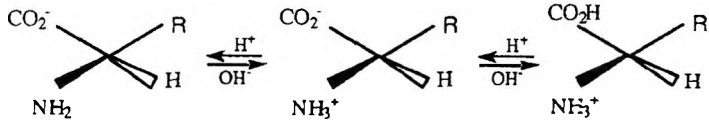


Figure 3. Effect of the injected redox reagents into PPDS/Si column on the chromatographic separation of theophylline (1) and caffeine (2). Mobile phase : 60 % MeOH/H_2O at 1 mL / min. A: before the reagents were injected; B: after 1.2 mL 0.1 M Na_2SO_3 was injected; C: after 1.0 ml 0.1 M $FeCl_3$ was injected (following step B).

chains of the amino acids, here, are hydrophobic in character. The chromatograms obtained suggest that, after the exposure of the polymeric phase to the reductant solution, the interaction between the amino acids and the phase became stronger due to increased hydrophobic interactions.



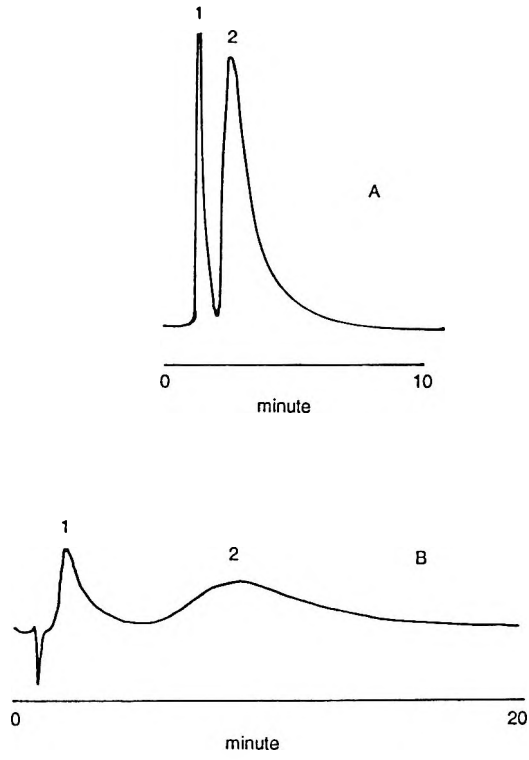


Figure 4. Chromatographic separation of tyrosine (1) and tryptophan (2) on PPCl/Si before (A) and after (B) the introduction of 1.0 mL 0.1 M Na_2SO_3 into the column. Mobile phase: 30 % MeOH / acetate buffer pH 3.8 at 1 mL/min.

The rationalization of the phenomenon is that, after the polymeric phase was exposed to the reagent, it was reduced so that its positive charges diminished, which resulted in the decrease of the repulsion effect between the polymeric phase and the amino acids.

At higher buffer pH (7.4) the amino acids should have net negative charges, which could enhance the electrostatic attraction between these and the polymeric phase.

The electrostatic interaction in combination with hydrophobic interactions resulted in longer retention times.

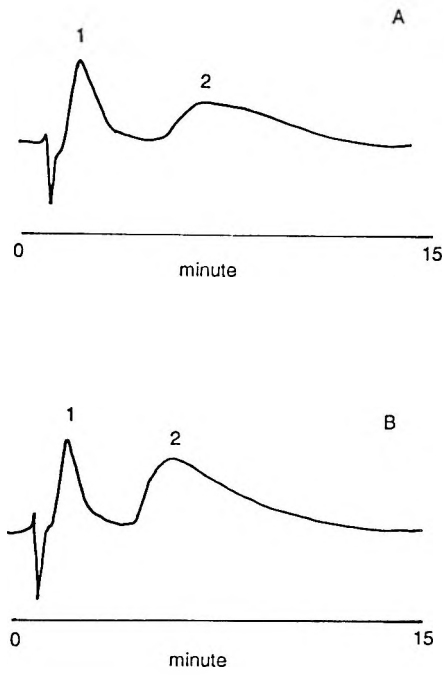


Figure 5a. Chromatographic separation of tyrosine (1) and tryptophan (2) on PPCl/Si before (A) and after (B) the introduction of 1.0 mL 0.1M Na_2SO_3 into the column. Mobile phase : 30 % MeOH/Tris-HCl, pH 7.4, at 1 mL/min.

A noticeable effect can be observed after the introduction of Na_2SO_3 solution, where the retention of the amino acids decreased. This effect was more marked for Trp, which is more hydrophobic than Tyr. The decrease in retention of the amino acids is attributed to reduction of the polymeric phase, removing the positive charges on the polymer backbone.

The effects of chemical manipulation on the chromatographic behaviour of the amino acids, using PPDS/Si, are illustrated by the chromatograms in Figures 5b and 5c. As can be seen in Figure 5b, the introduction of Na_2SO_3 solution into the column before the injection of the test compounds resulted in dramatic changes to the retention profile of the amino acids at low pH. Similar behaviour, as observed on PPCl/Si, occured on this column. On the original column, where the polymeric phase was positively charged, the electrostatic repulsion between this phase and the amino acids took effect, lowering the

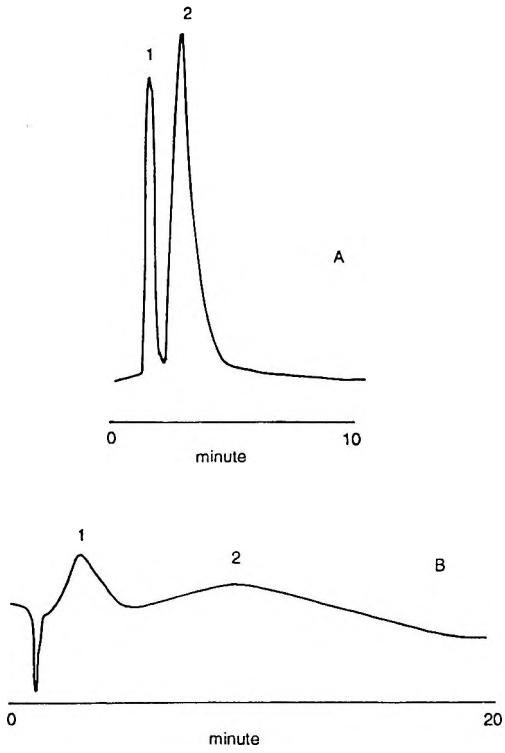


Figure 5b. Chromatographic separation of tyrosine (1) and tryptophan (2) on PPDS/Si before (A) and after (B) the introduction of 1.2 mL 0.1 M Na_2SO_3 into the column. Mobile phase : 30 % MeCN / acetate buffer, pH 3.8, at 1 mL/min.

retention time. Upon reduction with Na_2SO_3 solution, the positive charges on the polymeric phase are reduced. Because DS^- counterions had low mobility, which rendered them hard to expel on reduction,[17-19] the Na^+ cations could well be incorporated to compensate the negative charges of the counterions. When the amino acids were passing through the chemically treated column in low pH buffer, two different interactions could take place, i.e., cation-exchange and or hydrophobic interactions. The positively charged amino acids exchanged with sodium ions, a process that would be accompanied by hydrophobic interactions. As can be seen in Figure 5b, the amino acids were then more strongly retained.

In Figure 5c, the effect of chemical manipulation of PPDS/Si on the retention of the amino acids at neutral pH is shown. Under these conditions, the amino acids have a negative net charge, encouraging stronger interactions with

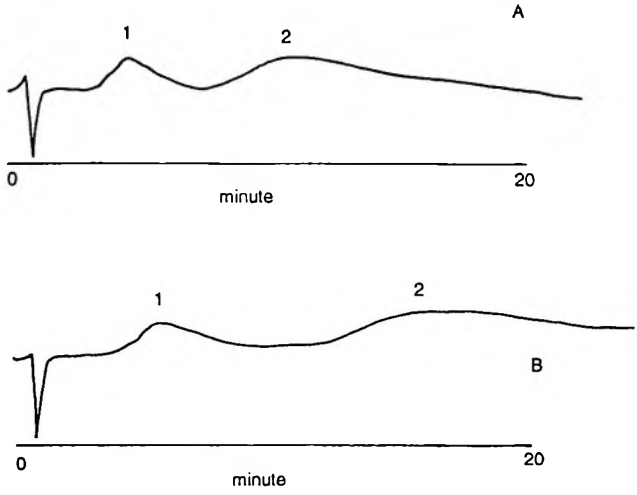


Figure 5c. Chromatographic separation of tyrosine (1) and tryptophan (2) on PPDS / Si before (A) and after (B) the introduction of 1.2 mL 0.1 M Na_2SO_3 into the column. Mobile phase : 30 % MeCN / Tris-HCl, pH 7.4, at 1 mL / min.

the untreated positively charged polymeric phase. After the column was treated with Na_2SO_3 solution, it was expected that the interaction between the polymeric phase and the amino acids would decrease. However, this was not observed. Instead of becoming weaker, the interactions were even stronger. The reason behind this phenomenon was not immediately obvious; the possibility of the inclusion of Na^+ was probably responsible for this behaviour. Perhaps the inclusion of these positively charged ions into the polymer increased the attractive interactions between the polymer and the negatively charged amino acids.

Reversibility of The Effect of The Chemical Treatment

It has been demonstrated, previously, that chemical treatment of the polypyrrole chloride column with redox reagents alters the properties of the polymeric phase, as revealed by the elution behaviour of theophylline and caffeine. The reversibility of this treatment was then considered. It is commonly believed that the oxidation state of conductive polymers, such as

Table 3

Effect of Redox Treatment of PPCl/Si on the Retention of Caffeine and Theophylline*

	Redox Reagent Injection (μL)	k′ Caffeine	k′ Theophylline
0.1M Na_2SO_3	0	2.5	1.7
	100	2.4	1.1
	200	2.6	0.8
	300	2.8	0.2
0.1M $FeCl_3$	100	2.3	1.6
	200	2.1	1.6

* Mobile phase: 60% MeOH/H_2O at 1 mL/min.

Table 4

Retention of Caffeine and Theophylline on PPCl/Si as a Function of the Treatment of the Column with Redox Reagent*

	k′ Caffeine	k′ Theophylline
Frc	2.6	2.2
red_1	2.7	0.1
oxd_1	2.3	2.0
red_2	2.4	0.1
oxd_2	2.1	1.8

*60% MeOH/H_2O at 1 mL/min. Frc: fresh column; red: after column was treated with 0.1M Na_2SO_3; oxd: after column reoxidation with 0.1M $FeCl_3$.

polypyrrole, can be reversibly altered by reduction-oxidation processes either chemically or electrochemically. In the following discussion, the reversibility was tested chromatographically and determined by the variation in the capacity factors observed for theophylline and caffeine.

Table 4 shows the result of reversibility test on PPCl/Si. The retention of theophylline and caffeine was first measured on the fresh column and remeasured after the column was alternately treated by the injection of Na_2SO_3

Table 5

Retention of Caffeine and Theophylline on PPD/Si as a Function of the Treatment of the Column with Redox Reagent*

	k′ Caffeine	k′ Theophylline
Frc	7.1	4.3
red$_1$	18.3	9.7
oxd$_1$	17.7	9.4
red$_2$	17.1	8.9
oxd$_2$	16.6	8.6

*60% MeOH/H_2O at 1 mL/min. Frc: fresh column; red: after column was treated with 0.1M Na_2SO_3; oxd: after column reoxidation with 0.1M $FeCl_3$.

and $FeCl_3$ solution. As can be seen, the reversibility of the effect of the chemical manipulation was verified. The retention of theophylline decreased to almost zero upon treatment with the reductant and back to almost normal following the treatment of the column with the oxidant. An opposite trend was observed for caffeine. The reversible behaviour observed with PPCl/Si was not observed with PPDS/Si (Table 5). This does not necessarily mean, however, that oxidation-reduction processes did not occur. As revealed by the retention behaviour of Tyr and Trp in the previous discussion, the lack of the fluctuation in retention on this column for the test compounds might be due to the different mechanism in the reduction-oxidation processes.

In this study, it was unclear which mechanism was more dominant. It could be through either an electron transfer or a protonation-deprotonation process. Both of these processes could lead to an increase or decrease of the conductivity of the polymer which, in turn, would lead to interaction with certain test compounds in different ways. Furthermore, the occurence of irreversible changes was also possible.

CONCLUSION

An examination of the effect of the exposure of polypyrrole chloride and polypyrrole dodecylsulfate to redox reagents has been carried out. It was found that the exposure brought changes in the properties of the polymers investigated

as indicated by the chromatographic elution behaviour of the test compounds in the columns packed with the polymer-coated packing materials. It was observed that the effect was more pronounced on the packing prepared from polypyrrole chloride. The changes observed on this material seem to be reversible.

The similar variation in electrode potential measured on polypyrrole chloride-coated platinum, that resulted from the exposure of the electrode to electrochemical and chemical redox manipulation, might suggest that reduction-oxidation process did occur during chemical exposure. Further investigation, however, might still be needed to be certain that other processes such as protonation-deprotonation were not involved.

The results of the study also suggest that, if the polypyrrole chloride polymer is used as a stationary phase for chromatography, the treatment with redox reagents would improve the column selectivity by exploiting the differences in the solutes' capability for producing electrostatic or charge-transfer interactions.

ACKNOWLEDGEMENTS

Prof. Gordon G. Wallace gratefully acknowledges the continued support of the Australian Research Council.

REFERENCES

1. G. G. Wallace, Chem. Brit., Nov., 1993, p. 967, and references cited therein.

2. P. R. Teasdale, G. G. Wallace, Analyst., **118**, 329 (1993), and references cited therein.

3. H. Mao, P. G. Pickup, J. Phys. Chem., **96**, 5604 (1992).

4. R. Casas, A. Dicko, J. M. Ribo, M. A. Valles, N. Ferrer-Anglada, R. Bonnett, N. Hanly, D. Bloor, Synth. Met., **39**, 275 (1990).

5. A. K. Bakhshi, J. Ladik, M. Seel, Phys. Rev., **35**, 704 (1987).

6. S. Kuwabata, S. Ito, H. Yoneyama, J. Electrochem. Soc., **135**, 1691 (1988).

7. R. C. D. Peres, J. M. Pernaut, M.-A. De Paoh, Synth. Met., **28**, C59 (1989).

8. F. I. A. Vork, B. C. A. M. Schuermans, E. Barendrecht, Electrochim. Acta, **35**, 567 (1990).

9. H. Ge, G. G. Wallace, Anal. Chem., **61**, 2391 (1989).

10. H. Ge, G. G. Wallace, J. Liq. Chromatogr., **13**, 3260 (1990).

11. R. S. Deinhammer, K. Shimazu, M. D. Porter, Anal. Chem., **63**, 1894 (1991).

12. T. Hanawa, T., H. Yoneyama, Synth. Met., **30**, 341 (1989).

13. J. J. Miasik, A. Hooper, B. C. Tofield, J. Chem. Soc., Faraday Trans., **1**, 1117 (1986).

14. J. Ratcliffe, Anal. Chim. Acta, **239**, 257 (1990).

15. G. B. Street, T. C. Clarke, M. Krounbi, K. Kanazawa, V. Lee, P. Pfluger, J. C. Scott, G. Weiser, Mol. Cryst. Liq. Cryst., **83**, 253 (1982).

16. W. Melander, Cs. Horvath, Arch. Biochem. Biophys., **183**, 200 (1977).

17. S. Panero, P. Prosperi, B. Scrosati, Electrochim. Acta, **37**, 419 (1992).

18. R. C. Martinez, F. B. Dominquez, F. M. Gonzales, J. H. Mendez, Anal. Chim. Acta, **279**, 299 (1993).

19. C. Zhong, K. Doblhofer, Electrochim. Acta, **35**, 1971 (1990).

20. T. Shimidzu, A. Ohtani, K. Honda, Bull. Chem. Soc. Japan, **61**, 2885 (1988).

21. M. Forsyth, V.-T. Truong, M. E. Smith, Polymer, **35**, 1593 (1994).

22. J. Heinze, Synth. Met., **41-43**, 2805 (1991).

23. H. Ge, G. G. Wallace, J. Chromatogr., **588**, 25 (1991).

24. H. Chriswanto, H. Ge, G. G. Wallace, Chromatographia, **37**, 423 (1993).

25. C. T. Mant, R. S. Hodges, Eds., **High-Performance Liquid Chromatography of Peptides and Proteins. Separation, Analysis, and Conformation**, CRC Press, Boca Raton, FL, 1991.

Received September 7, 1995
Accepted January 13, 1996
Manuscript 3966

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2477–2495 (1996)

PREDICTION AND MODELLING STUDIES FOR CAPACITY FACTORS OF A GROUP OF DIHYDROPYRIDINES IN MICELLAR LIQUID CHROMATOGRAPHY WITH HYBRID ELUENTS

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ABSTRACT

Empirical equations were used to predict the retention behaviour of a group of twenty-seven dihydropyridines in Micellar Liquid Chromatography with hybrid eluents on an octylsilica column. A theoretical model was also used to study their retention mechanism. Hexadecyltrimethylammonium bromide and sodium dodecyl sulphate were used as surfactants in the mobile phase and as organic modifiers n-propanol and n-butanol were employed.

INTRODUCTION

Micellar Liquid Chromatography (MLC) can be considered an attractive separation technique due to the special characteristics of the surfactant molecules. Ionic surfactants in aqueous solutions posses two zones, an ionic head group and a hydrophobic core, both capable of binding the solutes and the stationary phase,[1] and modifying the solute retention behaviour when introduced in the mobile phase of a chromatographic system.

Thus, micellar systems can bind a variety of solutes as a result of favourable electrostatic, pi, H-bonding, hydrophobic, or combination of such interactions, making such mobile phases much more versatile than any of the conventional mobile-phase systems.[2-3]

Moreover, different advantages that this technique presents when compared to conventional Reverse Phase High Performance Liquid Chromatography (RP-HPLC), can be cited. Among these, we can expect low cost and nontoxicity of the surfactants *versus* conventional solvents in RP-HPLC,[4-7] a unique selectivity,[3,7-11] compatibility of mobile phases with salts and water-insoluble compounds,[10] shorter equilibration times for gradient elution and the possibility of controlling retention times and selectivity,[12] modifying the mobile phase composition when hybrid eluents (micellar phases containing low percentages of an organic modifier, generally short-chain alcohols) are used.

To exploit the full advantages of MLC with hybrid eluents in a more judicious way, it should be desirable to know the equation that relates the solute capacity factor with surfactant and alcohol concentrations. As stated by Massart *et al.*,[13] mechanistic models should be used if systems are adequately understood and there is some guarantee that the systems will not deviate greatly from their expected behaviour. Otherwise, mechanistic models might be seriously misleading for predictive purposes; unbiased empirical models might prove to be better choices. Consequently, in this article some empirical models and a theoretical model have been employed from different points of view.

The empirical models are as follows:

$$1/k' = A\mu + B\varphi + C\mu\varphi + D \qquad (1)$$

$$1/k' = A\mu + B\varphi^2 + C\varphi + D\mu\varphi + E \qquad (2)$$

$$\lg k' = A\mu + B\varphi + C\mu\varphi + D \qquad (3)$$

where k' is the solute capacity factor, μ is the total surfactant concentration, φ is the volume fraction of organic modifier and A, B, C, D and E the model parameters.

These equations have been used earlier by Torres-Lapasió *et al.*[14] and by our research team,[15] to predict the retention behaviour of catecholamines (using sodium dodecyl sulphate (SDS) as the surfactant and n-propanol as the organic modifier),[14] benzene derivatives and polycyclic aromatic hydrocarbons (using hexadecyltrimetilammoniumbromide (CTAB) and SDS as the surfactants and n-propanol and n-butanol as the organic modifiers in the mobile phase).[15] For

catecholamines, the best results were obtained with equation (1) and for benzene derivatives and polycyclic aromatic hydrocarbons, the results showed that equation (2) was of more general applicability. In this work, one of our objectives is to expand the studies of solute retention prediction by means of empirical models with another family of organic compounds (dihydropyridines) and to propose, if it is possible, a general equation that permits to carry out the capacity factor prediction in any mobile phase (in MLC with hybrid eluents) for solutes of different nature with the minimum effort.

