JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 17 NUMBER 9 1994

Special Section on
CAPILLARY ZONE
ELECTROPHORESIS AND
RELATED TECHNIQUES

Edited by HALEEM J. ISSAQ
NCI-Frederick Cancer Research
& Development Center
Frederick, Maryland

JOURNAL OF LIQUID CHROMATOGRAPHY

May 1994

Aims and Scope. The journal publishes papers involving the applications of liquid chromatography to the solution of problems in all areas of science and technology, both analytical and preparative, as well as papers that deal specifically with liquid chromatography as a science within itself. Included will be thin-layer chromatography and all models of liquid chromatography.

Identification Statement. *Journal of Liquid Chromatography* (ISSN: 0148-3919) is published semimonthly except monthly in May, July, October, and December for the institutional rate of $1,350.00 and the individual rate of $675.00 by Marcel Dekker, Inc., P.O. Box 5005, Monticello, NY 12701-5185. Second Class postage paid at Monticello, NY. POSTMASTER: Send address changes to *Journal of Liquid Chromatography*, P.O. Box 5005, Monticello, NY 12701-5185.

Volume	Issues	Institutional Rate	Individual Professionals' and Student Rate	Foreign Postage		
				Surface	Airmail to Europe	Airmail to Asia
17	20	$1,350.00	$675.00	$75.00	$110.00	$130.00

Individual professionals' and student orders must be prepaid by personal check or may be charged to MasterCard, VISA, or American Express. Please mail payment with your order to: Marcel Dekker Journals, P.O. Box 5017, Monticello, New York 12701-5176.

CODEN: JLCHD8 17(9) i-x, 1861-2092 (1994)
ISSN: 0148-3919

Printed in the U.S.A.

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography* are selectively indexed or abstracted in:

■ Analytical Abstracts ■ ASCA ■ Berichte Pathologie ■ BioSciences Information Service of Biological Abstracts (BIOSIS) ■ Cambridge Scientific Abstracts ■ Chemical Abstracts ■ Chemical Reactions Documentation Service ■ Current Awareness in Biological Sciences ■ Current Contents/Life Sciences ■ Current Contents/Physical and Chemical Sciences ■ Engineering Index ■ Excerpta Medica ■ Journal of Abstracts of the All-Union Institute of Scientific and Technical Information of the USSR ■ Physikalische Berichte ■ Reference Update ■ Saltykov-Shchedrin State Public Library ■ Science Citation Index

Manuscript Preparation and Submission. See end of issue.

This journal is also available on CD-ROM through ADONIS™ beginning with the 1991 volume year. For information contact: ADONIS, Marketing Services, P.O. Box 839, Molenwerf 1, 1000 AV Amsterdam, The Netherlands, Tel: +31-20-6842206, Fax: +31-20-6880241.

The Journals of Marcel Dekker, Inc. are available in microform form: RESEARCH PUBLICATIONS, 12 Lunar Drive, Drawer AB, Woodbridge, Connecticut, 06525, (203) 397-2600 or Toll Free 1-800-REACH-RP(732-2477). Outside North and South America: P.O. Box 45, Reading, RG1 8HF, England, 0734-583247.

Contributions to this journal are published free of charge.

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

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 17, Number 9, 1994

CONTENTS

GENERAL PAPERS

CAPILLARY ZONE ELECTROPHORESIS AND RELATED TECHNIQUES

Edited by

Haleem J. Issaq
NCI-Frederick Cancer Research & Development Center
Frederick, Maryland

This is a special section of *Journal of Liquid Chromatography,* Volume 17, Number 9, 1994.

MARCEL DEKKER, INC. New York, Basel, Hong Kong

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1861-1875 (1994)

THE EFFECT OF ELECTRIC FIELDS ON THE DISPERSION OF OLIGONUCLEOTIDES USING A MULTI-POINT DETECTION METHOD IN CAPILLARY GEL ELECTROPHORESIS

PENG SUN AND RICHARD A. HARTWICK*
Department of Chemistry
State University of New York at Binghamton
Binghamton, New York 13902

ABSTRACT

A multi-point detection method is proposed to study the dispersion of oligonucleotides in polyacrylamide gel capillary. In this method a single gel filled capillary is curved into loops, with several detection points aligned with a common detector. In this manner, several electropherograms can be obtained at different migration times during one capillary electrophoresis (CE) run, thus permitting studies of changes in the spatial peak variance σ^2 as a function of time. Investigations of polydeoxyadenylic acids in the range of 40-60 bases indicate that the diffusion coefficient of these compounds increases at higher voltages in the polyacrylamide gel-filled capillaries. Using this simple multi-point detection method one can estimate the band broadening originating from sample injection (σ^2_{inj}), which is otherwise difficult to measure.

INTRODUCTION

Since the successful use of polyacrylamide-filled capillaries for the analysis of small oligonucleotides was first demonstrated (1,2), there has been considerable interest in the application of capillary gel electrophoresis (CGE) to DNA and

oligonucleotide separations. The resolving power and speed of CGE has been shown to be much better than that of slab gel electrophoresis (4-6) and HPLC (7). Compared to HPLC separations of charged large biopolymers, efficiencies in CGE are much higher. Using HPLC in the anion exchange mode for example, the slow diffusion of such large molecules prevents fast mass transfer between the mobile and stationary phases. In capillary gel electrophoresis the slow molecular diffusion of biopolymers is an advantage because the contribution of peak dispersion by axial diffusion decreases with increasing molecular weight of the oligonucleotides. Fast mass transfer in the direction perpendicular to that of the mass transport by the mobile phase as in HPLC is not required. It was presumed that the slow diffusion of large DNA molecules and the number of charges in such molecules, which increase proportionally to the number of base units, could be one of the reason for the high peak capacities which can be achieved with homologous oligonucleotide mixtures. Recently, Yin et al (8) have reported a method to measure the diffusion coefficients of oligonucleotides in gel capillaries without application of an electrical field. A relationship between such diffusion coefficients and molecular size of the oligonucleotides is achieved. However, the slow diffusion of large oligonucleotides in capillaries without high voltage cannot alone explain the high separation efficiencies of CGE. The diffusion coefficients of oligonucleotide molecules and their intermolecular interaction with the polyacrylamide gel may be altered considerably by the application of electrical fields. Thus, it was decided to be important to investigate the dispersive process of oligonucleotide molecules in the presence of strong (>100 V/cm) electrical fields.

Recently, Srichaiyo and Hjerten (9) used a simple multi-point detection method to record the progress of a separation by CZE. In this method a piece of fused-silica tubing coated with a monolayer of linear polyacrylamide was curved

into loops. Using a single detector, detection of the same sample at various points on the capillary could be obtained within a single CZE run. This method is more convenient than the multi-point monitoring method using several detectors ( )which has been reported previously since only one detector is needed.

In this paper, we present a multi-point detection method based on the techniques developed by Hjerten to study the dispersive process occuring to a series of oligonucleotides in gel capillaries in the presence of fields which might typically be applied during capillary electrophoretic separations.

EXPERIMENTAL

Apparatus

A new CZE apparatus which was designed and constructed in our laboratory was used. The modular instrument contains a Spectra 100 UV detector (Spectra Physics, Reno, NV), a CZE 1000 PN 30 high power supply (Spellman, Plainview, NY 11803) and a high power supply local control (Chamonix Industries, Binghamton, NY 13905) for electrokinetic sample introduction as well as adjustment of the actual separation voltage. A special cell fitted to the detector, was designed for multi-point detection (Fig.1). The cell body was constructed by adjoining two pieces of metal. There is a small hole in the center of the lower part of the cell and a ball lens is inserted. The capillary was bent into loops with the detection points inserted on top of each other in a slit on the upper part of the cell. There was a securing arm crossing the slit on each side of the cell that held the looped capillary in position. The top of the cell was covered with two pieces of black electrical tape and a channel (the width of the channel is less than 200 μm) was left between the two pieces of tape for light pass through. During a single CZE run, the solutes in the capillary could be detected several times as they passed the light path at different times. The electropherograms were processed on a SP

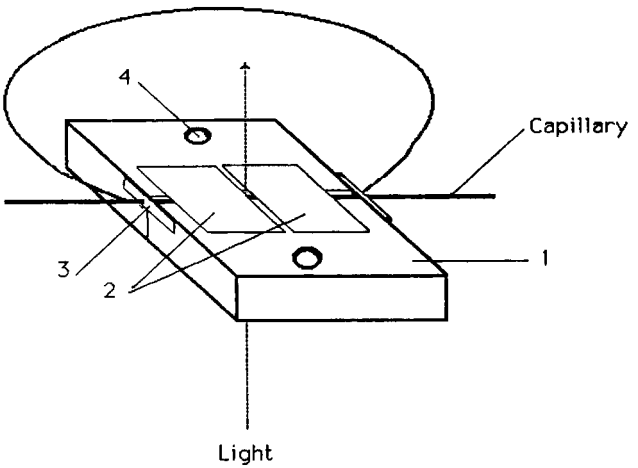


Figure 1. The schematic diagram of the multi-point detection cell. 1. cell body; 2. black tape; 3. securing arm and 4. screw hole.

4400 integrator (Spectra Physics, now a division of Thermo Separations, Fremont, CA).

Chemicals

Tris(Hydroxymethyl aminomethane), Urea ,acrylamide,N,N'-methylenebis(acrylamide)(BIS) and N,N,N',N'tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Richmond, CA). Ammonium persulfate was purchased from Sigma (Sigma,St Louis, MO). All of were of electrophoretic grade. Boric acid (Fisher Scientific Company, Fair Lawn, NJ) was of analytical grade. PdA_{40-60} was purchased from Pharmacia (Uppsala, Sweden). Adenosine 5'-monophosphate(AMP),adenosine 5'-diphosphate(ADP) and adenosine 5'-triphosphate(ATP) were obtained from Sigma (St Louis, MO). The buffer solution used for electrophoretic runs and gel preparation consisted of 0.10 M Tris, 0.25 M boric acid and 7 M urea (pH 7.6). The solution for the generation of gels contained

acrylamide/bis-acrylamide (6% T, 5% C), 0.10 M Tris, 0.25 M boric acid and 7 M urea. All solutions were filtered through a 0.45 μm membrane.

Methods

Bubble-free polyacrylamide gel filled capillaries without inner surface pretreatment were prepared by the following procedure: the transparent capillary (75 μm i.d. X 356 μm o.d. ,Polymicro Technology, Phoenix,AZ) was rinsed by passing 1 M NaOH for 1 hour and distilled water for 30 min. Five (5) mL of the acrylamide solution (6% T, 5% C) was carefully degassed in an ultrasonic bath for 5 min. Then 10 μL of 10% (w/v) ammonium persulfate and 20 μL of 10%(v/v) TEMED were added to this solution and mixed thoroughly. This final solution was passed through the capillary for 3 min. During the polymerization in the capillary, both ends of the capillary were placed in a buffer solution contained in a septum-topped vial which was pressurized using an injection of air via a 10 mL syringe. The first centimeter at both ends of the capillary was cut before the CZE runs since polymerization was incomplete in those regions. The capillary was equilibriated by running with buffer at 100-150 v/cm for 30 min. The samples were electrophoreticlly injected into the capillary by dipping the cathodic end of the capillary into the sample solution and applying a voltage for a predetermined time. Polynucleotides were detected at 260 nm. Detector rise time was 0.3 sec.

RESULTS AND DISCUSSION

Estimation of Diffusion Coefficients

A number of studies on the various sources of zone broadening in the CZE of molecular ions have recently been published (8, 10-13). The total spatial zone variance in capillary gel electrophoresis is given by:

$$\sigma^2_{total} = \sigma^2_{inj} + \sigma^2_{det} + \sigma^2_{D} + \sigma^2_{T} + \sigma^2_{con} + \sigma^2_{other} \quad (1)$$

where the terms on the right-hand side of the equation represent the variance contributions from injection (σ^2_{inj}), detection (σ^2_{det}), axial diffusion (σ^2_{D}), Joule heating (σ^2_{T}), conductivity changes through the solute zone (σ^2_{con}) and other effetcs (σ^2_{other}). For a carefully designed CGE experiment, the axial diffusion, injection volume and Joule heating terms are the dominant terms contributing to the zone broadening at low velocities (14,15). Thus,

$$\sigma^2_{total} \approx \sigma^2_{inj} + \sigma^2_{D} + \sigma^2_{T} \quad (2)$$

Except for σ^2_{inj}, both the σ^2_{D} and σ^2_{T} terms are directly proportional to the analysis time. Simple diffusion is described using the Einstein equation, as;

$$\sigma^2_{D} = 2Dt \quad (3)$$

The Joule heating (temperature) term can be estimated as (10,16):

$$\sigma^2_{T} = \left(\frac{k^2 B^2 \alpha^6 E^4 \gamma^2}{48 D (8 \lambda_b T_b^2)^2}\right) t \quad (4)$$

where D is the diffusion coefficient of the solute ion, k is the electrical conductivity of the buffer, B is the exponential coefficient relating viscosity to temperature, α is the capillary inner radius, λ_b is the thermal conductivity of the buffer, T_b is the temperature at the inner wall of capillary, and ν is zone velocity. Combining equations yields the relationship:

$$\sigma^2_{total} = \sigma^2_{inj} + 2D't \quad (5)$$

where:

$$D' = D + \frac{k^2 B^2 \alpha^6 E^4 \gamma^2}{96 D(8 \lambda_b T_b^2)^2} \quad (6)$$

D' is defined as the effective dispersive coefficient. In our experiments, the sample solutes pass the detector at several different times within a single CE run. At the first detection point:

$$(\sigma^2_{total})_1 = (\sigma^2_{inj})_1 + 2D't_1 \quad (7)$$

while at the second point:

$$(\sigma^2_{total})_2 = (\sigma^2_{inj})_2 + 2D't_2 \quad (8)$$

Since each detection point was obtained at different times in one run, the band broadening originating from injection was the same for both detection points. Thus, $(\sigma^2_{inj})_1 = (\sigma^2_{inj})_2$. By subtracting Eqn.7 from Eqn.8, we can get:

$$D' = \frac{(\sigma^2_{total})_2 - (\sigma^2_{total})_1}{2\,(t_2 - t_1)} = \frac{1}{2}\,k \quad (9)$$

where k is the slope of the line of σ^2_{total} vs. migration time.

Changes in Diffusion Coefficient with Field Strength

Fig. 2 shows two eletropherograms obtained at different field strengths (185 and 233 V cm^{-1} respectively) for four consecutive detections of $pdA_{40\text{-}60}$ by monitoring the solutes at different points in gel filled capillaries. From this figure the relationship between length and resolution is evident. Also, the reduced migration time as a function of field strength is evident.

One of the primary advantages of the multipoint detection technique is that of obtaining information not easily obtained using a conventional single point detection mode. An estimate of D' term of eq. 6 was made by measuring the σ^2 of

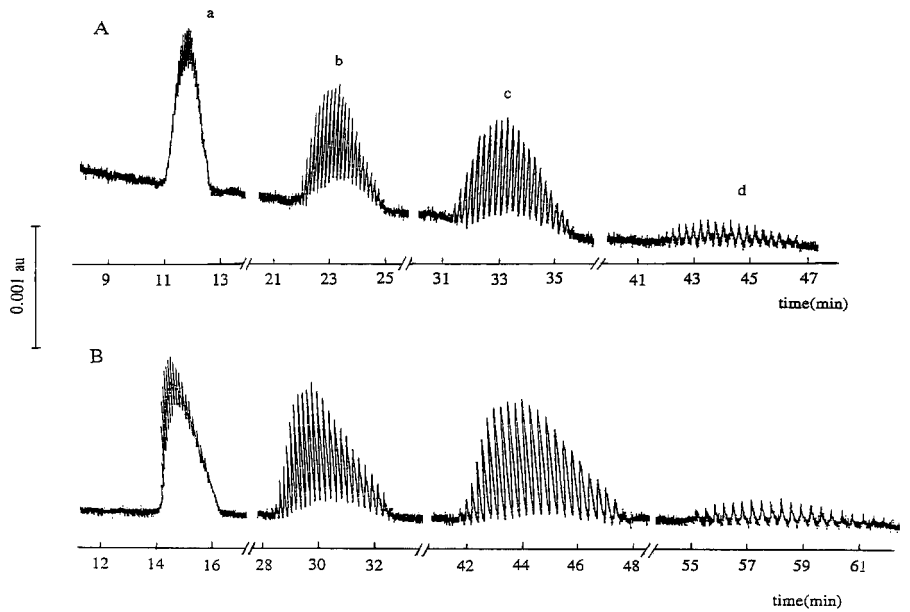


Figure 2. Electropherograms obtained from four consecutive detections of $pdA_{40\text{-}60}$ in gel filled capillary. Capillary: 75 μm i.d. X 356 μm o.d., total length 86 cm. Gel: polyacrylamide, 6% T, 5% C. Buffer: 0.1 M This; 0.25 M boric acid; 7 M urea (pH 7.6). Injection: 5 kv,2s. Sample: $pdA_{40\text{-}60}$ 5 μg/ml in water. Temperature: 24.5 C. **A.** high voltage: 185 v/cm; current:20 μA. **B.** high voltage: 233 v/cm; current: 29 μA. (a) first detection, effective length:18.4 cm; (b) second detection, effective length: 38.5 cm; (c) third detection, effective length: 56 cm; (d) fourth detection, effective length: 73.8 cm.

the peaks of oligomers with 42, 46, 50, and 54 bases at 3 different detection times, as shown in Figure 3. From the slope of the lines, the D' values (D' = 1/2 X slope) for the different oligomers was calculated (see Fig. 4).

There have been several previous studies on the effect of temperature on zone broadening in CZE (17-19). It was concluded that for capillary dimensions of 100 μm i.d. or less, and for a current of less than 30 μA (the same conditions of

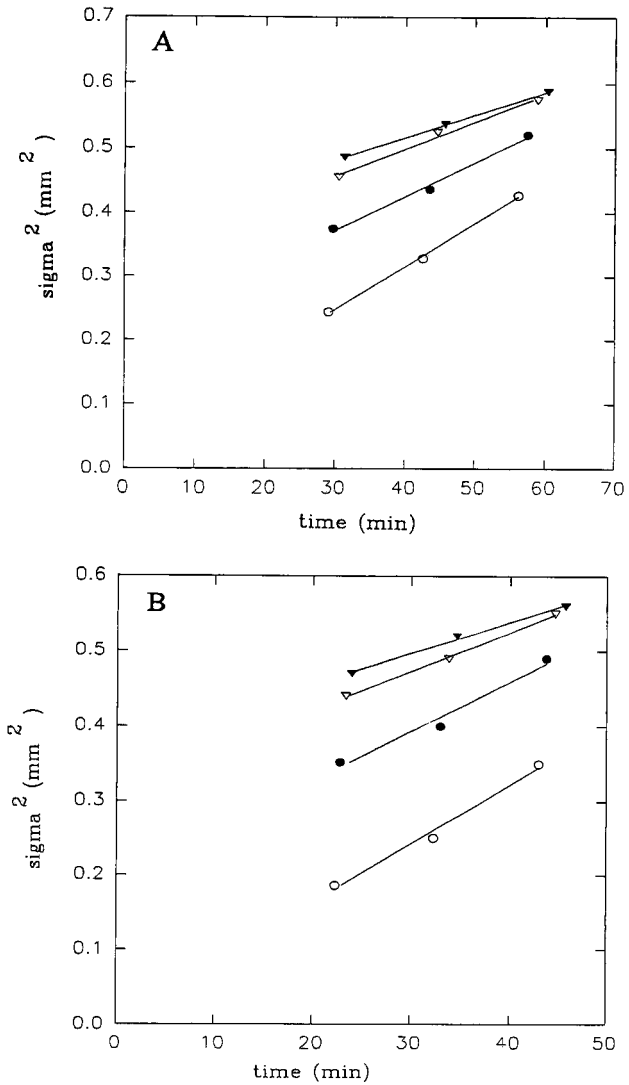


Figure 3. Plot of measured σ^2_{total} against migration time. Separation conditions were showed in Fig.2. Sample: $pdA_{40\text{-}60}$, 5 μg/ml in water. **A.** high voltage: 185 v/cm; **B.** high voltage: 233 v/cm. The four lines correspondent to four oligonucleotides with different number of base units in length: (○) base number: 42; (●) base number: 46; (△) base number: 50 and (▲) base number:54.

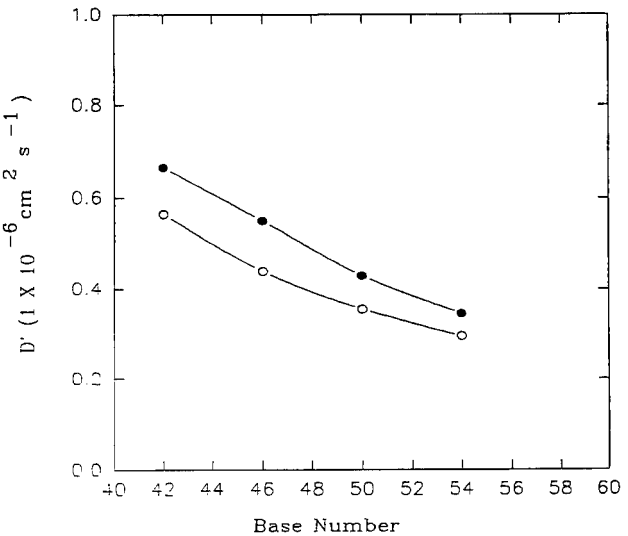


Figure 4. Dependence of dispersive coefficients (D') on the number of base units (i.e. molecular weight)of oligonucleotides(sample: $pdA_{40\text{-}60}$). (○) applied high voltage: 185 v/cm and (•) applied high voltage: 233 v/cm.

our experiments), the effect of heat generated within the capillary tube on the band broadening is much smaller than the effect of axial diffusion (11). Recently, Grossman (15) calculated the contributions of diffusion, injection and heat generated to the band broadening in CE. The σ^2_D value was 9.1 X 10^3 times higher than σ^2_T . The contribution of heat generated to the band broadening was therefore taken as negligible. The values they calculated were comparable to the published experimental values (13). The D' values obtained from our experiments are likewise presumed to be dominated by axial diffusion of the oligonucleotides in the gel capillary, even in the presence of the electrical field. In such a situation, D' approximates the diffusion coefficient (D) under a high electrical field. The diffusion coefficients (see Fig.4) obtained based on this assumption were about 2

to 3 times higher than the results obtained by Yin et al (8). The main reason for this difference might be that the D values they measured were the diffusion coefficients of oligonucleotides in a gel capillary in the absence of an electrical field. Based on theoretical considerations, they concluded that diffusion coefficients of the oligonucleotides might be considerably altered by the application of an electrical field. An electrical field-induced change of conformation of the oligonucleotide molecule may give rise to higher diffusion coefficients (8). The results of our experiments verify this assumption. Recently, Delinger et al (20) reported that the variation of analyte mobility and diffusion coefficients were not constant over a large range of voltages. From the results (Fig. 4), it can be concluded that the diffusion coefficients of the oligonucleotides in the gel capillary decrease nonlinearly with an increase in the number of base units or molecular weight. Yin et al (8) obtained the same results for oligonucleotides in a gel capillary without the application of an electrical field.

Estimation of Injection Variances

Another advantage of the multi-point detection method is that estimates of the σ^2_{inj} value from the plot of σ^2_{total} vs. migration time, which is difficult to measure by other means. In equation 8, it can be seen that $\sigma^2_{total} = \sigma^2_{inj}$ when $t=0$. Thus the value of σ^2_{inj} is given by the intercept of the line of σ^2_{total} vs. time. Fig. 5 shows the electropherograms of a two-point detection of AMP and ADP in a gel capillary with different injection times. The σ^2_{total} and calculated D' values for AMP are listed in Table I. The D' values obtained from the two runs under the same separation conditions with the exception of the injection time are very similar, giving confidence that temperature or other effects are minimal, as assumed in the derivations. Regression of σ^2 versus time yields σ^2_{inj} values for run 1 and 2 are

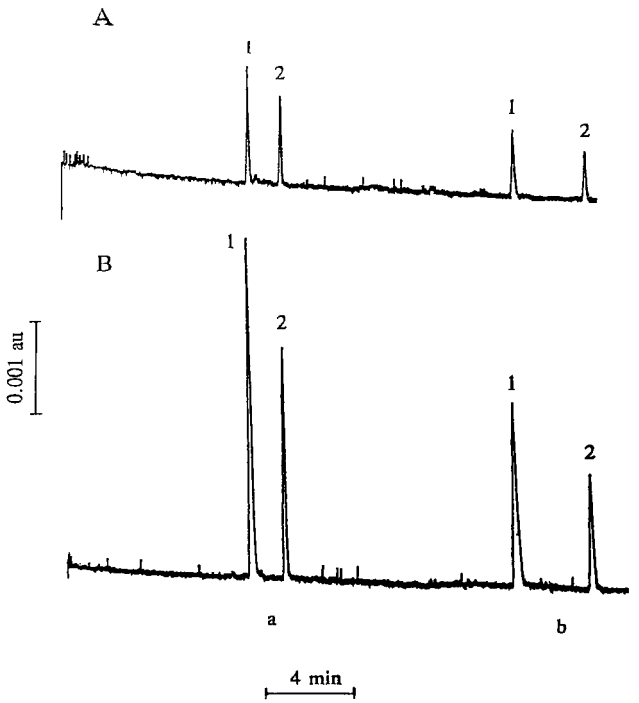


Figure 5. Electropherograms obtained from two consecutive detections of AMP and ADP in gel filled capillary. Capillary: 75 μm i.d. X 356 μm o.d., total length 55 cm, effective length: (a) 16 cm, (b) 40 cm. Gel: polyacrylamide, 6% T, 5% C. Buffer: 0.1 M this; 0.25 M boric acid; 7 M urea (pH 7.6). High voltage: 13.9 kv, 35 μA. Injection: **A.** 3 kv, 3 sec.; **B.** 3 kv, 5 sec.. 1 = ADP, 2 = AMP.