Some theoretical models to explain the solute retention behaviour in liquid chromatography with micellar mobile phases have been proposed in the literature,[16] but when hybrid eluents are considered, the complexity is greater because the alcohol can compete with the solutes for the interaction with micelles and with the stationary phase.[12,14]

In order to explain the mechanism of solute retention in such complicated media, our research team proposed a physico-chemical model[18] that accounts for the interactions between the solute and the stationary phase, the solute and the surfactant in the micelle and those between the alcohol (organic modifier) and the stationary phase and/or the surfactant in the micelle. The equation derived from this model that relates to the solute capacity factor and the micellized surfactant and alcohol concentrations in the mobile phase is as follows:

$$k' = \frac{\phi\, k_1\, [L_s]\,(1 + k_4\,[A_m])}{1 + (k_3 + k_4)\,[A_m] + k_2[M_m]\,(1 + k_3[A_m]) + k_3 k_4 [A_m]^2} \quad (4)$$

where k_1, k_2, k_3 and k_4 are different equilibrium constants (corresponding to solute/stationary phase, solute/micelle, alcohol/stationary phase and alcohol/micelle interactions, respectively), ϕ is the phase ratio, $[L_s]$ is the stationary phase sites concentration, $[A_m]$ is the alcohol concentration in the mobile phase and $[M_m]$ is the micellized surfactant concentration.

This equation can be reduced to more simple models depending on the equilibrium constant values. Thus, some of the simplified equations are the following:

$$\frac{1}{k'} = \frac{1}{a} + \frac{k_3}{a}[A_m] + \frac{k_2}{a}[M_m] + \frac{k_2 k_3}{a}[M_m][A_m] \quad (5)$$

$$\frac{1}{k'} = \frac{1}{a} + \frac{k_3 + k_4}{a}[A_m] + \frac{k_2}{a}[M_m] + \frac{k_2 k_3}{a}[M_m][A_m]$$

$$+\frac{k_3k_4}{a}[A_m]^2 \quad (6)$$

$$\frac{1}{k'}=\frac{k_2}{a}[M_m]+\frac{k_2k_3}{a}[M_m][A_m] \quad (7)$$

$$\frac{1}{k'}=\frac{1}{a}+\frac{k_3}{a}[A_m]+\frac{k_2}{a}[M_m]+\frac{k_2k_3}{a}[M_m][A_m]+\frac{k_3k_4}{a}[A_m]^2 \quad (8)$$

$$\frac{1}{k'}=\frac{1}{ak_4}\frac{1}{[A_m]}+\frac{k_3+k_4}{ak_4}+\frac{k_2}{ak_4}\frac{[M_m]}{[A_m]}+\frac{k_2k_3}{ak_4}[M_m]+\frac{k_3}{a}[A_m] \quad (9)$$

being $a = \phi k_1[L_s]$.

The model has been checked with a limited number of compounds (fifteen benzene derivatives and eight polycyclic aromatic hydrocarbons),[18] all of them with one or more aromatic rings, and employing CTAB and SDS as surfactants and n-propanol and n-butanol as organic modifiers. The second objective of this work is to check the model with solutes of different nature, such as dihydropyridines, and to extract information about their retention mechanism in MLC with hybrid eluents.

In order to achieve our purposes, retention data obtained in a MLC system for twenty-seven dihydropyridines by using CTAB and SDS as surfactants and n-propanol and n-butanol as organic modifiers, have been used.[19]

EXPERIMENTAL

Chromatographic Data

Retention data for 27 dihydropyridines[19] on a C_8 column (Technokroma, Barcelona, Spain) have been used. The solute capacity factors were determined in micellar mobile phases containing CTAB and SDS as surfactants and n-propanol and n-butanol as organic modifiers (all from Merck, Darmstad, Germany). The experimental retention data used have been summarized in Figure 1.

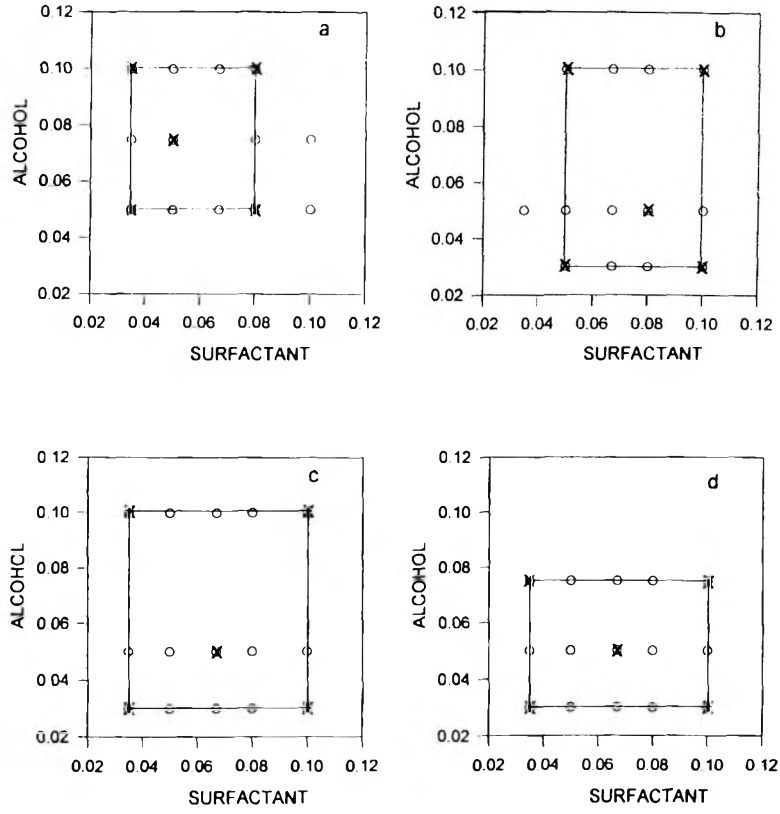
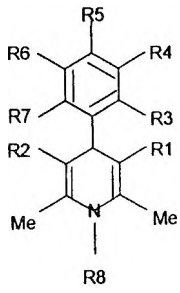


Figure 1. Mobile phase composition when CTAB-propanol (Fig. 1a), CTAB-butanol (Fig. 1b), SDS-propanol (Fig. 1c) and SDS-butanol (Fig. 1d) were used as hybrid eluents. Alcohol concentrations are expressed as the volume fraction of the organic modifier and the surfactant concentration is the total surfactant concentration in the mobile phase. Data points in and inside the square have been used in the studies with the empirical model. being the data marked with X the five mobile phases of the factorial design. All the data showed in these Figures have been employed in the studies with the theoretical model.

The structure of the dihydropyridines used in this work as well as their assigned numbers are shown in Figure 2. This Figure groups the four basic structures considered and in Tables 1, 2, 3, and 4, the assigned numbers for each solute and the substituting groups are tabulated for these four structures.



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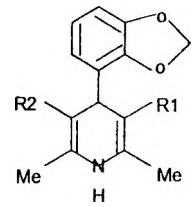

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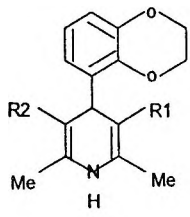
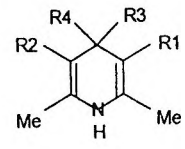


GROUP 3



GROUP 4

Figure 2. Structures of the dihydropyridinesused in this work and their assigned values.

Data Manipulation

First, some empirical models (equations (1), (2), and (3) in the introduction) have been applied to the chromatographic data, in order to check which of them was the best to predict the solute capacity factors. In this work, the model parameters were calculated[20] by using only five capacity factors according to the factorial design shown in Figure 1.

In this case, the capacity factors were calculated for all the mobile phases excepting those outside the squares shown in Fig. 1 and, in this way the prediction relative errors were obtained.

Table 1

Assigned Numbers and the Corresponding Substituting Groups for the Dihydropyridines Belonging to Group 1 in Figure 2

Solute	R1	R2	R3	R4	R5	R6	R7	R8
1	COOIsp	$COOCH_2CH_2OMe$	H	NO_2	H	H	H	H
9	COOEt	COOMe	H	NO_2	H	H	H	H
10	COOMe	COOEt	Cl	Cl	H	H	H	H
11	COOEt	COOMe	OCH_2OMe	H	H	H	H	H
12	COOEt	COOMe	H	OMe	H	OMe	H	H
14	COOEt	COOEt	H	H	H	H	H	H
16	COOEt	COOMe	H	OMe	OMe	H	H	H
17	COOEt	COOEt	OMe	OMe	H	H	H	H
19	COOEt	COOMe	H	OMe	OH	OMe	H	H
20	COOEt	COOMe	H	OMe	OMe	OMe	H	H
21	COOEt	COOEt	Cl	H	H	H	Cl	H
22	COOMe	COOMe	NO_2	H	H	H	H	CH_2OEt
23	COOEt	COOEt	Cl	H	H	H	H	H

Table 2

Assigned Numbers and the Corresponding Substituting Groups for the Dihydropyridines Belonging to Group 2 in Figure 2

Solute	R1	R2
2	$COOCH_2Py$	COOMe
3	COOMe	COOMe
4	$COOCH_2CH_2OMe$	$COOCH_2CH_2OMe$
6	COOEt	COOEt
7	$COOCH_2CH_2OMe$	COOIsp
8	COOIsp	COOEt
13	COOMe	COOEt
15	COOIsp	COOIsp
18	COOIsp	COOMe

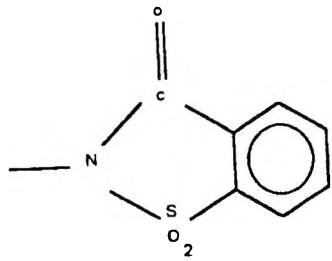
Second, the physico-chemical model reported by us in an earlier article[18] and the simplified equations derived from it, were applied to all the chromatographic data (shown in Figure 1). The equilibrium constants were calculated using the Sigma Plot System[21] and, with them, the relative errors were obtained for all the mobile phases studied and for all the equations under study (equations (4) to (9) in the introduction section).

Table 3

Assigned Numbers and the Corresponding Substituting Groups for the Dihydropyridines Belonging to Group 3 in Figure 2

Solute	R1	R2
5	COOEt	COOMe

Table 4
Assigned Numbers and the Corresponding Substituting Groups for the Dihydropyridines Belonging to Group 4 in Figure 2



Solute	R1	R2	R3	R4
24	CN	CN	Me	Me
25	COOMe	COOEt	H	Me
26	COO(1)	COOEt	H	Me
27	COOEt	COOEt	H	Me

RESULTS AND DISCUSSION

In this work, we have worked with the retention data for a group of twenty-seven dihydropyridines on a C_8 column using hybrid eluents in which CTAB and SDS (total concentration range: 0.035-0.100 M) are used as surfactants, and n-propanol and n-butanol are used as organic modifiers (concentration range: 0.399-1.331 M and 0.328-1.092 M, respectively). The empirical models presented in the introduction of this article have been checked, in order to clarify what is the best equation to predict the retention behaviour of dihydropyridines in a MLC system. Also, the theoretical physico-chemical model has been used because it can help us to understand the retention mechanism of these compounds.

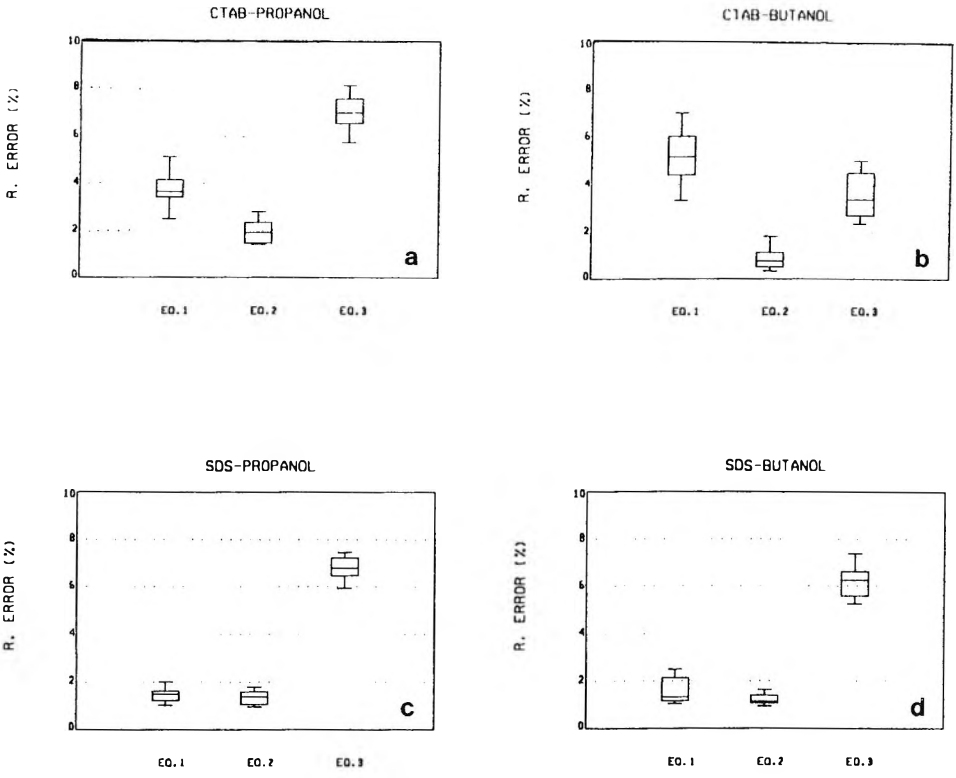


Figure 3. Mean relative errors (as absolute values) *versus* the equation used for mobile phases: CTAB-propanol (Fig. 3a), CTAB-butanol (Fig. 3b), SDS-propanol (Fig. 3c) and SDS-butanol (Fig. 3d).

A. Empirical Models

In this section, equations (1), (2), and (3) (see introduction) have been used to predict the solute capacity factors for the dihydropyridines. These equations relate the reciprocal of the capacity factors (1/k') or their logarithm (log k') with the total surfactant concentration in the mobile phase (μ) and the volume fraction of the organic modifier (φ).

The global results for these studies are shown in Figure 3. In this Figure, the mean relative errors of prediction are plotted *versus* the equation used for mobile phases containing CTAB-propanol (Figure 3a), CTAB-butanol (Figure 3b), SDS-propanol (Figure 3c) and SDS-butanol (Figure 3d), respectively.

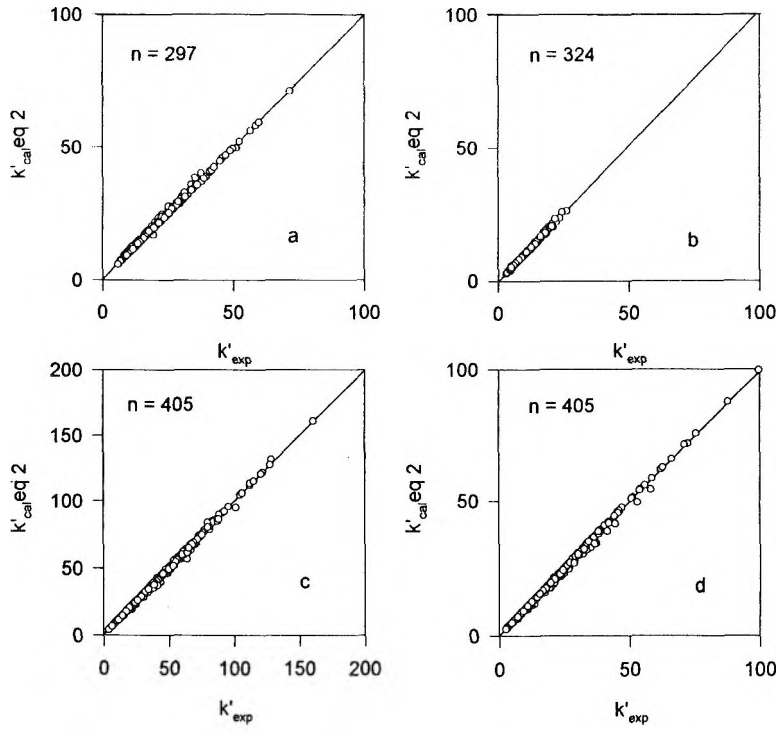


Figure 4. Calculated *versus* experimental capacity factors for systems: CTAB-propanol (Fig. 4a), CTAB-butanol (Fig. 4b), SDS-propanol (Fig. 4c) and SDS-butanol (Fig. 4d), being n the number of experimental data considered.

As can be observed in Figure 3, there are significative differences among the prediction errors for the three equations, with only two exceptions: SDS-propanol and SDS-butanol systems with equations (1) and (2). It seems that equation (2) predicts more adequately the capacity factors of these compounds because the errors obtained are the lowest. This fact is in agreement with an earlier report[15] in which the retention of benzene derivatives and polycyclic aromatic hydrocarbons was modellized. Always, the prediction errors with equation (2) are very low and they range from 1.29 to 3.3 %, 0.22 to 2.32 %, 0.8 to 2.13 % and 0.87 to 1.91 % for mobile phases containing CTAB-propanol, CTAB-butanol, SDS-propanol and SDS-butanol, respectively.

Comparable results were obtained in the case of SDS-propanol and SDS-butanol systems with equation (1) and (2), so, if we want to predict the retention behaviour we ought to use equation (1) because this later depends on a lower

number of parameters. To show the good agreement found between the calculated (k'_{cal}, predicted by means of equation (2)) and the experimental capacity factors (k'_{exp}), k'_{cal} *versus* k'_{exp} values for systems CTAB-propanol (Fig. 4a), CTAB-butanol (Fig. 4b), SDS-propanol (Fig. 4c) and SDS-butanol (Fig. 4d) are plotted in Figure 4. The equations for the straight lines corresponding to these systems are the following:

CTAB-propanol $k'_{cal} = 0.410 + 0.995\, k'_{exp}$; $r^2 = 0.9969$ $n = 297$
CTAB-butanol $k'_{cal} = 0.119 + 1.013\, k'_{exp}$; $r^2 = 0.9974$ $n = 324$
SDS-propanol $k'_{cal} = 0.164 + 0.996\, k'_{exp}$; $r^2 = 0.9984$ $n = 405$
SDS-butanol $k'_{cal} = 0.053 + 0.990\, k'_{exp}$; $r^2 = 0.9983$ $n = 405$

n being the number of experimental data considered.

The intercepts and the slopes of these straight lines are very close to zero and unity, respectively, which is a good indication of the validity of equation (2) to predict capacity factor values in such systems. Also, it is interesting to note that correlation coefficients are very close to unity. These facts make us to think that equation (2) is of more general applicability than equations (1) or (3), which is in agreement with the results reported by us earlier[15] with compounds of different nature.

B. Theoretical Model

In an earlier work,[18] a theoretical model that explains the retention behaviour as a function of micellized surfactant and alcohol concentrations was proposed. The solutes under study were benzene derivatives and polycyclic aromatic hydrocarbons (PAHs). For these compounds, a good agreement between the experimental and the calculated retention data was found and some important aspects about the retention mechanism could be extracted. In this study, our purpose is to check the validity of this model for compounds of different nature (dihydropyridines).

In a first step, the equilibrium constants by using equations (4) to (9) (in the introduction section) for the four hybrid eluents (CTAB-propanol, CTAB-butanol, SDS-propanol and SDS-butanol) were calculated. Then, the calculated capacity factors for the different solutes by means of those equations were obtained and the relative errors were calculated. In order to check the homocedasticity assumption of the model, the residuals obtained for every compound and for every phase composition *versus* the calculated capacity factors, the micellized surfactant concentration and the alcohol concentration in the mobile phase were plotted.