0.25 $mm^2$and 0.55mm^2, respectively. Given that $\sigma^2_{inj} = l^2 / 12$, where l is the width of the analyte plug (11, 14) and that l is proportional to the injection time (t_{inj}) (21), then σ^2_{inj} is proportional to t_{inj}^2. Thus;

$$\frac{(\sigma^2_{inj})_2}{(\sigma^2_{inj})_1} = \frac{(t^2_{inj})_2}{(t^2_{inj})_1} \qquad (10)$$

while taking the square root of both sides yields:

TABLE 1

Measured σ^2 Values for AMP at Two Different Migration Time

Injection time(sec.)	3		5	
Migration time(min)	9.88	23.49	9.71	23.35
σ^2_{total} (mm^2)	0.68	1.26	0.98	1.58
Calculated D' values* (1×10^{-6} cm^2/s)	3.5		3.7	

* D' values were calculated based on equation (9).

$$\frac{(\sigma_{inj})_2}{(\sigma_{inj})_1} = \frac{(t_{inj})_2}{(t_{inj})_1} \qquad (11)$$

The ratio of $(t_{inj})_2$ over $(t_{inj})_1$ for the two different injection times in Fig. 5 is 5/3 or 1.67. The ratio of $(\sigma_{inj})_2$ over $(\sigma_{inj})_1$ is $(.55/.25)^{1/2}$ or 1.48. The discrepency of ca. 12% between the two values could be caused by errors in controlling the exact injection times, and to measurement errors of the peak variances.

CONCLUSIONS

In addition to the application of this consecutive multi-point detection method on the study of dispersive processes of analytes during CZE, there are some other advantages of this method which were reported by Srichaiyo and Hjerten recently (9). For example, by using this technique it has been demonstrated that the appearance of the DNA pattern changes in an unexpected, discontinuous way during a CZE run. Also, using this multi-point detection method one can unambiguously study the relationship between the migration distance and

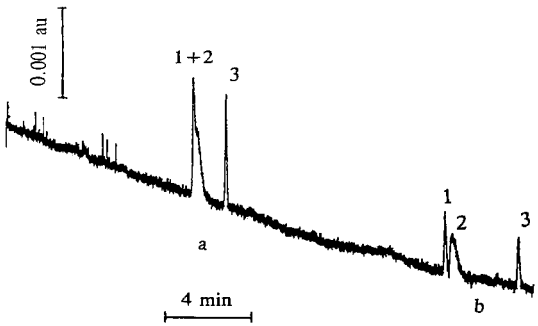


Figure 6. Electropherograms obtained from two consecutive detections of AMP, ADP and ATP in gel filled capillary. Separation conditions are same as those in Fig.5 except that the injection time is 2 sec at 3.5 kv. (a) first detection, effective length: 16 cm; (b) second detection, effective length: 40 cm. 1 = ADP; 2 = ATP and 3 = AMP.

the migration time. Also, it is possible to rapidly choose the optimum effective length for a certain preferred high voltage. This can minimize the time needed to optimize the separation conditions. In Fig. 6, one can see ADP and ATP cannot be separated at the first effective length distance but can be separated at the second detection point. In this way we can obtain good separation conditions in fewer runs than the single point detection method.

In conclusion, the simple multi-point detection method pioneered by Hjerten is very promising for the study of dispersive processes of oligonucleotides in a gel capillary which is subjected to an electric field. The method can help one to investigate the relationship of the diffusion coefficients with the number of base units of oligonucleotides, and to consequently understand the reason for the high resolution and efficiency which can be achieved with homologous oligonucleotide mixtures in gel filled capillaries. Also, one can estimate the σ^2_{inj} value with reasonable accuracy, which is difficult to measure by other methods.

ACKNOWLEDGEMENTS

This research was supported by a grant from a Biomedical Research Support Grant at Binghamton University, by Thermo Separation Products(formerly Spectra-Physics) and by the Center for Biotechnology, SUNY-Stonybrook.

REFERENCES

1 A.S Cohen, D.R. Najarian, A. Paulus, A. Guttman, J.A. Smith, B.L. Karger, *Proc. Natl. Acad. Sci. U.S.A.*, 85 9660 (1988)
2 A. Guttman, A.S. Cohen, D.N. Heiger, B.L. Karger, *Anal. Chem.*, 62 137 (1990)
3 G.L. Trainor, Anal. Chem., 62 418 (1990)
4 H. Drossman, J.A. Luckey, A.J Kostichka, J. D'Cunha, L.M. Smith, *Anal. Chem.*, 62 900 (1990)
5 H. Swerdlow, S. Wu, H. Harke, N.J. Dovichi, *J. Chromatogr.*, 516 61 (1990)
6 A.S. Cohen, D.R. Najarian, B.L. Karger, *J. Chromatogr.*, 516 49 (1990)
7 Y. Baba, T. Matsuura, K. Wakamoto, M. Tsuhako, *J. Chromatogr.*, 558 273 (1991)
8 H.F. Yin, M.H. Kleemiβ, J.A. Lux, G. Schomburg, *J. Microcol. Sep.*, 3 331 (1991)
9 T. Srichaiyo, S. Hjerten, *J. Chromatogr.*, 604 85 (1992)
10 F. Foret, M. Deml, P. Bocek, *J. Chromatogr.*, 452 601(1988)
11 X. Huang, W.F. Coleman, R.N. Zare, *J. Chromatogr.*, 480 95 (1989)
12 K. Otsuka, S. Terabe, *J. Chromatogr.*, 480 91 (1989)
13 H.K. Jones, N.T. Nguyen, R.D. Smith, *J. Chromatogr.*, 504 1 (1990)
14 S. Terabe, K. Otsuka, T. Ando, *Anal. Chem.*, 61 251 (1989)
15 P.D. Grossman, *Capillary Electrophoresis*, P.D. Grossman, J.C. Colbrn, eds., Academic Press, Inc. San Diego, CA., 1992, p3.
16 S.L. Petersen, N.E. Ballou, *Anal. Chem.*, 64 1676 (1992)
17 S. Hjerten, *Chromatogr. Rev.*, 9 122 (1967)
18 E.Grushka, R.M. McCormick, J.J. Kirkland, *Anal. Chem.*, 61 241 (1989)
19 D.S. Burgi, K. Salomon, R. Ghien, *J. Liq. Chromatogr.*, 14(5) 847 (1991)
20 S.L. Delinger, J.M. Davis, *Anal Chem.*, 64 1947 (1992)
21 D.J. Rose,Jr., J.W. Jorgenson, *Anal. Chem.*, 60 642 (1988)

Received: March 27, 1993
Accepted: September 28, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1877-1881 (1994)

A RAPID METHOD FOR ENKEPHALIN ANALYSIS IN TISSUES BY CAPILLARY ELECTROPHORESIS

W. JEFFREY HURST[1], PATRICIA J. McLAUGHLIN[2], AND IAN S. ZAGON[2]

[1]*Department of Comparative Medicine*
[2]*Department of Neuroscience and Anatomy*
M.S. Hershey Medical Center
Hershey, Pennsylvania 17033

Abstract

A rapid method for the determination of the pentapeptide, met enkephalin , in samples of rat retina is described. The method uses ultrafiltration as a sample preparation followed by free solution capillary electrophoresis with detection at 210 nm. The method is rapid with an analysis time of less than 10 minutes.

Introduction

There continues to be intense interest in methods for the determination of neurochemicals especially the pentapeptides met and leu enkephalin. Additionally, there is growing interest in the use of capillary electrophoresis to perform this assay. A number of researchers have reported the use of capillary electrophoresis (CE) for the determination of these compounds (1-4). Some of these reports focus on the development of the

appropriate conditions for the separation of the individual compounds while others report the analysis of complex samples. In those cases where complex samples were analyzed, the solid phase technique pioneered by Desiderio and others (5-7) was used to prepare samples for subsequent analysis . In this communication, we report the use of ultrafiltration (UF) coupled with CE using UV detection at 210 nm for the qualitative determination of met enkephalin in samples of retina obtained from a laboratory rat. This sample preparation is rapid and entails the use of no additional chemicals when compared to the solid phase extraction (SPE) protocol.

Materials and Methods

Sample Preparation

Retinas were obtained from 1 day and adult Sprague-Dawley rats (Charles River; Wilmington, MA). The material was homogenized in 50 mM Tris buffer containing protease inhibitors in a 1:10 ratio (v/v) using a Polytron tissue homogenizer (8) . After homogenization samples were centrifuged through a Centricon filter unit with a 10,000 Mw cutoff membrane (Amicon; Danvers, MA). Samples were stored at -70 ° C prior to analysis.

Equipment and Analytical Conditions

A SpectraPhoresis 1000 Capillary Electrophoresis Unit (Thermo Separation Products; Fremont, CA) operating under Version 1.04 of the SpectroPhoresis software was used in this study. A 44 cm x 75 um UVT Capillary (Polymicro; Phoenix, AZ) with a buffer system of 40 mM phosphate at pH 9.0 was used (9). The resulting electropherogram was monitored at 210 nm. The run time was 15 min. at a temperature of 30 °C with 15 kV applied voltage.
Samples of met and leu enkephalin (Sigma , St. Louis, MO) were dissolved in distilled water at concentrations of approximately 100 ug/ml. Samples and standards were injected using an electrokinetic injection of 10 kV for 1 s. The sample extract was compared with the standard to verify migration times.

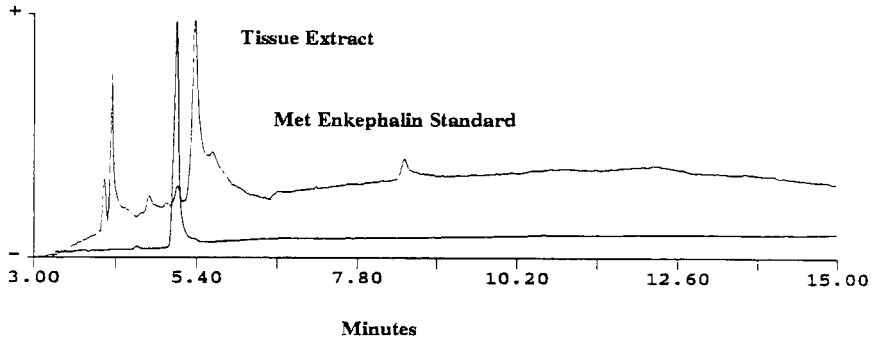


Figure 1
Overlay of Electropherogram of Met Enkephalin Standard and Tissue Extract
Conditions of Analysis: 44 cm x 75 um UVT Capillary with a 40 mM Phosphate Buffer @ pH 9.0 Detection @ 210 nm

Results and Discussion

Injections of met and leu enkephalin using the conditions described indicated that the two compounds migrated at differing rates as has been previously reported by others (1-3). A sample of retina from a developing rat was injected under the same conditions and a peak was seen at the same migration time as met enkephalin. To verify sample clean-up, samples of authentic standard were prepared using the same conditions as the sample and 100% recovery was seen by comparing peak areas of filtered and unfiltered met enkephalin standard. Additionally, the sample was spiked with met enkephalin standard to verify the migration time of the peak . The detector wavelength of 210 nm was chosen to monitor the peptide bond.Figure 1 illustrates this separation showing a comparison of the standard with the retina extract.

No quantitative data were developed in this experiment since the purpose was to illustrate the utility of ultrafiltration as a method of sample preparation for these compounds. UF has been widely used as a method of sample preparation for other biologically related compounds (10 -12) in similar systems so there is no reason not to expect success in these preliminary studies. Additionally, this sample preparation has only been applied initially to this one matrix but the results are encouraging enough to report on these preliminary studies.

Acknowledgements

The authors wish to thank Dr. Tomoki Isyama for assistance in tissue collection. This work is supported in part by NIH Grant NS-20500.

References

1) Dulffer,T.,Herb,R.,Herrmann,H. and Kobold, U. **Chromatographia 30** (1990) 675 .

2) Johansson, I. M. ,Huang, E. C. ,Henion, J.D. and Zweigenbaum, J. **J. Chromatog., 554** (1991) 311.

3) Muck, W. and Henion, J. **J. Chrom., 495** (1989) 41.

4) Mosley,M.A. , Deterding,L.J. , Tomer,K.B. and Jorgenson, J.W. **Anal. Chem., 63** (1991) 109.

5) Fleming, L.H. and Reynolds,N.C. Jr. **J. Chromatog., 431** (1988) 65.

6) Friland, G.H. and Desiderio, D.M. **Life Sci., 41** (1987) 809.

7) Desiderio,D.M. ,Onishi, H. ,Friland, G. , Wood, G. and Pagidipati, D. **Biomed Chromatog., 2** (1987) 47.

8) Zagon, I.S. , Gibo, D.M. and McLaughlin, P. J. **Brain Res., 596** (1992) 149.

9) Calbiochem Corp, Buffers: A Guide for the Preparation and Use of Buffers in Biological Systems (1975).

10) Beresford, A.L. , Jones, S.R. , and Nelson, R.G. **J. Biol. Chem., 262** (1987) 35.

11) Updyke, T.V. and Nicolson, G.L. **J. Immunol Methods, 73** (1984) 83.

12) Tsomides, T.J. **BioTechniques, 14** (!993) 656.

Received: December 3, 1993
Accepted: January 14, 1994

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1883-1897 (1994)

FULLY AUTOMATED ANALYSIS OF AMINO ACID ENANTIOMERS BY DERIVATIZATION AND CHIRAL SEPARATION ON A CAPILLARY ELECTROPHORESIS INSTRUMENT

H. RUYTERS AND SJ. VAN DER WAL
DSM Research
P.O. Box 18, 6160 MD Geleen
The Netherlands

ABSTRACT

A completely automated method for determining the chiral purity of amino acids at large enantiomeric excess was developed based on automated pre-separation derivatization with 4-fluoro-7-nitrobenz-2,1,3-oxadiazole, separation by capillary electrophoresis with cyclodextrin chiral selectors and detection by laser-induced fluorescence at 488 nm excitation.

The detection limit is 140 ppm L-phenylalanine in D-phenylalanine, linearity is more than three orders of magnitude and the coefficient of variation is 3 % at the 0.5 % enantiomer level.

INTRODUCTION

High voltage capillary electrophoresis (CE) is a very efficient separation technique, but it suffers from a lack of detector sensitivity. Pre-separation derivatization can alleviate this problem without adversely affecting the efficiency. In HPLC

pre-column derivatization was not widely used until the introduction of standard protocols on fully automated instrumentation.

We therefore decided to explore the benefits of automated pre-separation derivatization on a commercially available CE-instrument. We shall focus here on enantiomeric analyses of some amino acids.

Two approaches can be used:

A. Derivatization with a chiral reagent and separation of the diastereomers.

B. Derivatization with a non-chiral reagent and separation of the enantiomers.

The LDR can not be extended by increasing the buffer capacity of the MECC system, because of current limitations. A different derivative or wavelength for UV-detection could increase the LDR by a factor of three at the expense of derivatization speed, using Marfey's reagent, or selectivity, using 214 nm as the detection wavelength (1).

A variety of chiral reagents can be utilized not only with absorbance detection but also with fluorescence detection, which extends the LDR by several orders of magnitude. However, at extreme enantiomer ratios the enantiomeric impurity of the reagent can cause an error in the enantiomeric excess (ee) of the analyte. Approach B thus appears more appropriate for performing enantiomeric analyses at large ee.

Several off-line pre- and post-separation reactions with fluorogenic reagents were compared by Albin et al. (2). Fluorescein is accompanied by artifact and blank reagent problems, fluorescamine derivatives are generally too unstable to be used in an off-line method, so 9-fluorenylmethyl chloroformate (FMOC) was their preferred reagent, although the reagent itself is fluorescent and was extracted with pentane (2). This last drawback and the lack of a suitable laser for excitation (at 260 nm) of these derivatives of primary and secondary amino acids made us search for an alternative to be used in automated derivatization. Such a reagent - for primary amino acids - is naphtalene dicarboxaldehyde (3), which is included in our study. Liu et al. (4) demonstrated with a similar, new reagent that they synthesised a three order of

magnitude linearity in the determination of amino acid derivatives using laser induced fluorescence at 442 nm and MECC.
In 1989 thermooptical absorbance detection of 4-(dimethylamino) azobenzene-4'-sulfonylchloride derivatives was reported to be even superior over fluorescence detection (5). Unfortunately this detection mode is not available to us.

In this paper we explore the determination of the enantiomeric purity of some amino acids at large ee by fully automated pre-separation derivatization with 4-fluoro-7-nitrobenz-2,1,3-oxadiazole (NBD-F), separation by CE with β-cyclodextrin (βCD) and dimethyl βCD as chiral selectors, and detection by laser-induced fluorescence (LIF) with an argon ion laser.

THEORY

The purpose of any separation is to obtain adequate resolution. In CE the resolution of two components i and j, R_{ji}, can be expressed as:

$$R_{ji} = \frac{\Delta\mu_{ji} \cdot E}{\sqrt{\sigma^2_{L,inj} + 2Dt_j}} t_j \qquad (1)$$

in which:

R_{ji} = resolution between components j and i, expressed in units of the average standard deviation of peaks j and i

$\Delta\mu_{ji}$ = selectivity, difference in electrophoretic mobility between component j and i ($m^2/V.s$)

E = electric field strength (V/m)

$\sigma^2_{L,inj}$ = injection contribution to the variance of the peaks (m^2)

D = average diffusion coefficient of components j and i (m^2/s)

t_j = elution time of the last component (s)

The dependence of the selectivity on the selector concentration in an enantiomeric separation with chiral selectors was given by Wren and Rowe (6):

$$\Delta\mu = \frac{[CD]\ (\mu_o - \mu_\infty)\ (K_B - K_A)}{1 + [CD]\ (K_A + K_B) + K_A K_B [CD]^2} \qquad (2)$$

where:

[CD] = chiral selector concentration

μ_o = mobility of the enantiomers

μ_∞ = mobility of the enantiomer-selector complex

K_A, K_B = equilibrium constants for enantiomers A and B, respectively

The apparent electrophoretic mobility of an enantiomer in this system is:

$$\mu = \frac{\mu_o + \mu_\infty K[CD]}{1 + K[CD]} \qquad (3)$$

The maximum selectivity is obtained at a selector concentration equal to the reciprocal average equilibrium constant (6), so with

equation 3 the optimum selector concentration can be evaluated from mobility data:

$$\mu_{opt} = \frac{\mu_o + \mu_\infty}{2} \qquad (4)$$

This means that for any analyte the optimum selector concentration can be obtained in a few experiments, but on the other hand each analyte will have a different optimum selector concentration depending on its affinity to the selector. This was demonstrated by Wren and Rowe (7) for compounds differing in hydrophobicity.

The analyte concentrations are much lower than the selector concentrations used so the selector concentration in the equations above can be considered constant.

Penn et al. (8) stressed that in order for the μ_∞ determination to be accurate the viscosity of the separation medium should be taken into account. At the relatively low selector concentrations used in our research viscosity corrections were not applied: they have only a minor effect on μ_{opt} (equation 4), and most optima are rugged (see table 2) and insensitive to small deviations. In addition μ_∞ is often not reached due to solubility limitations of the chiral selector in the run buffer.

EXPERIMENTAL

Experiments were done on a PACE 2050 system (Beckman Instruments, Fullerton, CA, USA) equipped with a 3 mW argon-ion

laser-induced fluorescence detector. A 488 nm rejection filter and a 520 ± 20 nm bandpass filter were supplied with the detector and inserted in the filter holder on the emission side of the detector. The separations were carried out on a 970 x 0.05 mm (length x ID) fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The program for performing the analysis is given in table 1.

Except for the reaction microvials (30 µl, Beckman) standard 4 ml vials (Beckman) were used.
Triacetyl-β-cyclodextrin was obtained from Aldrich (Milwaukee, WI, USA), α-, β-, γ-, and 2,6-di-O-methyl-β-cyclodextrin from Fluka (Buchs, Switzerland).
4-Chloro-7-nitrobenzofurazan (NBD-Cl) was obtained from Janssen (Beerse, Belgium), its fluoro analog (NBD-F) from Sigma (St. Louis, MO, USA), naphthalene-2,3-dicarboxaldehyde (NDA) from Polysciences (Warrington, PA, USA) and the amino acids used were from a variety of sources.

Sodium hydroxide, tris(hydroxymethyl)aminomethane (TRIS), boric acid, potassium cyanide and ethanol were purchased from Merck (Darmstadt, Germany) and all water was produced by a Milli-Q apparatus (Millipore, Bedford, MA, USA). The 0.1 M TRIS-borate buffer used throughout this paper was prepared by adjusting a 0.1 M boric acid solution to pH 8.3 by addition of a 0.1 M TRIS solution. The reagent for derivatization (autosampler vial # 4) was prepared daily by dissolving 0.5 mg NBD-F in 310 mg of ethanol.

TABLE 1

Program for Automated Derivatization Followed by Separation of the Derivatives.

inlet vial[a]	contents	outlet vial	contents
11	separation buffer	1	separation buffer
12-x	analyte	3	water
(x+1)-30	empty microvial	4	reagent
		5	ethanol
31	1M sodium hydroxide	8	empty
		10	empty
32	water		
33	empty		
34	ethanol		

[a] x = 20 for single analysis of nine samples; x = 14 for fivefold analysis of three samples.

Separation buffer = 0.1 M TRIS-borate pH 8.3

step	process	duration	inlet	outlet	control summary
1	set detector				LIF: 488:520 nm Rate: 5 Hz Zero 2.0 min
2	set temp.				Temp.: 25 °C
3	rinse	1.0 min	31	8	Forward: High Pressure
4	rinse	1.0 min	32	8	Forward: High Pressure
5	rinse	1.0 min	33	8	Forward: High Pressure
6	rinse	2.5 min	12	8	Forward: High Pressure
7	wait	0.0 min	32	3	
8	rinse	0.1 min	22	5	Reverse
9	rinse	2.0 min	22	4	Reverse
10	rinse	0.1 min	22	5	Reverse
11	rinse	2.0 min	22	10	Reverse
12	wait	5.0 min	34	3	
13	rinse	3.0 min	11	8	Forward: High Pressure
14	inject	3.0 sec	22	8	Pressure
15	wait	0.0 min	34	3	
16	inject	3.0 sec	32	10	Pressure
17	separate	18.0 min	11	1	Constant Voltage: 30.00kV Current Limit: 100.0 µA Integrator On
18	wait	0.0 min	34	3	

RESULTS AND DISCUSSION

Selection of Derivatization Reagent and Chiral Selector

Our selection of reagent was determined by the following limitations:

- fast reaction with amines,
- derivatives to be detected by LIF using 442 nm (He-Cd laser) or 488 nm (Ar-ion laser) excitation,
- enantiomeric derivatives separable by CE or MECC.

Two reagents fulfilled these requirements: naphthalene-2,3-dicarboxaldehyde (NDA) (3,9) and NBD-F (10). In experiments using 0.1 M TRIS-borate buffer pH 8.3 containing α-, β-, γ-, dimethylβ- or triacetylβ-cyclodextrin we found that the optimum selector concentration and the selectivity were much lower with NDA-derivatized amino acids than with NBD-derivatives. Selectivities at the optimum cyclodextrin concentrations (see equation 4) for some NBD-amino acid derivatives using β-CD and dimethylβ-CD are given in table 2.

Applying a buffer of lower pH (11) and/or MECC (10) offers opportunities for resolution enhancement, but were considered to be outside the scope of this paper.

Derivatization Protocol

The autosampler program is similar to that used before (1, c.f. table 1) except for ethanol enclosed transport of reagent to

TABLE 2

Selectivities at Optimum Cyclodextrin Concentrations

amino acid	$\Delta\mu$ 10^{-10} $m^2/V.s$	[βCD] mM	$\Delta\mu$ 10^{-10} $m^2/V.s$	[dimethylβCD] mM
alanine	4.2	≥ 14		
leucine	5.5	≥ 8	2	≥ 4
valine	3.7	> 14		
proline	5.5	≥ 14	3.3	> 8
phenylglycine	3.1	2	7	2
phenylalanine	5.7	2	10	1.5
methionine	3.5	> 14	2	> 8
Phe-amide	3.5	> 14	7	2

the reaction vial. If this is omitted the reagent tends to adhere to the wall and precipitates during the mixing-step.

To obtain sufficient resolution the temperature during separation should not exceed 35 °C. With our instrument the derivatization is performed at the same temperature as the separation. Derivatization with NBD-CL takes a few minutes at 60 °C.
Therefore, the reaction was done with NBD-F, which reacts much faster (10). Unfortunately, the NBD-F solution is not very stable and has to be prepared daily.

In a more recent version of the CE-instrument the vials of the autosampler can be heated separately so that the reaction might be performed with the more stable NBD-Cl at elevated temperature while separation takes place at lower temperature.

TABLE 3

Precision of the determination of enantiomeric purity of phenylglycine at 0.5 % level.

A.

duration of injection (s)	L area (counts)	D area (counts)	D/L (%)
2	20399.5	99.4	0.58
2	21398.0	107.1	0.60
1	9287.5	46.8	0.60
1	12034.2	62.0	0.62
5	41046.1	205.4	0.60
5	32074.3	168.8	0.63

cv (%) 2.7

B.

D area (counts)	L area (counts)	L/D (%)
12177	78.7	.54
24904	164.7	.55
27232	172.9	.53
24900	167.0	.56
21236	136.8	.54
19032	117.0	.51

cv (%) 3.1

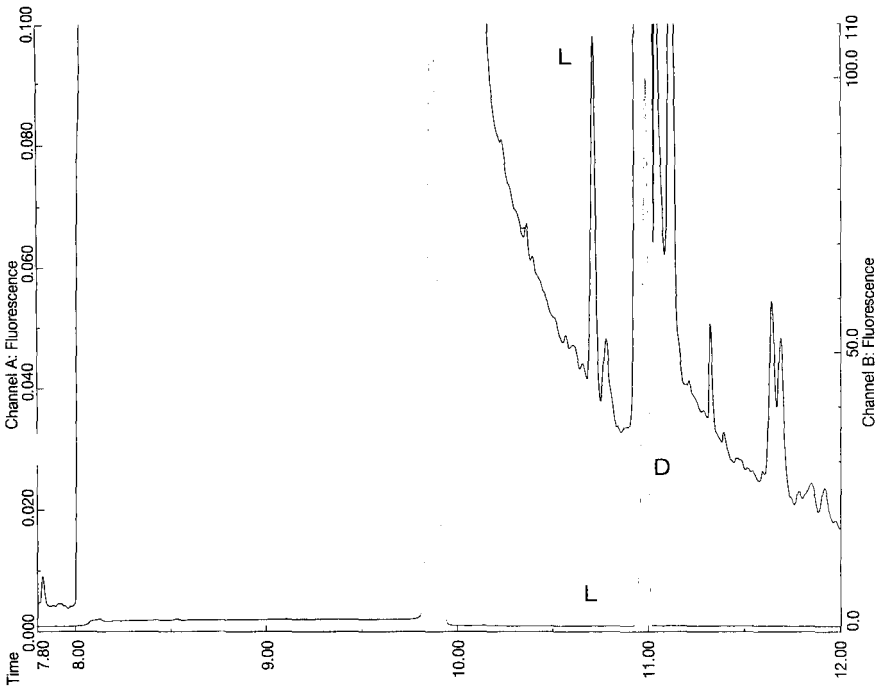


FIGURE 1. Electropherogram for determination of enantiomeric purity of D-phenylalanine.
Derivatization and separation program as in table 1. The scale on the right belongs to the lower tracing, the expanded scale on the left to the upper trace of the same electropherogram.
The wide band eluting at 8-10' is caused by the reagent.
The run buffer contains 2 mM dimethyl βCD, the sample 0.5 mg/ml D-Phe.

Determination of Enantiomeric Purity of Phenylglycine and Phenylalanine

In chiral analysis the content of one of the enantiomers in the sample can be used as an internal standard if the LDR of the

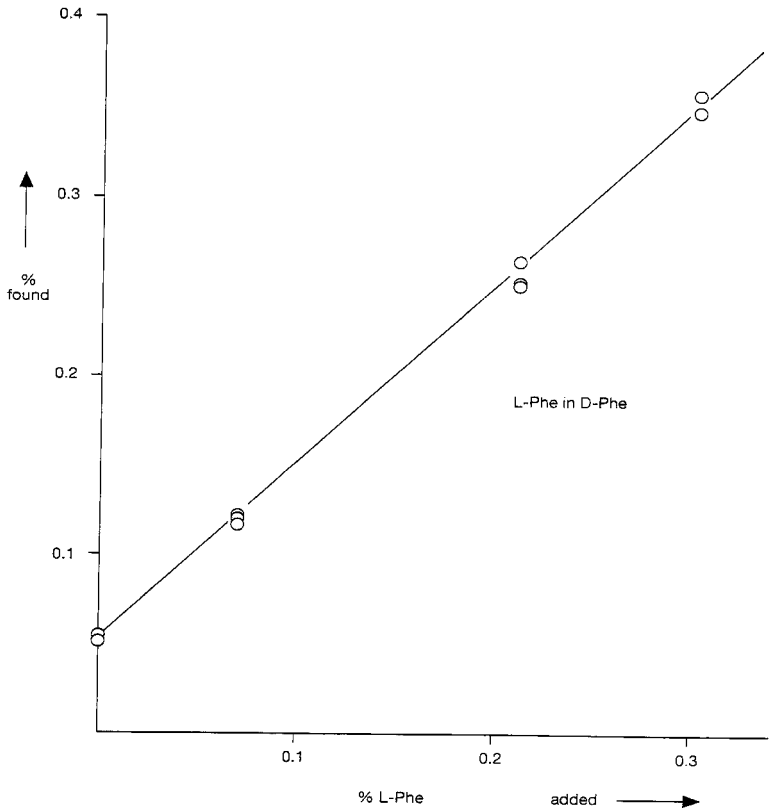


FIGURE 2. Calibration curve for determination of L-Phe in D-Phe by standard addition.
Derivatization and separation program as in table 1; sample as in fig. 1.

system is sufficient. The determination of L-phenylglycine in D-phenylglycine and D-phenylglycine in L-phenylglycine under these circumstances (data in tables 3A and 3B) shows that the precision at the 0.5 % level is about 3 % CV.

Analysis of a racemic mixture indicates that the intrinsic fluorescence sensitivities of the cyclodextrin complexes of the derivatives of D- and L-phenylglycine are different. This difference is taken into account in table 3.
In table 3A an amount of derivative varying over a factor of four was introduced into the system. The result indicates that any non-linearity of the system is limited to less than the three percent variation coefficient of this table. A calibration curve for L-Phe in D-Phe was made by standard-addition to determine the detection limit and the purity of D-phenylalanine (see fig. 1). The D-phenylalanine sample used contained 0.05 % L-phenyl-alanine (fig. 2), which is well above the detection limit calculated from the calibration curve: 0.014 % L- in D-phenylalanine (95 % confidence interval).

ACKNOWLEDGEMENT

The authors gratefully acknowledge the help of Beckman Instruments, who lent us an Ar-ion laser which produced better signal-to-noise ratios with the NBD-derivatives than the He/Cd-laser we originally planned to use.

REFERENCES

1. R.J.H. Houben, H. Gielen and Sj. van der Wal, J. Chromatogr., 634, 317 (1993).

2. M. Albin, R. Weinberger, E. Sapp and S. Moring, Anal. Chem., 63 417 (1991).

3. B. Nickerson and J.W. Jorgenson, J. High Resolut. Chromatogr. Chromatogr. COmmun. 11, 533, 878 (1988).

4. J. Liu, Y-Z.Hsieh, D. Wiesler and M. Novotny, Anal. Chem., 63 408 (1991).

5. M. Yu and N.J. Dovichi, Anal. Chem. 61 37 (1989).

6. S.A.C. Wren and R.C. Rowe, J. Chromatogr., 603, 235 (1991).

7. S.A.C. Wren and R.C. Rowe, J. Chromatogr., 635, 113 (1993).

8. S.G. Penn and D.M. Goodall, J. Chromatogr., 636, 149 (1993).

9. T. Ueda, R. Mitchell, F. Kitamura and T. Metcalf, J. Chromatogr., 593 265 (1992).

10. K. Imai and Y. Watanabe, Anal. Chim. Acta, 130 377 (1981).

11. Y.Y. Rawjee, D.U. Staerk and G. Vigh, J. Chromatogr., 635 291 (1993).

Received: December 29, 1993
Accepted: January 7, 1994

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1899-1916 (1994)

FREE-SOLUTION CAPILLARY ELECTROPHORESIS OF TRYPTIC DIGEST FRAGMENTS OF A RECOMBINANT PORCINE PRO-GROWTH HORMONE RELEASING HORMONE (2-76)OH

J. P. CHANG*, D. L. SMILEY, AND M. R. COLEMAN
Eli Lilly and Company
Lilly Research Laboratories
2001 West Main Street
Greenfield, Indiana 46140

ABSTRACT

The application of free-solution capillary electrophoresis (FSCE) to the separation of peptide fragments produced by tryptic digestion of a new recombinant porcine pro-growth hormone releasing hormone (2-76)OH (rpGHRH) composed of 75 amino acid residues is presented. It was found that 11 digest peptide fragments of rpGHRH could be separated by FSCE using an uncoated fused silica capillary with 0.1 M phosphate buffer, pH 3.3, used as the separating electrolyte. Individual peptide fragments prepared by solid phase synthesis method were spiked into the tryptic digest mixture to verify structure and fragment assignments of the polypeptide in electrophoresis. Optimal separation conditions were obtained by studying the effect of pH in the separating electrolyte and applied voltage. An excellent

* To whom correspondence should be addressed.

correlation was found for the electrophoretic migration time, t_m, of the digest peptides versus $q/MW^{2/3}$, where q is the calculated charge, and MW is molecular weight of peptides.

INTRODUCTION

Characterization of the identity and purity of recombinant peptides used as drugs is necessary for manufacturing and recovery processes. Tryptic digest mapping of proteins has been used extensively to provide information about protein structure and purity. One of the useful approaches to characterization is reversed phase high performance liquid chromatography (RP-HPLC) (1,2). Recently, capillary zone electrophoresis (CZE) has been widely employed to separate and characterize biomolecules due to its simplicity, high resolving power, and ability to automate the analyses (3,4). Some papers have reported the use of CZE in separation of peptide mixtures containing 19 peptide fragments produced from the tryptic digest of biosynthetic human growth hormone (5,6). Nielsen et al. (7) compared capillary zone electrophoresis and RP-HPLC to characterize tryptic digests of recombinant human growth hormone. Peaks were identified and the differences in selectivity in the two techniques were reported.

In this paper, an application of free-solution capillary electrophoresis (FSCE) to the separation of the tryptic digest peptides of the recombinant porcine pro-growth hormone releasing hormone (2-76)OH (rpGHRH) (8), composed of 75 amino acid residues, is reported. Phosphate buffer and acidic pH were selected as the separating electrolyte because of its low ultraviolet absorbance at 200 nm and low osmotic flow. Optimal separation conditions have been found by studying the effect of pH and applied voltage in electrophoresis. Correlation of electrophoretic migration time of digest peptides with $q/MW^{2/3}$ was studied.

EXPERIMENTAL

Instrument

High performance capillary electrophoresis was performed on the P/ACE system 2000 (Beckman Instruments, Palo Alto, California, USA) in an uncoated fused silica capillary (75 μm i.d.x 57 cm long to the window) mounted in a cartridge with on-column flow cell for optical detection. Another coated capillary CElect-P175 (57 cm long to the window x 75 μm i.d.) (Supelco Inc., Bellefonte, PA, USA) was mounted in a Beckman cartridge. P/ACE system 2000 version 1.5 software was used to collect data and the System Gold chromatography data system (Beckman) was used to analyze results. The peptides were monitored by UV absorbance at 214 nm.

RP-HPLC was performed on a Waters 625 LC System equipped with a 991+ photodiode array detector (Waters Associates, Bedford, MA, USA) and a Brownlee Aquapore OD-300 column (250 x 7 mm i.d., 7 μm particle size).

Reagents and Materials

rpGHRH was produced at Eli Lilly & Company (Indianapolis, IN, USA). TPCK[L-(tosylamido-2-phenyl)ethylchloromethyl ketone] treated trypsin was purchased from Fluka Chemie AG (Switzerland). Reagent-grade water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA) and was used to prepare all solutions. All other reagents were analytical grade without further purification. All amino acids and resins for solid phase peptides synthesis were purchased from Applied Biosystems, Inc. (Foster City, CA, USA) and Peptides International (Louisville, KY, USA).

Tris-acetate buffer used in the tryptic digestion was prepared by adjusting the pH of 0.1 M Tris solution to 8.6 using acetic acid. Phosphate buffer solutions of different pH were prepared by mixing 0.1 M phosphoric acid and 0.1 M

sodium monobasic phosphate in different ratios. A 0.6 M phosphate buffer (pH 2.4) was used as a rinsing buffer in electrophoresis. Borate buffer solution used as a running electrolyte was purchased from Beckman (Palo Alto, CA, USA).

Method

Synthesis of polypeptide fragments. Peptide fragments were prepared by solid phase peptide synthesis using an Applied Biosystems 430A peptide synthesizer (Applied Biosystems Inc., Foster City, CA, USA). Protected amino acids used in the synthesis were of the L-configuration and the alpha amino groups were exclusively Boc-protected. Side chain functionalities were protected as follows: benzyl for threonine and serine, 2-bromobenzyloxy carbonyl for tyrosine, p-toluene-sulfonyl for arginine, cyclohexyl for aspartic and glutamic acids, benzyloxymethyl for histidine, and formyl for tryptophan. The side chains of asparagine and glutamine were unprotected. All amino acids were singly coupled except for arginine, asparagine, glutamine, and histidine which were doubly coupled. The N-terminal Boc group of each peptidyl resin was removed on treatment with trifluoroacetic acid in dichloromethane and neutralized with N,N-diisopropylethyl-amine in dichloromethane. Each peptidyl resin was dried and treated with 1 mL m-cresol and 10 mL anhydrous hydrogen fluoride per gram of resin. 1,2-ethanedithiol was added to the cleavage mixture for fragment 9. Each cleavage-deprotection was stirred in an ice bath for 60 min. After removal of the hydrogen flouride in vacuo, the free peptides were precipi-tated with ether, filtered, washed with ether, extracted with aqueous acetic acid, and lyophilized. Most products (solubility permitting) were desalted over a Sephadex G-10 column. All peptides fragments were analyzed on a 0.45 x 15 cm Vydac C18 column by gradient elution, and characterized by amino acid analysis (6N hydrochloric acid hydrolysis) and by fast atom bombardment mass spectrometry.

Trypsin digest. One mg of polypeptide rpGHRH was dissolved in 0.5 mL water and then mixed with 0.5 mL Tris-

acetate buffer. Periodically, the solution became cloudy because of poor solubility of the polypeptide in this buffer solution, but digestion results were not affected. A 40 μL of TPCK-treated trypsin solution (1 mg /mL in tris-acetate buffer) was added in the solution and vortexed 1 min. The mixture was then incubated for 4 hrs at 37°C. Aliquots of the digest mixture were frozen (-20°C) for use at a later time.

Capillary zone electrophoresis. The digested peptide mixture was electrophoresed in an uncoated fused silica capillary. Peaks in the peptide map were identified by electrophoresis using a sample solution spiked with relative peptide fragment prepared by solid phase synthesis. Before each run, the capillary was rinsed for 1 min with 0.6 M sodium phosphate buffer, pH 2.4, and then for 1 min with separating electrolyte, 0.1 M phosphate, at the appropriate pH. At the end of each run the capillary was rinsed again with 0.6 M phosphate buffer and conditioned over night with this buffer. A pressure injection was used to apply the sample into the capillary, and the temperature of the capillary cartridge was kept at 22°C.

Reversed phase HPLC. Reversed-phase chromatographic analysis of the digest peptides was performed using a Brownlee Aquapore OD-300 column at room temperature. To enhance separation efficiency, a 7.5 mm I.D. column at a flow rate of 2.3 mL/min was used in this study. Mobile phase consisted of 0.1% aqueous TFA (v/v) (A solvent) and 0.1% TFA in 60% acetonitrile (B solvent). A gradient profile, 0 to 85% B in 50 min, hold 85% B for 5 min, and return to initial condition immediately, was applied. The injection volume was 100 μL and the peptides were detected at 215 nm.

RESULTS AND DISCUSSION

The sequence and molecular weight of 12 tryptic digest peptide fragments of polypeptide rpGHRH are shown in Table 1.

Table 1

TRYPTIC DIGEST OF BIOSYNTHETIC POLYPEPTIDE

Fragment	Amino acid residues	Sequence	MW	Calculated charge
1	10	ADAIFTNNYR	1184.3	1.06
2	1	R	174.2	1.00
3	8	VLTQLSAR	887.1	1.44
4	1	R	174.2	1.00
5	8	LLQDILSR	957.2	1.06
6	5	QQGER	616.6	1.38
7	7	NQEQGAR	801.8	1.38
8	2	VR	273.3	1.44
9	3	LGR	344.4	1.44
10	10	QVDSLWADQR	1217.3	0.67
11	16	QLALESILATLLQEHR	1835.2	2.32
12	4	NSQG	404.4	0.44

MW = Molecular weight

The digest mixture containing all of the peptide fragments were separated by FSCE. It was found that poor resolution and fewer peaks were obtained in electrophoresis using pH 9 borate buffer as a separating electrolyte (Fig. 1), but the digest peptides could be completely separated by FSCE when a 0.1 M phosphate buffer (pH 3.3) was used (Fig. 2).

All separations in this study were performed on an uncoated capillary using acidic phosphate buffers. Use of low pH buffer significantly reduces the magnitude of the electroosmotic flow through the silica capillaries since silanols on the capillary wall become more protonated. In addition, low pH values minimized wall interaction because of

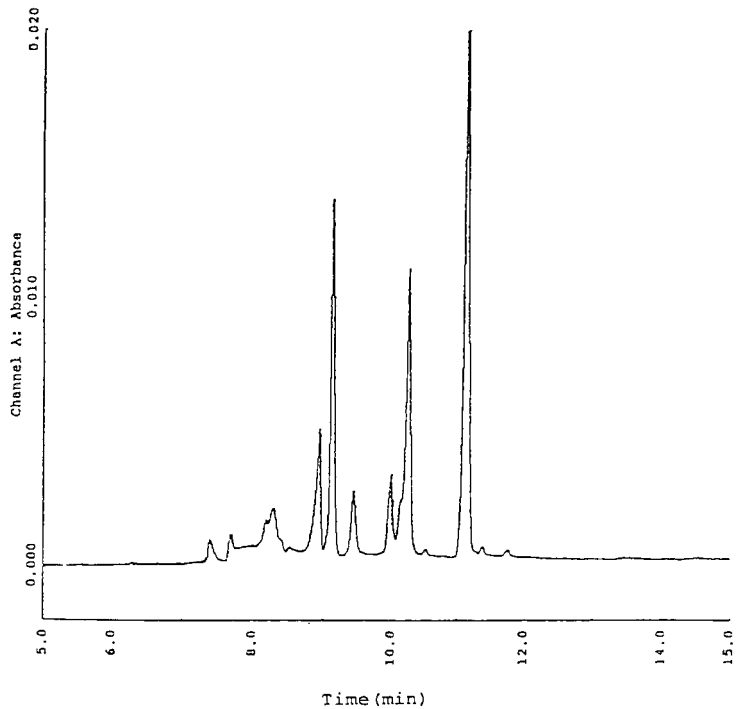


Fig. 1
Electropherogram of peptide fragments from tryptic digestion of polypeptide rpGHRH. Electrophoresis condition: field, 350V/cm; current, 9 μA; Injection, 3 seconds by pressure; separating electrolyte, 0.1 M borate pH 8.6; other conditions given in text.

proteins with higher positive charge (9). Phosphate has also been reported to reduce the interaction of proteins with polysilicic acid (10). A study was conducted to determine the optimal pH for separation of the digest peptides in 0.1 M phosphate buffer at 15 kV applied voltage. Five pH values ranging from 2.0 to 3.3 were selected for this study. As

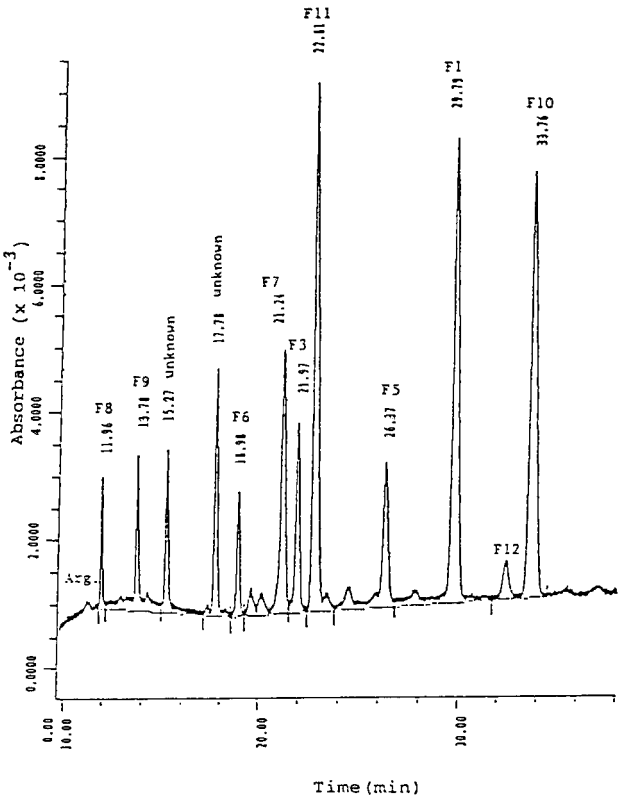


Fig. 2
Electropherogram of peptide fragments from tryptic digestion of polypeptide rpGHRH. Electrophoresis conditions: same as in Figure 1 except field, 263 V/cm; current, 100 μA and separating electrolyte, 0.1 M phosphate, pH 3.3.

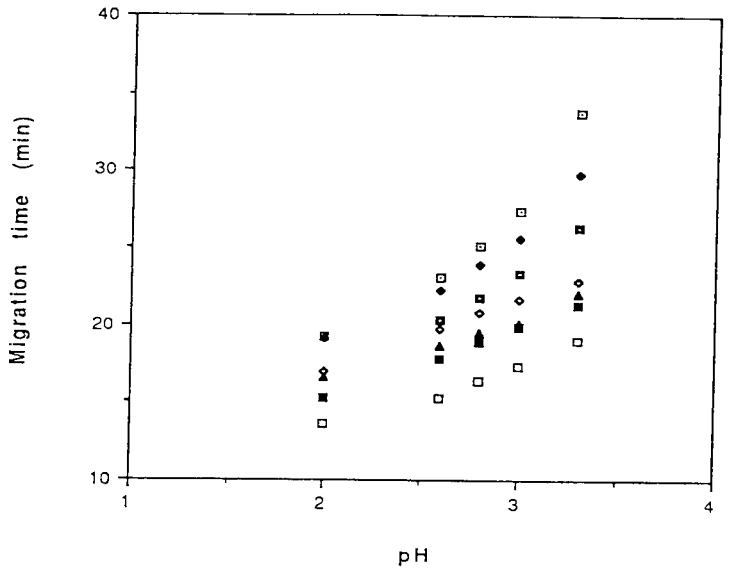


Fig. 3
Effect of pH on the migration time of digest peptide fragments. Electrophoresis conditions: same as in Figure 2 except pH was changed. ⊡—F10; ◆—F1; ◘—F5; ◇—F11; ▲—F7; ■—F6; □—F3.

shown in Fig. 3, the migration times t_m of digest peptides increased with increasing pH of separating electrolyte. It was observed that poor resolving power for fragments F1 and F10; F11 and F5; F6, F8, and F9 was obtained at pH 2.0. The best separation was reached in the separating electrolyte of 0.1 M phosphate buffer at pH 3.3. Under these conditions, all 11 peptides can be excellently resolved as shown in Fig. 2. The peak assignment was accomplished by spiking individual synthesized fragments. There are two unknown peaks on the electropherogram which will be studied in future work.

The effect of applied voltage in capillary electrophoresis has been studied. From Fig. 4, it can be seen that the resolution of peptide separation at four different voltages is similar, but F12 can only be resolved with F10 under the condition of 15kV applied voltage.

In order to improve the reproducibility of migration time in electrophoresis, a capillary rinsing procedure (11) was used in this study. The capillary was rinsed with 0.6 M phosphate buffer (pH 2.4) prior to run and stored overnight in the same buffer. Results of reproducibility in migration time are shown in Table 2.

In most cases, polymer-coated capillaries for capillary electrophoresis can reduce electroendosmosis and eliminate electro-interaction between silanols on the wall surface and proteins or peptides. In this work, we have compared the resolution of peptide fragments on the uncoated and the capillary coated with hydrophilic layer on the wall surface under the identical experiment conditions. From Fig. 5, it can be seen that a comparable result in the separation of digest peptide mixture was obtained on the coated capillary, but fragments F3 and F7 could not be resolved.

Comparing the tryptic digest peptide map by FSCE with that obtained from RP-HPLC, some peptides such as F6, F12, F2, F4 (Arginine) were co-eluted on RPHPLC (Fig. 6), but these peptides can be completely separated by FSCE. Assignment of peptide fragments was carried out by spiking individual peptides prepared by solid phase synthesis into the unseparated digest mixture under the same separation conditions.

Differences in peak elution order between RP-HPLC and FSCE are apparent. In RP-HPLC, the smaller the more hydrophilic peptide, generally, the lower the retention time is. For example, F2 and F4 are arginine, a single amino

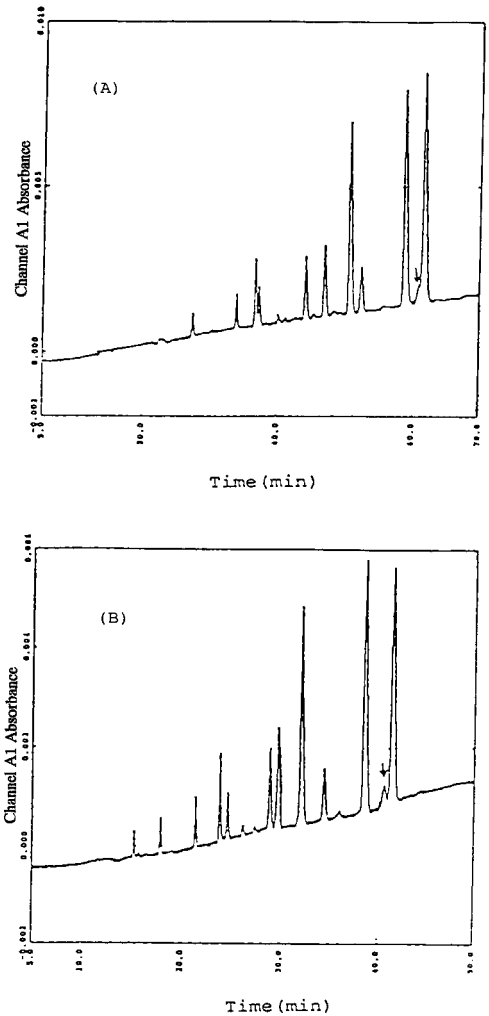


Fig. 4
Effect of field voltage on resolution of digest peptide fragments in FSCE. Electrophoresis conditions: same as in Figure 2, field applied voltage (A) 10kV; (B) 15 kV; (C) 20 kV; (D) 25 kV.

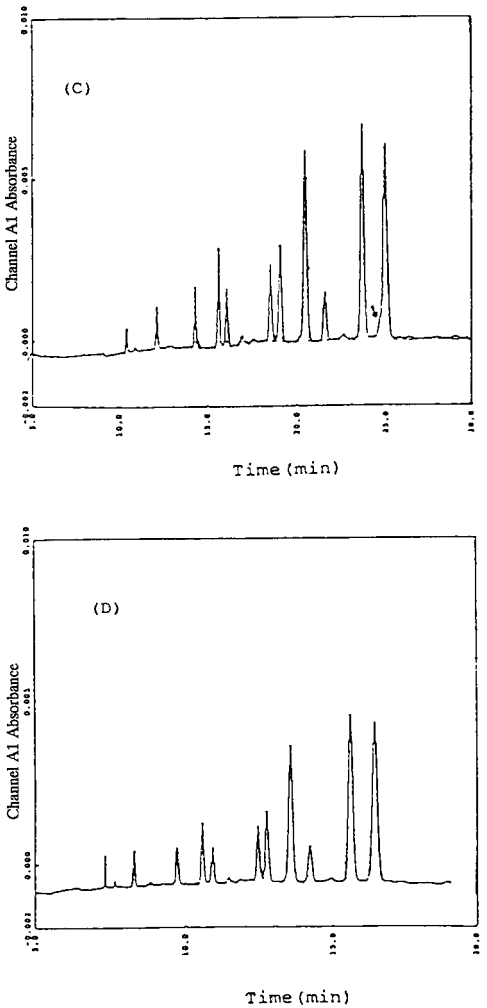


Fig. 4 (continued)

Table 2

REPRODUCIBILITY OF MIGRATION TIME t_m

Fragment	N	Average t_m	RSD %
F8	5	8.22	0.46
F9	5	9.81	0.53
F6	5	12.63	0.57
F7	5	14.34	0.57
F3	5	15.05	0.53
F11	5	15.82	0.56
F5	5	16.23	0.60
F1	5	17.66	0.57
F10	5	18.18	0.62

acid; consequently, it was eluted early in the void volume. F1 and F10 are decapeptides and F11 is a hexadecapeptide which are more hydrophobic than arginine; therefore, those peptides were eluted later in RPHPLC. In CZE, under the same experimental condition, the electrophoretic mobility depends on the net charge and molecular size of the analyte. From Fig. 2 and Table 1 we can see that the migration time of F10 is smaller than that of F1 due to a lower charge of F10 (0.67) even though the molecular weight of both peptides are similar. F11 has the largest molecular weight, 1837, and more charge, 2.32, so that it shows a short migration time. F12 , a tetrapeptide, has the least calculated charge (0.44) among digested peptides. As a consequence, it shows a longer migration time in electrophoresis. The values of calculated charge in Table 1 were calculated by using the same approach reported in the literature (12).