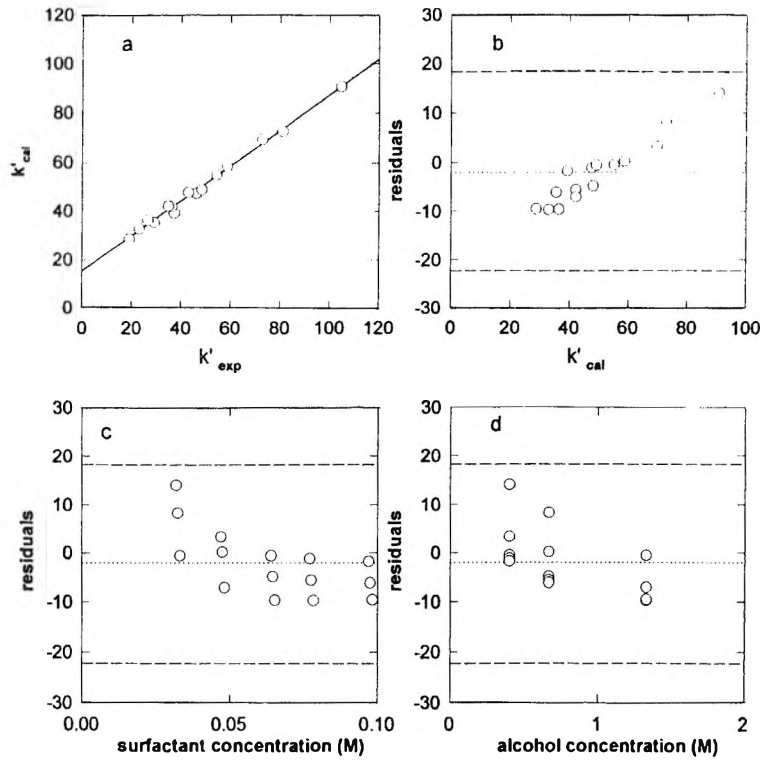


Figure 5. Calculated capacity factor by eq. (9) *versus* experimental capacity factor (Fig. 5a) and residuals *versus* k'_{cal}, micellized surfactant concentration and alcohol concentration (Fig. 5b, 5c. and 5d. respectively) for compound number 1 and for mobile phases containing SDS as the surfactant and n-propanol as the organic modifier.

With these Figures (not shown), together with the plot of the calculated *versus* the experimental capacity factors, we were able to decide if the model was adequate to explain the retention behaviour and what was the equation that, with the minimum number of parameters, could explain the retention data.

Thus, it can be cited that, for example, these Figures show the inadequacy of the equation (9) in all the cases studied. To illustrate this fact in Figure 5, the plots of k'_{cal} (calculated capacity factor by eq. (9)) *versus* k'_{exp} (experimental capacity factor) (Figure 5a), residuals versus k'_{cal}, micellized surfactant concentration and alcohol concentration in the mobile phase (Figures 5b, 5c and 5d, respectively), are shown for compound 1 when mobile phases containing SDS and n-propanol are considered. The slope and the intercept shown in Figure 5a do not coincide with

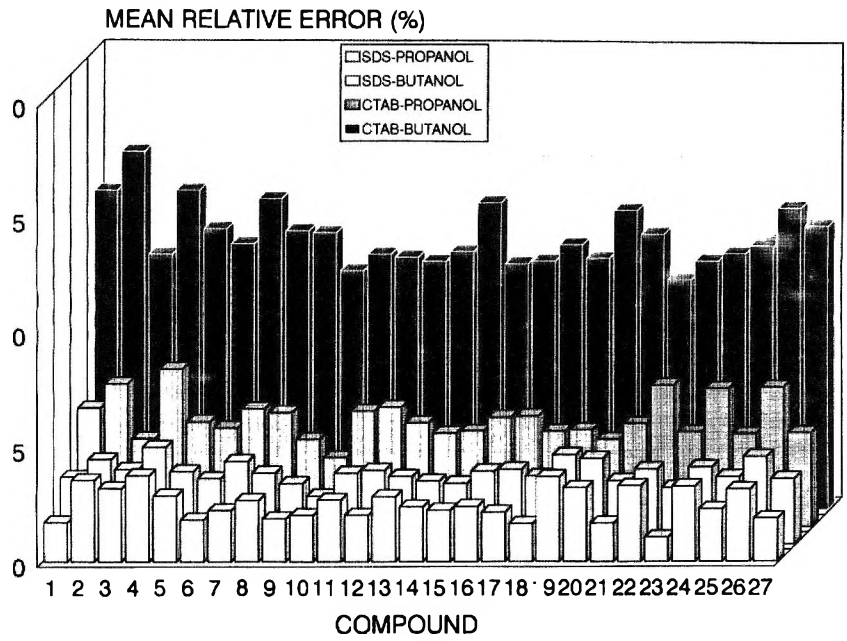


Figure 6. Mean relative errors (in absolute value) obtained for every compound with the best equation in CTAB-propanol, CTAB-butanol, SDS-propanol and SDS-butanol systems.

the unity and zero values, respectively, although the correlation coefficient is very close to unity. Figures 5b, 5c and 5d show that there is not a random distribution of the residuals with the dependent or the independent variables.

In Tables 5, 6, 7 and 8 the calculated values of a ($ok_1[L_s]$), k_2, k_3 and k_4 or related parameters (by means of the most adequate equation), for hybrid eluents containing CTAB and propanol, CTAB and butanol, SDS and propanol, and SDS and butanol, respectively, are presented.

For mobile phases containing CTAB as the surfactant, the more adequate equation for explaining the dihydropyridine retention behaviour is generally equation (7). This fact suggests, that in these systems the solutes suffer a direct transfer from the micellar mobile phase to the modified stationary phase. In these cases, the alcohol influences the solute retention due to its interaction with the stationary phase. The values of k_4 are negligible, so we can consider that the interaction of the alcohol with the micelle is of little importance with respect to the others.

Table 5

Calculated Parameter Values for Compounds in Systems Containing CTAB and n-Propanol and the Best Equation Used. Data Between Parentheses are the Parameter Standard Error

Compound	A*	B**	C***	Equation
1	0.034 (0.043)	0.724 (0.056)		(7)
2	0.161 (0.067)	0.866 (0.084)		(7)
3	0.169 (0.054)	1.095 (0.068)		(7)
4	0.00 (0.12)	1.45 (0.16)		(7)
5	0.139 (0.060)	1.014 (0.075)		(7)
6	0.091 (0.041)	0.764 (0.053)		(7)
7	0.000 (0.048)	0.847 (0.063)		(7)
8	0.048 (0.041)	0.674 (0.052)		(7)
9	0.152 (0.036)	0.708 (0.045)		(7)
10	0.169 (0.029)	0.709 (0.037)		(7)
11	0.095(0.063)	1.032 (0.081)		(7)
12	0.075 (0.057)	0.942 (0.073)		(7)
13	0.102 (0.053)	0.931 (0.068)		(7)
14	0.163 (0.041)	0.771 (0.051)		(7)
15	0.024 (0.022)	0.567 (0.029)		(7)
16	0.099 (0.066)	1.110 (0.085)		(7)
17	0.099 (0.066)	1.106 (0.085)		(7)
18	0.108 (0.040)	0.768 (0.051)		(7)
19	552 (215)	241 (96)	2.71 (0.45)	(5)
20	0.000 (0.069)	1.372 (0.091)		(7)
21	0.084 (0.034)	0.604 (0.043)		(7)
22	0.079 (0.055)	0.782 (0.070)		(7)
23	0.117 (0.039)	0.721 (0.050)		(7)
24	161 (16)	99 (11)	1.55 (0.12)	(5)
25	0.107 (0.053)	1.053 (0.068)		(7)
26	0.117 (0.061)	0.857 (0.077)		(7)
27	0.095 (0.039)	0.825 (0.049)		(7)

* A means k_2/a or a when equation (7) or (5) is considered, respectively.
** B means k_2k_3/a or k_2 when equation (7) or (5) is considered, respectively
*** C means k_3 when equation (5) is considered

When we consider mobile phases containing SDS as the surfactant again, the constant k_4 is low and the equation that best explains the experimental capacity factors is, in general, the equation (5). The k_3 values shown in Tables 7 and 8 are

Table 6

Calculated Parameter Values for Compounds in Systems Containing CTAB & n-Butanol. Data Between Parentheses are the Parameter Standard Error

Compound	A*	B**	C***	Equation
1	0.34 (0.13)	1.60 (0.29)		(7)
2	0.59 (0.20)	1.88 (0.42)		(7)
3	0.53 (0.16)	2.48 (0.36)		(7)
4	0.42 (0.26)	3.78 (0.62)		(7)
5	0.54 (0.17)	2.26 (0.36)		(7)
6	0.38 (0.12)	1.71 (0.26)		(7)
7	0.36 (0.14)	1.87 (0.32)		(7)
8	0.34 (0.11)	1.45 (0.24)		(7)
9	0.40 (0.12)	1.67 (0.27)		(7)
10	0.36 (0.11)	1.70 (0.23)		(7)
11	0.53 (0.16)	2.25 (0.34)		(7)
12	0.44 (0.14)	2.14 (0.31)		(7)
13	0.45 (0.13)	2.04 (0.29)		(7)
14	0.46 (0.13)	1.78 (0.28)		(7)
15	0.27 (0.10)	1.26 (0.22)		(7)
16	0.53 (0.16)	2.55 (0.36)		(7)
17	0.54 (0.17)	2.54 (0.37)		(7)
18	0.41 (0.12)	1.70 (0.27)		(7)
19	64 (15)	43 (11)	3.33 (0.62)	(5)
20	0.45 (0.23)	3.32 (0.52)		(7)
21	0.34 (0.10)	1.31 (0.22)		(7)
22	0.79 (0.14)	1.75 (0.28)		(7)
23	0.37 (0.11)	1.63 (0.24)		(7)
24	0.96 (0.22)	2.91 (0.47)		(7)
25	0.47 (0.16)	2.32 (0.36)		(7)
26	0.54 (0.17)	1.90 (0.36)		(7)
27	0.40(0.14)	1.85 (0.31)		(7)

* A means k_2/a or a when equation (7) or (5) is considered, respectively.
** B means k_2k_3/a or k_2 when equation (7) or (5) is considered, respectively.
*** C means k_3 when equation (5) is considered.

low, but we can observe that they are greater in the case that butanol is the organic modifier in the mobile phase. That is, the interaction alcohol/stationary phase is greater when butanol is considered. This fact is in agreement with Borgerding *et al.*,[12] who reported that the amount of surfactant desorbed by such additives

Table 7

Calculated Parameter Values for Compounds in Systems Containing SDS and n-Propanol and the Best Equation Used. Data Between Parentheses are the Parameter Standard Error

Compound	a	k_2	k_3	Equation
1	1603 (278)	240 (45)	1.917 (0.098)	(5)
2	883 (337)	267 (111)	1.33 (0.13)	(5)
3	475 (117)	192 (53)	1.36 (0.12)	(5)
4	276 (52)	120 (26)	1.96 (0.20)	(5)
5	568 (140)	208 (57)	1.32 (0.10)	(5)
6	1369 (249)	256 (51)	1.616 (0.079)	(5)
7	1030 (241)	224 (57)	1.57 (0.11)	(5)
8	2626 (1308)	369 (195)	1.65 (0.16)	(5)
9	1572 (314)	262 (57)	1.88 (0.10)	(5)
10	4117 (2011)	574 (292)	2.22 (0.15)	(5)
11	843 (191)	217 (54)	1.57 (0.11)	(5)
12	800 (109)	158 (24)	1.99 (0.12)	(5)
13	923 (254)	253 (76)	1.50 (0.11)	(5)
14	1707 (675)	387 (162)	1.44 (0.10)	(5)
15	2543 (698)	255 (76)	1.79 (0.13)	(5)
16	811 (156)	217 (46)	1.99 (0.12)	(5)
17	679 (102)	181 (30)	1.94 (0.11)	(5)
18	1044 (149)	219 (34)	1.385 (0.060)	(5)
19	201 (46)	145 (38)	1.98 (0.21)	(5)
20	583 (138)	189 (49)	2.58 (0.24)	(5)
21	2011 (507)	294 (80)	1.261 (0.074)	(5)
22	2688 (1117)	329 (146)	2.43 (0.24)	(5)
23	2332 (365)	327 (55)	1.92 (0.067)	(5)
24	124 (21)	83 (17)	0.93 (0.11)	(5)
25	437 (89)	180 (41)	1.059 (0.078)	(5)
26	524 (146)	204 (63)	1.060 (0.096)	(5)
27	621 (91)	176 (29)	1.143 (0.062)	(5)

increases as the hydrophobicity of the alcohol increases. If we compare the a and k_2 values shown in Tables 7 and 8, we can observe that solute/stationary phase and solute/micelle interactions decrease when the hydrophobicity of the alcohol increases. This means that butanol can compete in a greater extent with the stationary phase and the surfactant in the micelle, which has been reported earlier by Borgerding *et al.*[12] and by Khaledi *et al.*[17]

Table 8

Calculated Parameter Values for Compounds in Systems Containing SDS and n-Butanol and the Best Equation Used. Data Between Parentheses are the Parameter Standard Error

Compound	a	k_2	k_3	Equation
1	1717 (375)	98 (12)	18.7 (4.3)	(5)
2	355 (50)	77 (11)	6.10 (0.86)	(5)
3	237 (28)	62.7 (7.1)	6.30 (0.81)	(5)
4	203 (42)	48.9 (6.3)	11.2 (2.6)	(5)
5	275 (32)	67.2 (7.6)	6.11 (0.74)	(5)
6	941 (134)	92 (10)	10.9 (1.6)	(5)
7	631 (99)	87 (12)	8.6 (1.4)	(5)
8	211 (44)	108 (14)	17.3 (3.8)	(5)
9	1429 (251)	96 (10)	16.4 (3.1)	(5)
10*	3439 (485)	192 (64)	18.5 (7.4)	(6)
11	473 (60)	75.8 (8.3)	8.4 (1.1)	(5)
12	773 (133)	79.3 (9.2)	12.9 (2.4)	(5)
13	431 (49)	78.2 (8.5)	7.09 (0.83)	(5)
14	646 (73)	86.2 (9.0)	7.80 (0.88)	(5)
15	5580 (1923)	102 (11)	39 (14)	(5)
16	337 (41)	71.8 (7.9)	7.53 (0.97)	(5)
17	343 (44)	71.8 (8.2)	7.7 (1.0)	(5)
18	764 (100)	94 (11)	8.4 (1.1)	(5)
19	122 (20)	53 (6.4)	9.5 (1.8)	(5)
20	468 (133)	61.0 (7.5)	19.7 (6.2)	(5)
21	2160 (345)	135 (18)	12.0 (1.8)	(5)
22	1736 (452)	81.7 (9.7)	21.8 (6.1)	(5)
23	2460 (466)	111 (11)	21.6 (4.3)	(5)
24	84.6 (7.4)	38.6 (3.8)	3.63 (0.39)	(5)
25	239 (24)	67.6 (7.1)	4.75 (0.48)	(5)
26	240 (38)	105 (20)	2.88 (3.34)	(5)
27	442 (47)	84.7 (9.3)	5.64 (0.56)	(5)

* k_4 = 1.9 (1.4)

In order to show the adequacy of the theoretical model and the equations from it derived in Figure 6, the mean relative errors (in absolute value) obtained with the best equation for every compound studied is plotted. The errors obtained were generally low and ranged from 3.04 to 6.88 (CTAB-propanol), from 10.06 to

15.70 (CTAB-butanol), from 1.06 to 3.74 (SDS-propanol) and from 2.11 to 4.23 (SDS-butanol). In the case of hybrid eluents containing CTAB and butanol, the errors are not good enough, probably due to that at high alcohol concentrations a change in the mechanism can be produced. It is important to note that in the assays with CTAB-butanol systems the alcohol concentration ranged from 3% to 10% and with eluents containing SDS and butanol it ranged from 3% to 7.5%.

CONCLUSIONS

From the results obtained in this work and for the group of dihydropyridines studied, the following statements can be established:

* Equation (2) is of more general applicability than equation (1) to predict solute capacity factors and significatively better than equation (3)

* In general, capacity factors can be related to micellized surfactant and alcohol concentrations in the mobile phase by means of only two or three equilibrium constants or related parameters and the best equation depends on the system considered.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support of this work through a research project from DGICYT (Spain) (reference PS90-0026). Also, we wish to acknowledge to Dr. Alvarez Builla for the kind gift of the dihydropyridines and Dr. Saz Díaz for offering us the idea of one of the Figures presented.

REFERENCES

1. J. K. Strasters, E. D. Breyer, A. H. Rodgers. M. G. Khaledi, J. Chromatogr., **11**, 17 (1990).

2. D. W. Armstrong, G. Y. Stine, Anal. Chem., **55**, 2317 (1983).

3. A. Berthod, I. Girard, C. Gonnet, Anal. Chem., **58**, 1362 (1986).

4. D. W. Armstrong, F. Nome, Anal. Chem., **53**, 1662 (1981).

5. P. Yarmchuck, R. Weinberger, R.F. Hirsch, L. J. Cline Love, Anal. Chem., **54**, 2233 (1982).

6. R. A. Barford, B. J. Sliwinski, Anal. Chem., **56**, 1554 (1984).

7. A. Berthod, I. Girard, C. Gonnet, Anal. Chem., **58**, 1359 (1986).

8. J. G. Dorsey, M. T. De Echegaray, J. S. Landy, Anal. Chem., **55**, 924 (1983).
9. A. Berthod, M. F. Borgerding, W. L. Hinze, J. Chromatogr., **556**, 263 (1991).

10. R. Bailey, R. M. Cassidy, Anal. Chem., **64**, 2277 (1992).

11. A. Berthod, I. Girard, C. Gonnet, Anal. Chem., **58**, 1356 (1986).

12. M .F. Borgerding, R L. Williams, Jr., W. L. Hinze, F. H. Quina, J. Liq. Chromatogr., **12**, 1367 (1989).

13. D. L. Massart, B. G. M. Vandeginste, S. N. Deming, Y. Michotte, L. Kaufman, **Chemometrics: A Textbook**, Elsevier 1988.

14. J. R. Torres-Lapasió, R. M. Villanueva-Camañas, J. M. Sanchis-Mallols, M. J. Medina-Hernández, M. G. García-Alvarez-Coque, J. Chromatogr., **639**, 87 (1993).

15. M. A. García, O. Jiménez, M. L. Marina, J. Chromatogr., **675**, 1 (1994).

16. M. Arunyanart, L. J. Cline Love, Anal. Chem., **56**, 1557 (1984).

17. M. G. Khaledi, J. K. Strasters, A. H. Rodgers, E. D. Breyer, Anal. Chem., **62**, 130 (1990).

18. O. Jiménez, M. A. García, M. L. Marina, J. Chromatogr., **719**, 15 (1996).

19. I. Benito, J. C. Díez-Masa, M.L. Marina, *in preparation.*

20. **SOLO Statistical System**, BMDP Statistical Software. Los Angeles, CA USA, 1991.

21. **Sigma Plot**, Scientific Graphing Software, Jandel Scientific, Corte Madera, CA, USA, 1989.

22. M. L. Marina, S. Vera, A. R. Rodríguez, Chromatographia, **28**, 379 (1989).

December 15, 1995
Accepted February 12, 1996
Manuscript 4057

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2497–2503 (1996)

DETERMINATION OF TRIACYLGLYCEROLS IN *PANAX PSEUDO-GINSENG* BY HPLC POLYMERIC COLUMN

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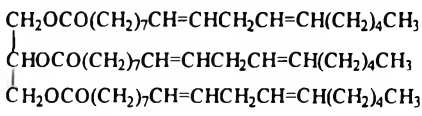
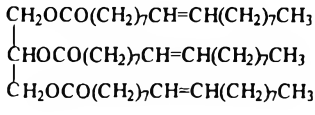
ABSTRACT

An improved high performance liquid chromatographic method was developed to determinate the active triacylglycerol principles of *Panax pseudo-ginseng* Wall. var. notoginseng Hoo & Tseng extracts. Chromatographic analysis is achieved on an isocratic system consisting of a polymeric reverse phase C_{18} column with a mobile phase of acetonitrile-methanol (50:50, v/v) to elute the trilinolein and triolein. The eluted triacylglycerol are detected at 205 nm. The linear calibration range was 0.5-20 and 20-500 μg/mL for trilinolein and triolein, respectively. The detection limit was 0.1 μg/mL for trilinolein and 10 μg/mL for triolein. It was found that n-hexane extracts of *Panax pseudo-ginseng* Wall. var. notoginseng Hoo & Tseng contained 0.21±0.021 mg/g of trilinolein. However, triolein was not detectable in the same herbal extract.