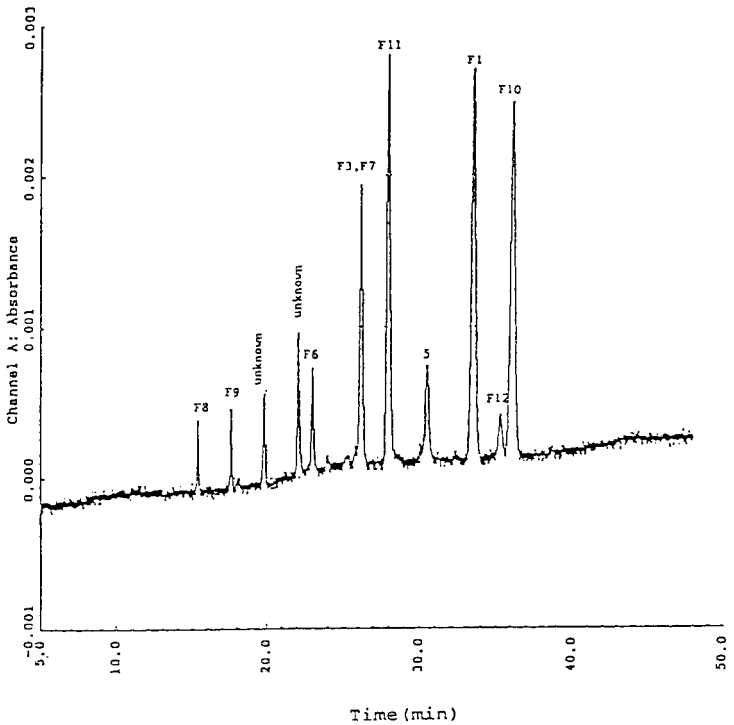


Fig. 5
Electropherogram of tryptic digest peptides of rpGHRH on the coated fused silica capillary. Conditions: same as in Figure 2.

Recently, a linear correlation between the electrophoretic mobility measured by FSCE and the ratio of the net charge to molecular weight of peptides has been reported (12). These results indicated that the mobility of peptides is proportional to the surface area (2/3 power of the molecular weight) of peptides. Based upon that result, the reciprocal of migration time, $1/t_m$, of peptide fragments,

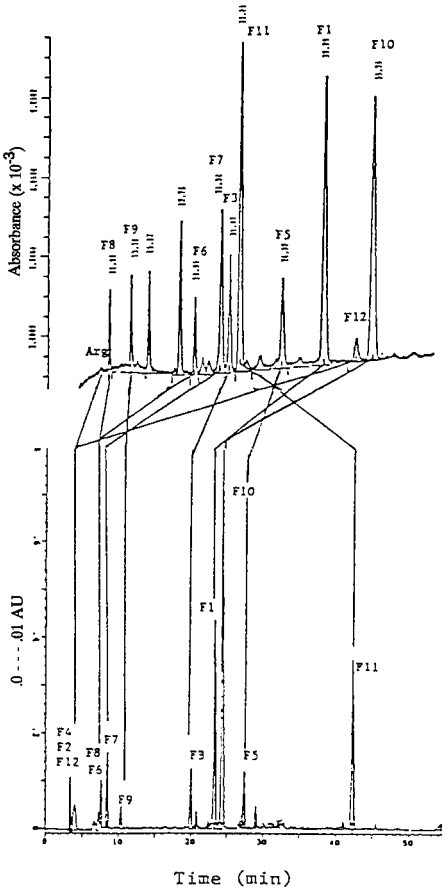


Fig. 6
Comparison of FSCE (A) and RPHPLC tryptic digest peptide maps. Electrophoresis conditions: same as in Figure 2. Chromatographic conditions: as described in the text.

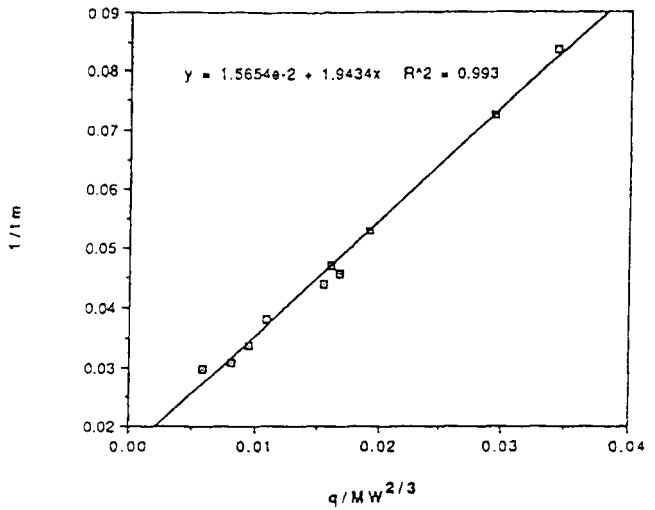


Fig. 7
Linear correlation curve of the reciprocal of electrophoretic migration time $1/t_m$ vs calculated charge to molecular size for digest peptides separated in pH 3.3, 0.1 M phosphate buffer. Equation and correlation coefficient for the linear least squares line are given.

obtained from FSCE in this study, were correlated with the ratio of q/MW, where q is the calculated charge (see Table 1), and MW is molecular weight of peptide fragments. A similar conclusion as in the literature (12) was reached from the results in this study. An excellent linear correlation was found for the $1/t_m$ in electrophoresis versus $q/MW^{2/3}$ (Fig. 7). The correlation coefficient (r) was 0.993. A slightly lower correlation coefficient (r = 0.944) was obtained in the correlation with $MW^{-1/2}$. The correlation coefficient for the Stoke's radius ($MW^{-1/3}$) of peptide molecule was only 0.780.

CONCLUSION

CZE is a valuable method for primary structure determination and uses as a "finger-print" to identify a recombinant peptide rpGHRH. FSCE in a system of the uncoated capillary-acidic phosphate medium provides high efficiency for the tryptic peptide separation with resolving power comparable to that on the coated capillary. The separation obtained in this study demonstrated that the variation of pH may provide optimal efficiency and resolution for the CZE of tryptic peptides of rpGHRH. The resolving power in the CZE separation is slightly better than that in the RPHPLC.

An excellent correlation for the $1/t_m$ versus $q/MW^{2/3}$ was found in this study. It is consistent with the results reported in the literature.

ACKNOWLEDGMENTS

The authors thank Dr. E. C. Rickard for helpful discussion and the calculation of physical parameters.

REFERENCES

1. P. A. Hartmen, J. D. Stodola, G. C. Harbour, and J. G. Hoogerheide, *J. Chromatogr.*, 360: 385-395 (1986).

2. J. J. Dougherty, L. M. Snyder, R. L. Sinclair, and R. H. Robins, *Anal. Biochem.*, 190: 7-20 (1990).

3. W. G. Kuhr, *Anal. Chem.*, 62: 403R-414R (1990).

4. P. D. Grossman, J. C. Colburn, H. H. Lauer, R. G. Nielsen, R. M. Riggin, G. S. Sittampalam, and E. C. Rickard, *Anal. Chem.*, 61: 1186-1195.

5. J. Frenz, S. Wu, and W. S. Hancock, *J. Chromatogr.*, 480: 379-391 (1989).

6. R. G. Nielsen, and E. C. Rickard, *J. Chromatogr.*, 516: 99-114 (1990).

7. R. G. Nielsen, R. H. Riggin, and E. C. Rickard, *J. Chromatogr.*, 480: 393-401 (1989).

8. R. D. Dimarchi, M. L. Heiman, J. E. Shields, and D. J. Smiley, European Patent Application 511003.

9. R. M. McCormick, *Anal. Chem.*, 60: 2322-2328 (1988).

10. R. J. Emerick, *J. Nultr.*, 117: 1924-1928 (1987).

11. M. Strickland, and N. Strickland, *Amer. Laboratory* No.11, 60-65 (1990).

12. E. C. Rickard, M. M. Strohl, and R. G. Nielsen, *Anal. Biochem.*, 197: 197-207 (1991).

Received: September 24, 1993
Accepted: October 8, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1917-1927 (1994)

RECENT DEVELOPMENTS IN PORPHYRIN SEPARATIONS USING CAPILLARY ELECTROPHORESIS WITH NATIVE FLUORESCENCE DETECTION

NIAN WU, BIHUA LI, AND JONATHAN V. SWEEDLER*
Department of Chemistry
University of Illinois at Urbana-Champaign
Urbana, Illinois 61801

ABSTRACT

A mixed anionic surfactant buffer system is used in the micellar electrokinetic chromatography (MEKC) separation of porphyrin carboxylic acids as well as hematoporphyrin derivatives (HPD) at physiological pH with untreated capillaries. The resolution of type I II, III and IV isomers of coproporphyrin is achieved in bile salt micellar solution. The effects of altering the composition of sodium taurodexyocholate (TDC) and triton QS-15 (QS) on the separation efficiency of the porphyrins are presented. The results show that separation efficiencies are enhanced by using the mixture of bile salt and triton micelles. The limits of detection (LOD) for the porphyrin acids are significantly improved with laser-induced fluorescence (LIF) detection of the native porphyrin fluorescence to the 1 - 10 nM range.

INTRODUCTION

The separation and determination of tetrapyrrole molecules, such as porphyrins, is an interesting and important area for the application of capillary electrophoresis (CE). The determination of total content as well as individual component concentration of porphyrins in biological materials is important for the diagnosis of a family of disease known as porphyria [1, 2]. In addition, their use as therapeutic drugs has increased dramatically in the last decade [3]. The

MEKC separation of urinary porphyrins as well as hematoporphyrin derivatives has been reported previously [4-7]. The porphyrins can be separated by CE using a SDS-CAPS run buffer under rigorous pH conditions; the SDS acts more to compete with the samples for wall sites than as a means of enhancing selectivity of the system [4]. The purposes of this study are twofold: (1) to investigate the feasibility of the combining bile salt with anionic triton (QS) micelles to enhance the selectivity of MEKC and improve the solubility of selected porphyrins and (2), to improve the detectability by using laser-induced fluorescence of their native fluorescence.

Bile salts are biological surfactants possessing substituted steroidal structures which have been postulated to form rigid helical micellar aggregates with the hydrophobic portions of the monomer facing the aqueous solution while the hydrophilic portions turn inward [8, 9]. Bile salt micelles have been successfully applied to the resolution of optical isomers and hydrophobic compounds in HPLC and MEKC [10, 11]. The mixed bile salt and QS micellar system is necessary to improve the capability of bile salt micelle for separating negatively charged or highly polar species [12] and minimizing the phenomena of solute-wall interaction.

Currently, LIF is one of the most sensitive detection methods available for CE and detection limits under 1000 molecules have been reported [13, 14]. However, relatively few biologically important molecules fluorescence, and so large research efforts are involved with attaching fluorescent probes to the analytes of interest [15-17]. In an earlier paper, we reported using the LIF detection method for capillary electrophoretic separation of bilirubin species [18], a group of naturally fluorescent tetrapyrrole molecules with an open-ring porphyrin-like structure. Similarly, porphyrins also are naturally fluorescent. This offers the potential for a sensitive and selective detection scheme without the need to modify the analyte. The detection of the native fluorescence of porphyrins has been demonstrated previously using a Xe arc lamp at the absorption maxima of the porphyrins of 395 - 405 nm [4]. The absorption maximum of the porphyrins is caused by an intense Soret band around 400 nm, however, several smaller absorption bands exist. The second most intense band, the δ band,

absorbs in the 490 - 510 region, depending on porphyrin substituents and solution pH [19]. We report the detection of porphyrins using laser-induced fluorescence detection with the 488-nm Argon-ion line.

MATERIALS AND METHODS

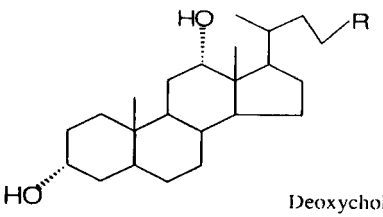
Chemicals Porphobilinogen, Hematoporphyrin, hematoporphyrin derivative, copro- (I and III), penta-, meso-, hexa-, hepta-, and uro- (I and III) porphyrin are from Porphyrin Products (Logan, Utah). A mixture of type I to IV isomers of coproporphyrin are prepared by previously described procedures [20, 21]. TDC (taurodeoxycholic acid, sodium salt), DCA (deoxycholic acid, sodium salt), SDS and triton QS-15 are from Sigma (St. Louis, MO). The structures of the porphyrin carboxylic acids and bile salts are shown in Table I. All other chemicals are of analytical grade from Fisher (Springfield, NJ) or Aldrich (Milwaukee, WI). The run buffer solutions are prepared in doubly deionized water and filtered through a 0.2 μm membrane.

Apparatus All CE experiments are performed on a commercially available LIF/CE instrument (P/ACE system 2100, Beckman Instruments, Fullerton, CA). Approximately 15 mW of the 488 nm emission line from an air-cooled argon ion laser (Omnichrome, Chino, CA) is focused onto the 140 μm input fiber supplied with the P/ACE using a 25 mm f.l. fused silica lens (Newport Research Corporation, Fountain Valley, CA). The emission filter used inside the P/ACE detector head is a 595-nm long-pass fluorescence filter (Omega Optical, Brattleboro, VT). The electropherograms are obtained from the P/ACE and all data processing accomplished using System Gold® software supplied with the P/ACE Model 2100.

CE Instrument Conditions The parameters employed for operation of the P/ACE 2100 instrument follow: the detector is set at LIF mode, injection is set at 8 kV for electrokinetic mode at 4.0 s

TABLE. I

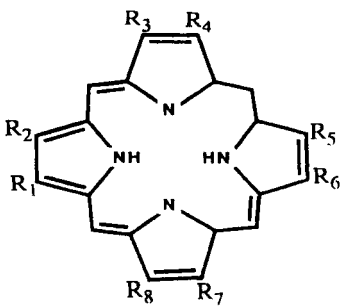
a) Structures of bile salts



Deoxycholate: R = COO^-

Taurodeoxycholate: R = $CONH(C_2H_4)SO_3$

b) Structures of Porphyrin



#	Porphyrin (isomer I)	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1	Coproporphyrin	M	P	M	P	M	P	M	P
2	Pentaporphyrin	M	P	M	P	A	P	P	M
3	Mesoporphyrin	M	E	M	E	M	P	P	M
4	Hexaporphyrin	M	P	M	P	A	P	P	A
5	Heptaporphyrin	M	P	A	P	A	P	A	P
6	Uroporphyrin	A	P	A	P	A	P	A	P

Substituents: M = $-CH_3$; E = $-C_2H_5$; A = $-CH_2COOH$; P = $-CH_2CH_2COOH$.

injection time, column temperature is maintained at 20° C, voltage is set to 16 kV with a positive polarity. The run time is 25 min and the current draw is 85 μA using 60-mM DCA and 10-mM borax buffer solutions. The capillary is washed between runs with 0.1 M NaOH for 0.5 min, followed by a rinse with the borax buffer for 0.5 min, and then with the run buffer for another 0.5 min.

Capillary Conditions Untreated 55-cm x 75-μm I.D. fused-silica capillary tubes (Polymicro Technologies, Phoenix, AZ) are used for all separations. New capillaries are conditioned by purging with 0.5-M NaOH for about 0.5 h and then filling capillary with the run buffer for 10 h before use.

RESULTS AND DISCUSSION

Figure 1 shows the electropherograms of six porphyrins that have three to eight carboxylic acid side-chains in TDC, QS and a mixture of TDC and QS micellar solutions. As shown in Figure 1(a) and 1(b), attempts to separate the mixture of six porphyrins have been unsuccessful using TDC or QS micelle alone in the running buffer solutions. Because of the hydrophobicity of the bile salt and triton, these micelles have poor retentivity for hydrophilic molecules such as uro-porphyrin. Figure 1(c) shows the separation of the six porphyrins with excellent efficiency using the mobile phase containing triton QS-15 (a sulfonated anionic surfactant) and sodium taurodeoxycholate at pH 7.4 in a bare silica capillary. As shown in Figure 1 (c), three peaks are observed for hexacarboxyl-porphyrin in the mixed micellar solution. Similar results are also obtained by Weinberger et. al [4] who suggests that these additional peaks arise from geometrical isomers of hexacarboxyl-porphyrin produced during the manufacturing process. In an attempt to understand the mechanisms that contribute to the successful separation of various hydrophilic and hydrophobic porphyrins as shown in Figure 1 (c), the retention behavior in the TDC-QS micellar solution needs to be better understood. The major interactions between bile salt and QS are thought to be the Van der Waals

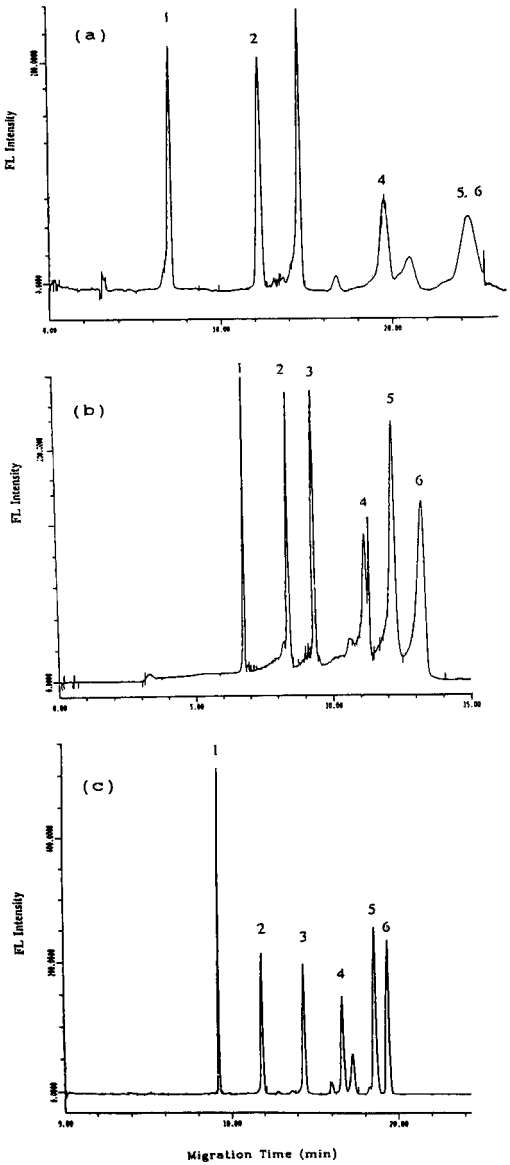


Figure 1. Electropherograms of six porphyrin acid standards (2 μM each) in (a) 60 mM DCA, 15% acetonitrile (v/v), 10 mM sodium borate buffer (pH = 9), (b) 0.5% QS (w/v), 15% acetonitrile, 10 mM sodium borate buffer (pH = 9.0) and (c) 60 mM TDC, 0.2% QS, 15% acetonitrile, 10 mM H_3PO_4 buffer (pH = 7.4). Peaks: 1. copro-, 2. pentacarboxyl-, 3. meso-, 4. hexacarboxyl-, 5.heptacarboxyl- and 6. uro-porphyrin.

forces acting through the hydrophobic face of the bile salt molecule and hydrophobic polyoxyethylene backbone of triton. Although there is little direct evidence, the hydrophobic lateral external surface of the bile salt micelle can be adsorbed onto the QS phase by hydrophobic interactions while maintaining its micellar structure. This suggests that the retention behavior characteristic of electrokinetic chromatography has been maintained in the mixed micelle solution formed in the system. Therefore, it is possible that specific TDC-QS complex(es) may exist in the run buffer and that these provide solubility and selectivity for the separation of the six porphyrin standards with good efficiency and resolution at physiological pH. A possible explanation for the distorted peak shapes for heptacarboxyl-, and uro-porphyrins as shown in Figure 1 (a) and (b) may be attributed to the adsorption of the porphyrins at the capillary wall.

The average of concentration limits of detection (LOD) for the porphyrin acids shown in Figure 1 is ~ 4 nM using the commercially available CE/LIF system. This is very close to the concentration limits of detection reported for HPLC/FL and ~ 50-fold better than previous MEKC LODs [4]. The mass limits of detection are ~ 10 amol, approximately 40-fold lower than previously reported fluorescence method [4]. These improved LODs make detection of nM concentrations of porphyrins in biological sample systems possible; i. e., the total amount of uroporphyrin in normal urine specimens is in a range of 5 - 35 nM [19].

The simultaneous separation of copro-porphyrin type I to IV isomers using MEKC has not been described before. The analysis and separation of pure isomers is important in the fields of porphyrin chemistry and biochemistry. The separation of a mixture of synthetic copro-porphyrin I to IV isomers is achieved with good efficiency using the mobile phase containing 60 mM sodium deoxycholate (DCA) and acetonitrile at pH 9 (Figure 2). The attempt to separate uro-porphyrin isomers of type I to IV is unsuccessful using this method, presumably due to their hydrophilic nature.

Figure 3 shows the electropherograms of the separation for hematoporphyrin derivative (HPD), a clinically important group of molecules for photodynamic therapy [3]. As shown in Figure

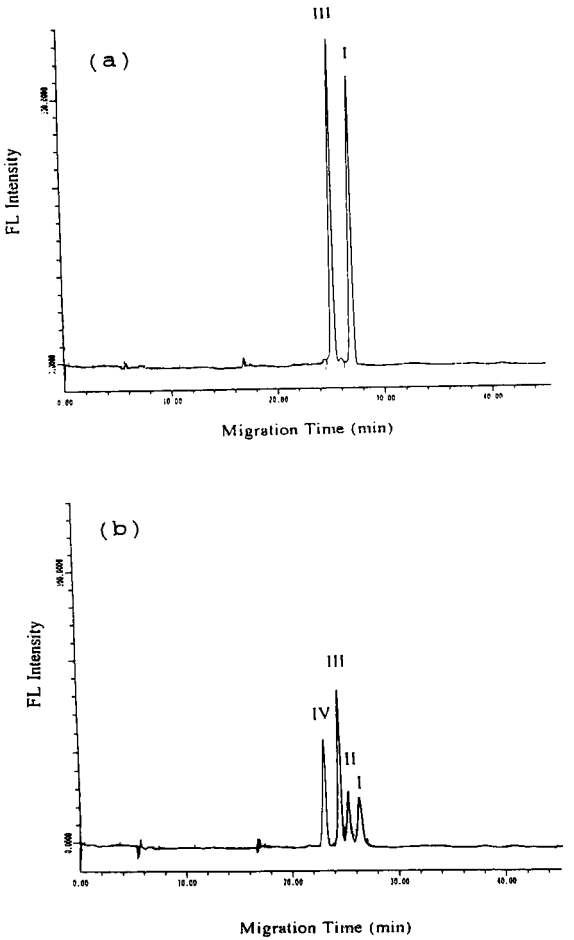


Figure 2. Electropherograms of (a) coproporphyrin III (1.9 μM) and I (1.8 μM) isomers (commercially available standards); (b) synthetic coproporphyrin IV, III, II and I (2.5 μMtotal) isomers in 60 mM DCA-15% acetonitrile-10 mM sodium borate buffer (pH = 9.0).

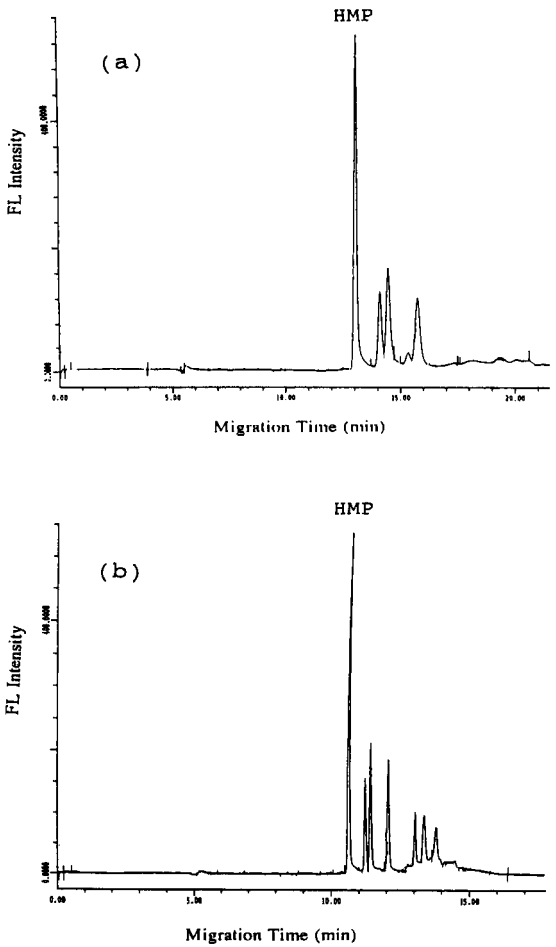


Figure 3. Electropherograms of hematoporphyrin derivative (25 μg / ml) in (a) 50 mM SDS-10% acetonitrile-10 mM sodium borate buffer (pH = 9.0) and (b) 60 mM TDC - 0.2% QS-10% acetonitrile-10 mM H_3PO_4 buffer (pH = 7.4). Peak "HMP" is referred to the migration times of hematoporphyrin standard under the same experimental conditions.

3 (b), there are seven peaks observed using a mobile phase containing a mixture of TDC-QS micelles but only four peaks are observed in an SDS micellar solution (Figure 3 (a)) under similar conditions as previously reported [5]. Although we are unable to identify the additional peaks because the precise compositions and structures of HPD are not known with certainty, the MEKC method has been shown to be a very efficient separation technique to assist in the search for the critical photosensitizing species of hematoporphyrin derivatives.

In summary, we have demonstrated the use of a mixture of anionic surfactant buffer additives in capillary electrophoresis that allows for the separation of various hydrophobic and hydrophilic porphyrins and the hematoporphyrin derivative as well as the simultaneous resolution of several clinically important geometrical porphyrin isomers using untreated capillaries. Using small amounts of an anionic triton micelle as a run buffer modifier in bile salt micellar electrokinetic chromatography can significantly improve separation efficiency and increase sample solubility. More research is necessary to elucidate the mechanisms in which separation performance is improved by various ionic surfactant-surfactant systems; in particular, the separation efficiency and the retention behavior in the TDC - QS solution will be investigated in future studies.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the loan of the P/ACE 2100 capillary electrophoresis system from Beckman Instruments. This research is supported by a National Science Foundation Young Investigator Award, a Henry and Camille Dreyfus Foundation New Faculty Award, the National Institutes of Health and the Searle Scholars Award/Chicago Community Trust.