Trilinolein



Triolein

Figure 1. Chemical structures of trilinolein and triolein.

INTRODUCTION

Trilinolein and triolein (Fig. 1) are triacylglycerol in all three esterified positions of glycerol with linoleic acid and oleic acid, respectively. Trilinolein is also the active principle of the root of *Panax pseudo-ginseng* Wall. var. notoginseng (Burk.) Hoo & Tseng (Chinese name: Sanchi).[1] Sanchi is a herbal drug widely used in traditional Chinese medicine for the treatment of cardiovascular diseases.[2]

It was recently reported that, both saponins[3] and trilinolein[4] of Sanchi inhibit adrenaline-induced human platelet aggregation. This inhibition of trilinolein was accompanied by reduced ATP release and thromboxane B_2 formation[1]. During the cardiopulmonary bypass, trilinolein also improves the erythrocyte deformability.[5,6]

To determine fatty acids or triacylglycerols, a straight phase with silica column,[7] cyanopropyl column,[8] reverse phase[9] HPLC with UV detection, pre-column[10] and post-column[11,12] with fluorescent detection and HPLC-mass[13] have been reported. However, most of the methods for lipids measurement are not generally specific for trilinolein or triolein. Although the deuterium-labeled of [$^2H_{12}$]trilinolein and [$^2H_{18}$]triolein have been synthesized,[1] the contamination of radioisotope-labeled concerns prohibit their applications. In this work, we used a polymeric reverse phase method for the determination of trilinolein and triolein in the content of *Panax pseudo-ginseng* Wall. var. notoginseng (Burk.) Hoo & Tseng.

MATERIALS AND METHODS

Chemicals and Reagents

Panax pseudo-ginseng Wall. var. notoginseng (Burk.) Hoo & Tseng was purchased from a traditional Chinese herbal drug store in Taipei. Authentic compounds, trilinolein and triolein were obtained from Sigma Chem. (St. Louis, MO, USA). Acetonitrile (HPLC far UV grade), n-hexane, ethanol and methanol (HPLC grade) were obtained from LabScan Chem. (Dublin, Ireland). The stock solutions of trilinolein and triolein were dissolved in n-hexane at concentration of 1 mg/mL.

Apparatus and Chromatography

The HPLC system consisted of an injector (Rheodyne 7125, Cotati, CA, USA), a variable wavelength UV-VIS detector (Soma, Tokyo, Japan) and a chromatographic pump (Waters model 510). Separation was achieved on a HEMA reverse phased C_{18} polymer column, 250 x 4 mm, particle size 10 μm (P.J. Cobert Asso. St. Louis, MO, USA). The mobile phase was acetonitrile-methanol (50:50, v/v), and the flow rate was 1.0 mL/min. Triacylglycerols were monitored at a wavelength of 205 nm throughout the experiments. The system was operated at room temperature (25 °C).

Extraction

Panax pseudo-ginseng Wall. var. notoginseng Hoo & Tseng powder (0.5 g) was boiled with 50 mL of extraction solvent [n-hexane, methanol, ethanol (99.5 %), ethanol (50 %) or water] for 10 min. This procedure was repeated twice. The two filtrates were combined and diluted to 100 mL in a volumetric flask.

Precision

To determine the intra-assay variance, quadruplicate assays were carried out on the same concentrations (1, 5 or 20 μg/mL) at different times during the day. Inter-assay variance was determined by assaying in quadruplicate, on days one, two, four and six. Coefficients of variation (C.V.s) were calculated from these values.

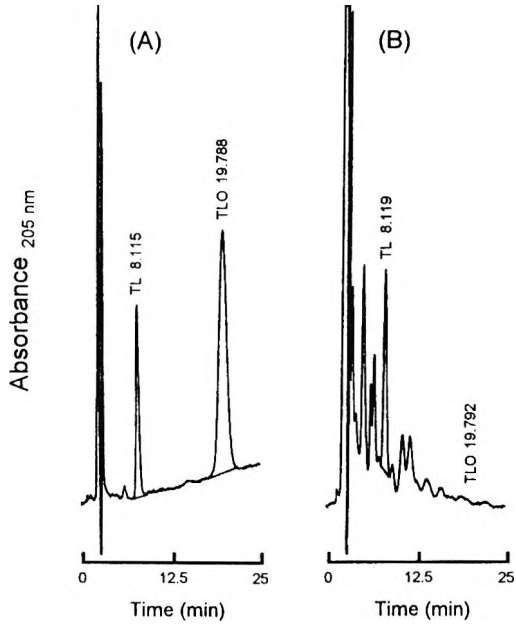


Figure 2. Elution profile of the injection 20 μL of (A) mixture of TL (trilinolein; 10 μg/mL) and TLO (triolein; 250 μg/mL), (B) extract of *Panax pseudo-ginseng* Wall. var. notoginseng Hoo & Tseng (20 mL/g) was separated by a polymeric reverse phase C_{18} column with 205 nm.

Determination of Trilinolein and Triolein

Calibration graphs for trilinolein and triolein dissolved in n-hexane were constructed by HPLC of various known amounts of these compounds (0.5, 1, 5, 10 and 20 μg/mL). The contents of trilinolein and triolein in the crude extract of *Panax pseudo-ginseng* Wall. var. notoginseng Hoo & Tseng were determined by the regression equation for the area under the curve versus concentration of these two compounds.

RESULTS AND DISCUSSION

Ultraviolet detector was a popular HPLC detector for lipids analysis[15]. However, the only functional groups detectable in the UV region (190-210 nm) are the carbonyl (C=O) and double bonds in the fatty acid chains (C=C)[16]. Thus, monitoring the elution of triacyl glycerols UV detection at 205 nm is

Table 1

Contents of Trilinolein and Triolein in Different Extracts of 1 Gm. of *Panax Pseudo-Ginseng* Wall. Var. Notoginseng Hoo & Tseng.

Extraction Solvents	Trilinolein	Triolein
n-Hexane	0.21 ± 0.021	n.d.
Methanol	n.d	n.d
Ethanol (99.5 %)	n.d.	n.d.
Ethanol (50 %)	n.d.	n.d.
Water	n.d.	n.d.

Data are expressed as mean ± SEM (mg/g, n=6).
n.d.: not detectable.

often used. But the solvents with significant absorption above 200 nm cannot be used in the mobile phase.

Under the conditions described above, the retention times of trilinolein and triolein were found to be 8.12 and 19.79 min, respectively (Fig. 2). The triolein content in the herbal extracts was under the detection limit (Fig. 2), thus, its validation was omitted. The detection limit for trilinolein and triolein, at signal-to-noise ratio of 4, were 0.1 and 10 μg/mL, respectively.

The content of trilinolein in the crude herbal extract was determined from the linear regression equation of the calibration graph for the compound. The equation for trilinolein was $Y = 5.301E\text{-}5\ X + 0.037$ ($r^2 = 0.999$), here, X is peak-area response and Y is amount of compound. The linearity range of trilinolein was 0.5-20 μg/mL.

The recovery tests were carried out by adding trilinolein (20 μg/mL) and triolein (100 μg/mL) to crude extract of herbal drug. The recoveries were 99.5 % and 99.3 % for trilinolein and triolein, respectively.

The intra-assay variation for the determination of trilinolein at concentrations of 1, 5, and 20 μg/mL were acceptable with C.V.s of less than 8 %. The inter-assay C.V.s for trilinolein at the same concentration were less than 10 %.

Table 1 summarized the contents of trilinolein (triolein is not detectable) in extracts of Panax pseudo-ginseng Wall. var. notoginseng Hoo & Tseng obtained with different solvents. Highest yield of trilinolein was founded from n-hexane extraction.

In conclusion, the proposed technique should be useful for the quality control of triacylglycerol in the herbal drug of *Panax pseudo-ginseng* Wall. var. notoginseng Hoo & Tseng for stability and for pharmacokinetic study of trilinolein.

ACKNOWLEDGMENTS

This study was supported by a research grant from Department of Health (DOH-86) and National Science Council (NSC 85-2331-B-077-004), Taiwan.

REFERENCES

1. C. Y. Hong, L. J. Lai, S. F. Yeh, Planta Med., **59**, 323-325 (1993).

2. X. L. Lei, G. C. Y. Chiou, Amer. J. Chin. Med., **14**, 145-152 (1986).

3. Y. Y. Guan, C. Y. Kwan, H. He, E. E. Daniel, Blood Vessels, **25**, 312-315 (1988).

4. L. J. Lai, Y. C. Shen, C. Y. Hong, Thromb. Res., **76**, 281-287 (1994).

5. S. K. Tsai, P. Chan, T. Y. Lee, J. M. Yung, C. Y. Hong, Br. J. Clin. Pharmacol., **37**, 457-459 (1994).

6. C. Y. Hong, L. J. Lai, M. S. Shiao, B. N. Chiang, Prostaglandins Leukot. Essent. Fatty Acids, **48**, 351-353 (1993).

7. J. G. Hamilton, K. Comai, Lipids, **23**, 1150-1153 (1988).

8. M. D. Greenspan, C. Y. L. Lo, D. P. Hanf, J. B. Yudkovitz, J. Lipid Res., **29**, 971-975 (1988).

9. R. Vercaemst, A. Union, M. Rosseneu, J. Chromatogr., **494**, 43-47 (1989).

10. Y. Tsuruta, T. Teranishi, Y. Date, K. Kohashi, J. Chromatogr., **617**, 213-220 (1993).

11. K. Akasaka, H. Ohrui, H. Meguro, J. Chromatogr., **617**, 205-211 (1993).

12. K. Akasaka, H. Ohrui, H. Meguro, J. Chromatogr., **622**, 153-159 (1993).

13. A. Kuksis, L. Marai, J. J. Myher, Lipids, **26**, 240-244 (1991).

14. D. L. Hachey, M. R. Thomas, E. A. Emken, C. Garza, L. Brown-Booth, R. O. Adlof, P. D. Klein, J. Lipid Res., **28**, 1185-1192 (1987).

15. J. J. Myher, A. Kuksis, J. Chromatogr. B, **671**, 3-33 (1995).

16. E. B. Hoving, J. Chromatogr. B, **671**, 341-362 (1995).

Received February 19, 1996
Accepted March 13, 1996
Manuscript 4095

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2505–2515 (1996)

ELECTROCHEMICAL DETECTION OF UNDERIVATIZED AMINO ACIDS WITH A Ni-Cr ALLOY ELECTRODE

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ABSTRACT

A nickel-chromium (80:20) alloy is employed as electrode material in the amperometric detection of underivatized amino acids in flow injection and high performance liquid chromatographic experiments. Cyclic voltammetric results show that amino acids are oxidized by a surface catalyzed process, proposed to involve Ni(III) oxyhydroxides, which are formed on the electrode surface at approximately 0.43 V (Ag/AgCl reference electrode) in 0.10 N NaOH. The hydrodynamic voltammograms of different amino acids show current plateaus at potentials above ca. 0.48 V. Preliminary HPLC experiments show that the nickel-chromium alloy is useful for the amperometric detection of underivatized amino acids following anion exchange separations.

INTRODUCTION

Determination of amino acids has a great importance in food analysis, clinical chemistry, biotechnology and fermentation control. Several strategies for amino acids determination have been developed, including direct detection by ultraviolet absorbance, refractive index and fluorescence.

However, derivatization of these molecules is necessary to attain high sensitivities in ultraviolet absorbance and fluorescence detection because most of the amino acids lack a chromophore group.

Electrochemical detection in Flow Injection Analysis (FIA) and High Performance Liquid Chromatography (HPLC) has become widely accepted nowadays. There are various modes for the electrochemical detection of amino acids.[1] Direct oxidation of amino acids can be performed with varying degree of success using constant potential amperometric detection[2] or pulsed amperometric detection.[3,4] Chemically modified electrodes can also be used for the electrochemical detection of amino acids.[5] Still, derivatization has become important in improving their electrochemical response at conventional electrode materials (e.g. glassy carbon) because a very few amino acids are electrochemically active.

Derivatization approaches involving the use of nitroaryl reagents,[6,7] o-phthaldehyde,[8,9] phenylisothiocyanate,[10] and ferrocene derivatives[11] have been described in the literature. However, pre- or post-column derivatization methods add complexity to the detection scheme.

Nickel electrodes have been previously used for the amperometric detection of organic molecules in alkaline solutions.[2,12,13,14] However, the sensitivity and the long term response reproducibility of the electrode was poor when compared with copper electrodes.[2] One advantage of the nickel electrode is its relatively low background current at the usual working potentials (approximately 4-6 $\mu A/cm^2$ at 0.5 V vs Ag/AgCl).

Nickel alloys have demonstrated good catalytic activity and proved useful in the amperometric detection of carbohydrates after high performance anion exchange separations.[15] Moreover, high sensitivity and good response reproducibility were achieved using the nickel-chromium (80:20) (Ni-Cr) alloy.[16] An increase in sensitivity by a factor of approximately ten was observed at the Ni-Cr alloy electrode as compared with pure nickel electrodes. Easy implementation, very low background current, and short settling time are another advantages of the Ni-Cr alloy electrode.

In this paper, we report the use of Ni-Cr alloy electrodes for the electrochemical detection of underivatized amino acids following anion exchange separations. Cyclic voltammetric (CV) experiments show that oxidation of amino acids is associated with the Ni(II)/Ni(III) redox couple. The set potential for the electrochemical detection of amino acids is optimized on a Signal-to-Noise ratio (S/N) analysis at different potential values. Good electrode performance was obtained in preliminary HPLC experiments with an anion exchange column for amino acids separation.

MATERIALS AND METHODS

Reagents

Standard amino acid (Sigma, USA) and sodium hydroxide (Merck, Argentina) solutions were daily prepared with triply distilled water. All reagents were analytical degree.

Equipment

Nickel-chromium (80:20) (Goodfellow, England) working electrodes for CV experiments were prepared with 0.1 cm diameter wires embedded in Teflon shrinkable tubes. The working electrodes for the liquid chromatographic experiments were made of the same Ni-Cr wires embedded in Kel-F blocks of 0.5x1.0x1.0 inches.

The electrodes were polished successively with emery paper of 400, 600 and 0000 grit, and finished to a mirror surface with 1, 0.3 and 0.05 μm alumina particles suspended in water on a microcloth pad, sonicated, and then thoroughly washed with triply distilled water.

A Ag/AgCl (3 M KCl) reference electrode was used in the CV experiments.

Cyclic voltammetric experiments were performed with an EG&G PARC Model 273 computerized potentiostat. An EG&G PARC model 175 Universal Programmer was used as the waveform generator.

The current-voltage (i-E) output was recorded with either the PC's printer or with a Houston 2000 XY recorder. A conventional three electrode glass cell was used for the CV experiments.

The chromatographic system consisted of a model 307 pump (Gilson, France), a model 7125 inyector (Rheodyne, USA) with a 20 μL injection loop, and a Wescan Anion-R, 250x4.6 mm (Hamilton Co., USA). A home made potentiostat was used as an amperometric detector.

The electrochemical signal was fed to a Personal Computer (PC) equipped with Peak Simple (SRI, USA) data processing software. The electrochemical cell for the flow experiments consisted of a home made working electrode, a stainless steel auxiliary electrode and a Ag/AgCl (3 M KCl) reference electrode. The dead volume of the flow cell was approximately 5 μL.

RESULTS AND DISCUSSION

Previous studies carried out in our laboratory showed that the voltammetric behavior of Ni-Cr electrodes in alkaline solutions is complex.[17] A featureless i-E trace is obtained in the potential range from 0.00 V to -1.10 V after the first few 3-4 potential cycles at 0.050 V/s. A reversible redox system is observed in the potential range from 0.00 V to 0.60 V.

The complexity of both oxidative and reductive waves in this potential range increases as the number of CV cycles increases. An increment of the peak current, peak potential, and peak charge is observed on the oxidative wave during approximately 50 CV cycles. After this number of cycles, only the peak current and peak charge of the same wave increase. The peak remains at approximately 0.43 V

The peak potential of the reductive wave on the reverse scan is approximately 0.35 V. A decrease of the peak current and electrochemical charge of this wave is observed as the number of CV cycles increases. Simultaneously, a shoulder develops on this reductive wave at approximately 0.29 V. After approximately 500 CV cycles at 0.050 V/s the shoulder has changed into a totally defined peak, and the original reductive peak is almost not detected.

Finally, a steady i-E response is obtained after approximately 600 CV cycles, using the same potentiodynamic conditions. A square reaction diagram, with different Ni oxy-hydroxide species, was proposed[17] to explain the peak multiplicity observed at Ni-Cr electrodes in the potential range from 0.00 V to 0.60 V.

The steady i-E trace obtained with Ni-Cr electrodes after nearly 600 CV cycles in the background solution, is shown in Figure 1 (solid line). The oxidative wave observed at approximately 0.43 V was attributed to the oxidation of Ni(II) to Ni(III).

A reductive wave, which may be attributed to Ni(III)/Ni(II) reduction, is obtained at approximately 0.29 V on the reverse scan. No other features are observed in the cyclic voltammogram of Ni-Cr electrodes in this potential range.

Cyclic voltammograms, of different glycine concentrations at the Ni-Cr electrode, are also shown in Figure 1 (dotted lines). An increment in the charge and peak current under the anodic wave on the positive scan, and a decrease of these parameters during the negative scan are the main effects due to the addition of glycine.

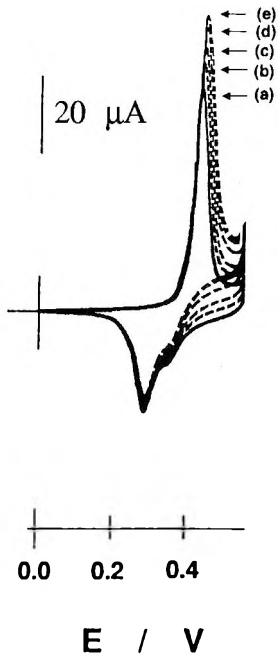

Figure 1. Cyclic voltammogram of a Ni-Cr electrode in 0.10 N NaOH (a), and with different glycine concentrations: (b) = 0.5 mM; (c) = 1.0 mM; (d) = 1.5 mM; (e) = 2.0 mM. v = 0.050 V/s, T = 298 K.

This behaviour is typical of electrocatalytic mechanisms,[18] and was previously observed in the oxidation of carbohydrates at Ni-Cr electrodes in alkaline solutions.[16] The proposed mechanism is shown below in the general pathway indicated in reactions (1)-(2):

$$Ni(OH)_2 + OH^- \overset{k(E)}{\rightleftharpoons} NiOOH + H_2O + e \quad (1)$$

$$NiOOH + \text{organic molecule} \xrightarrow{k} Ni(OH)_2 + \text{products} \quad (2)$$

The active species, Ni(III), is generated from Ni(II) species on the electrode surface at approximately 0.43 V via electron transfer (reaction (1)). Electrogenerated Ni(III) species then oxidize the analyte via redox reactions (reaction (2)). The rate constant for the first reaction is potential dependent. The analytically important experimental observable is that for an electron

transfer which is kinetically slow (k(E) small), the application of a greater potential difference will increase the reaction rate. On the other side, reaction (2) has been proposed[19] as the rate limiting step of the reaction pathway.

The same basic mechanism may be proposed for the oxidation of glycine (and other amino acids) at the Ni-Cr alloy electrode since only the Ni(II)/Ni(III) redox couple seems to be involved in the reaction. However, mixed oxides and hydroxides of both Ni(II) and Ni(III) may be involved, as well as their different crystalline forms (α, β and γ). Thus, not one species, such as NiO, $Ni(OH)_2$, or NiO(OH) suffices to describe the surface chemical entities involved in the reaction.