REFERENCES

1. U. A. Meyer, "Porphyria" in Harrison's **Principles of Internal Medicine**, 9th Ed., K. J. Isselbacher et. al Eds., McGraw-Hill, N. Y., 494-508, (1980).

2. N. Evans, A. H. Jackson, S. A. Matlin and R. Towill, *J. Chromatogr.*, 77, 167-178 (1977).

3. J. B. Cannon, *J. Pharm. Sci.*, **83**, 435-446 (1993)

4. R. Weinberger and E. Sapp, *J. Chromatogr.*, **516**, 271-285 (1990).

5. E. N. L. Chen and D. M. Goodall, *J. Chromatogr.*, **636**, 171-178 (1993).

6. C. Kiyohara, K. Saitoh and N. Suzuki, *J. Chromatogr.*, **646,** 397-403 (1993).

7. N. Wu. G. E. Barker and C. W. Huie, *J. Chromatogr.*, **659**, 435-442 (1994).

8. G. A. Pico and C. Houssier, *Biochim. Biophys. Acta*, **999**, 128-134 (1989).

9. M. C. Carey and D. M. Small, *Arch. Intern. Med.*, **130**, 506-518 (1972).

10. S. Terabe, M. Skibata and Y. Miyashita, *J. Chromatogr.*, **480**, 403-411 (1989).

11. R. O. Cole, M. J. Sepaniak, W. L. Hinze, *J. Chromatogr.*, **557**, 113-123 (1991).

12. R. O. Cole and M. J. Sepaniak, *LC-GC*, **10,** 380-385 (1992).

13. D. Y. Chen, H. P. Swerdlow, H. P. Harke, J. Z. Zhang and N. J. Dovichi, *J.Chromatogr.*, **559**, 237-246 (1991).

14. L. Hernandez, J. Escalona, N. Joshi and N. Guzman, *J. Chromatogr.*, **559**, 183-196 (1991).

15. S. L. Pentoney and J. V. Sweedler, "Optical Detection Techniques for CE", in **Handbook of Capillary Electrophoresis**, J.P. Landers, Ed., CRC Press, Boca-Raton, Chapter 7 (1993).

16. J. V. Sweedler, R. Fuller, S. Tracht, A. Timperman, V. Toma and K. Khatib, *J. Microcol. Separations*, **5**, 403-412 (1993).

17. L. N. Amankwa, M. Albin and W. G. Kuhr, *Trends in Anal. Chem.*, **11**, 114-120 (1992).

18. N. Wu, J. V. Sweedler and M. Lin. *J. Chromatogr.*, in press.

19. L. Eales, "Clinical Chemistry of the Porphyrins" in **The Porphyrins**, D. Dolphin Ed., Academic Press, N. Y., 665-804, (1978).

20. D. Mauzerall, *J. Am. Chem. Soc.*, **82**, 2605-2609 (1960).

21. T. K. With, *Enzyme*, **17** 76-80 (1974).

Received: October 29, 1993
Accepted: February 10, 1994

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1929-1939 (1994)

DETERMINATION OF N-METHYLNICOTINAMIDE IN URINE WITH CAPILLARY ZONE ELECTROPHORESIS

EUGÈNE H. J. M. JANSEN AND PETRA DE FLUITER
Laboratory for Toxicology
National Institute of Public Health and Environmental Protection
P.O. Box 1
3720 BA Bilthoven, The Netherlands

ABSTRACT

In the present study a method is described for the quantitation of N-methylnicotinamide (NMN) in rat and human urine with capillary zone electrophoresis (CZE). Because NMN is considered as a possible biomarker for nitrite exposure, a quantitative method of analysis is required for further evaluation studies. The CZE method for NMN analysis turned out to be very simple and practicable. No pretreatment of the human urine sample was needed before analysis and large series of samples can be handled. Although only a few human urine samples have been analyzed at present, the CZE method looks very promising.

In addition, rat urines from a toxicity experiment have been analyzed. At this point it is questionable whether a dose-response relationship exists in the low concentration exposure range because the statistical significance disappeared after correction for the produced urine volume or creatinin content.

INTRODUCTION

In a toxicity experiment in which rats have been exposed to nitrite and nitrate, it was found with 1H-nuclear magnetic resonance (NMR) that the concentration of N-methylnicotinamide (NMN) was increased in urines of rats exposed to nitrite [1]. To prove the hypothesis that NMN was a metabolite of tryptophan, experiments have been performed with labelled tryptophan which confirmed that NMN indeed originated from tryptophan [2]. Because NMN was raised only in the nitrite group, the use of this compound as a biomarker for nitrite exposure in human biomonitoring experiments was further investigated.

Because NMR is a relatively insensitive technique, the quantitation of NMN in rat urines with NMR was not satisfactory. Especially in urines from control animals the quantitation of low concentrations of NMN could only be performed in a semi-quantitative manner. Therefore, more sensitive detection methods are required for a better quantitation of NMN. Besides high performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE), which is a very suitable technique for the separation and detection of charged small molecules, can be used for the quantitation of NMN.

In the present report we describe the detection and quantitation of NMN in both rat and human urine with CZE.

MATERIALS AND METHODS

MATERIALS

Citric acid, sodium citrate, sodium hydroxide, ammonium sulfate, ethanol and trichloroacetic acid were obtained from Merck-Schuchardt (Darmstadt, Germany). Cytosine, 1-methylnicotinamide iodine salt, nicotinic acid, tryptophan, kynurenine, 3-hydroxyanthranilic acid and 3-[cyclohexylamino]-1-propaansulfonic acid (CAPS) were obtained from Sigma (St. Louis, U.S.A.). A

kit for measuring creatinine in urine was obtained from Hoffmann - La Roche Diagnostica (Basel, Switzerland). Separation buffers were made by mixing a solution of 20 mM citric acid in water (from a Milli-Q system) and a solution of 20 mM sodium citrate to obtain the desired pH. The rinsing solution which was used for the capillary column was 1 M sodium hydroxide. A CAPS buffer (50 mM, pH 10) and a cytosine solution (500 mg/l CAPS) were used for testing the system with regard to the plate height and reproducibility. Other standard solutions were made by dissolving the compound of interest in 20 mM citrate buffer with a pH of 5. 3-Hydroxy anthranilic acid did not dissolve in the buffer. The suspension was filtered and used as a standard solution. The concentration of this standard is therefore unknown.

EQUIPMENT

The Capillary Zone Electrophoresis (CZE) system consisted of a Prince injection system with power supply, an Applied Biosystems 759A absorbance detector suitable for CE , and a GynkoSoft Chromatography datasystem V4.10 (Separations, H.I. Ambacht, The Netherlands). Chromatograms were printed on a Laserjet IIP printer (Hewlett Packard, Amstelveen, The Netherlands). SGE deactivated silica with an I.D. of 75 μm was used as column (Bester, Amstelveen, The Netherlands). The effective length of the column was about 0.8 m. From a part of the column which was situated in the light path of the detector the polyimide coating was removed with a cigarette lighter.
Creatinine was measured with a Cobas Bio centrifugal analyzer (Hoffmann-La Roche Diagnostica, Basel, Switzerland).

SAMPLES

SPF male rats (Wistar Riv:TOX strain) were about 3.5 weeks old at the start of the experiment with a body weight range of 55-70 grams. The animals were housed individually in a one wire cage. During the two weeks of the experiment, the animals were placed in a cage for metabolic studies for 24

hours every 5 days. The urine was collected, and the volume of the urine was determined. In this experiment, 12 animals had free access to a grounded semi-purified diet (SSP-TOX flour). The animals (four per group) were exposed to 35.25 mmol KCl, 35.25 mmol KNO_2 or 35.25 mmol KNO_3 per liter tapwater. The total study will be reported in detail elsewhere [2]. The 24-hours urine of the rats exposed for two weeks to KCl, to KNO_2 and to KNO_3 were used for the determination of NMN with CZE.

The human urine was a void from a healthy female volunteer.

SAMPLE PRETREATMENT

The advantage of CZE is the relatively simple sample pretreatment. The human urine was only filtered through a HV13 0.45 μm filter (Millipore, Etten-Leur, The Netherlands). The urine from rat origin contained more proteins which had to be removed. The following methods for removing the proteins were tested: the addition of 120 mg ammoniumsulfate to 200 μl urine, the addition of ethanol to a total of 50% in the urine and the addition of trichloroacetic acid to a total of 10% in the urine. After centrifugation the supernatant was filtered through the filter mentioned before.

CZE METHOD

Before each injection, the column was rinsed with 1 M sodium hydroxide for 1 minute with a pressure of 1000 mbar. The column was equilibrated with the buffer (20 mM citric acid/citrate buffer, pH 3) during 1 minute with a pressure of 2000 mbar. 30 kV was applied during 0.3 minute before introducing the sample. Sample introduction was done by applying a pressure of 40 mbar during 0.1 minute. The analysis was performed in the constant voltage mode of 30 kV applied at a ramp speed of 6 kV/s. The column was kept at a temperature of 30 degrees and no pressure was applied during the analysis. Compounds were detected by measuring the absorbance at 264 nm (at this wavelength NMN has an absorbance maximum). The signal of the detector was collected and integrated with the GynkoSoft software package.

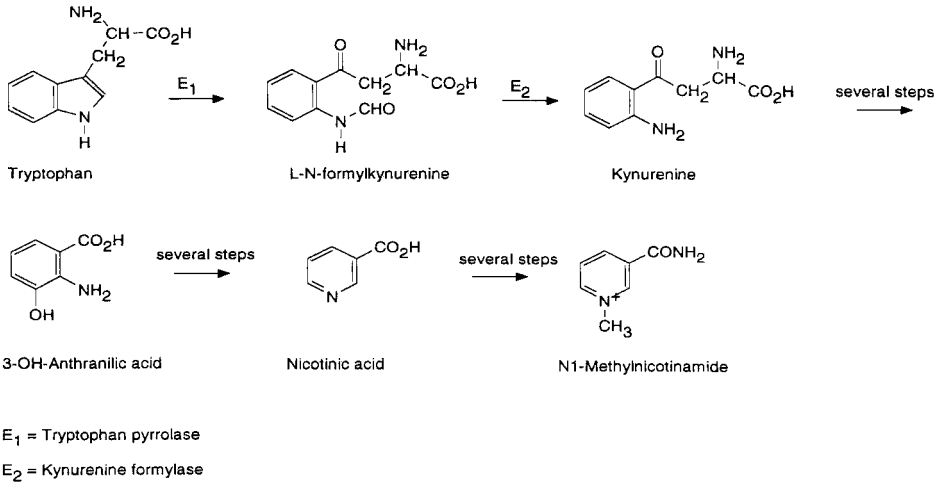


Figure 1: Simplified metabolic pathway of tryptophan

RESULTS AND DISCUSSION

SEPARATION OF TRYPTOPHAN METABOLITES

NMN is a metabolite in the tryptophan dioxygenase metabolisme. A simplified representation of the metabolic pathway is shown in figure 1. The first step is a ring opening catalyzed by the enzyme tryptophan pyrrolase. This enzyme can be influenced by several physiological parameters, such as corticosteroids. Since there is some evidence that nitrite can act on the adrenal gland [3], which can result in the release of corticosteroids, a possible indirect effect of nitrite on the tryptophan metabolism can be expected.

The separation of the main compounds, being NMN, kynurenine, tryptophan, nicotinic acid and 3-hydroxyanthranilic acid was tested with 20 mM citrate buffers with different pH values, being 3.0, 4.0 and 5.0. The other variables were kept the same as with the analysis of NMN. At pH 5.0, kynure-

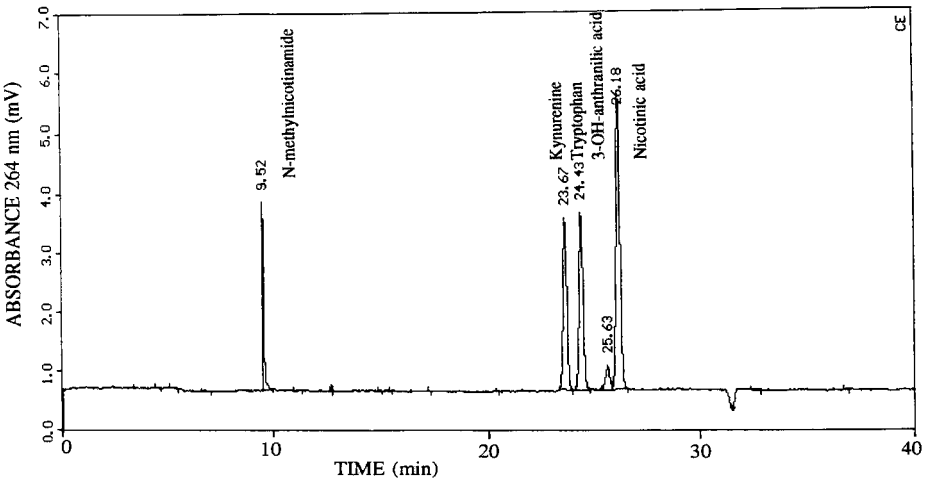


Figure 2: Separation of different tryptophan metabolites with a 20 mM citrate buffer, pH 3.

nine and tryptophan were not separated. The total run time was 30 minutes. At pH 4.0, only three peaks were visible after 40 minutes. The optimum separation of all compounds was achieved with the buffer with a pH of 3.0. All the compounds were separated within 30 minutes. An example of a representative chromatogram is shown in figure 2.

REPRODUCIBILITY

With the above described optimum separation, nine injections were made to determine the reproducibility of the technique. Both the reproducibility of the migration time and the peak height and the peak area were considered. The variation in the migration time is within 2%, but the system is only stable after six injections. Although the column is thermostatted, the initial instability is probably due to the warming up of the whole system. Since with CZE high

plate numbers can be achieved, the migration time must be constant for adequate peak identification and quantitation based on peak height. An auto-sampler can overcome the problem of initial instability.

The variation in the peak area is within 7.5% and in the peak height within 5%. The use of an internal standard can further decrease the variation in the analyses.

DETERMINATION OF NMN IN HUMAN URINE

Filtered human urine was analyzed upon the presence and concentration of NMN. The resulting chromatogram can be seen in figure 3. A small, very sharp peak appeared at the migration time of a standard of NMN. Since this peak was so small (the corresponding plate number was 11 million) and the chromatogram was very clean even without prepurification of the urine, we had some doubt upon the origin of this peak. Therefore, NMN standard addition to the urine was done which indeed increases the peak area. The correspondending plate number dropped to 4.5 million plates. No other peak appeared, so it was concluded that the peak at a migration time of 9.5 minutes was indeed NMN. To confirm this conclusion, the pH of the buffer was changed from 3 to 5. The NMN moved both in the standard and in the urine from 9.5 minutes to 8.3 minutes. Also the peak area remained the same, although the plate number for NMN in the urine dropped to 50,000. Attempts to determine an UV-spectrum by varying the absorbance wavelength was only successfull with the standard solution. The NMN peak in urine had a too low intensity for a reliable UV-spectrum.

DETERMINATION OF NMN IN RAT URINE

Rat urine contains more proteins than human urine. To remove these proteins, three methods were used. It appeared that with ammonium sulfate the peak moved 3 minutes backwards and the peak area dropped to 35% of the original standard. Also precipitation with trichloroacetic acid was not succes-

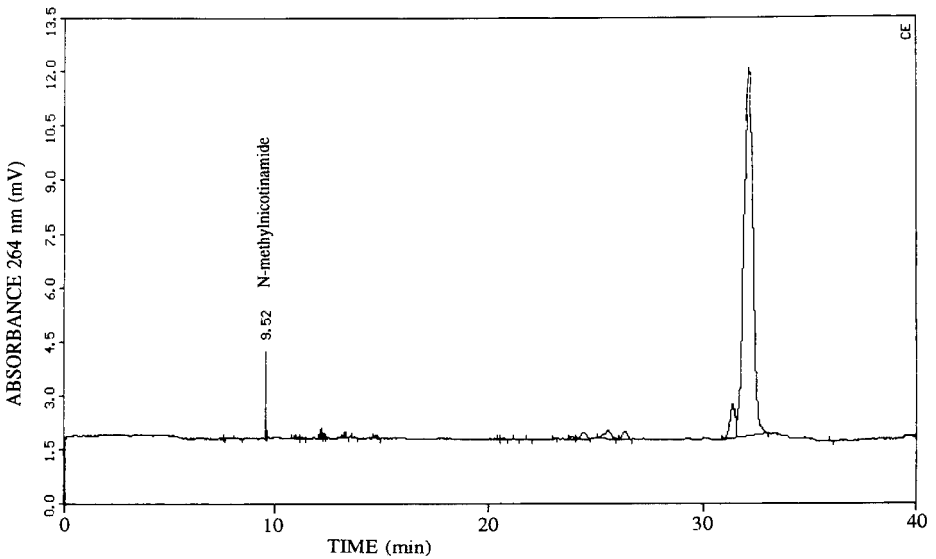


Figure 3: CZE-chromatogram of normal human urine.

full. The peak doubled and became very broad. The addition of 50% ethanol was the best method because the migration time remained the same. Therefore, this procedure was used for removing the proteins from rat urine.

All the rat urines were measured twice. The mean values of the urinary NMN concentration of the animals of the three groups which have been exposed to KCl, KNO_2 or KNO_3 have been listed in table 1. A statistical difference is observed between the nitrite and the control (chloride) group. The nitrate group shows similar NMN concentrations as the nitrite group. If the concentration of NMN is corrected, however, for the dilution factor the statistical difference disappears. As is shown in Table 1 in the third and fourth column, upon correction both for the total volume of the produced urine within 24 hours and for the creatinine content of the urine, still a difference is

Table 1:

Summary of the NMN determinations (mean ± standard deviation) of the three groups. NMN values have been expressed as mg/l urine (uncorrected), in mg/24 hr urine and in g/mol creatinine.

Exposure group	NMN (mg/l)	NMN (mg) per 24 hr	NMN (g) per mol creat.
KCl	13 ± 2	0.13 ± 0.02	2.8 ± 0.6
KNO_2	29 ± 8 **	0.18 ± 0.08	3.9 ± 2.0
KNO_3	13 ± 4	0.10 ± 0.02	2.1 ± 0.5

** $p < 0.01$.

observed between the nitrite and the two other groups but the statistical significance has disappeared.

NMN AS BIOMARKER FOR NITRATE/NITRITE EXPOSURE

The human population is exposed to very low amounts of nitrite in comparison to the higher amounts of nitrate. The intake of nitrate, however, results in an internal nitrite exposure caused by the blood-salivary cycle in the human body [4]. In addition, it must be taken into account that humans have a relatively high endogenous production of nitrate [5] which can make it difficult to distuiguish between variations in the endogenous nitrate levels and low exogenous exposure levels of nitrate.

From the present study it can be concluded that NMN is promising as a biomarker for nitrite exposure, although additional experiments are required.

Because of the relatively high concentrations of the biomarker NMN in urine, the control levels can be measured without any problem with probably a number of analytical methods. In the present study CZE was evaluated as

method of analysis which turned out to be very suitable. No pretreatment of the urine sample was needed before analysis and as a result large series of samples can be handled. Although only a few urine samples have been analyzed at present, the CZE method looks very promising. To develop this technique into a routine-like method of analysis for NMN more experience must be obtained especially in the quantitation of the very small peaks that are obtained with this technique.

The use of NMN as a biomarker for nitrite exposure, a clear dose-response relationship between nitrite and the biomarker NMN should exist. This has at present not yet adequately been assessed. In addition, the sensitivity must be determined for small changes in nitrite exposure. Also the disappearence of the statistical significance after correction of the produced urine volume must be further investigated.

At present experiments with human volunteers are undertaken under controlled conditions which probably will give an answer to the still existing questions.

ACKNOWLEDGEMENT

This study is part of project 623830 in order of the Medical Chief Inspectorate of Public Health (GHI).

REFERENCES

1. G. Zomer et al., to be published.
2. M.J. Zeilmaker et al., to be published.
3. H.P. Til, H.E. Falke, C.F. Kuper and M.I. Willems, Fd. Chem. Toxic., 26: 851-859 (1988).

4. B. Spiegelhalder, G. Eisenbrand and R. Preussmann, Fd. Cosmet. Toxicol., 14: 545-548 (1976).

5. L.C. Green, K. Ruiz de Luzuriaga, D.A. Wagner, W. Rand, N. Istfan, V.R. Young and S.T. Tannenbaum, Proc. Natl. Acad. Sci., 78: 7764-7768 (1981).

Received: February 1, 1994
Accepted: February 10, 1994

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1941-1951 (1994)

DETERMINATION OF POTATO GLYCOALKALOIDS USING ISOTACHOPHORESIS AND COMPARISON WITH A HPLC METHOD

F. KVASNICKA[1], K. R. PRICE[2], K. NG[2], AND G. R. FENWICK[2]

[1]*Institute of Chemical Technology*
Department of Carbohydrate Chemistry and Technology
Technická 5
166 28 Prague, Czech Republic
[2]*Institute of Food Research*
Food Molecular Biochemistry Department
Norwich Research Park
Colney, Norwich NR4 7UA
United Kingdom

ABSTRACT

A method for the determination of potato glycoalkaloids (PGAs) and their aglycone, solanidine, by capillary isotachophoresis (ITP) was developed. The PGAs were extracted by methanol or by a mixture of methanol : water : acetic acid and then purified by solid phase extraction (SPE). The leading electrolyte consisted of 2 mM HCl solution in 99% methanol and 5 mM $Zn(NO_3)_2$ in 99% methanol served as terminating electrolyte. The average recoveries were 88 - 101% for solanidine and 90 - 103% for α-PGAs at a level of 50 mg/kg. The detection limit of solanidine and α-PGAs was 1 mg/kg and 2 mg/kg, respectively. A comparison of the ITP and a HPLC method for the determination of PGAs in commercial products was carried out. The HPLC determination of PGAs was carried out on a Nucleosil 5-NH_2 column using a mixture of acetonitrile and 20 mM KH_2PO_4 (75 : 25, v/v) as the mobile phase with UV detection at 208 nm.

Comparative studies showed that the methods give similar figures for total glycoalkaloid content (sum of α-solanine and α-chaconine). The HPLC method provided both the separation and quantification of both α-solanine and α-chaconine but this technique under conditions used did not allow the determination of the aglycone solanidine. ITP does not provide the separation of individual glycoalkaloids differing only in sugar moiety such as α-solanine and α-chaconine but does allow the separation and determination of aglycone solanidine and the α-glycoalkaloids in one run.

INTRODUCTION

Potato tubers contain a mixture of the steroidal triglycosides α-solanine and α-chaconine accounting for about 95% of the total glycoalkaloid content. Both possess the same aglycone, solanidine, but differ in their sugar moieties. Because of their known toxicity (gastrointestinal disturbances and neurological disorders), PGAs must be analysed in all new potato varieties before they can be released commercially. Although the exact toxic dose in humans has not been established, potatoes with glycoalkaloid levels not exceeding 200 mg/kg fresh weight are generally regarded as being safe for human consumption.

Methodologies[1-7] for analysis of PGAs and their hydrolysis products have been extensively studied. Among a number of published methods HPLC is increasingly used to analyse both individual glycoalkaloids and hydrolysis products. Typically, an analytical method comprises three major steps, namely extraction of the alkaloids with a suitable solvent (aqueous or non aqueous), removal of any interfering impurities and analysis by either HPLC or GLC.

In this paper the determination of PGAs using a capillary isotachophoretic method being developed at Department of Carbohydrate Chemistry and Technology of Prague's Institute of Chemical Technology is described. The ITP technique is compared with a HPLC method routinely used for glycoalkaloid determination in potato and potato products.

EXPERIMENTAL

Chemicals and reagents

Methanol (AR grade), hexane (AR grade) and acetonitrile (HPLC Far UV grade) were purchased from Koch-Light Ltd. The methanol was demineralized by treatment with strong anion exchanger Amberlite IR 120 (d_p 0.3 - 1.2 mm) and strong cation exchanger Duolite A113 (d_p 0.3 - 1.2 mm) before use. Water was from a Millia 185

TABLE I **Operational electrolyte system**

Parameter	Leading electrolyte	Terminating electrolyte
Solvent	99% methanol	99% methanol
Ion	H^+	Zn^{++}
Concentration (mM)	2	5
Counter ion	Chloride	Nitrate
Concentration (mM)	2	10
Additive	None	None

deioniser. Stock solutions of glycoalkaloids (1 mg/ml) and solanidine (0.5 mg/ml) were prepared by dissolving 5 mg of α-solanine, 5 mg of α-chaconine and 2.5 mg of solanidine (Sigma, USA) respectively, in 5 ml of methanol. The leading electrolyte was prepared by diluting a solution of 1 M hydrochloric acid (BDH, UK) in methanol and the terminating electrolyte by dissolving zinc nitrate hexahydrate AR grade (Sigma, USA) in methanol. The operational electrolyte system is shown in Table I. The HPLC mobile phase was prepared by mixing 20 mM potassium dihydrogenphosphate (AR grade, BDH, UK) buffer solution and acetonitrile (25 : 75, v/v). For SPE sample cleanup Sep-Pak C18 (Millipore, USA) and TechElut NH_2 (500 mg/3 ml syringe; HPLC-Technology, UK) columns were used.

Apparatus

A single capillary isotachophoregraph IONOSEP 900.1 was used (RECMAN - laboratorní technika, Czech Republic) equipped with a contactless high-frequency conductimeter. The separation was carried out in a PTFE capillary (150 mm x 0.45 mm ID) thermostatted at ambient temperature by a built-in ventilator. The initial driving current of 20 μA was automatically decreased to 5 μA during detection. A 20 μl sample was automatically injected into the isotachophoregraph. ITPgrams were evaluated on-line via a personal computer. The computer-controlled isotachophoregraph uses a software package for both analysis control and ITP data evaluation. Each analysis took 20 - 25 minutes.

HPLC was carried out using a modular chromatograph consisting of PU 4100 Liquid Chromatograph pump, PU 4025 spectrophotometer (both Phillips, UK) and a Gilson autosampler Model 231. The analytical column was Nucleosil 5-NH_2 (250 mm x 4.6 mm ID, Macherey-Nagel, Germany). The mobile phase was run isocratically at 30°C with a flow rate of 1 ml/min (pressure drop 90 bar). The sample (20 μl) was injected by Rheodyne valve. The detection wavelength and the sensitivity were set at 208 nm and 0.02 AUFS respectively. Each analysis took 20 minutes.

Calibration

An external standard calibration method was used for both ITP and HPLC. The α-PGA (equimolar mixture of α-solanine and α-chaconine) and solanidine were injected into the isotachophoregraph at five concentration levels 5 - 25 μg/ml and 2.5 - 12.5 μg/ml, respectively and the relationship evaluated between concentration and step length.

A standard mixture of α-solanine and α-chaconine (1 - 50 μg/ml each) was injected into the HPLC system at five levels. A calibration graph was constructed by measuring the peak heights of the PGAs.

Sample preparation

Potato samples I to III were extracted either sliced and freeze-dried raw or quartered unpeeled and boiled in water for 15 minutes, drained and then freeze-dried. Samples IV to IX were extracted fresh. The varieties used for samples I to IX were Estima (UK), King Edward (UK), Ostara (NL), Eba (NL), Bintje (NL), Korima (Czech Rep.), Lukava (Czech Rep.) and Karim (Czech Rep.) respectively.

Samples with low lipid levels (potato tubers, potato starch, mashed potato) were extracted prior to quantification of the PGAs using the method of Saito *et al.*[6]. The sample (5 g) was homogenised with *ca* 30 ml of methanol for 2 minutes in an ultrasonic bath, followed by filtration through a sinter (S4). The residue was rinsed twice with *ca* 5 ml of methanol and the rinsings were combined with the original filtrate and made up to

50 ml with methanol. An aliquot (5 ml) was mixed with water (8 ml) and applied to a Sep-Pak C18 cartridge (flow rate about 5 ml/min), which was then washed with 5 ml of 40% methanol. In the case of ITP determination of PGAs the cartridge was rinsed with 10 ml of 10 mM acetic acid. The cartridge was then dried with suction using a water pump. The PGAs and solanidine were eluted with 10 ml of methanol, evaporated to dryness using a rotovapor (40°C) and redissolved in 1 ml of methanol. An aliquot (20 μl) of the solution was injected into the isotachophoregraph and/or the chromatograph. Samples with high lipid levels (potato crisps, chips and snacks) were treated in two different ways.

i) The sample (5 g) was mixed with 100 ml of hexane and boiled for 30 minutes, the hexane removed by decantation and the defatting procedure repeated with fresh hexane (100 ml). After decantation, the remaining hexane was removed on a rotovapor under vacuum. The defatted sample was extracted with 30 ml of a mixture of methanol : water : acetic acid (94 : 6 : 1, v/v/v) for 2 minutes in an ultrasonic bath. After filtration the residue was rinsed twice with *ca* 5 ml of the mixture, the rinsings were combined with the original filtrate and made up to a volume of 50 ml. The procedure then followed that used for low lipid content samples.

ii) The method of Saito *et al.*[6] was used for treatment of high fat content samples. A 5 g sample was treated in a manner described for low lipid content samples. The residue after SPE on Sep-Pak C18 treatment was dissolved in 1 ml of methanol and the solution mixed with 19 ml of acetonitrile. The mixture was applied to a TechElut NH_2 cartridge (flow rate about 5 ml/min), washed with 5 ml of acetonitrile and the PGAs and solanidine eluted with 10 ml of methanol. The eluate was evaporated to dryness using a rotovapor (40°C) and redissolved in 1 ml of methanol. An aliquot (20 μl) of the solution was injected into the isotachophoregraph and/or the chromatograph.

RESULTS AND DISCUSSION

The ITP analysis is based on the fact that both PGAs and solanidine are weak bases and migrate towards the cathode under certain conditions. For this migration an acidic

electrolyte system was used and several cations were examined as leading and/or terminating ion (hydroxonium, potassium, sodium, β-alanine, etc.). Water, methanol and water-methanol mixture were tested as electrolyte solvents. The best electrolyte system is described in Table I. The effective mobilities of α-PGA and solanidine are sufficiently different to give a good separation, but α-solanine and α-chaconine are not separated due to their having a common aglycone, solanidine, bearing a positive charge and also having practically the same molecular weight (867 and 851, respectively).

Results of qualitative and quantitative ITP calibration analyses are summarised in Table II. The relationship between concentration and step length was linear in the case of both the PGAs and solanidine. Similarly in the case of HPLC calibration analyses a linearity between glycoalkaloids (both α-chaconine and α-solanine) concentration (1 - 50 μg/ml) and peak height was confirmed (r = 0.998).

The calibration results obtained showed that both α-solanine and α-chaconine gave almost the same step height and step length and thus either could be used for ITP calibration analyses. Figure 1 shows an isotachophoregram of a standard mixture of solanidine (7.5 μg/ml) and of α-PGAs (15 μg/ml).

The ITP detection limit corresponding to a step length of 50 counts was 0.5 mg/ml and 1 mg/ml for solanidine and α-PGAs, respectively. A similar detection limit was achieved by the HPLC technique. A chromatogram of a standard mixture of β_2-chaconine, α-chaconine and α-solanine (15 ppm each) is shown in Figure 2.

TABLE II **ITP calibration results**

Species	RSH (-)	Intercept (counts^{+})	Slope (counts μg^{-1}.ml)	Correlation coefficient	Range (μg/ml)
hydroxonium	0.0	-	-	-	-
solanidine	65.7	25	42.5	0.999	2.5 - 12.5
α-solanine	82.0	28	26.8	0.999	5 - 25
α-chaconine	81.5	30	27.4	0.997	5 - 25
α-PGAs^{++}	82.5	30	27.0	0.998	5 - 25
zinc	100.0	-	-	-	-

$^{+}$ - 1 count = 50 ms (sampling frequency 20 Hz)

$^{++}$ - equimolar mixture of α-solanine and α-chaconine

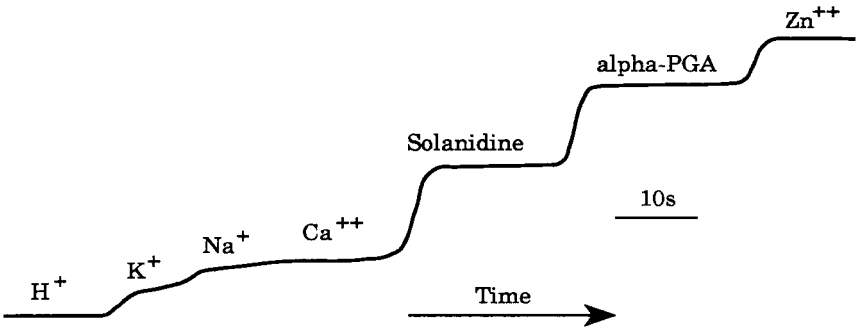


FIGURE 1 **Isotachophoregram of standard mixture of solanidine (7.5 μg/ml) and α-PGAs (15 μg/ml) - for analysis conditions see text**

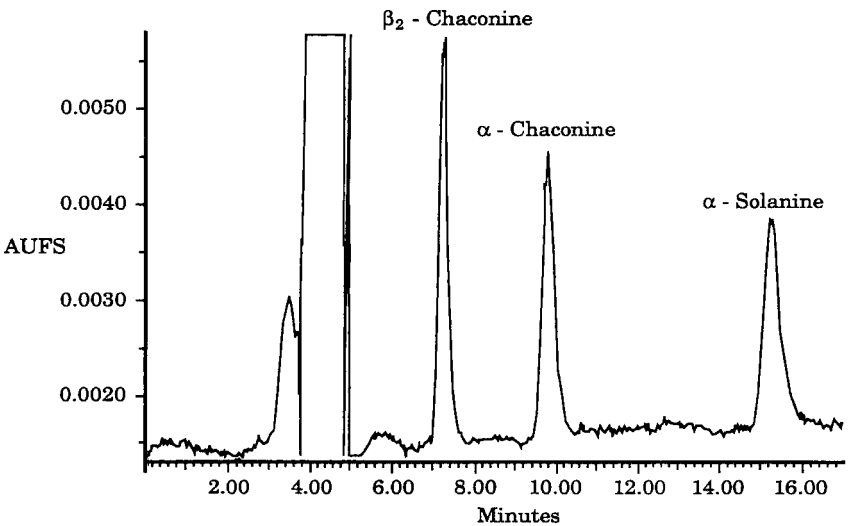


FIGURE 2 **Chromatogram of standard mixture of β_2-chaconine, α-chaconine and α-solanine (15 μg/ml each) - analysis condition see in text.**

Two different sample treatments (see in Sample preparation) of samples with high lipid content were compared and the results obtained showed that both techniques gave almost the same values of glycoalkaloid content (9 samples; correl. coeff. 0.995). When the determination of lipid content is carried out simultaneously with PGAs determination the technique based on sample defatting by hexane prior to PGAs extraction is recommended. In other cases the sample treatment based on two step SPE cleanup on Sep-Pak C18 and NH_2 cartridges is considered more suitable because it is less labour intensive and uses less solvent. These two SPE cleanup steps are necessary in the case of HPLC but not for ITP although it was found that ITP does need the second cleanup step by Tech Elut NH_2 in the case of high lipid containing samples; this makes ITP determination of PGAs simpler than HPLC.

The method developed using ITP for determination of PGAs was compared with the HPLC method and the results obtained are summarised in Table III. The data were handled by regression analysis to evaluate the relationship between ITP and HPLC values of α-PGA content. The following equation was obtained:

$$\text{ITP} = 1.077 * \text{HPLC} - 0.7 \quad (r = 0.989)$$

From this equation it is clear that the ITP technique gave a slightly higher figure for α-PGA content than that from HPLC analysis. The relatively high correlation coefficient confirms the linearity between these two techniques. On the basis of experiments described here it is possible to state that both techniques give similar values of α-PGA content in the samples of potato and potato products.

Although both the techniques described here give comparable total values, their particular strengths lie in their ability to measure individual components within the total glycoalkaloid mixture. It should be noted however that a more rapid technique, ELISA, has found considerable use in the measurement of total glycoalkaloid content of potato and its products.

The PGA content, as shown in Table III, varied from 16.6 to 169.9 mg/kg in the raw tissue of the commercial varieties, reducing to 47.0, 42.4 and 32.5% respectively by

TABLE III **Comparison of HPLC and ITP methods on PGAs (sum of α-solanine and α-chaconine) content**

SAMPLE	HPLC			ITP	
	(mg/kg) α-chaconine	(mg/kg) α-solanine	(mg/kg) α-PGAs	(mg/kg) α-PGAs	(mg/kg) Solanidine
Raw Potato I	63.8	18.4	82.2	91.4	-
Cooked Potato I	30	8.6	38.6	35.4	-
Raw Potato II	70	38	108.0	131.6	6.5
Cooked Potato II	29.7	14.9	44.6	40.7	-
Raw Potato III	125.7	44.2	169.9	182.6	-
Cooked Potato III	40.8	12.3	53.1	46.2	-
Raw Potato IV	33.5	19	52.5	44.4	2.5
Raw Potato V	29.4	12.7	42.1	33.4	-
Raw Potato VI	27.8	12	39.8	44.7	-
Raw Potato VII	39.8	20.4	60.2	55.4	-
Raw Potato VIII	9.2	7.4	16.6	28.1	-
Raw Potato IX	33.6	16	49.6	55.8	-
Snacks I	2.1	6.9	8.0	5.3	-
Snacks II	3	2.2	5.2	9.8	-
Dried Potato I	1.5	1.3	2.8	3.7	-
Dried Potato II	2	1.9	3.9	8.0	-
Unpeeled Crisps I	3.3	1.8	5.1	3.3	-
Unpeeled Crisps II	6.4	2.3	8.7	10.2	1.1
Unpeeled Crisps III	15.6	5.7	21.3	27.2	-
Unpeeled Crisps IV	10.5	6	16.5	15.4	-
Peeled Crisps I	4.1	2.2	6.3	9.6	-
Peeled Crisps II	2.9	1.3	4.2	5.2	-
Chips	2.3	1.6	3.9	2.8	-
Potato Starch I	1.1	0.8	1.9	2.2	-
Potato Starch II	0.5	0.4	0.9	0.89	-

- = not detected

the cooking procedure which presumably leached the remainder from the tissue into the cooking water. It is difficult to make direct comparison with data from other publications due to differences in cooking conditions, although, smaller but significant losses have been observed from boiled whole tubers. Bushway and Ponnampalam[8] measured changes in commercial whole unpeeled tubers after boiling in water and found the PGAs reduced to 71% of the original level (161 mg/kg). Similarly Maga[9] demonstrated a reduction to 74% from a level of 43 mg/kg in the raw unpeeled whole tuber.

From the data presented here, the ratio of α-chaconine to α-solanine varied between 1.24 and 3.49 and did not appear to be affected by cooking. Processed material

contained much lower levels ranging from 2.8 to 21.3 mg/kg with the ratio of the individual glycoalkaloids also lower in the range 0.3 to 2.78. The higher levels of glycoalkaloids found in crisps made from unpeeled tubers (upto 21.3 mg/kg) when compared with the lower levels in those made from peeled tubers (upto 6.3 mg/kg) is consistent with the fact that 90% of the glycoalkaloid is known to reside in the peel of the tuber.

The incidence of the aglycone solanidine in only 3 samples with a PGA content in excess of 10 mg/kg is interesting to note because if its presence was due to degradation of the PGAs during extraction then some would be expected in all samples, especially in those with high PGA content. Since this is not the case, its occurrence is presumably due to genetic factors relevant to those particular varieties.

CONCLUSIONS

The results show that the ITP technique is suitable for the determination of α-PGA in potato and products such as potato crisps, snacks or in dried potato products. The ITP method gives a slightly higher figure ($< 10\%$) of α-PGA than HPLC. By this technique it is not possible under the given analysis conditions to determine an individual α-PGA such as α-chaconine or α-solanine as in the case of HPLC. However ITP has some advantages in comparison with HPLC such as lower chemical consumption (only ca 1 ml of both leading and terminating electrolytes per analysis) resulting in very low running costs and the ability to determine the solanidine together with α-PGA. One step SPE cleanup of high lipid content samples by C18 cartridge is another benefit since HPLC needs an additional cleanup step using a NH_2 cartridge. Preliminary experiments on the separation of glycoalkaloids differing in the number of sugar molecules bounded to the aglycone (using a mixture of solanidine, β_2-chaconine and α-chaconine or products of the hydrolysis of tomatine) demonstrated the potential value of ITP.

ACKNOWLEDGEMENTS

F. Kvasnicka would like to acknowledge the award of an AFRC ISIS fellowship.

REFERENCES

1. R.J. Bushway. High performance liquid chromatographic separation of potato glycoalkaloids using radial compressed amino column. J. Chromatogr., 247, 180 - 183 (1982).

2. M.A. Filadelfi and A. Zitnak. A simple TLC standard for identification of potato glycoalkaloids. Can. Inst. Food Sci. Technol. J., 16, 151 - 153 (1983).

3. K.E. Hellenäs. A simplified procedure for quantification of potato glycoalkaloids in tuber extracts by HPLC; comparison with ELISA and a colorimetric method. J. Sci. Food Agric., 37, 776 - 782 (1986).

4. R.J. Bushway, J.L. Bureau and J. King. Modification of the rapid HPLC method for the determination of potato glycoalkaloids. J. Agric. Food Chem., 34, 277 - 279 (1986).

5. K. Kobayashi, A.D. Powell, M. Toyoda and Y. Saito. HPLC method for simultaneous analysis of α-solanine and α-chaconine in potato plants cultured *in vitro*. J. Chromatogr., 462, 357 - 364 (1989).

6. K. Saito, M. Horie, Y. Hoshino, N. Nose and H. Nakazawa. HPLC determination of glycoalkaloids in potato products. J. Chromatogr., 508, 141 - 147 (1990).

7. M.R.A. Morgan, R. McNerney, J.A. Matthew, D.T. Coxon and H.W-S. Chan. An enzyme-linked immunosorbent assay for total glycoalkaloids in potato tubers. J. Sci. Food Agric., 34, 593 - 598 (1983).

8. R.J. Bushway and R. Ponnampalam. α-chaconine and α-solanine content of potato products and their stability during several modes of cooking. J. Agric. Food Chem., 29, 814 - 817 (1981).

9. J.A. Maga. Potato glycoalkaloids. CRC Crit. Rev. in Food Sci. and Nutr., 371 - 405 (1980).

Received: December 28, 1993
Accepted: January 7, 1994

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1953-1969 (1994)

STUDY ON PHOSPHATE OF ETHYLENE-DIAMINE, 1,3-DIAMINOPROPANE AND 1,4-DIAMINOBUTANE AS CARRYING ELECTROLYTE IN OPEN-TUBULAR CAPILLARY ELECTROPHORESIS

LIGUO SONG, QINGYU OU, AND WEILE YU
Lanzhou Institute of Chemical Physics
Chinese Academy of Sciences
Lanzhou, Gansu 730000
The People's Republic of China

ABSTRACT

The use of ethylenediamine, 1,3–diaminopropane and 1,4 –diminobutane phosphate as carrying electrolyte in open– tubular capillary electrophoresis is proposed. The advantage of them as carrying electrolyte was found in the ability to slow electroosmotic mobility because of adsorption of their ions to the capillary inner wall which caused decrease of ζ potential of the wall. Difference among ethylenediamine, 1,3–diminopropane and 1,4–diaminobutane lies in their adsorption, and enthylene diamine gave higher electroosmotic mobility than other two at same concentration maybe due to its weak adsorption. The dependence of electroosmotic mobility on diamine concentration, pH, neutral salt concentration and applied voltage was determined. Increased diamine concentration caused decrease of electroosmotic mobility, but increased pH, neutral salt concentration and applied voltage gave reversed effects. The

addition of neutral salt caused decreased adsorption of diamine ions, and the increased applied voltage resulted in temperature rise. The usefulness of diamine phosphate as carrying electrolyte was demonstrated in the improved separation of basic proteins in CZE .

INTRODUCTION

The heart of any separation technique is found in the ability to adjust experimental parameters to optimize a specific analysis. With respect to efficiency, selectivity, and time, electrophoretic separation in open-tubular can be optimized by adjusting operation voltage, capillary dimension and buffer system. Much of early work employed phosphate buffers. Zwitterionic buffer systems developed by Good have become popular now because of their low conductivity.

Buffer type, buffer concentration, buffer ionic strength and buffer pH must be considered in buffer selection. The concentration of the buffer ions should be appromately 1000 times larger than that of the solute ions in order to minimize distortion of the solute zone in the applied electric field(1,2). The tailing of cationic solutes can be minimized through the addition of neutral and zwitterionic salts to the operating buffer to compete for the adsorption sites(3, 4, 5) . Excessive Jeale heat which can not dissipated sufficiently by the capillary tube precludes the use of buffer systems with high concentration and high ionic strength.

Separation of special compounds such as neutral species, chiral compounds, and proteins needs special buffer systems which often have special buffer additives. Various compounds such as organic solvents(6,7) , ionic surfactants, nonionic surfactants, chiral surfctants, metal complexes, cyclodextrins, modified cyclodextrins and bile salts were used as buffer additives to achieve desired separation (8).

Amines such as spermine(9), morpholine(10), ethylenediamine (11), 1,3–diaminopropane(12), 1,4 –diaminobutane(13, 14) , 1, 5 –diaminopentane(15) have been used as buffer additives, but no systematic investigations on their application as carrying electrolyte were published. In this report, the use of ethylenediamine, 1, 3– diminopropane and 1, 4– diminobutane phosphate as carrying electrolyte in open– tubular capillary electrophoresis is proposed. The advantage of them as carrying electrolyte was found in the ability to slow electroosmotic mobility. The dependence of electroosmotic mobility on experimental parameters such as diamine concentration, pH, neutral salt and appllied voltage was determined. The usefulness of diamine phosphate as carrying electrolyte was demonstrated in the improved separation of basic proteins in capillary zone electrophoresis (CZE).

EXPERIMENTAL

Apparatus

The capillary electrophoresis apparatus used in this study resembles that reported earlier by Jorgenson and Lukacs(1). It was constructed by Peking Institute of Technology Application (Peking, China). It consists of a high voltage d. c. power supply delivering up to +/–30kV, and a UV detector which has several wavelengths optional with a fixed and removable device for on column detection, a plexiglass box with a safety interlock and a syringe installation used to flush capillaries. The electrophoregrams were recorded with a hp3390A integrator (Hewlett–Packard, Avondale, PA, USA).

Reagents and materials

Ethylenediamine anhydrous, 1, 3– diaminopropane, sodium sulfate anhydrous, phosphoric acid, dimethyl sulphoxide were

purchased in China. 1,4-diaminobutane was purchased from Fluka (CH-9470 Buchs, Switzerland).

Carrying solutions were prepared by using the correspondent amount of diamine, then adjusting the pH value with phosphoric acid. These solutions were used to determine electroosmotic mobility of the capillary at different conditions. In some cases, sodium sulphate anhydrous was added to them in order to examine the effect of neutral salt on electroosmotic mobility. 1% dimethyl sulphoxide (DMSO) was prepared as neutral marker of the electroosmotic mobility.

Lysozyme from egg white (pI11.0) was purchased from Fluka (CH-9470 Buchs, Switzerland). Cytochrome C (pI10.5) from horse heart, trypsinogen from bovine pancreas (pI9. 3) and α -chymotrypsinogen A from bovine pancreas (pI9.1) were purchased from Sigma (St.Louis, MO, USA) . Stock protein mixture with individual concentration of about 2.0 mg/ml were prepared and separated in indicated carrying solutions.

Sample injection was accomplished by syphoning for 5s- 10s at 8cm. In all experiments, deionized water was used.

Electrophoresis

Fused silica capillary tubes (Yongnian Photoconductive Fibre Factory, Hebei, China) of 50um i. d. and 375um o.d. were used with a total length 70cm or 64cm in which the detector was placed at 20cm from the capillary outlet. Detection was monitored at 214nm. Before using, a new capillary was flushed with 1M KOH for 1 hour and then equilibrated with carrying solution overnight using a syringe to force the solution through it. When change of carrying solution was needed, the same step was followed. Between two runs, the capillary was first flushed with one capillary volume of 1M KOH, then with carrying solution for 5 minutes. All analyses were run at ambient temperature without temperature control.

RESULTS AND DISCUSSION

Electric double layer and electroosmotic mobility of the capillary

Because of the ionization of silanol groups, the inner wall of fused silica capillary always carries negative charge when filled with aqueous solution. At the interface between the wall and the solution, negative charges are balanced by positive ions in the solution. A part of counterions will be absorbed by the wall, giving rise to an immobilized compact layer. The remaining counterions are distributed in a diffuse layer, their concentration approaching the bulk value as the distance from the wall increased to infinity. Then an electric double layer adjacent to the capillary inner wall is generated, which is represented in Figure 1. The potential drop is linear in the compact layer, but decreases exponentially within the diffuse layer. When an electric field is applied to the capillary, positive counterions in the diffuse layer migrate toward the cathode and pull solvent molecules with them, giving rise to a flow of the carrying solution through the capillary which is called electroosmotic flow. On the other hand, the compact layer will not move towards the cathode. The corresponding potential in the plane between the compact layer and the diffuse layer is called ζ potential. The relation between the electroosmotic mobility μ_{eom} and the ζ potential is given by(16)

$$\mu_{eom} = \frac{\varepsilon_o \varepsilon \zeta}{\eta} \quad \cdots\cdots\cdots\cdots (1)$$

where ε_o is the permittivity of free space, ε is the permittivity of the carrying solution, and η is the viscosity of the carrying solution. The relation between the ζ potential and the thickness of diffusion layer δ is given by(17):

$$\zeta = \frac{4\pi\delta e}{D} \quad \cdots\cdots\cdots\cdots (2)$$

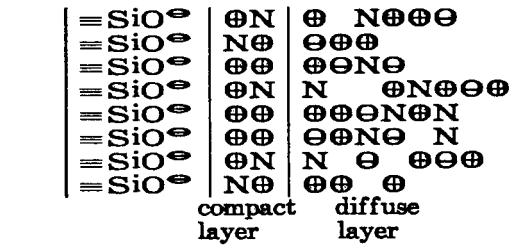


FIGURE 1. Representation of electric double layer, N represent water molecule.

$$\delta = [3\times10^{7}\,|Z|\,C^{1/2}]^{-1} \quad \ldots \quad (3)$$

where e is the amount of charge per unit surface area in the plane between the compact layer and the diffuse layer, and D is the diffusion coefficent, Z is the number of valence electrons, C is concentration of the counterion in carrying solution respectively.

In our study, the electroosmotic mobility was caculated from the migration time of an electrically neutral marker substance from equation (4)

$$\mu_{eom} = lL / t_o V \quad \ldots \quad (4)$$

where l is the distance between the inlet of the capillary tube and the detector, L in the total length of the capillary tube, t_o is the retention time of the neutral marker and V is the applied voltage, respectively. In CZE mode, dimethyl suphoxide was used as neutral marker, whereas in MECC mode, methanol was used instead.

Dependence of electroosmotic mobility on diamine concentration

At a constant pH8. 5, the dependence of electroosmotic mobility on diamine concentration was determined and shown in

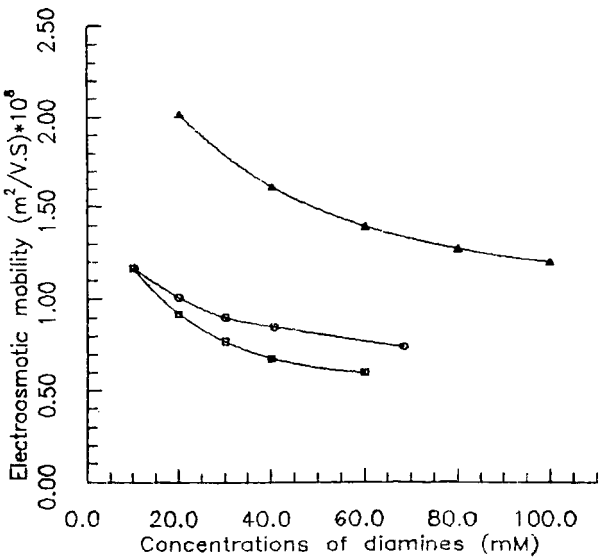


FIGURE 2. Dependence of electroosmotic mobility on diamine concentration with diamine phosphate as carrying electrolyte. Conditions: applied voltage, 13.5kV; capillary, 70cm total length, 50cm to the detector, 50um i. d. ; buffer, pH8. 5; ambient temperature, 14–16℃. △ ethylenediamine, □ 1,3– diaminopropane, ○ 1,4–diaminobutane.