Both peak current and oxidative charge have a linear relationship with the concentration of glycine. Calibration plots of the anodic peak current as a function of glycine concentration give straight lines. The linearity of the plot is extended up to approximately $3x10^{-3}$ M. These results indicate that the Ni-Cr alloy may be used as an amperometric detector for amino acids in flowing systems (e.g. HPLC and FIA).

Sensitivity is a detector parameter of major analytical importance, and it is defined as the ratio of current generated to concentration present. Sensitivity depends mainly on two sorts of processes: mass transfer and charge transfer.[20] Mass transfer refers to how easily a solute can reach the electrode surface. Charge transfer is related to the ease of the analyte to undergo electron transfer once it has reached the electrode surface.

The oxidation of amino acids at Ni-Cr electrodes in alkaline solutions was previously proposed as an electrocatalytic process. The whole process of electron transfer from the analyte to the electrode is composed of two steps, reactions (1) and (2) in the reaction scheme. The rate constant for reaction (1) is potential dependent. Thus, the rate for this process may be controlled by selecting the appropriate working potential.

On the other side, the rate at which reaction (2) proceeds, may not compromise the detector's sensitivity, assuming that the analytes remain enough time into the flow cell chamber. This assumption is valid for most of the usual chromatographic working conditions. For example, a 25-cm column with 15,000 theoretical plates will have a peak width of about 10 s for k'=1.[21]

Hydrodynamic voltammetric studies provide information about the working potential range in which the detector's sensitivity is only dependent on the mass transfer rate. Thus, we performed hydrodynamic voltammograms of various amino acids by FIA experiments using the Ni-Cr electrode in alkaline solutions (Figure 2). The initial working potential was 0.40 V. After obtaining

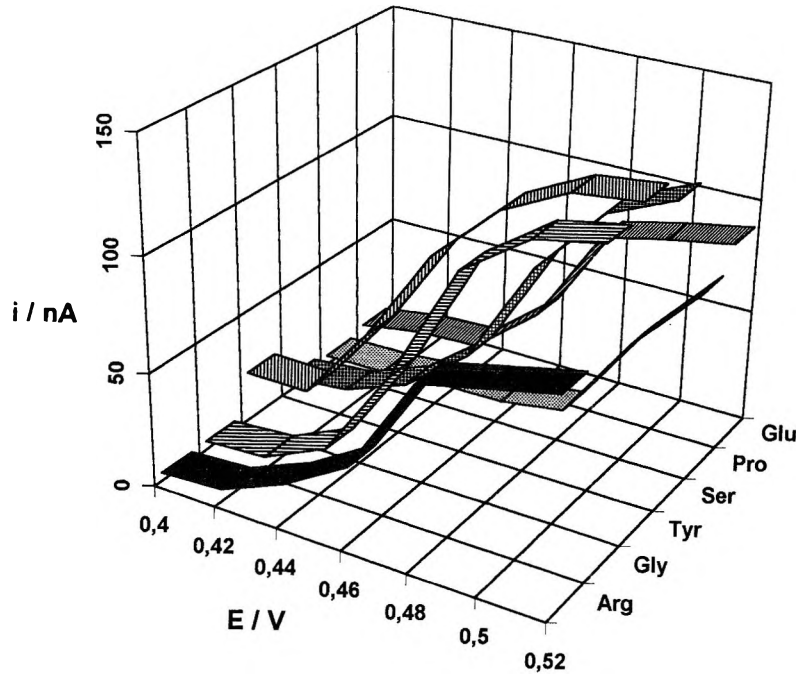

Figure 2. Hydrodynamic voltammogram of various amino acids at Ni-Cr electrodes in 0.10 N NaOH. Glutamic acid (Glu), Proline (Pro), Serine (Ser), Tyrosine (Tyr), Glycine (Gly), and Arginine (Arg). Flow rate = 0.50 mL/min; concentration of the analytes: 1.0×10^{-4} M each; injection amount: 2 nmol each.

a stable, drift-free baseline at the set potential, ten separate injections of each amino acid were analyzed. The working potential was then increased by increments of 0.02 V, and the FIA experiments repeated. In the region between 0.40 V to approximately 0.44 V, there is essentially no evidence for amino acid oxidation (except for tyrosine, which gives a small oxidative current at this low potential). At potentials greater than 0.44 V the current increases and, then, plateaus at values above ca. 0.48 V. The current for Proline oxidation increases only after 0.48 V, and this trend seems to be an exception for the behavior of the amino acids in this study.

At potentials above 0.52 V, oxygen evolution starts as characterized by an increase in the background noise level. Thus, the optimum set potential for amino acid detection with the Ni-Cr electrode in alkaline solution, should lay in the potential range between 0.44 V and 0.50 V.

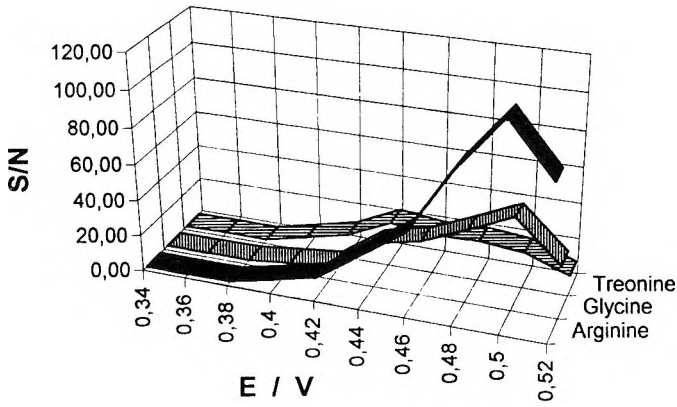

Figure 3. Signal to Noise ratio analysis of different amino acids at Ni-Cr electrodes in 0.10 N NaOH. Flow rate = 0.50 mL/min. Injection amount = 2 nmol each amino acid. T = 298 K.

In order to be able to use any theoretical expression for signal intensity in the calculation of optimum conditions, some knowledge of the noise in the system is needed. Poppe et al. studied the noise in a flow-through channel electrode.[22] The sources which they gave for noise showed a linear relationship between noise intensity and electrode area. Taking these findings into account, the optimum set potential for electrochemical detection should be that, where the highest Signal-to-Noise ratio is observed.

The S/N analysis for different amino acids was performed by HPLC experiments using the Ni-Cr electrode in alkaline solution. Figure 3 shows the results of this analysis at various working potentials. The initial working potential was 0.34 V. After obtaining a stable, drift free baseline, a record of this background current was obtained at high sensitivity, in order to analyze the noise. Then, various separate injections of the amino acid mixture were analyzed, and the S/N ratio for each amino acid computed. The working potential was then increased by 0.02 V and the study repeated.

The potential for the highest Signal-to-Noise ratio in the analysis of amino acids with Ni-Cr electrodes in 0.10 N NaOH is approximately 0.48-0.50 V (Figure 3). This working potential range, can be taken as the best[23] for HPLC analysis of amino acids. Although, the current plateaus at potentials over 0.48V in the hydrodynamic voltammograms, it has to be noticed that, the S/N ratio for each analyte has a maximum value in the above mentioned

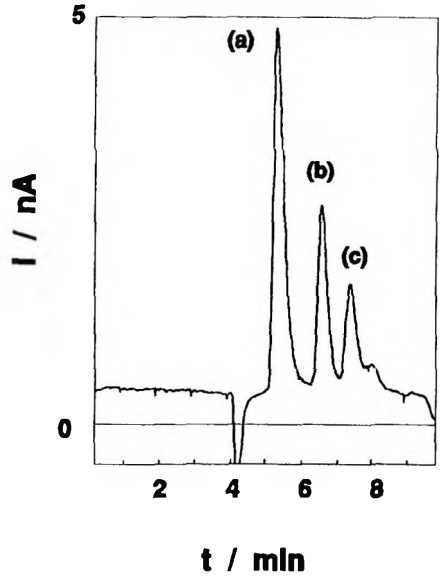

Figure 4. High Performance Anion Exchange chromatogram of three amino acids separated with an anion exchange column. Injection amount = 2 nmol each amino acid. Flow rate - 0.50 mL min. T = 298K. (a) Arginine, (b) Glycine, (c) Treonine.

potential range. This phenomena may be attributed to the increased background noise observed at potentials over 0.48 V. Thus, the S/N ratio is lowered over 0.50 V.

An anion exchange high performance chromatogram of glycine, treonine and arginine at a working potential of 0.48 V is shown in Figure 4. These compounds can be easily separated with the anion exchange column, and detected with the Ni-Cr alloy electrode. The effectiveness of the chromatographic separation is indicated by the excellent resolution at a flow rate of 0.5 mL/min.

CONCLUSIONS

Amino acids separated by anion exchange columns, are easily detected with a Ni-Cr alloy electrode in 0.10 N NaOH. It is believed, that the target molecules are oxidatively detected by a surface catalyzed process, proposed to involve Ni(III) species. The hydrodynamic voltammogram of various amino acids show that the mass-transfer control of the current is obtained at potentials above 0.48 V.

The signal-to-noise ratio analysis at different working potentials, shows that the range from 0.48 V to 0.50 V can be taken as the best for the analysis of amino acids with Ni-Cr electrodes.

ACKNOWLEDGEMENTS

The authors want to thank Fundacion Antorchas and SECyT (Universidad Nacional de Rio Cuarto) for supporting this work.

REFERENCES

1. L. Dou, J. Mazzeo, I. S. Krull, BioChromatography, **5**, 74 (1990).

2. P. Luo, F. Zhang, R. P. Baldwin, Anal. Chim. Acta., **244**, 169 (1991).

3. J. A. Polta, D. C. Johnson, J. Liq. Chromatogr., **6**, 1727 (1983).

4. D. C. Johnson, W. R. LaCourse, Anal. Chem., **62**, 589A (1990).

5. M. K. Halbert, R. P. Baldwin, J. Chromatogr., **345**, 43 (1985).

6. W. L. Caudill, G. P. Houck, R. M. Wightman, J. Chromatogr., **227**, 331 (1982).

7. J. L. Meek, J. Chromatogr., **266**, 401 (1983).

8. L. A. Allison, G. S. Mayer, R. E. Shoup, Anal. Chem., **56**, 1089 (1984).

9. W. A. Jacobs, J. Chromatogr., **392**, 435 (1987).

10. R. R. Granberg, LC Magazine, **2**, 776 (1984).

11. K. Shimada, Y. Kawai, T. Oe, T. Nambara, J. Liq. Chromatogr., **12**, 359 (1989).

12. J. B. Kafil, C. O. Huber, Anal. Chim. Acta, **175**, 275 (1985).

13. W. Buchberger, K. Winsauer, Ch. Breitwieser, Fresenius Z. Anal. Chem., **315**, 518 (1983).

14. M. Goto, H. Miyahara, D. Ishi, Anal. Chim. Acta, **515**, 213 (1990).

15. J. M. Marioli, T. Kuwana, Electroanalysis, **5**, 11 (1993).

16. J. M. Marioli, T. Kuwana, Anal. Chim. Acta, **282**, 571 (1993).

17. J. M. Marioli, L. E. Sereno, Electrochim. Acta, **40**, 983 (1995).

18. A. J. Bard, L. R. Faulkner, **Electrochemical Methods, Fundamental and Applications**, J. Wiley and Sons, New York, 1980, ch. 11.

19. M. Fleischman, K. Korinek, D. Pletcher, Electroanal. Chem., **31**, 39 (1971).

20. S. G. Weber, W. C. Purdy, Ind. Eng. Chem. Prod. Res. Dev., **20**, 593 (1981).

21. J. W. Dolan, L. R. Snyder, **Troubleshooting LC Systems**, Humana Press, New Jersey, USA, 1989, ch. 13.

22. J. Lankelma, H. Poppe, J. Chromatogr., **125**, 375 (1976).

23. K. Stulik, V. Pacakova, **Electroanalytical Measurements in Flowing Liquids**, Ellis Horwood Ltd., Chichester, United Kingdom, 1987, p. 29.

Received January 18, 1996
Accepted February 7, 1996
Manuscript 4070

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2517–2525 (1996)

RAPID ASSAY FOR THE DETERMINATION OF RESIDUES OF AMPROLIUM AND ETHOPABATE IN CHICKEN MEAT BY HPLC

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ABSTRACT

A simple, rapid and sensitive HPLC method for the determination of amprolium and ethopabate in chicken meat, is presented. The samples were extracted with acetone, the organic layer then being separated and evaporated to dryness (ethopabate analyse). Employing traditional liquid-liquid extraction, a clean extract was obtained from the water phase (amprolium analyse). The limits of quantification were 5 and 1 ng/g for amprolium and ethopabate, respectively.

INTRODUCTION

The combination of ethopabate (ETB) and amprolium (AMP) is frequently used in the prophylaxis and treatment of coccidiosis and leukocytozoonosis in chickens.[1,12]

Several analytical methods for the determination of AMP and ETB in different biological materials based on colorimetry,[2] gas chromatography,[3,4,5] and high performance liquid chromatography with ultraviolet and fluorescence detection[6,7,8,9,10,11,12] have been published. The methods are, however, time-consuming and require the use of large quantities of chemical reagents.

The purpose of the present study was to develop a rapid, simple and sufficiently sensitive method, for the determination of AMP and ETB, which required only small quantities of chemical reagents.

MATERIALS AND METHODS

Materials and Reagents

Samples of chicken meat were used.

All chemicals and solvents were of analytical or HPLC grade. AMP was supplied by Sigma Co. (St. Louis, MO, USA), and ETB by Merck Frosst Canada, Pointe - Claire, Dorval, Quebec. AMP stock solution (1mg/mL) and working standards were prepared by dilution with 0.02 M KH_2PO_4. The ETB stock solution (1mg/mL) was made by dissolving 25 mg ETB in 5 mL acetone and diluting to 25 mL with water. The working standards were prepared by dilution with distilled water. The standards solution were stored in the refrigerator.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, an ISS 100 sampling system equipped with a Lauda RMT6 cooler (12°C) from Messgeräte Werk Lauda, (Lauda Königshafen, Germany), and a LC 240 fluorescence detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at an excitation wavelength of 365 nm and emission wavelength of 470 nm for AMP, and an excitation wavelength of 300 nm and emission wavelength of 350 for ETB, with a response of 5 and a factor of 256. The integration was carried out using the software programme Turbochrom 4.0 (Perkin-Elmer), which was operated on a Brick personal computer connected to a BJ-330 printer (Canon).

The analytical column (stainless steel, 15 cm x 4.6 mm I. D.), operated at room temperature and guard column (stainless steel, 2 cm x 4.6 mm I. D.) were packed with 5-μm particles of Supelcosil LC-ABZ, (Supelco) for AMP. The analytical column for ETB (stainless steel, 25 cm x 4.6 mm I. D.), operated at

room temperature, and guard column (stainless steel, 2 cm x 4.6 mm I. D.), were packed with 5-μm particles of Supelcosil LC-ABZ+Plus (Supelco, Bellefonte, PA, USA). The guard column was connected to a A.318 precolumn filters with a A-102 frits (Upchurch Scientific, USA).

The mobile phase for AMP was a mixture of the solutions A and B (90 : 10). Solution A was 0.2 M KH_2PO_4, 0.005 M hexane sulfonic acid Na salt, made by dissolving 27.2 g/L KH_2PO_4 and 0.94 g/L hexane sulfonic acid Na salt in ca. 750 mL water when making 1 litre of solution. The solution was made up to volume with water . Solution B was acetonitrile. The flow rate was 0.8 mL/min. The column effluent was introduced into a vortex mixer (a low dead volume tee (1.2μL)) from a system for HPLC post column reactions (PCRS 520 - Kratos). A Series 10 Liquid Chromatograf (Perkin-Elmer), (fitted with an extra pulse-dampened pump) was used, with a mobile phase of 1.25 M NaOH, 0.025 M $K_3Fe(CN)_6$ made by dissolving 50 g/L NaOH in ca. 75 mL water, adding ca. 700 mL water (waiting until the solution had achieved room temperature), and then adding and dissolving 0.8 g/L potassium ferricyanide. The solution was made up to volume with water. The mobile phase had a flow rate of 0.7 mL/min., and was coupled to the vortex mixer, and a reaction coil (Beam Boost Photochemical Reactor Unit, PTFE 10 m x 0.3 mm I. D. - ITC Dandelsgesellschaft m.b.H.Frankfurt, Germany), operated at room temperature. The solvent stream was then coupled to the fluorescence detector. The system was equilibrated with the mobile phase and the derivating reagents for about 20 min. (0.8 and 0.7 mL/min. respectively) prior to injecting the sample into the HPLC.

The mobile phase for ETB was a mixture of water - acetronitrile (65 : 35). The flow rate was 0.8 mL/ min.

Sample Pretreatment

The sample, (3g) was weighed into a 50 mL centrifuge tube with a screw cap (NUNC, Roskilde, Denmark). Volumes of 1 mL water (or standard) and 4 mL acetone were added. The mixture was homogenized for approx. 6 sec. in an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Ika Werk, Staufen, F. R. G.), and then centrifuged for 3 min. (5000 rpm). Four mL of the supernatant (corresponding to 1.5 g) were transferred into a glass-stoppered centrifuge tube. A volume of 5 mL CH2Cl2 was added and mixed for approx. 5 sec. After centrifugation for 3 min. (3000 rpm), the upper (water) layer was transferred to another glass-stoppered tube (AMP analysis). The organic layer dichloromethane-acetone for ETB analysis was transferred to another glass-stoppered tube, possible water residues in the tube being transferred back to the water phase tube (AMP analysis). The organic layer was evaporated to dryness under a stream of nitrogen using a Reacti-Therm heating module at 60°C and

Reacti-Vap evaporating unit (Pierce, Rockford, IL, USA). The dry residue was dissolved in 500 µl of a mixture of two solutions A and B (70 : 30). Solution A was methanol and B was 0.02 M 1- heptane sulfonic acid sodium salt (Supelco, USA) - 0.01 M di-sodium hydrogenphosphate-2-hydrate (Ferax, Germany), made by dissolving 4.45 g/L heptane sulphonate and 1.8 g/L di-sodium hydrogen phosphate 2-hydrate in ca. 750 mL of water when preparing 1 litre of solution. The pH was then adjusted to ca. 6.3 with 5 M H_3PO_4 and to 6.0 with 1 M H_3PO_4, and the solution made up to volume (1 L) with water, the pH again being adjusted to 6.0 with 1 M H_3PO_4. The dissolved residue was kept in a freezer (-20°C) for 5 min. The methanol-based phase was filtered through a Costar Spin-X centrifuge filter unit with 0.2 µm nylon membrane, and centrifuged for 3 min. at 10000 rpm. (5600g). Aliquots of 20 µL were injected into the HPLC at intervals of 10 min. for the determination of ETB.

1g NaCl, 3mL CH3CN and 1 mL 0.3 M NaOH were added to the water phase. The sample was shaken vigorously for 20 sec., and centrifuged for 2 min. at 3000 rpm. The upper layer (CH_3CN) was transferred to another glass-stoppered tube. The sample was extracted twice with 3 mL CH_3CN, and the water layer discarded. The CH_3CN was evaporated to dryness at 60°C under a stream of nitrogen. The dry residue was dissolved in 500 µL 0.02 M KH_2PO_4 and filtered through a Spin-X by centrifugation for 3 min. at 10000 rpm. (5600g). Aliquots of the filtrate (20µL) were injected into the HPLC at intervals of 8 min. for the determination of AMP.

Calibration Curves And Recovery Studies

The calibration curves for AMP and ETB were obtained by spiking muscle tissue samples with standard solutions, to yield 5, 10, 15, 20, 30, 50, 100, 200, 300 and 500 ng/g and 1, 2, 5, 10, 20, 30 and 50 ng/g of AMP and ETB, respectively. Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked muscle samples with those of standard solution. The linearity of the standard curves for AMP and ETB in muscle were tested using peak-height measurements.