Figure 2. It is observed that diamine phosphate made slow electroosmotic mobility compared with common carrying electrolyte(18), and increased diamine concentration resulted in decrease of electroosmotic mobility.

Change on diamine concentration caused two effects on the electric double layer. First, incrasing the concentration decreased thickness of the diffusion layer δ according to equation (3), so decreased ζ potential of the electric double layer and the eletroosmotic mobility μ_{eom} according to equation

(2) and equation (1). Second, increasing the concentration decreased the amount of charge per unit surface area in the plane between the compact layer and diffuse layer, that is e in equation (2). Unlike metal ions, positively charged diamine ions were absorbed by the capillary inner wall much stronger, and caused reduction of e, then caused reduction in ζ potential and μ_{endo} according to equation (2) and equation (1).

Difference among ethylenediamine, 1,3–diminopropane and 1,4–diminobutane lies in their adsorption to the capillary inner wall. It seemed that ethylenediamine ions were absorbed weaker than other two maybe because of its short carbon chain, so the electroosmotic mobility of the capillary with it was the highest at same concentration. There was not very much difference between 1,3–diminopropane and 1,4–diminobutane.

Dependence of electroosmotic mobility on pH

At constant diamine concentration, the dependence of electroosmotic mobility on pH from 5.5 to 10. 5 with diamine phosphate as carrying electrolyte was determined and shown in Figure 3. It is observed that increasing pH caused increase in electroosmotic mobility as a half of titration curve in the experimental pH range.

The dependence of electroosmotic mobility on pH with common carrying electrolyte is already known from the literature(18) , and it resembles a titration curve in which there is a 10– fold increase over the pH range from 3 to 11 with the largest increase being from 4 to 8. The reason for this curve is ionization of silanol groups on the capillary inner wall which can give a silmilar curve between $\equiv SiO^{\ominus}$ concentration and pH, and the amount of charge per unit surface area e is determined by $\equiv SiO^{\ominus}$ concentration. When diamine phosphate was used as carrying electrolyte, e was determined not only by the concentration of $\equiv SiO^{\ominus}$ group, but also by diamine ions absorbed by the capillary inner wall. At low pH, increased pH

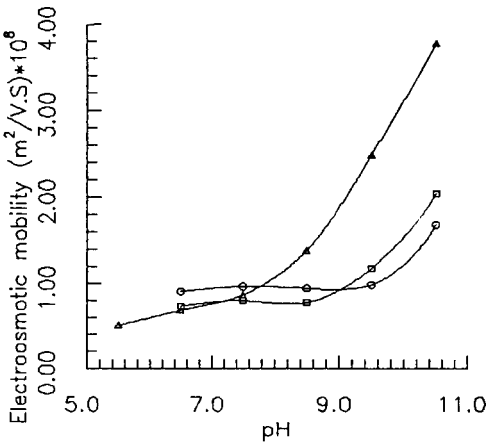


FIGURE 3. Dependence of electroosmotic mobility on pH with diamine phosphate as carrying electrolyte. △ 60mM ethylenediamine and □ 30mM 1, 3- diaminopropane, coditions: applied voltage, 13.5kV; capillary, 70cm total length, 50cm to the detector, 50um i.d.. ○ 30mM 1,4-diaminobutane, conditions: applied voltage, 12kV; capillary, 64cm total length, 44cm to the detector, 50um i.d.. Ambient temperature, 14-16℃.

caused increase of $\equiv SiO^{\ominus}$ concentration, but increased concentration of $\equiv SiO^{\ominus}$ also caused more absorbed diamine ions which made e almost unchanged, so there were pH ranges with almost constant electroosmotic mobility in Figure 3. With increasing pH, absorbed diamine ions may not be large enough to make e unchanged anymore, then a titration curve relation between electroosmotic mobility and pH was observed. From ethylenediamine, 1,3-diaminopropane to 1,4-diaminobutane, the pH range with almost constant electroosmotic mobility value became wider and wider, maybe attributed to the adsorption becoming stronger and stronger.

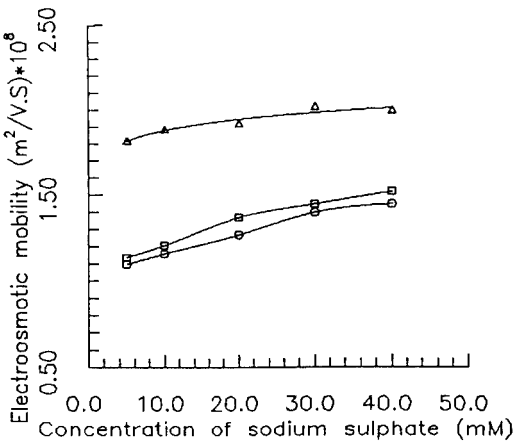


FIGURE 4. Dependence of electroosmotic mobility on sodium sulphate concentration with diamine phosphate as carrying electrolyte. Conditions: applied voltage, 9kV; capillary, 64cm total length, 44cm to the detector, 50um i.d.; buffer, pH8. 5; ambient temperature, 23–24℃. △ ethylenediamine; □ 1, 3 –diaminopropane; ○ 1,4–diaminobutane.

Effect of neutral salt on electroosmotic mobility

As shown above, the advantage of diamine phosphate as carrying electrolyte was found in the ability to slow electroosmotic mobility. More results showed that the adddition of neutral salt can speed the electroosmotic mobility, as demonstrated in Figure 4.

As predicted above, incrasing the concentration of carrying electrolyte decrease thickness of the diffusion layer δ according to equation (3), so decrease ζ potential of the electric double layer and the eletroosmotic mobility μ according to equation (2) and equation (1). So there seemed to be some conflict between theory and experimental results. This

may be also ascribed to the adsorption of diamine ions to the capillary inner wall. Because neutral salt ions were absorbed by the capillary inner wall much looser than diamine ions, they maybe served to increase the amount of charge per unit urface area e through entering the compact layer. In addition, increasing its concentration caused increase of current through the capillary tube which maybe also caused temperature rise of the carrying solution, and then increase of the electroosmotic mobility.

Effect of applied voltage on electroosmotic mobility

The effect of applied voltage on electroosmotic mobility is shown in Figure 5. It was not anticipated that increasing the applied voltage caused increase in eletroosmotic mobility.

It was supposed that increasing applied voltage maybe increased the amount of charge per unit surface area in the plane between the compact layer and diffuse layer. That was because positively charged diamine ions were absorbed by negatively charged capillary inner wall through electric attraction, and increasing applied voltage maybe weakened this attraction, therefore increased e, ζ, μ_{eom}. But more results denied this hypothesis.

Although increased applied voltage caused increase of the electroosmotic mobility, linear relationship between electroosmotic flow and current through the capillary tube was obtained, as shown in Figure 6. This linear relationhip was well recognized theoretically by Terabe et al(19) and Tsuda et al(20) with common carrying eletrolyte. Their works also revealed nonlinear relationship between electroosmotic flow and applied voltage, and similiar relationship between electroosmotic mobility and applied voltage just like ours can be derived. So there seemed no difference between diamine phosphate carrying electrolyte solution and commmmon carrying electrolyte solution in this respect, and the effect of applied voltage on

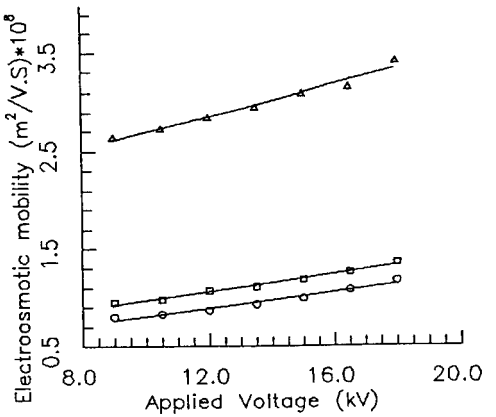


FIGURE 5. Dependence of electroosmotic mobility on applied voltage with diamine phosphate as carrying electrolyte. △ ethylenediamine, □ 1,3–diaminopropane, ○ 1, 4– diaminobutane. Conditions: capillary, 70cm total length, 50cm to the detector, 50um i.d.; buffer, pH9.5 60mM diamine + 30mM Na_2SO_4 ; ambient temperature, about 17℃.

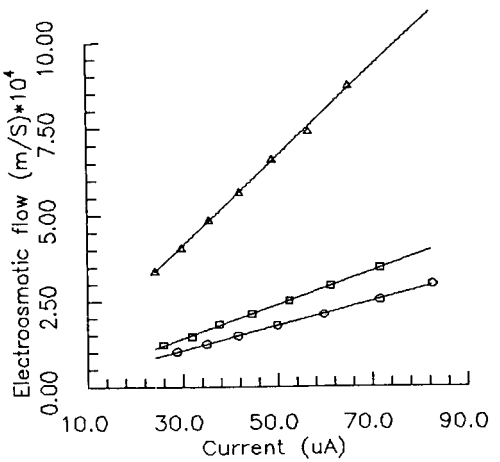


FIGURE 6. Linear relationship between electroosmotic flow and current through the capillary tube with diamine phosphate as carrying electrolyte. Conditions are the same as those given on Figure 5.

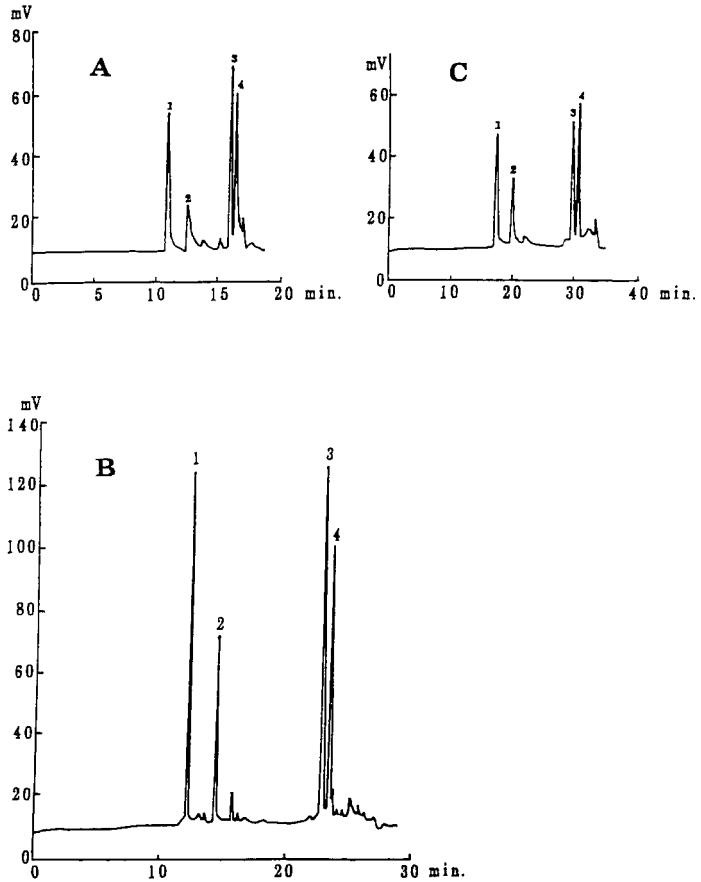


FIGURE 7. Improved separation of basic proteins with diamine phosphate as carrying electrolyte. (1) lysozyme (2) cytochrome C (3) trypsinogen (4) α-chymotrypsinogen A. (A) buffer, pH8. 5 60mM ethylenediamine; applied voltage, 15.5kV; current, 35uA; ambient temperature, 18℃; (B) buffer, pH8. 5 30mM 1, 3 -diaminopropane; applied voltage, 18kV; current, 65 - 68uA; ambient temperature, 20℃; (C) buffer, pH8. 5 30mM 1, 4 -diaminobutane; applied voltage, 18kV; current, 34.5uA; ambient temperature, 15℃.

TABLE 1

Separation Efficency of Every Peak in Figure 7.

Carrying electrolyte	*Thereotical plate number × 10^{-4}			
	peak 1	peak 2	peak 3	peak 4
ethylenediamine	1.4	0.5	7.7	2.8
1,3-diaminopropane	4.9	5.1	14.9	13.6
1,4-diaminobutane	1.2	1.1	3.3	4.9

*N=5.53(RT/WI), where RT is the retention time, WI is the peak width at half height.

TABLE 2

Retention Time Reproducibility of Every Peak in Figure 7.

Carrying electrolyte	RSD%(n=6)			
	peak 1	peak 2	peak 3	peak 4
ethylenediamine	1.22	0.95	1.53	1.51
1,3-diaminopropane	0.26	0.21	0.54	0.86
1,4-diaminobutane	0.81	0.73	0.61	0.57

electroosmotic mobility can also be arributed to excessive Jeale heat which caused temperature rise of the capillary tube and then viscosity reduction of the carrying solution although sometimes the applied voltage was not very high.

It should be noted that linear relationship between electroosmotic mobility and applied voltage was also obtained as

demonstrated in Figure 5, which meaned linear relationship between the reciprocal of viscocity and applied voltage.

Usefulness of ethylenediamine, 1, 3- diaminopropane and 1, 4 -diaminobutane as phophate as carrying electrolyte

In capillary zone electrophoresis (CZE) , electroosmotic mobility affects separation efficency and resolution. The use of ethylenediamine, 1, 3- diaminopropane and 1, 4- diaminobutane phosphate as carrying electrolyte gave slow electroosmotic mobility which can make low separption efficency but high resolution. In a limited range, the slowed electroosmotic mobility can be optimized to a approriate value by adjusting diamine concentration, pH and neutral salt concentration to make a desired separation. The dependence of electroosmotic mobility on these experimental parameters was well depicted in this study.

Besides the advantage above, the use of diamine phosphate as carrying electrolyte can also improve the separation of basic proteins, which is demonstrated in Figure 7. At operating pH8.5, the selected basic proteins possess positive charges, then they are absorbed by capillary inner wall through electric attraction in common carrying electrolyte solution, as indicated in reference (21). With diamine phosphate as carrying electrolyte, positive diamine ions competed for adsorption of basic proteins to capillary inner wall through electric attraction, then the separation of basic proteins was improved. The separation efficency and retention time reproducibility of every peak demonstrated in Figure 7 were shown in Table 1 and Table 2, respectively.

ACKNOWLEDGEMENT

This work is financially supported by National Natural Science Foundation of China.

REFERENCES

1 J.Jorgenson and K.D.Lukacs, Anal.Chem., 53: 1298(1981).

2 H.Lauer and D.McManigill, Trends Anal.Chem., 5: 11(1986).

3 J.S.Green and J.W.Jorgenson, J.Chromatogr., 478: 63(1989).

4 M.Bushey and J.W.Jorgenson, J.Chromatogr., 480: 301(1989).

5 F.A.Chen, L.Kelly, R.Palmieri, R.Biehler and H.Schwartz, J. Liquid Chromatogr., 15: 1143(1992).

6 S.Fujiwara and S.Honda, Anal.Chem., 59: 487(1987).

7 C.Schwer and E.Kenndler, Anal.Chem., 63: 1801(1991).

8 G.M.Janini and H.J.Issaq, J.Liquid Chromatogr., 15: 927(1992).

9 V.Dolnik, J.Liu, J.F.Banks, J.Milos, V.Novotny and P.Bocek, J.Chromatogr., 480: 321(1989).

10 R.G.Nielsen and E.C.Rickard, J.Chromatogr., 470: 241(1989).

11 L.Song, Q.Ou and W.Yu, to be published.

12 J.A.Bullock and L.C.Yuan, J.Microcol.Sep., 3: 241(1990).

13 H.H.Laucer and D.McManigill, Anal.Chem., 58: 166(1986).

14 F.S.Stover, B.L.Haymore and R.J.McBeath, J.Chromatogr., 470: 241(1989).

15 V.Rohlicek and Z.Deyl, J.Chromatogr.(Biochemm.Appl.), 494: 87(1989).

16 R.J.Hunter, Zeta Potential in Colloid Science, Academic Press, London, Chapter 3.

17 P.Velaha, Double Layer and Electrode Kinetics, Interscience, New York, 3rd ed., 1976.

18 J.K.Towns and F.E.Regnier, Anal.Chem., 63: 1126(1991).

19 S.Terabe, K.Otsuka and T.Ando, Anal.Chem., 57: 534(1985).

20 T.Tsuda and G.Showa, J. Liquid Chromatogr., 12: 250(1989).

21 M.Zhu, R.Rodriguez, D.Hansen and T.Wehr, J.Chromatogr., 516: 123(1990).

Received: September 4, 1993
Accepted: November 15, 1993

GENERAL PAPERS

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1971-1980 (1994)

RAPID FLUORIMETRIC ASSAY FOR PLASMA NEFOPAM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

LI-CHIEN CHANG AND DA-PENG WANG
School of Pharmacy
National Defense Medical Center
Taipei, Taiwan
The Republic of China

ABSTRACT

A simple and rapid liquid chromatographic method for the determination of nefopam in plasma is presented. Plasma samples after de-protein management were directly analyzed by HPLC system with fluorimetric detector. The separation was achieved on a NOVA-PAK C_{18} column with a mixture of acetonitrile and 0.05M phosphate buffer at pH 3.0 using sodium propanesulfonate as the ion-pair agent. Low detection limit (0.5 ng) and linearity of calibration curve validate the suitability. Peak purity of nefopam in the chromatograms was checked by an additional photodiode-array detector (PAD). Recoveries of nefopam in plasma by de-protein and liquid-liquid extraction were 94.79 and 94.31%, respectively. In the in vivo iontophoresis study, remarkable differences of penetration effect were obtained .

It is suggested that this HPLC method could be used for pharmacokinetic study of nefopam.

INTRODUCTION

Nefopam hydrochloride (Fig 1) , structurally related to antiparkinson (orphenadrine) and antihistamine (diphenhydramine) drugs, is a non-narcotic

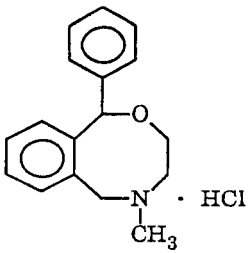


FIGURE 1 Structure of Nefopam Hydrochloride

analgesia with an analgesic potency lying in the range of 0.2 to 0.6 times that of morphine sulfate but without the propensity for addiction and respiratory depression[1]. Currently, it is comprehensively used in hospitals for the treatment of a wide range of painful conditions and has been relatively well tolerated by most patients.

For more detail documenting its pharmacokinetic properties, there had been many reports of application to analyze the drug content in human fluid such as GLC[2], GC-FID[3], HPLC-UV[4] and HPLC-ECD[5] methodologies. Some of them show relatively high sensitivity, precision and accuracy. However, these analytical methods generally require a cumbersome extraction protocol and are also time consuming. Besides, HPLC-UV method is simpler and more suitable for routine practice in the laboratory and hospital but the low selectivity of conventional UV techniques near 200 nm leads to the difficulty of determining nefopam in plasma.

In this paper, a simple high-performance liquid chromatographic (HPLC) fluorimetric detection method has been developed to eliminate these drawbacks and enables the rapid determination of plasma nefopam concentration. Moreover, an advanced photodiode-array UV detector in HPLC system also has been used to describe the peak purity and qualitation in this method. In our laboratory, the method has been successfully applied to an in vivo iontophoresis study.

MATERIALS AND METHODS

Chemicals and Reagents

Nefopam hydrochloride was purchased from Sigma (St. Louis, MO, U.S.A.) Glycine, Sodium hydroxide and dibasic potassium hydrogen phosphate (E. Merck, Darmstadt, F.R.G.) were used to prepare different pH buffers. Sodium propanesulfonate was purchased from TCI (Tokyo, Japan). Hipersolv-grade cyclohexane for liquid-liquid extraction was from BDH (Poole, U.K.). Acetonitrile (E. Merck, Darmstadt, F.R.G.) was HPLC grade and was used as the mobile phase and de-protein solvent. Milli-Q water was prepared through a Milli-RO 60 water purification system (Millipore, Bedford, MA, U.S.A.).

Chromatography

HPLC was performed with a Shimadzu liquid chromatographic system (Shimadzu Corp., Kyoto, Japan) with a Model LC-6A pump, a Model SIL-9A autosampler, a Model RF-551 spectrofluorometric detector set at the high sensitivity (Ex 284nm, Em 302nm) and a Model C-R6A CHROMATOPAC integrator. An additional Model SPD-M6A photodiode-array detector (PAD) for assay validation was placed in series between the column and spectrofluorometric detector. Nefopam was eluted isocratically with the mixture of acetonitrile and 0.05M phosphate buffer (pH=3.0) (20:80) containing 0.02M sodium propanesulfonate salt through a NOVA-PAK C_{18} analytical column (5μm, 3.9*150 mm) at a flow rate of 1.0 mL/min. Peak areas were integrated and recorded by the C-R6A CHROMATOPAC integrator. The PAD data were stored and processed by a 386DX personal computer (Aveen, Taipei, Taiwan) provided with a chromatographic software (Shimadzu). Mobile phase always filtered through a 0.22 μm millipore filter followed by degassing.

Sample Preparation

Blood bank plasma for the preparation was provided by the Tri-Service General Hospital (Taipei, Taiwan). Other blood samples were drawn into

heparinized tubes, plasma was separated by centrifugation and stored at -20°C until assay.

Standard curve in plasma: 0.5 mL of plasma containing varied amount of nefopam with a range of 0.1 to 2.0 μg/mL were prepared in centrifuge tubes, then 0.5 mL of acetonitrile was added. The mixture was thoroughly mixed and centrifuged at 3000 rpm for 15 minutes. Following centrifugation the upper layer was filtrated by 0.45 μm millipore filter and injected into the HPLC for analysis.

De-protein management: A 0.5-mL spiked sample of plasma was placed into a 20*15 mm centrifuge tube containing 0.5 mL of acetonitrile and mixed thoroughly, then centrifuged at 3000 rpm for 15 minutes. The final solution was filtrated by 0.45 μm millipore filter and injected into the HPLC for analysis.

Liquid-liquid extraction: In order to compare with conventional cumbersome method, a liquid-liquid extraction management was also taken place. Following Liu's method[4], a 2.0-mL spiked sample of plasma was alkalinized by 200.0 μL of 1.0M glycine buffer (pH 11.0) and extracted by cyclohexane (6.0 mL). Then through acidified (0.1M HCl, 3.0mL) and alkalinized (0.5 M NaOH, 1.0mL) manipulation, the final extract in cyclohexane (6.0mL) was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100.0μL of mobile phase for injection into the HPLC column.

In vivo iontophoresis study

An in-vivo evaluation provides information about the applicability of the method for analysis of rat plasma samples. In this study 6-8 week-aged female hairless mouse (Animal Center, Tri-Service General Hospital, Taipei, Taiwan) were fixed in consciousness, then 1.5% nefopam gel through a self-synthesis polymer membrane was applied on the skin of abdomen. Nefopam within the gel administered into the body was based on the iontophoresis impulse and another group was only based on the passive transmission used as the control. Blood samples were collected by the direct heart transfixion at the 0.5, 1.0, 2.0 and 3.0

hours postdose. Plasma were separated by the centrifugation and stored in -20°C until analysis

RESULTS AND DISCUSSION

Precision, accuracy and sensitivity

The highly selective and simple procedure described in this paper gives a clear separation of nefopam in blank and spiked samples. (Fig 2). Its efficacy demonstrated the two advantages: (1) the elution monitored by fluorescence detection allowing the direct assay of nefopam content without interference from other components of the assay plasma. (2) no requirement of compound modification and extremely simple de-protein management achieving the rapid determination of plasma nefopam.

The precision and linearity of the HPLC method were examined. The repetitive analyses (n=3) of the nefopam standard solution (0.1-2.0 μg/mL) in plasma gave a corresponding coefficient of variation (both intra- and inter-day coefficients of variation less than 10%) (Table 1). The retention time of nefopam in the HPLC system was 10.30 min. The C.V. (n=15) of the retention times was 0.5%.

The calibration graph of peak area versus nefopam concentration showed excellent linearity over the range 0.1-2 μg/mL(Fig. 3). The detection limit was 0.5 ng (signal-to-noise ratio=3).

Photodiode-array detection validation

For additional validating this assay method, HPLC of nefopam spiked with plasma was carried out with the on line photodiode-array detector to provide the spectrum, the absorbance ratio and the criteria for assessing the purity of peaks. Data on the peak purity of spiked plasma was obtained by comparing the spectra in the ascending, apex and descending portion of nefopam containing peak. Spectrum

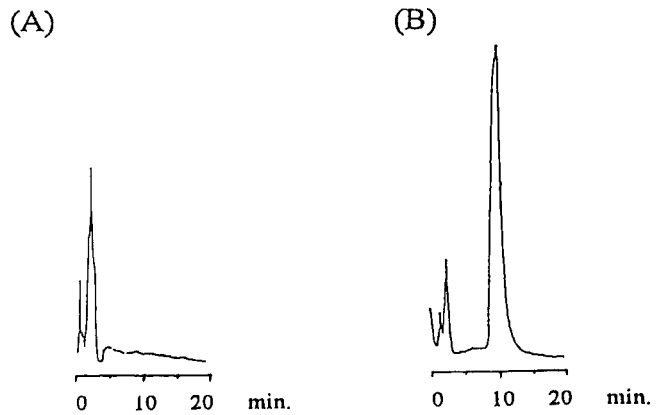


FIGURE 2 Chromatograms of (A) blank plasma, (B) plasma spiked with nefopam (0.4μg/mL).

TABLE 1 Intra- and Inter-day Standard Curve Data of Nefopam in Plasma

Concentration	Intra-day		Inter-day	
(μg/mL)	Found (μg/mL)	C.V. (%)	Found (μg/mL)	C.V. (%)
0.1	0.12	8.41	0.09	5.78
0.2	0.25	4.41	0.20	7.21
0.4	0.41	2.92	0.39	3.44
0.8	0.86	3.27	0.83	1.31
1.6	1.59	2.46	1.64	2.52
2.0	2.12	1.60	1.98	3.17

n=3

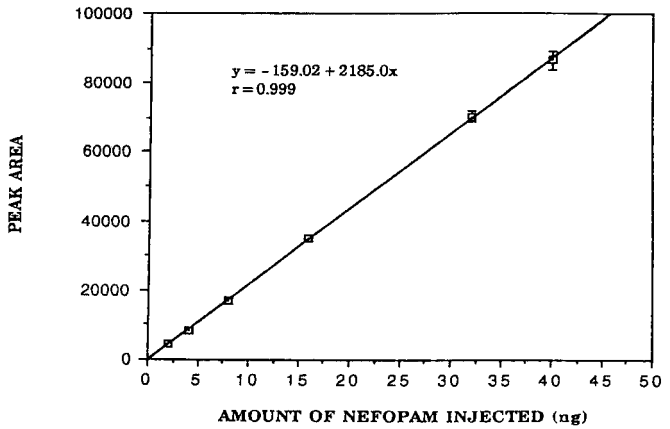


FIGURE 3 A plot of concentration vs peak area of nefopam in plasma.

of the three portions from 195 to 380 nm were superimposable, indicating the absence of impurities and that the corresponding purity index was 0.9998 (Fig 4). In this identification, the retention time and absorbance spectra were also taken into account. Different wavelength chromatograms in the time window were normalized and overlaid. Homogeneity of chromatograms showed no co-eluted interference and a high quality of the separation.