RESULTS AND DISCUSSION

Chromatograms of extract of blank samples, and spiked samples from chicken meat are shown in Fig. 1 and 2. The standard curves were linear in the investigated areas; 5 - 500 and 1 - 50 ng/g for AMP and ETB in meat, respectively. The linearity of the standard curves was 0.9998 for AMP and

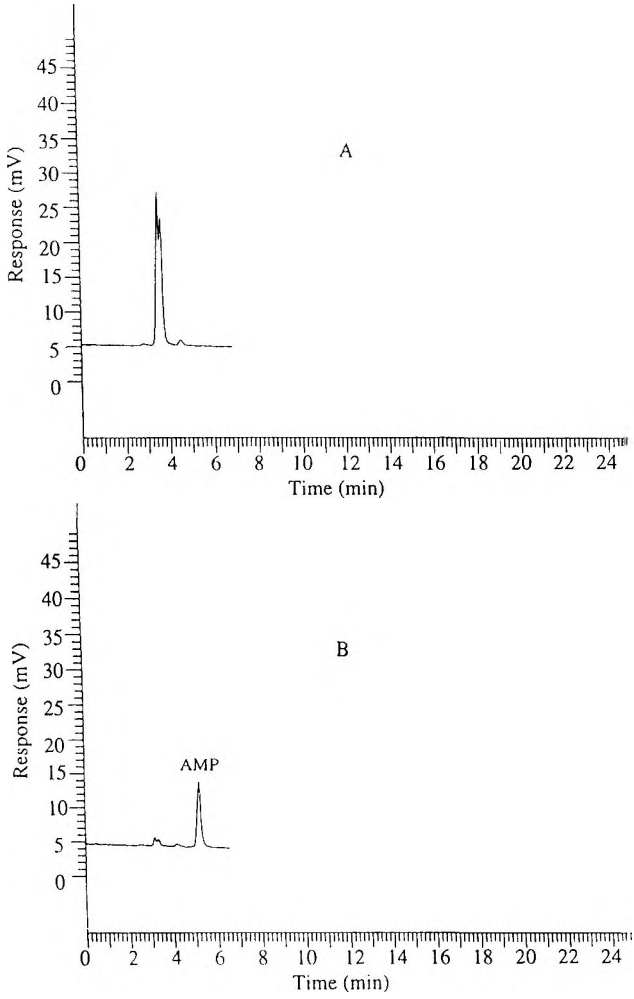

Figure 1. Chromatograms of extracts from chicken meat. **A:** drug-free meat. **B:** meat spiked with amprolium (200 ng/g).

0.999 for ETB in meat, when using the external standard method of calculation. The precision and recovery for AMP and ETB from meat were also calculated and are shown in Table 1.

The extraction procedures were validated, and showed good recovery of AMP and ETB. The recovery was 99% for AMP and varied from 98 to 99% for ETB in meat. The precision of these recovery studies varied from 0.8 to

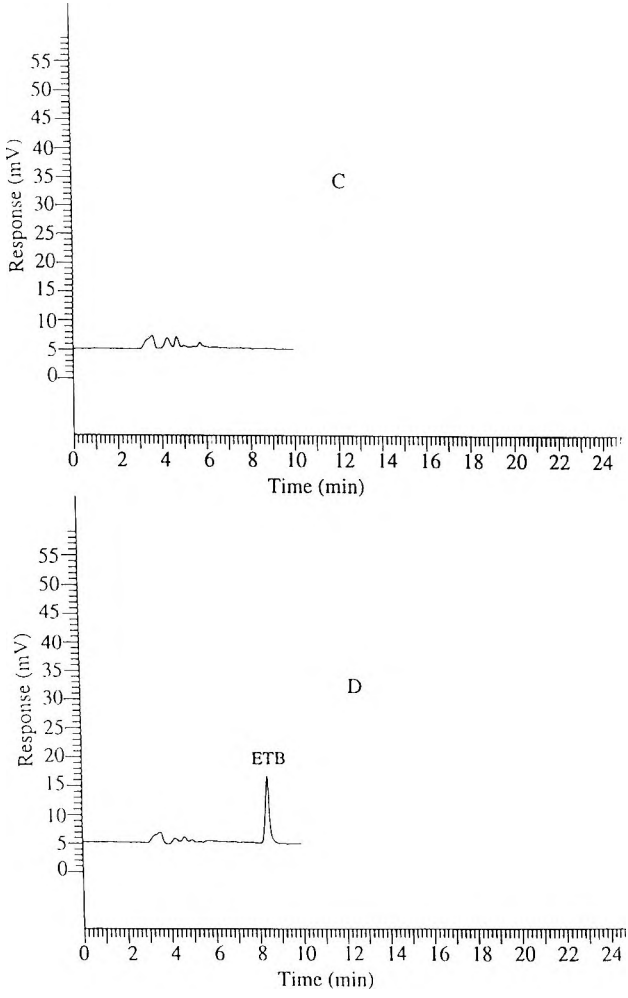

Figure 2. Chromatograms of extracts from chicken meat. **C:** drug-free meat, **D:** meat spiked with ethopabate (20ng/g).

1.0% and from 0.2 to 0.8% for AMP and ETB in meat, respectively. The limit of quantification was 5 ng/g and 1ng/g for AMP and ETB in meat, respectively.

The method was tested under practical conditions by analysing about 50 samples from different chickens, with no interfering peaks being observed. No interference was seen during analysis, when calibrating the curves, nor when performing recovery studies.

Table 1

Precision and Recovery of AMP and ETB

Tissue 3g	No. of Samples	Amount in Spiked Samples (μg/g)	Recovery% AMP Mean	AMP SD	ETB Mean	ETB SD
Meat	8	0.05	99	0.8		
3g	8	0.30	99	1.0		
Meat	8	0.005			98	0.8
3g	8	0.030			99	0.2

S.D. = standard deviation

AMP was separated from interfering substances, using a reverse phase ion-pair system, and was post-column oxidized to amprochrome with ferricyanide in alkaline solution, giving a fluorophore that can be determined with greater sensitivity than by U.V. detection.[2,8] The LC operating conditions, *i.* e., mobile phase composition, flow rate, column temperature, concentrations of NaOH and ferricyanide, and detection wavelength were investigated, each of these parameters being optimized to provide maximum drug response.

Conditions were as reported by T. Nagata and M. Saeki[8] apart from column temperature which do not have any particular effect between 20 - 40°C, and the mobile phase composition which was adjusted to a 15 cm ABZ analytical column. The reaction coil length used gave a satisfactory result.

Because of the high salt concentration in the mobile phase (0.2M KH_2PO_4 - 0.005M hexane sulfonic acid Na salt) the HPLC system, including the analytical column (15 cm) for AMP, was flushed with water-acetonitrile (90 : 10) for 15 min. (1 mL/min.) prior to and after running a serie of samples.

It is also important to follow the recommendations of the producer to store Supelcosil LC-ABZ column in 100% acetonitrile, to avoid serious retention loss.

Regarding the ETB analytical column (25 cm) it is not necessary to take special precautions since the mobile phase is water - acetonitrile. However, the producers recommendations concerning the storage of Supelcosil LC-ABZ+Plus in 100% methanol must be followed.

This study has shown that residues of the coccidiostatic compounds AMP and ETB in chicken meat can be determined using minimal sample manipulation. The cost of chemicals is reduced and the manual work-up procedures are less laborious, compared to previously published methods.

An experienced technician can carry out sample clean-up of about 18 samples per day. The pretreatment of tissues by liquid-liquid extractions combined with centrifugation filters, is preferable to solid-phase extraction columns when performing the pretreatment manually. The assay shows good precision when using the external standard method. The method is robust, sensitive and is efficient for quantification of residues of AMP and ETB. The quantification is linear over a wide concentration range.

ACKNOWLEDGEMENTS

We are grateful to the Norwegian Research Council for financial support.

REFERENCES

1. E. Takabatake, Eisei Kagaku, **27**, 127-143 (1982).

2. Official Methods of Analysis 14th Ed., AOAC, Arlington, VA, 42.011-42.015 and 42.016-42.020, 1984.

3. T. Okada, M. Uno, Y. Onji, T. Ohmae, K. Tanigawa, H. Akagi, E. Takabatake, J. Food Hyg. Sci., **22 (4)**, 279-284 (1981).

4. N. Nose, Y. Hoshino, F. Yamada, Y. Kikuchi, S. Kawauchi, J. Food Hyg. Sci., **22 (6)**, 508-511 (1981).

5. N. Nose, Y. Hoshino, Y. Kikuchi, S. Kawauchi, J. Food Hyg. Sci., **23 (2)**, 176-183 (1982).

6. Y. Hori, J. Food Hyg. Sci., **24 (5)**, 447-453 (1983).

7. T. Nagata, M. Saecki, H. Nakazawa, M. Fujita, E. Takabatake, J. Assoc. Off. Anal. Chem., **68 (1)**, 27-28 (1985).

8. T. Nagata, M. Saecki, J. Assoc. Off. Anal. Chem. **69 (6)**, 941-943 (1986).

9. T. Nagata, M. Saecki, J. Food Hyg. Sci., **29 (1)**, 13-19 (1988).

10. D. G. Keith, P. Hyun, Y. John, M. Y. Larry, J. Assoc. Off. Anal. Chem., **71 (1)**, 48-50 (1988).

11. M. Murayama, S. Uchiyama, Y. Saito, J. Food Hyg. Sci., **32 (3)**, 155-160 (1991).

12. Y. Takahashi, T. Sekiya, M. Nishikawa, Y. S. Endoh, J. Liq. Chromatogr., **17 (20)**, 4489-4512 (1994).

Received December 1, 1995
Accepted March 18, 1996
Manuscript 4043

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2527–2540 (1996)

VERSATILE MULTIDIMENSIONAL CHROMATOGRAPHIC SYSTEM FOR DRUG DISCOVERY AS EXEMPLIFIED BY THE ANALYSIS OF A NONPEPTIDIC INHIBITOR OF HUMAN LEUKOCYTE ELASTASE

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ABSTRACT

A direct injection method for the determination of nonpeptidic inhibitors of human leukocyte elastase in plasma, using column switching techniques, has been developed. In the technique, a heart-cut fraction is collected from a size exclusion column to separate the drug and metabolites from the plasma protein. Peak compression, concentration and additional purification are performed by a collection column. The components are flushed forward onto an analytical column for the separation of the drug component from the metabolites. The parallel processing of the technique gives it a number of advantages over conventional extraction procedures and many column switching procedures in terms of speed and effort required in the analysis of samples. The technique's advantages and the precision and accuracy of the assay are discussed.

INTRODUCTION

In the development of pharmaceuticals, an analysis for the drug in plasma is generally required. High-performance liquid chromatography (HPLC) is one of the most common methods for analyzing these drugs. The organic modifiers in most conventional HPLC mobile phases require the removal of proteins from the plasma or serum before analysis, in order to avoid the precipitation caused by denaturing the protein. Commonly, a pretreatment step is performed using liquid or solid phase extraction procedures to remove the protein before HPLC analysis. These procedures are laborious and introduce additional sources of errors. Efforts to automate solid phase extraction techniques have met with some success using robotics and commercial instrumentation.[1] However, the expense, set-up time and labor involved has limited the general application.

Column switching techniques that allow for direct analysis of plasma or serum without a separate pretreatment step are being used more frequently. There have been a number of review articles that discuss applications and configurations for column switching.[1-4] Problems encountered with the direct injection of plasma onto HPLC columns are rapid clogging, accumulation of proteinaceous materials and long equilibration times. These problems often require frequent column changes or larger column particles that deteriorates performance.[5-8]

Probably the most common column switching configuration is the use of alkyl-bonded phases for the pre-column. The drug is adsorbed onto the column and the proteinaceous material is allowed to pass through the column. This approach generally results in the need for higher concentrations of organic modifiers to remove the drug (increasing the probability of protein precipitation) or requires back-flushing (which can increase the equilibration times). Using this approach, proteinaceous material can accumulate on the column resulting in the deterioration of the column's performance.

Hagestam and Pinkerton[9] introduced internal surface reversed-phase (ISRP) supports having a hydrophobic internal surface and a hydrophilic outer surface. The concept was that the proteinaceous material would be excluded from the internal surface avoiding the adsorption problem and allowing for the direct analysis of plasma samples on a single column. There are limitations on the amount and types of organic modifiers that can be used with the ISRP approach. This may be one reason why the ISRP technique has not been more widely used. Shintani[10] used an ISRP column as a pre-column in a multidimensional chromatography system. Back-flushing was still necessary to recover the drug, but the accumulation of proteinaceous material was not reported.

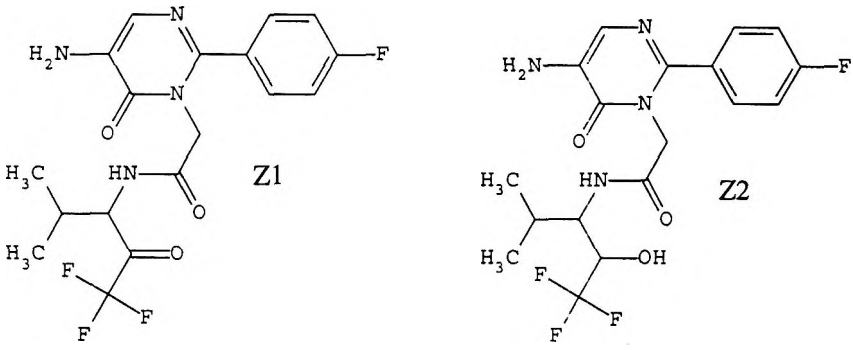

Figure 1. Structure of parent compound (Z1) and its major metabolite (Z2).

Few papers[11,12] have been found where size exclusion columns (SEC) were used as a pre-column in multidimensional chromatography systems. The use of SEC to separate proteinaceous material from drugs in plasma would avoid many of the problems previously discussed. The author of this paper has used SEC as a pre-separation column for a number of development drugs.

In this paper, the use of SEC as a pre-separation technique in the assay of a development drug will be discussed. The column switching configuration to be discussed is very versatile and has been used for a number of different types of exploratory drug candidates. This configuration was used extensively in the evaluation of the nonpeptidic inhibitors of human leukocyte elastase (NIHLE).[13-15] This paper illustrates the column switching configuration and exemplifies its performance with one of the NIHLE compounds evaluated. The example compound is presented in Figure 1 and the synthesis has been previously reported.[15]

EQUIPMENT AND MATERIALS

Chemicals

Z1 and a mixture of the erythro and threo alcohols, Z2 (major metabolite), were obtained from the Medicinal Chemistry Department of ZENECA Pharmaceuticals (Wilmington, DE, USA). Acetonitrile, methanol and water were all HPLC grade and obtained from J. T. Baker (Phillpsburg, NJ, USA). The potassium phosphate, monobasic was Ultrapure Pure and the potassium hydroxide (for pH adjustment) was ACS reagent grade; both were obtained from J. T. Baker (Phillpsburg, NJ, USA). Tetra butyl ammonium phosphate (TBAP)

was prepared in-house from a 40% solution of tetra butyl ammonium hydroxide obtained from Aldrich Chemical Company (Milwaukee, WI, USA). A 0.25 M solution of tetra butyl ammonium hydroxide was adjusted with 0.2 M phosphoric acid (ACS reagent grade J. T. Baker, Phillpsburg, NJ, USA) to a pH of 7.5. The TBAP solution was purified by passing the solution through an ODS column at 1 mL/min.

Instrumentation

The main HPLC system consisted of a Hewlett-Packard (HP)1090M with ChemStation and column switching valve. Additional equipment used included two HP 1050 programmable HPLC pumps, a 1050 variable UV detector (set at 306 nm), a second six port switching value (Rheodyne Inc., Cotati, CA, USA) and a HP 3350 data acquisition system. A Hewlett-Packard 1090L HPLC, HP 1050 variable UV detector (set at 306 nm) and HP 3350 data acquisition systems were used for the non-column switching comparison. All Hewlett-Packard equipment was obtained from Hewlett-Packard Company (Wilmington, DE, USA).

The following columns, as indicated in Figure 2, were used: Two optional conditioning columns (C1 & C5) 5 µm, 150 mm x 4.6 mm I.D., C_{18} Rainin Instrument Co. (Ridgefield, NJ, USA), one collection column (C4) 7 µm, 15 mm x 3.2 mm I.D., RP-18 Brownlee (San Jose, CA, USA), one size exclusion column (C3) 10 µm, 300 mm x 7.5 mm, TSK-125 BioRad (Richmond, CA, USA) with a guard column (C2) 7 µm, 15 mm x 3.2 mm I.D., diol Brownlee (San Jose, CA) and one analytical column (C6) 5µm, 250 mm x 4.6 mm, LC-18 Supelco, Inc. (Bellefonte, PA, USA). However, a number of substitute columns can be and have been used with this technique.

METHOD

Column-Switching Procedure

The interconnection of the apparatus is illustrated in Figure 2. One of the HP 1050 pumps (P1, Bio pump) delivers a constant flow of size exclusion mobile phase through the conditioning column (C1), injector (I), guard column (C2) and size exclusion column (C3). The effluent from this column is directed by switching valve (V1), which is controlled from the 1090M time table as contact # 2. A detector is generally connected (optional manual switching valve may be installed) to waste 1 during method development to establish the cut times from the size exclusion column. The other HP 1050 pump (P2, wash pump) delivers a wash solution through conditioning column (C5) to collection

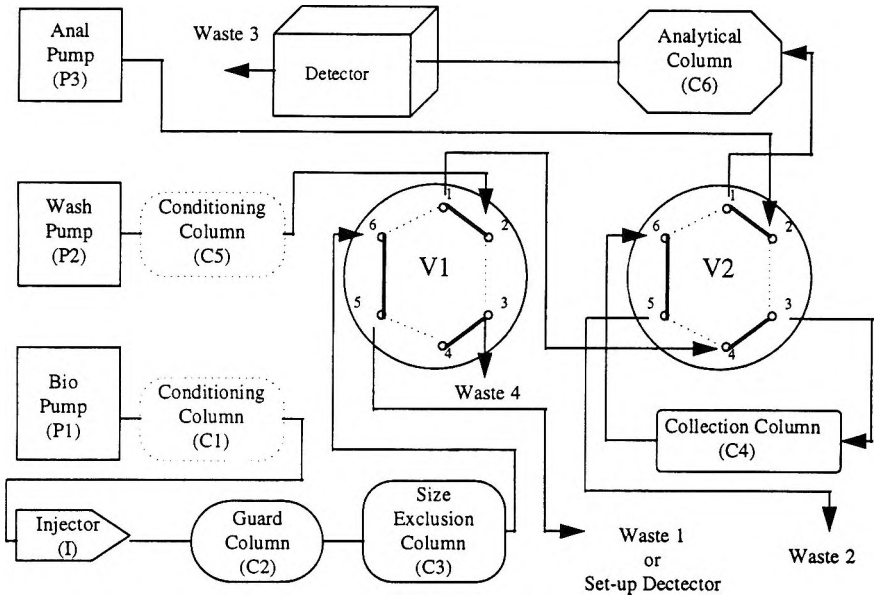

Figure 2. Column switching configuration and interconnections.

column (C4). The flow is programmed using the controls on the HP 1050 pump (P2). Typically, the flow is 0.2 mL/min except when the collection column (C4) is being actively washed, then the flow is 2.0 mL/min. The flow is directed through column (C4) via valves (V1) and (V2). The mobile phase for the analytical column (C6) is supplied by the 1090M pumping system (P3, Anal pump). The sample is transferred from collection column (C4) to the analytical column (C6) via valve (V2), which is controlled from the time table as event "column".

HPLC Parameters

The analysis parameters for Z1 and Z2 are as follows: The size exclusion mobile phase was 0.025 M potassium phosphate (pH 6.8) with 5% (v/v) acetonitrile and the flow rate was 1.5 mL/min. The wash mobile phase was 1% methanol (v/v) and flow rate was 0.2 mL/min. The flow rate was increased linearly to 2 mL/min over a 1 minute period for the active wash cycle then decreased to 0.2 mL/min over a 1 min period. The analytical mobile phase was 0.01 M tetra butyl ammonium phosphate at pH 7.5 with 31.5% acetonitrile (v/v). The analytical column flow rate was typically 1.2 mL/min and the column temperature was maintained at 28 °C using the 1090M column heater.

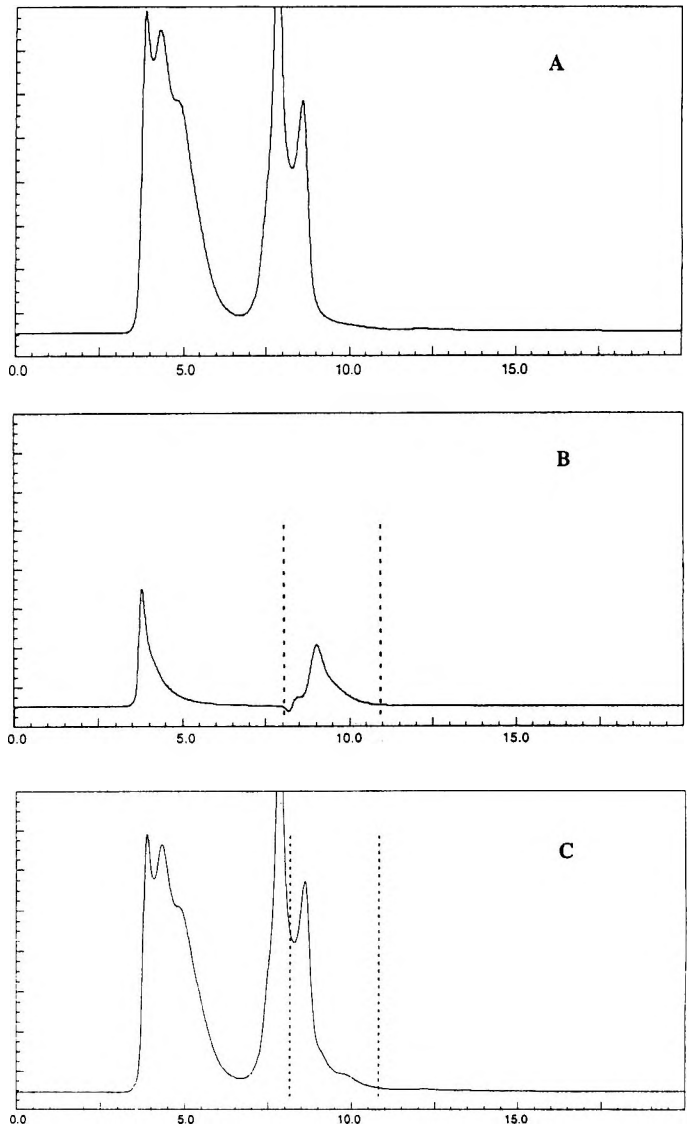

Figure 3. (a) Chromatogram of blank plasma. (B) Chromatogram of a 10 μg/mL aqueous standard of Z1. (C) Chromatogram of a 10 μg/mL standard of Z1. The chromatograms were obtained from the effluent of the size exclusion column (C3). The dotted lines show the approximate times of the heart cut from the size exclusion column.

Sample Analysis Procedure

Plasma samples (100-200 µl) were filtered by centrifugation through a 0.45 µm Utrafree®-MC filter, Millipore Corp. (Bedford, MA, USA) using an Eppendorf micro centrifuge model 5415C, Brinkmann Instruments, Inc. (Westbury, NY, USA). The time and speed are not critical and are dictated by the conditions needed to obtain a filtrate. The filtered plasma sample is injected (typically 50 µL) onto the size exclusion column. The proteins elute first, then the parent (Z1) and metabolites (Z2) elute together in the 8-11 minute zone. At 8 minutes (V1) is switched to the activate position (dotted lines) and the components from the size exclusion column are transferred to the collection column. At the end of 11 minutes, (V1) is returned to the non active position. Starting at 10 minutes the wash flow rate was increased linearly over a one minute period to achieve a flow rate of 2 mL/min at 11 minutes. The collection column is then washed for 1 minute at 2 mL/min. At 12 minutes, (V2) is activated (dotted lines) and the collection column and analytical column are in series. The analytical mobile phase transfers the compounds from the collection column to the analytical column. Contact #4 on 1090M is activated on/off for 0.01 minutes to start the electronic data acquisition. The collection column remains in series with the analytical column for 1 minute and is then returned to the non active position (solid lines) and is now being washed at 0.2 mL/min. The next sample is injected; thereby, the processing of the second sample is started while the first sample is eluting from the analytical column. Three chromatograms illustrating the separation on the size exclusion column are given in Figure 3. In the plasma containing chromatograms (A and C), the earlier peaks represent the higher molecular weight proteins. The second set of major peaks is endogenous components, some of which are trapped and eliminated in the next purification step.

A typical elution time for Z1 is about 8 minutes on the analytical column. The erythro alcohol (Z2) elutes about 1 minute before Z1 and the threo alcohol elutes about 1 minute after Z1. The alcohol metabolites are the most difficult separation and create the need for the long elution time of Z1. A chromatogram of a plasma standard with 2.5 µg/mL of Z1 and 7.5 µg/mL of the alcohol mix (Z2) is illustrated in Figure 4. The separation between the peaks can be increased by decreasing the acetonitrile concentration, which will increase the elution time. However, good quantitative results can be achieved with the electronic area measurement for these peaks

Preparation of Standards

A series of aqueous standards (30% acetonitrile) was prepared by weighing 10 mg of Z1 into a 10 mL volumetric flask and dissolving in acetonitrile. This solution was serially diluted to obtain the following standards 10,000, 5,000,

1000, 500, 100 and 50 ng/mL. A rat plasma standard series was prepared by evaporating one of the intermediate standards (100,000 ng/mL) with a stream of nitrogen and quantitatively diluting it with rat plasma. The spiked plasma standard was then diluted serially with rat plasma to obtain the following standards 10,000, 5,000, 1,000, 500, 100 and 50 ng/mL. The plasma samples were filtered through 0.45 micron filters before injecting into the size exclusion column.

Determination of Assay Linearity and Precision

The area of the Z1 peak was measured using the HP 3350 data acquisition system with the Genie integrator software. The linearity and precision of the aqueous standard (30% acetonitrile) series were first analyzed on the HP 1090L by injecting directly onto an analytical column without column switching. This establishes the linearity and precision of the method without column switching. Each experiment represents the 5 standards stated above and 3 experiments were run each day. The same series was re-evaluated in the same manner on with the 1090M column switching configuration. The plasma series was then analyzed on the 1090M with column switching. Three sets of the five plasma standards were analyzed for two days.

Determination of Assay Recovery and Accuracy

An important evaluation is the effect that plasma has on the recovery of the components of interest. A comparison of standards prepared with plasma and standards containing no plasma will allow for the effects of plasma on the system to be determined. Therefore, standards prepared in plasma and the corresponding aqueous standards were analyzed and compared. The system was calibrated to yield the prepared aqueous standards concentrations. The corresponding plasma standards were analyzed and the concentration determined by comparing to the respective aqueous calibration.

RESULTS

The linearity was evaluated using non weighted linear regression analysis. The slope is the standard's concentration divided by the chromatographic area counts. The intercept is concentration. The results from the direct analysis of the aqueous series are tabulated in Table 1. The results from the column switching analysis of the aqueous standards are tabulated in Table 2. The analyses of the plasma standards using the column switching analysis are tabulated in Table 3. The correlation coefficient was similar for all three

Table 1

Linearity of Aqueous Standards Without the use of Column Switching (50 ng/mL to 10,000 ng/mL)

Experiment	Slope	Intercept	Correlation Coefficient
1	0.007387	-0.1571	0.99999
2	0.007365	11.40	0.99998
3	0.007353	10.86	0.99998
4	0.007271	-2.820	0.99999
5	0.007446	33.74	0.99996
6	0.007589	-0.4131	0.99997
7	0.007504	12.93	0.99999
8	0.007516	5.776	0.99997
9	0.007492	-18.34	0.99998
mean	0.007436	5.886	0.99998
SD	0.000104	11.71	0.00001
RSD %	1.40	199	0.00105

Table 2

Linearity of Aqueous Standards using Column Switching (50 ng/mL to 10,000 ng/mL)

Experiment	Slope	Intercept	Correlation Coefficient
1	0.006485	21.53	0.99999
2	0.006495	22.73	0.99999
3	0.006425	36.11	0.99998
4	0.006709	51.13	0.99991
5	0.006673	51.58	0.99993
6	0.006777	51.22	0.99996
7	0.006660	71.21	0.99996
8	0.006687	48.45	0.99995
9	0.006698	54.53	0.99998
mean	0.006623	45.39	0.99996
SD	0.000122	15.95	0.00003
RSD %	1.84	35.1	0.00276

Table 3

Linearity of Plasma Standards using Column Switching (50 ng/mL to 10,000 ng/mL)

Experiment	Slope	Intercept	Correlation Coefficient
1	0.006899	35.10	0.99996
2	0.006905	33.60	0.99997
3	0.007002	29.95	0.99992
4	0.006744	41.46	0.99990
5	0.006614	32.49	0.99996
6	0.006582	40.32	0.99995
mean	0.006791	35.49	0.99994
SD	0.000171	4.23	0.00003
RSD %	2.51	11.9	0.00273

Table 4

Precision and Accuracy of Plasma Standards using Column Switching (n=6)

Aqueous Standards Concentration (ng/mL)	Mean Plasma Assay Concentration (ng/mL)	SD	Mean Percentage Recovery	SD
10000	9647	175	96.5	1.76
5000	4859	66	97.2	1.33
1000	946	14	94.6	1.35
500	456	7	91.1	1.39
100	102	6	102	5.63
50	52	3	104	6.24

determinations (Tables 1-3) and was greater than 0.9999. The percentage recoveries for plasma samples are tabulated in Table 4. The average recovery was 97.6 % for the series. There is no obvious difference in the peak shape or the separation of the metabolites between the switching and the non-switching chromatograms.

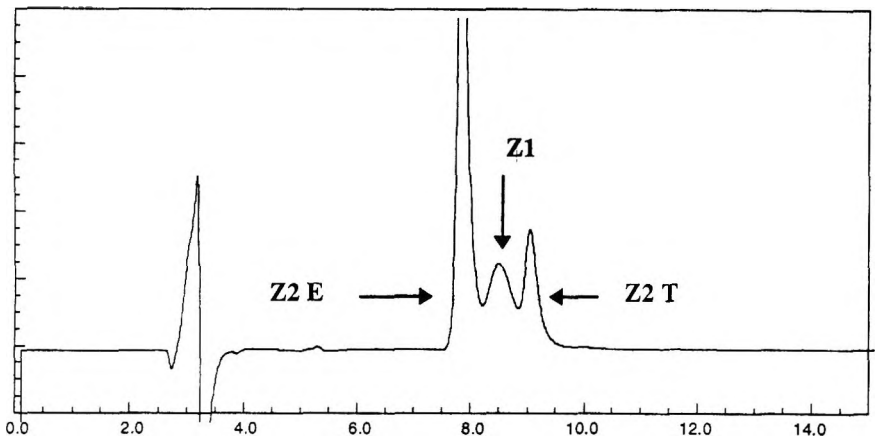

Figure 4. Chromatogram of 50 µL plasma standard (2.5 µg/mL of Z1 and 7.5 µg/mL Z2 mix, erythro and threo) processed by the multidimensional chromatographic system and detected eluting from the analytical column (C6).

A comparison of a 10 µg/mL aqueous standard peak injected directly onto an analytical column and the same standard processed through the multidimensional system are illustrated in Figure 5.

DISCUSSION

The use of a size exclusion column to separate proteins from drug components simplifies the development of new analyses. Generally, the adjustment of the pH is not a critical factor nor the percentage of organics needed to achieve enough solubility for the components to elute. The pH is maintained between 3 and 7.5 and the level of organics is keep below 15 % in order to avoid the precipitation of proteins. The use of a high salt content mobile phase, as is frequently used with size exclusion columns, was not found to be necessary. The achievement of a true size separation mechanism is not necessary. The retention time observed for Z1 eluting from the size exclusion column is greater than would be predicted for a true size separation mechanism. Presumably, the separation is enhanced by the presence of some reverse-phase interaction.

The use of the collection column solves many of the problems associated with multidimensional systems. A number of investigators[7] (including this author) believe the clogging problem many investigators have experienced using direct plasma injections has been caused by the high percentage of organics in the analytical mobile phase being switched onto the pre-separation

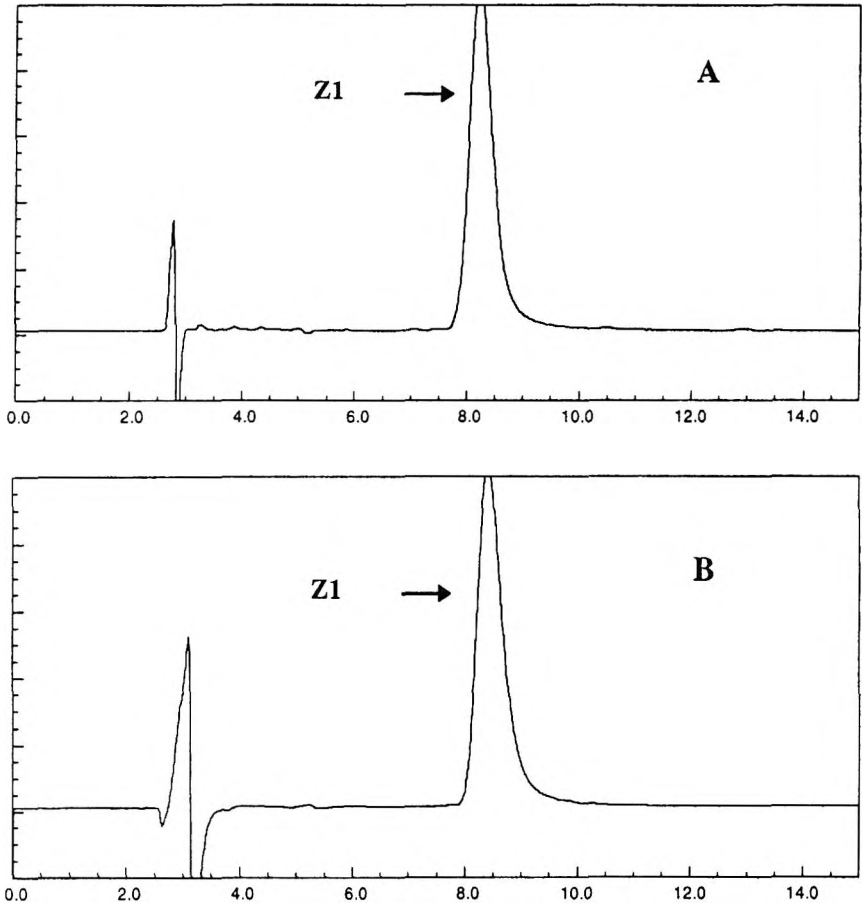

Figure 5. (A) Chromatogram of a 10 μg/mL aqueous standard injected directly onto the analytical column. (B) Chromatogram of a 10μg/mL aqueous standard processed through the multidimensional system.

column. This may cause residual proteins on the pre-separation column to be precipitated. The collection column assures that the size exclusion column mobile phase and the analytical mobile phase do not come into contact. Clogging of the size exclusion and collection column has not been a problem over the numerous years that this technique has been in use, even when using 5 micron size exclusion columns. The collection column de-couples the size exclusion column and the analytical column allowing a wide range of mobile phases to be used and the separation on both columns to be optimized. It compresses the broad peak from the size exclusion column; thereby, yielding

peak widths similar to non-switching techniques (see Figure 5). This column also makes it possible to inject large sample volumes unto the size exclusion column and allows for the concentration of samples by collecting multiple injections before switching to the analytical column. This technique makes the processing of two samples at the same time possible. This keeps the analysis time similar to off line sample preparation techniques. Additional processing of the sample by washing the collection column with different solutions is also possible using this technique.

The collection column should be as small as possible, as the volume of the column becomes the injection volume and the guard column for the analytical column. The packing material is generally the same or of a weaker column strength than the analytical column. These columns do not clog, but the retention capacity decreases after about 200 injections. This is probably caused by a combination of switching abruptly between different mobile phases and the small amount of packing material present in the 15 mm guard columns normally used for this purpose.

In the configuration presented, the collection column is washed with a neutral mobile phase when not in use and unfortunately impurities are concentrated from the mobile phase. The conditioning columns (C1 and C2) and the flow programming of the wash mobile phase are all being utilized to minimize the concentration of impurities.

The three modular parts of this system allow each component to be optimized individually. This greatly speeds up method development time. The analytical separation from non-switching HPLC methods can generally be used with very few modifications. Once the system is operational, the only sample preparation required is the filtration of the plasma through a 0.45 μm filter. The system is normally operated overnight unattended. The unattended operation and ease of sample preparation have made a significant contribution to the number of analyses that can be accomplished by an individual operator. Pharmacokinetic evaluations for Z1 in hamster, rat and dog have previously been reported[15] using this column switching technique.

REFERENCES

1. D. C. Turnell, J. D. H. Cooper, J. Chromatogr., **492**, 59-83 (1989).

2. K. A. Ramsteiner, J. Chromatogr., **456**, 3-20 (1988).

3. P. Campins-Falco, R. Herraez, A. Seillano-Cabeza, J. Chromatogr., **619**, 177-190 (1993).

4. M. J. Koenigbauer, J. Chromatogr., **531**, 79-99 (1990).

5. U. Juergens, J Chromatogr., **310**, 97-106 (1984).

6. T. Arvidsoon, K. G. Wahlund, N. Daoud, J. Chromatogr., **317**, 213-226 (1984).

7. I. H. Hagestam, T. C. Pinkerton, J. Chromatogr., 351, 239-248 (1986).

8. L. R. Snyder, J.W. Dolan, Sj. Van Der Wal, J. Chromatogr., **203**, 3-17 (1981).

9. I. H. Hagestam, T. C. Pinerton, Anal. Chem., **57**, 1757 (1982).

10. T. Shintani, M. Takamoto, M. Sawada, H. Aishita and T. Nakagawa, J. Pharm. Biomed. Anal., **12**, 397-405 (1994).

11. J. A. Apffel, T. V. Alfredson, R. E. Majors, J. Chromatogr., **206**, 43-57 (1981).

12. M. J. B. Mengelers, A. M. M. Polman, M. M. L. Aerts, H. A. Kuiper, A. S. J. P. A. M. Van Miert, J. Liq. Chromatogr., **16**, 257-278 (1993).

13. F. J. Brown, D. W. Andisik, P. R. Bernstein, C. B. Bryant, C. Ceccarelli, J. R. Damewood, Jr., P. D. Edwards, R. A. Earley, S. Feeney, R. C. Green, B. Gomes, B. J. Kosmider, R. D. Krell, A. Shaw, G. B. Steelman, R. M. Thomas, E. P. Vacek, C. A. Veale, P. A. Tuthill, P. Warner, J. C. Williams, D. J. Wolanin and S. A. Woolson, J. Med. Chem., **37**, 1259-1261 (1994).

14. P. R. Bernstein, D. W. Andisik, P. K. Bradley, C. B. Bryant, C. Ceccarelli, J. R. Damewood, Jr., R. A. Earley, P. D. Edwards, S. Feeney, B. Gomes, B. J. Kosmider, , G. B. Steelman, R. M. Thomas, E. P. Vacek, C. A. Veale, J. C. Williams, D. J. Wolanin, S. A. Woolson, J. Med. Chem., **37**, 3313-3326 (1994).

15. C. A. Veale, P. R. Bernstein, C. B. Bryant, C. Ceccarelli, J. R. Damewood, Jr., R. A. Earley, S. W. Feeney, B. Gomes, B. J. Kosmider, G. B. Steelman, R. M. Thomas, E. P. Vacek, J. C. Williams, D. J. Wolanin, S. A. Woolson, J. Med. Chem., **38**, 98-108 (1995).

Received March 15, 1996
Accepted April 8, 1996
Manuscript 4110

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2541–2542 (1996)

ANNOUNCEMENTS

BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

A Two-Day In-House Training Course

The course, which is offered for presentation at corporate laboratories, is aimed at chemists, engineers and technicians who use, or plan to use, high performance liquid chromatography in their work. The training covers HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC hardware module and system problems.

The following topics are covered in depth:

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- Modes of HPLC Separation
- Developing and Controlling Resolution
- Mobile Phase Selection and Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration and Quantitation
- Logical HPLC System Troubleshooting

The instructor, Dr. Jack Cazes, is founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Series Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on Gel Permeation Chromatography for many years.

Details of this course may be obtained from Dr. Jack Cazes, P. O. Box 2180, Cherry Hill, NJ 08034-0162, USA. Tel: (609) 424-3505; FAX: (609) 751-8724; E-Mail: jcazes@voicenet.com.

APPLICATIONS OF HIGH-PERFORMANCE TLC IN THE PHARMACEUTICAL INDUSTRY

A Mini-Symposium at the Eastern Analytical Symposium

November 17 - 22, 1996

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This premier symposium will feature state-of-the-art technology and applications of high-performance thin-layer chromatography, presented by invited experts in their respective fields of endeavor and chaired by Dr. Juan G. Alvarez of Harvard Medical School..

Program

"Introduction and General Remarks," Juan G. Alvarez, Harvard Medical School

"Planar Chromatography: An Invisible Workhorse/Ultimated Now?," Keith Palmer, Chromatography Consultant

"Analytical Issues in TLC: Impurities or Artifacts?," Nelu Grinberg, Merck Research Laboratories

"Development and Validation Issues for the TLC Analysis of COX-2 Inhibitors," Bin Li, Zhi Hong Ge, Richard Thompson, Merck Research Laboratories

"Analysis of Surfactants by Enzymatic Hydrolysis and Micro-HPTLC-Reflectance Spectrodensitometry," Juan G. Alvarez, Harvard Medical School

Further information may be obtained from Dr. Juan G. Alvarez, Beth Israel Hospital, 879 Research West, Boston, MA 02215; Tel: (617) 667-4230; FAX: (617) 667-7939; Email: jalvarez@bih.harvard.edu

J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2543-2548 (1996)

THE BOOK CORNER

PHARMACEUTICAL AND BIOMEDICAL APPLICATIONS OF LIQUID CHROMATOGRAPHY, Edited by C. M. Riley, W. J. Lough and I. W. Wainer, Elsevier Science Ltd, Oxford, England, 1994, x + 379 pp., £ 85.00 ($ 136.00); ISBN: 008041009 X

This book represents Volume 1 in the new series entitled *"Progress in Pharmaceutical and Biomedical Analysis,"* which is edited by distinguished scientists in the field. The book provides an update on recent developments in liquid chromatography, with topics that quickly progressed in the last decades such as chirality, biotechnical substances and the need for more rigorous documentation and validation of analytical procedures.

The book consists of four parts:

Part one deals with the application of new technology to pharmaceutical and biomedical analysis and consists of three chapters, discussing the pharmaceutical and biomedical applications of capillary electrophoresis, novel approaches for the analysis of primary amines, amino acids and peptides by liquid chromatography, while chapter 3 deals with analysis of enantiomers.

Part two describes the recent advances in the area of solid phase extraction, restricted access media for direct injection, microdialysis and coupled column chromatography. This part consists of 4 chapters.

Part three contains two chapters concerned with liquid chromatographic methods for the isolation of drug substances on preparative scale and purification of peptides and proteins.

Part four describes the development of validation of analytical methods in pharmaceutical and biomedical research, with a chapter devoted to validation of liquid chromatography methods of pharmaceuticals in bulk form, formulations and biological fluids. The final chapter describes the strategy for method validation for bioanalytical applications in the pharmaceutical industry.

Each chapter ends with a list of references related to the topic discussed, with citations as recent as 1993. This book is highly recommended for analytical chemists, pharmaceutical chemists, biochemists in both industrial and academic affiliations, and also drug regulatory centers.

This book is the start of a new series, with two more volumes to be published in the near future; I anticipate their place as valuable references in the areas of pharmaceutical and biomedical analysis.

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GAS CHROMATOGRAPHY, Edited by Ian A. Fowlis, second edition, in Analytical Chemistry by Open Learning Series, John Wiley & Sons Inc., New York, 1995, 258 pages.

In the second edition of Gas Chromatography, the author expanded the treatment of column systems into packed column systems (chapter three) and capillary column systems (chapter four) while, in the first edition, they were discussed together. Also, the detector systems are discussed independently (chapter six). Furthermore, this second edition includes two new topics: injection systems for high resolution gas chromatography, HRGC (chapter five), and environmental analysis systems (chapter ten).

Other subjects in the first edition were expanded and revised in the second edition and are discussed in greater depth and detail; these include: the fundamental chromatograph (chapter two), data handling systems and quantitative analysis (chapter seven), qualitative analysis (chapter eight), and analysis of less volatile samples (chapter nine).

This book is divided into ten chapters. Chapter one is an introduction. Chapter two includes the topics of gas supply systems, columns, oven, injection systems, detectors, data systems, and starting up the complete system. Chapter three covers packed column dimensions, column packing and preparation, solid supports, stationary phases, column performance, carrier gases, the injection systems, system optimization and evaluation of chromatography. Chapter four discusses the structures and dimensions of capillary columns, handling of capillary columns, fittings and connectors, optimization and separation, and column maintenance, while chapter five deals with injection systems such as, the split/splitless injectors, on-column injector, automatic injection in HRGC, and large volume injection in HRGC. In chapter six, the emphasis is on the types of detectors used in GC. Chapter seven discusses the chromatography and the data systems, injection and quantitation, integration methods, and method validation. Chapter eight is an interesting one which discusses retention times and multidimensional systems, such as, HRGC-MS, HRGC-IR, HRGC-IR-MS

and HPLC-HRGC-MS. Chapter nine discusses the use of high temperature stationary phases, derivatization and pyrolysis gas chromatography. Chapter ten covers solvent extraction, on-line focusing, headspace analysis in HRGC, and purge and trap systems.

The book is well written and illustrated. It is recommended for students, technicians, and chemists who want to gain fundamental working and practical knowledge in gas chromatography.

Table of Contents:

Reviewed by
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EXPLOITING BIOTECHNOLOGY, edited by Vivian and Sheila Moses, Harwood Academic Publishers, London, UK, 1995, 332 pages, $28.00.

This book is written in a way that is easy to understand and with some business orientation. It is suitable for both biotechnology students as well as those whom in their education and training does not include the science and engineering on which the technology is based. The book is divided into thirteen chapters. Chapter one is an introduction, which briefly describes the definition, prospects, applications, and industrial products of biotechnology. Chapters two through four deal with the basis of biotechnology in sciences, engineering, and feedstocks. In the science, they discuss the basic concepts of biochemistry, genetics, DNA, genetic codes, replication of DNA, and genetic engineering; in engineering, the book focusses on choice of organisms, growth of genetically engineered organisms, production, packaging and marketing; and in feedstocks, deals with the role of feedstocks in biotechnology. Chapter five discusses the patenting of products from biotechnology and the protection of intellectual

property. Chapter six emphasizes the management aspects of a biotechnology company, including research, strategy, product development, human resources, finance, marketing and decision making. Chapter seven discusses the applications and products of biotechnology in human health care, which includes production of vaccines, monoclonal antibodies, diagnostic products, therapeutic drugs, and gene therapy. Chapter eight focuses on the use of biotechnology for the production of antibiotics, vitamins, drugs, amino acids, organic acids, proteins, enzymes, starches, sugars, methanol and ethanol. Chapter nine emphasizes the manipulation of plant genetics results in improvement of crop quality, plants' resistance to insects, herbicides and plant pathogens; production of new ornamental plants; plants which produce pharmaceutical compounds, chemicals for agriculture, perfumes components, pigments, and food additives. Introduction of nitrogen fixation genes into rice, corn, wheat, and other cereal crops to reduce the use of nitrogen fertilizers. Chapter ten deals with the exploitation of biotechnology for improvement of techniques in mining and recovery of crude oil. Chapter eleven discusses the application of genetically engineered micro-organisms for removal of pollutants in water, soil, air, oil spills, disposal of sewage sludges, solid wastes, toxic organics, heavy metals and hazardous wastes. Chapter twelve describes the use of biosensors in immunoassay for screening of bacteria, pathogens, pesticides, or pollutants in meats, fishes, waters, and human blood. Chapter thirteen is an overview of future applications of biotechnology.

The authors stated that "while mainly scientific and technical in its approach and in the material it covers, the book hopes to satisfy both scientific and business interests. Primarily, the book seeks to explain to people with no special knowledge of chemistry, and with no more than a layman's appreciation of biology, both the promise and the limitations of using living organisms and their products in industry."

I think they have accomplished not only their objective, but the purpose of the book, which is to make accessible a general understanding of the technical base on which biotechnology rests. It also offers a broad view of the commercial and industrial applications which have already been made or are likely to be developed before too long. A few things that I liked about the book: it is easy to read, well written and costs only $28.00. I also liked the honesty of the authors who stated, "In no sense is this book a comprehensive catalogue of all that is going on." To satisfy that objective would have required a volume many times longer and would have demanded of its readers an extensive knowledge of the underlying sciences. Moreover, the subject is moving so fast that, however hard the authors might have tried to include every last bit of news, publication delays would inevitably have led to the omission of many interesting items. "We have tried, instead, to offer a balanced presentation of current activity and, where it might lead in the short- and medium-term." The book is recommended to all those interested in the biotechnology field.

Table of Contents:

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NEW METHODS IN PEPTIDE MAPPING FOR THE CHARACTERIZATION OF PROTEINS, edited by William S. Hancock, CRC Series in Analytical Biotechnology, CEC Press, Boca Raton, FL, 1995, 246 pages.

This book is devoted to the characterization of recombinant DNA-derived proteins by peptide mapping. It describes new technological procedures, including capillary electrophoresis, analysis of glycopeptides, and the use of electrospray- and matrix-assisted laser desorption mass spectrometry. The book presents practical procedures for preparing a protein sample, enzyme digestion, choice of separation method, and procedures for the structural analysis of the separated species.

The book, which is written by experts in this field, and edited by William S. Hancock, is divided into eight chapters. Chapter one describes the use of tryptic mapping of peptides as the key method for characterization of biotechnological protein products. Chapter two gives a useful discussion of approaches for the use of computer simulation for the rapid optimization of the peptide map. Chapter three demonstrates the power of spectra in the interpretation of peptide maps. Chapter four gives an account of the application of HPCE to peptide mapping and gives practical tips on developing the most effective conditions. Chapters five through seven discuss the power of combined

LC/MS techniques for analysis of complex proteins such as glycosylated samples. The extra dimension of mass spectrometry may be required to fully characterize a peptide map. With LC/MS, the mass spectrometer allows on-line identification of peptides and coelutions are readily detected. Chapter five gives an excellent description of the power of electrospray mass spectrometry (ESI-MS) as an on-line HPLC detector. Chapter six describes how ESI-MS can be successfully coupled to capillary electrophoresis. Chapter seven describes the application of ESI-MS to analyze complex glycoproteins via on-line analysis of HPLC-tryptic maps. Matrix-assisted laser desorption mass spectrometry, combined with time of flight MS (MALDI-TOF), a promising new approach to characterize polypeptides, is discussed in chapter eight.

In general, the book is well written and edited. It is informative, to the point and free from errors. Each chapter is rich with illustrations and references. The book is recommended to all those interested in peptide and protein separation and characterization.

Table of Contents:

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J. LIQ. CHROM. & REL. TECHNOL., 19(15), 2549–2558 (1996)

LIQUID CHROMATOGRAPHY CALENDAR

1996

AUGUST 18 - 23: 17th International Conference on Magnetic Resonance in Biological Systems, Keystone, Colorado, USA. Contact: Conference Office, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4735; FAX: (505) 989-1073.

AUGUST 21 - 23: 4th International Symposium on Capillary Electrophoresis, York, UK. Contact: Dr. T. Threlfall, Industrial Liaison Executive, Dept of Chem, University of York, Heslington, York, YO1 5DD, UK.

AUGUST 25 - 29: 212th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; Email: natlmtgs@acs,org.

AUGUST 25 - 30: International Symposium on Metal Hydrogen Systems: Fundamentals and Applications, Les Diablerets, Switzerland. Contact: MH-96, Inst of Physics, Univ of Fribourg, Perolles, CH-1700 Fribourg, Switzerland. Tel: 41 37 299 113; FAX: 41 37 299 772.

AUGUST 25 - 30: 12th International Congress on Chemical & Process Engineering, Praha, Czech Republic. Contact: Organizing Committe, CHISA'96, P. O. Box 857, 111 21 Praha, Czech Republic. Tel: 42 2 353287; FAX: 42 2 3116138.

SEPTEMBER 1 - 4: 4th Inetrnational Symposium on Preparative & Industrial Chromatography & Related Techniques, Basel, Switzerland. Contact: Secretariat Prep'96, Messeplatz 25, CH-4021 Basel, Switzerland. Tel: 41 61 686 28 28; FAX: 41 61 686 21 85.

SEPTEMBER 1 - 6: IUPAC Chemrawn IX, Seoul, Korea. Contact: IUPAC Chemrawn IX, Secretariat, Tongwon B/D 6th Floor, 128-27 Tangjudong, Chongro-ku, Seoul 110-071, Korea. FAX: 82 2 739-6187.

SEPTEMBER 1 - 6: 11th International Symposium on Organosilicon Chemistry, Monpellier, France. Contact: R. Corriu, University of Montpellier II, Place Eugene Batallon, cc007, 34095 Montpellier cedex 05, France. Tel: 67 14 38 01.

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

SEPTEMBER 4 - 9: International "Thermophiles '96" Symposium: Biology, Ecology & Biotechnology of Thermophilic Organisms, Athens, Georgia. Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; FAX: (706) 542-2651.

SPETEMBER 7: Field-Flow Fractionation Workshop VIII, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy. Tel: 39 532 291154; FAX: 39 532 240709.

SEPTEMBER 9 - 11: Sixth International Symposium on Field-Flow Fractionation, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy. Tel: 39 532 291154; FAX: 39 532 240709.

SEPTEMBER 9 - 11: 110th AOAC International Annual Meeting & Expo, Hyatt, Orlando, Florida. Contact: AOAC International, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, USA. Tel: (703) 522-3032; FAX: (703) 522-5468.

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

SEPTEMBER 10 - 12: International Thermophiles Workshop: Keys to Molecular Evolution & the Origin of Life, Athens, Georgia. Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; FAX: (706) 542-2651.

SEPTEMBER 11 - 13: Corn Refiners Assn Scientific Conference, Bloomindale, Illinois. Contact: Corn Refiners Assn, 1701 Pennsylvania Ave, NW, Washington, DC 20036, USA. Tel: (202) 331-1634; FAX: (202) 331-2054.

SEPTEMBER 11 - 13: 2nd Workshop on Biosensors & Biol Techniques in Environmental Analysis, Lund, Sweden. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland.

SEPTEMBER 15 - 20: 21st International Symposium on Chromatography, Stuttgart, Germany. Contact: Dr. L. Kiessling, Geselleschaft Deutscher Chemiker, Postfach 900440, D-60444 Frankfurt/Main, Germany.

SEPTEMBER 16 - 18: 3rd International Conference on Applications of Magnetic Resonance in Food Science, Nantes, France. Contact: Laboratoire de RMN-RC, 2 rue de la Houssiniere, 44072 Nantes cedex 03, France. Tel: 33 40 74 98 06; FAX: 33 40 37 31 69.

SEPTEMBER 16 - 19: International Ion Chromatography Symposium 1996, University of Reading, Reading, UK. Contact: J. R. Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052, USA. Tel: (508) 359-8777; FAX: (508) 359-8778.

SEPTEMBER 17 - 20: 10th International Symposium on Capillary Electrophoresis, Prague, Czech Republic. Contact: Dr. B. Gas, Dept of Physical Chem, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic.

SEPTEMBER 19 - 21: 19th Annual Gulf Coast Chemistry Conference, Pensacola, Florida. Contact: J. Gurst, Chem Dept, University of West Florida, Pensacola, FL 32514, USA. Tel: (904) 474-2744; FAX: 904) 474-2621.

SEPTEMBER 20 - 24: 12th Asilomar Conference on Mass Spectrometry, Pacific Grove, California. Contact: ASMS, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4517; FAX: (505) 989-1073.

SEPTEMBER 28 - OCTOBER 5: Federation of Analytical Chem & Spectroscopy Societies (FACSS), Kansas City. Contact: J.A. Brown, FACSS, 198 Thomas Johnson Dr., Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 17 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

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

OCTOBER 27 - 31: American Assn of Pharmaceutical Scientists Annual Meeting & Expo, Seattle, Washington. Contact: AAPS, 1650 King St, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000; FAX: (703) 684-7349.

OCTOBER 29 - 30: ASTM Symposium on Pesticide Formulations & Application Systems, New Orleans, Louisiana. Contact: G. R. Goss, Oil-Dri Corp, 777 Forest Edge Dr, Vrenon Hills, IL 60061, USA. Tel: (708) 634-3090; FAX: (708) 634-4595.

OCTOBER 29 - 31: Cphl Pharmaceutical Ingredients Worldwide '96 Conference, Milan, Italy. Contact: Miller Freeman BV, Industrieweg 54, P. O. Box 200, 3600 AE Maarssen, The Netherlands. Tel: 31 3465 73777; FAX: 31 3465 73811.

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

NOVEMBER 6 - 9: 24th Biennial International Conference on Application of Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan or B. Stippec, University of North Texas, Physics Department, Denton, TX 76203, USA. Tel: (817) 565-3252; FAX: (817) 565-2227.

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

NOVEMBER 10 - 13: 4th North American Research Conference on Organic Coatings Science & Technology, Hilton Head, South Carolina. Contact: A. V. Patsis, SUNY, New Paltz, NY 12561, USA. Tel: (914) 255-0757; FAX: (914) 255-0978.

NOVEMBER 10 - 14: 10th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Kluczynski, Electrosynthesis Co, 72 Ward Road, Lancaster, NY 14086, USA. Tel: (716) 684-0513; FAX: (716) 684-0511.

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

NOVEMBER 11 - 20: 2nd Latin-American Conference on Biomedical, Biopharmaceutical and Industrial Applications of Capillary Electrophoresis, Santiago, Chile. Dr. E. Guerrero, Servicio Medico Legal, Avenida de la Paz 1012, Santiago, Chile.

NOVEMBER 12 - 14: Plastics Fair, Charlotte, North Carolina. Contact: Becky Lerew, Plastics Fair, 7500 Old Oak Blvd, Cleveland, OH 44130, USA. Tel: (216) 826-2844; FAX: (216) 826-2801.

NOVEMBER 13 - 15: 13th Montreux Symposium on Liquid Chromatography-Mass Spectrometry (LC/MS; SFC/MS; CE/MS; MS/MS), Maison des Congres, Montreux, Switzerland. Contact: M. Frei-Hausler, Postfach 46, CH-4123 Allschwill 2, Switzerland. Tel: 41-61-4812789; FAX: 41-61-4820805.

NOVEMBER 18 - 20: 3rd International Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: Chem in Industry Conf, P. O. Box 1723, Dhahran 31311, Saudi Arabia. Tel: 966 3 867 4409; FAX: 966 3 876 2812.

NOVEMBER 17 - 22: 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; FAX: (302) 738-5275.

NOVEMBER 24 - 27: Industrial Research for the 21st Century, 1996 Biennial Meeting, Red Lion Resort, Santa Barbara, California. Contact: R. Ikeda, DuPont Central Research Dept., P. O. Box 80356, Wilmington, DE 19880-0356, USA. Tel: (302) 695-4382; FAX: (302) 695-8207; Email: ikeda@esvax.dnet.dupont.com.

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

1997

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

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

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

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

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

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

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

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

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

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

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

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

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

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

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

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

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; FAX: (302) 738-5275.

1998

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

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

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

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

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

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

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

1999

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

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

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

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, 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. Tel: (800) 825-3221; FAX: (412) 825-3224.

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

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

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 23: 222nd 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.

2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. 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 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2003

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

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

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, 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 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2005

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

AUGUST 28 - SEPTEMBER 1: 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.

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520 Wakara Way
Salt Lake City, Utah 84108

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Chapter in a Book:

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

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Volume 19, Number 15, 1996

CONTENTS

(continued on inside back cover)

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