De-protein and liquid-liquid extraction

Table 2 summarized the recoveries of the nefopam from human plasma by ACN de-protein and liquid-liquid extraction at 100, 10 and 1 μg/mL concentration. Triplicate at each concentration gave overall recoveries (Mean ± S.D.) of 94.79 ± 0.92 and 94.31 ± 0.75, respectively. The fact that there was no significant difference between the two methods reveals that high selectivity of fluorescence determination could effectively eliminate the complexity in the sample preparation.

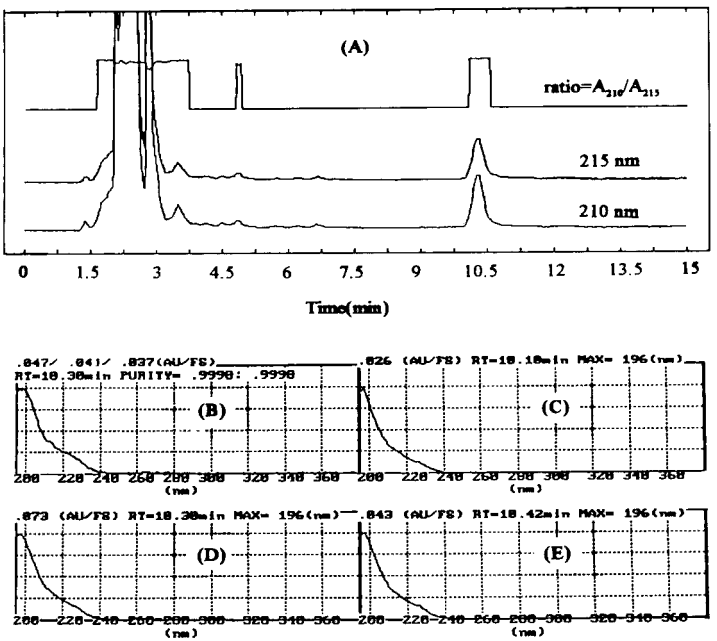


FIGURE 4 (A) Chromatograms of plasma spiked with nefopam (0.4 μg/mL) detected by diode-array detector at 210 and 215 nm. (B) Overlay spectra of nefopam for assessing peak purity. (C), (D),(E) UV spectra acquired in the ascending, apex and descending portion in the range of 195-380 nm.

In vivo iontophoresis study

A plasma concentration-time curve presented in Fig. 5 clearly depicted the iontophoresis efficacy. In comparison with the control group, applying current in skin increased the penetration of nefopam from gel up to seven fold after 3 hours postdose. The significant effect of penetration increment appeared after a half hour. Large scale studies were undertaken in our laboratory applying this analytical method.

TABLE 2 Recoveries of Nefopam from Human Plasma by Different Management

Nefopam	De-protein		Liquid-liquid extraction	
Added (μg/mL)	Found (μg/mL)	Recovery (%)	Found (μg/mL)	Recovery (%)
100	95.24	95.2	93.61	93.6
	95.82	95.8	92.97	93.0
	94.57	94.6	94.62	94.6
10	9.42	94.2	9.43	94.3
	9.53	95.3	9.49	94.9
	9.35	93.5	9.52	95.2
1	0.94	94.6	0.94	94.1
	0.93	93.7	0.95	95.2
	0.96	96.2	0.93	93.9
Mean ± S.D.		94.79 ± 0.92		94.31 ± 0.75

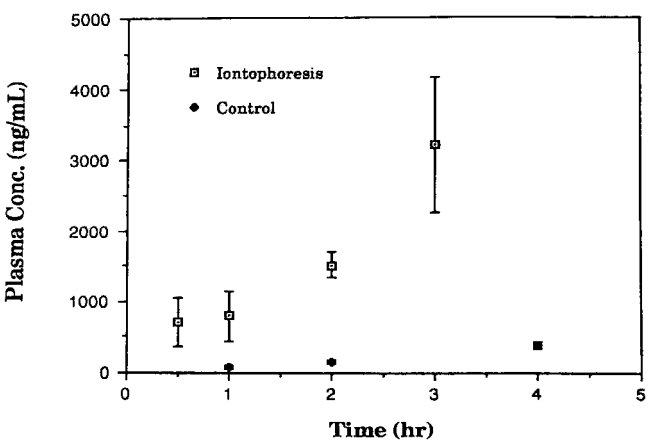


FIGURE 5 Comparison of the plasma nefopam concentration-time profiles in iontophoresis and control groups.

Conclusion

In summary, the fluorimetric method described here is simple, highly selective, specific and routinely useful for the quantitative analysis of nefopam in plasma. Moreover, it is convenient and can be readily incorporated into existing laboratory HPLC system as a novel method.

REFERENCES

1. R. C. Heel, R. N. Brogden, G. E. Parkes, T. M. Speight, G. S. Avery, Drugs, 19: 249(1980).

2. D. Schuppan, C. S. Hansen, R. E. Ober, J. Pharm. Sci., 67, 1720(1978).

3. H. Ehrsson, S. Eksborg, J. Chromatogr., 136, 154(1977).

4. D. T. Y. Liu, J. Savage, D. Donnell, Br. J. Clin. Pharmac., 23, 99(1987).

5. L. C. Burton, N. J. Loftus, D. W. Vere, R. Whelpton, J. Chromatogr., 526, 159(1990).

Received: December 6, 1993
Accepted: January 14, 1994

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1981-1993 (1994)

A RAPID PROCEDURE FOR PURIFYING LARGE AMOUNTS OF PYRIDINOLINE CROSSLINKS OF BONE

P. ARBAULT[1]*, E. GINEYTS[2], M. GRIMAUX[1], P. SEGUIN[1], AND P. D. DELMAS[2]

[1]Cis Bio International
BP 175
30203 Bagnols sur Cèze Cedex, France
[2]INSERM Unité 234
Hôpital E. Herriot
69437 Lyon Cedex 03, France

ABSTRACT

HPLC assessment of urinary Pyridinoline (Pyr) and Deoxypyridinoline (Dpyr) requires the use of large amounts of purified Pyr and Dpyr as external standards. We have developped a procedure for large-scale pyridinoline (Pyr and Dpyr) purification from sheep bone combining successively gel filtration, partition chromatography and semi-preparative HPLC. After bone powder (500 g) hydrolysis in 6N HCl (5 liters), the concentrated hydrolysate (600 ml) was separated by gel filtration on a Biogel P2 column (2.4 liters), allowing the elimination of 95% of impurities and the reduction of the pyridinolines solution to 150 ml. Then partition chromatography was carried out on CF1 cellulose where non-polar contaminants were suppressed. Finally, drawing on analytical HPLC knowledge, an isocratic semi-preparative HPLC was developed using a reversed phase C_{18} column (250 mm x 10 mm) with HFBA as the ion pairing agent. The last impurities were thus eliminated, and the Pyr was separated from the Dpyr. By this sequence of processes, 15 mg of pyridinoline and 1.8 mg of deoxypyridinoline were purified. This optimized procedure allows the large-scale production of Pyr and Dpyr from large amounts of bone or other tissue in a relatively short time, and requires only conventional biochemical reagents.

* *Author to whom correspondence should be addressed.*

INTRODUCTION

In recent years, new markers have been developed for the diagnosis and management of metabolic bone diseases. Bone formation can be evaluated by serum measurement of osteocalcin (1,2) or alkaline phosphatase (2). Bone resorption was first assessed through the determination of urinary hydroxyproline whose level increases in cases of bone degradation, such as in osteoporosis or Paget's disease (3). However, this marker cannot be considered as bone-specific since it may be influenced by gelatin in the diet, by extra-osseous collagen synthesis, and by complement activation (4,5). This lack of specificity makes hydroxyproline a poorly sensitive marker for monitoring bone resorption. Since the identification of pyridinoline (Pyr) by Fujimoto (6,7) and of deoxypyridinoline (Dpyr) by Ogawa (8), several authors have investigated the interest of these collagen pyridinium crosslinks as markers for metabolic bone disease.

Deoxypyridinoline is not specific for type I collagen of bone, but it is found in large amounts in bone only. Pyridinoline is present in large concentrations in tissues like bone (type I collagen), cartilage (type II collagen), and in several other connective tissues such as aorta and tendon (9,10). In adult human bone, the molar ratio of Pyr to Dpyr is about 3.5 : 1. In urine, the Pyr/Dpyr ratio is usually similar to that found in bone, indicating that urinary pyridinolines seem essentially to originate from bone collagen breakdown (11). First clinical results demonstrated that urinary Pyr and Dpyr increase occured in many diseases such as Paget's disease (12,13), osteoporosis (14,15), hyperparathyroidism (12,13,16), or cancers with bone metastasis (17).

Urinary Pyr and Dpyr are currently quantified, after acid hydrolysis and cellulose separation, by reversed phase high performance liquid chromatography (HPLC), where both crosslinks are detected by their natural fluorescence (9,10,12-19). For Pyr and Dpyr measurements in tissues, Eyre (9,10) and Black (18) developed HPLC methods with internal standards, respectively pyridoxamine and pyridoxine. But for urinary assays all the authors work with Pyr and Dpyr as external standards (12-19) which require large amounts of both molecules.

This paper describes a procedure for large-scale purification of pyridinoline and deoxypyridinoline, involving three steps with successively gel filtration, partition chromatography and semi-preparative HPLC.

MATERIALS AND METHODS

Bone powder preparation

Metacarpal bone was collected from a nine-year old sheep. Bone (500 g) was frozen in liquid nitrogen, broken into one centimeter square pieces with a hammer and crushed into a fine powder with a Retsch mill (Bioblock, Illkirch, France), under liquid nitrogen. Lipids were eliminated by washing the powder several times with acetone and water. Demineralization was carried out by stirring the powder in a 0.5 M EDTA solution pH 7.4 (five liters) during 24 hours. This solution was centrifuged and the pellet was pooled and dried.

Bone powder hydrolysis

Envisaging large-scale hydrolysis, we adjusted the bone powder concentration in the hydrolysis solution and measured the pyridinoline release: one to ten grams of bone powder were mixed with 100 ml of 6N HCl solution, and hydrolysis was performed in a glass bottle at 110 °C under agitation for 24 hours. After hydrolysis, one milliliter samples were taken and analyzed for their free pyridinoline (Pyr and Dpyr) contents by HPLC after analytical cellulose purification (described hereafter).

Large scale production: five hundred grams of bone powder in five liters of 6N HCl solution were heated at 110 °C under reflux during 24 hours. The hydrolysate was concentrated under reduced pressure and by heating at 45°C, to 600 ml of solution.

Gel filtration

A first analytical run was carried out to monitor the pyridinoline elution: 50 ml of concentrated hydrolysate were injected into a K 50/30 column (Pharmacia, St Quentin en Yvelines, France),containing 400 ml of Biogel P2 superfine gel (400

mesh) (Biorad, Paris, France). The elution was performed with a 250 ml/hour flow rate using ultraviolet (UV) detection at 280 nm. Twelve milliliter fractions were collected and analyzed by analytical HPLC to measure their pyridinoline concentration.

Large-scale process: about 300 ml of hydrolysate were processed in a BP 113 column (Pharmacia) containing 2.4 liters of Biogel P2 fine gel (200-400 mesh). Elution was carried out at a 900 ml/hour flow rate and followed up by UV detection at 280 nm. The 600 ml of hydrolysate was purified in two runs.

Pyridinoline fractions were pooled and concentrated under reduced pressure by heating at 45°C, to 150 ml of solution.

Partition chromatography

For these steps, a CF1 cellulose slurry was prepared by mixing 1000 ml of 1-butanol, 250 ml of acetic acid, 250 ml of water (4:1:1 solvent mixture), and 100 grams of Whatman CF1 cellulose powder (Touzart et Matignon, Vitry sur Seine, France).

Analytical cellulose column: successively, five hundred microliter fractions of hydrolysate, gel filtration solutions, or of cellulose solutions were mixed with 500 µl of acetic acid and 2 ml of 1-butanol. They were processed in a ten milliliter polypropylene column (Bioblock) containing 5 ml of cellulose slurry. After three washes with 5 ml of 4:1:1 solvent mixture, the pyridinoline fraction was collected with 2 ml of water. The residual 1-butanol fraction was eliminated by pipetting, and the water fraction was removed by evaporation using a Savant speed-vac apparatus (Bioblock).

Large scale cellulose: one hundred and fifty milliliters of pyridinoline solution were mixed together with 450 ml of CF1 cellulose slurry, 600 ml of 1-butanol, and 150 ml of acetic acid in a polypropylene bottle, and left for three hours under shaking. The slurry was passed through a G2 filter, then the recovered cellulose was suspended in 600 ml of 4:1:1 solvent mixture, left under shaking for 30 min, and refiltered on a G2 filter. After repeating this washing step three times, the

cellulose was suspended in 400 ml of water to release pyridinolines, left thirty minutes under shaking, and passed through a G2 filter after which the solution was collected for pyridinoline analysis by HPLC. This release step was repeated once more and the pyridinoline fractions were pooled and their water content evaporated. Filtrate from each step was analyzed by HPLC for pyridinoline yield assessment.

HPLC

Analytical HPLC: pyridinoline and deoxypyridinoline were assayed by the HPLC method, as previously described by Uebelhart (1990)(12). The HPLC system included LKB 2150 pumps and LKB 2152 LC controller (Pharmacia). The reversed-phase column was a Lichrospher 100 RP 18 endcapped, 5 μm beads diameter, 125 mm x 4 mm column (Merck, Nogent sur Marne, France). The detector was a LS II Filter Fluorimeter (Perkin-Elmer, St Quentin en Yvelines, France) with a 300 nm excitation filter and a 395 nm emission monochromator. The samples were diluted in a 1% heptafluorobutyric acid (HFBA) (Sigma, La Verpilliere, France) solution and eluted isocratically with a 15% acetonitrile - 85% water -10 mM HFBA solution, at a flow rate of 1 ml/min. Purified human Pyr and Dpyr prepared and calibrated as previously described (12) were used as external standards.

Semi-preparative HPLC: the preparative HPLC system consisted of two Shimadzu LC8A pumps (Touzart et Matignon), a SCL6B system controller (Touzart et Matignon) and a LKB 2510 Uvicord SD detector (Pharmacia). The semi-preparative HPLC conditions were previously optimized using a Lichrospher RP 18 endcapped 250 mm x 4 mm column (Merck).

The dried pyridinoline fraction was dissolved in 15 ml of a 20% HFBA solution. Pyridinoline and deoxypyridinoline were separated from final endogenous contaminants by reversed-phase HPLC on a Lichrospher RP 18 endcapped, 5μm bead diameter, 250 mm x 10 mm column (Merck). The separation was performed isocratically at a 5 ml/min flow rate, using a solvent mixture of 15% acetonitrile - 85 % water 10mM HFBA. After 25 min, the acetonitrile gradient was brought up to 100% in 10 min to wash the column. The elution was monitored with a 280 nm

UV detector. Finally, Pyr and Dpyr were quantified using the analytical HPLC procedure and their respective spectral characteristics were controled by excitation and emission spectra with an LS 50 luminescence spectrometer (Perkin Elmer).

Statistical analysis

Results are expressed as mean ± 1 standard deviation (SD). Student's t test was used for comparison of two means.

RESULTS AND DISCUSSION

In his report, D. Eyre (9) described the purification of Pyr from 40 g of cartilage which had been hydrolyzed in 2 liters of 3N HCl. Using the same experimental conditions, 25 l of 6N HCl solution would have been necessary to carry out the hydrolysis of 500 g of bone powder. Our investigations demonstrate that the hydrolysis can be performed at a concentration of 100 g of bone/l with a good pyridinoline release yield compared to classical conditions at 10 g/l concentration (see table n° 1). The hydrolysis solution volume can thus be reduced to 5 liters for 500 g of bone.

After this step, the hydrolyzate contained a mixture made of amino acids and mineral salts. Prior to the CF1 cellulose step, a gel filtration on Biogel P2 was added to eliminate the smallest molecular weight particles and to reduce volume before the cellulose step. The Biogel P2 chromatograms (see figure n° 1) were similar for the large scale application and the Biogel P2 development, showing a good extrapolation of this step. Pyridinolines were eluted from the first fractions and most of the smallest molecules could be eliminated. At 280 nm, the peak area calculation showed that 95% of impurities were eliminated. In the large scale production, the volume was reduced to 150 ml. Thus the cellulose purification could be performed with small 1-butanol volume and container, which would allow manipulation under a classical laboratory hood and so avoid inhalation of 1-butanol, a toxic solvent. Although the volume was reduced to 150 ml, the final HPLC step could not take place with so much solution. Therefore, a CF1 cellulose step had to be performed to eliminate other contaminants and to reduce volume.

TABLE 1

Hydrolysis Solution: Influence of Bone Powder Concentration on the Amount of Pyridinoline Released.

Hydrolysis conditions	*n*	mg of released Pyr for 100g of hydrolyzed bone (Mean ± 1 SD)
1g of bone powder /100 ml HCl 6N	4	4.58 ± 0.59
10g of bone powder /100ml HCl 6N	4	5.39 ± 0.76[a]

a: non significant difference.

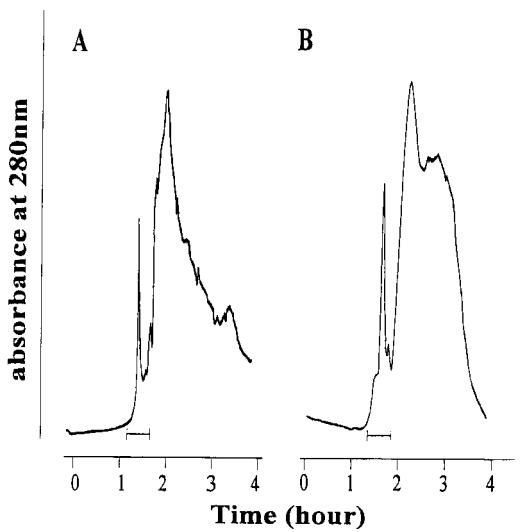


FIGURE 1. Typical chromatograms of gel filtration step. **A**: Biogel P2 development. **B**: large-scale Biogel P2. Bars indicate fractions containing pyridinolines.

TABLE 2

Cellulose Purification: Amount of Pyridinoline Present in each Solution (Fixation, Washes, Release).

The Pyr amount was assessed after each step of this purification. HPLC measurements were performed in the aqueous fractions of the 4:1:1 solutions after fixation, washing, and in the purified pyridinoline solution.

	Aqueous fraction recovered in the 4:1:1 solution after fixation	Aqueous fraction recovered in the 4:1:1 solution after washes	Aqueous fraction containing purified pyridinolines
Pyridinoline	10.6%	11.0%	78.4%

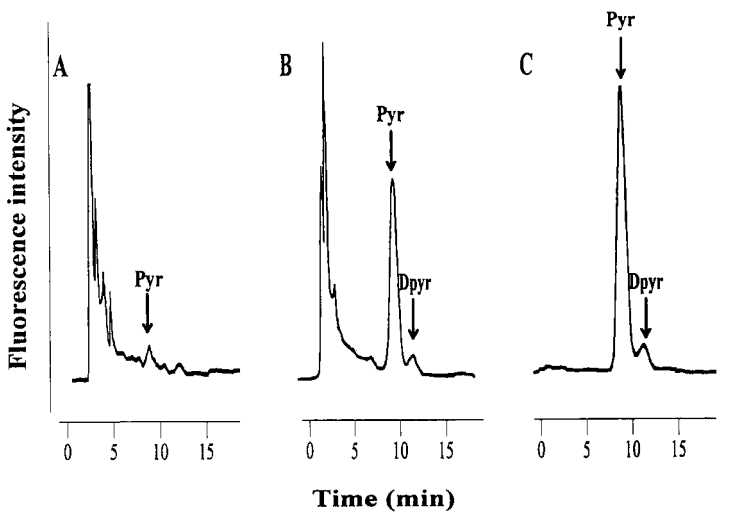


FIGURE 2. Analytical HPLC chromatograms for pyridinoline fractions after bone powder hydrolysis (**A**), gel filtration (**B**) and partition chromatography (**C**). Column: Lichrospher 100 RP 18 endcapped, beads diameter 5 μm (125 mm x 4 mm). Solvent: 85% water-15% acetonitrile 10 mM HFBA.

TABLE 3

Semi-preparative HPLC: Influence of Quantity of HFBA in the Injection Solution.

The amount of HFBA in the injection solution was increased and the pyridinoline rate in the first fractions (Elution time: 2-5 min) of the semi-preparative HPLC was monitored by analytical HPLC method.

HFBA	Pyridinoline (% of total) in the first HPLC fractions
1%	42%
3%	30%
5%	22%
10%	10%
20%	5%

Yields were studied (table n° 2) demonstrating that about 78% of Pyr was recovered after this step. Analytical HPLC before and after cellulose showed that many contaminants were eliminated (figure n° 2).

At this stage, some contaminants were still present, and pyridinoline purification needed an HPLC step. Extrapolating the conditions of the analytical method, a large-scale HPLC was performed isocratically with the same solvent. First of all, the column length was increased two-fold, in order to obtain a good resolution for the separation between Pyr, Dpyr and other products. Similarly, HFBA was adjusted in the injection solution to improve the separation of pyridinolines from the remaining impurities. Using 1% HFBA, only 50% of the expected Pyr and Dpyr were separated from the contaminants. The remaining fraction of Pyr-Dpyr was eluted simultaneously in the first fractions (elution time 2-5 min) with the usual contaminants. When the amount of HFBA was brought up

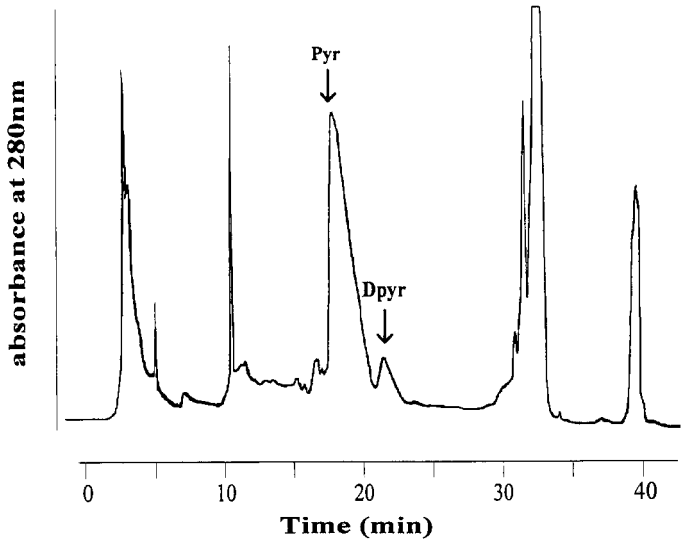


FIGURE 3. A typical chromatogram of semi-preparative HPLC for final step of pyridinoline purification. Column: Lichrospher 100 RP 18 endcapped, beads diameter 5µm (250 mm x 10 mm). Solvent A: 85% water-15% acetonitrile 10 mM HFBA. Solvent B: 100% acetonitrile. From 0 to 20 min: 100% A, then a linear gradient over 15 min from 0 to 100 % B.

from 3% to 20% (table n° 3), the Pyr-Dpyr presence in the first fractions decreased to reach 5%, which allowed a good pyridinoline recovery. It must be stressed that the amount of HFBA had to be increased either when the preparation contained a lot of impurities or when the quantity to be injected was greater. In our large-scale HPLC conditions (figure n°3), HFBA was fixed at 20%. Finally, in order to separate pyridinoline from deoxypyridinoline, the amount of HFBA in the solvent was studied. At 1.3 ml for 1 liter of 15% acetonitrile solution, Pyr and Dpyr were eluted respectively at 19.5 min and 21 min. These elution times became respectively 23 min and 26 min with 2 ml HFBA for 1 liter of solution, allowing perfect separation of the two crosslinks (see figure n° 4). The increasing amount of HFBA in the solvent mixture delayed the pyridinolines' elution by enhancing their reversed phase interactions.

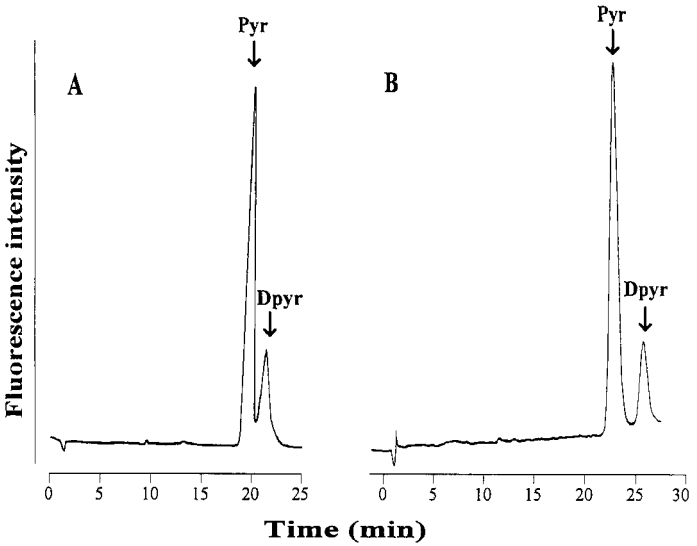


FIGURE 4. Pyridinoline (Pyr) and deoxypyridinoline (Dpyr) separation at 1.3 ml HFBA (**A**) or at 2 ml HFBA (**B**) in 15% acetonitrile solution.

Using this pyridinoline purification process, about 15 mg of pyridinoline and 1.8 mg of deoxypyridinoline were purified from 500 g of bone powder. A brief estimation after hydrolysis showed that 500 g of sheep metacarpal bone contained potentially about 25 mg of Pyr and 2.5 mg of Dpyr. Thus, using the complete process, the final purification yield was about 60% for Pyr and 72% for Dpyr. Whilst, pyridinoline losses were generated throughout the three-step process, due to the analytical investigations carried out to optimize the purification conditions, the CF1 cellulose was indeed shown to be crucial for pyridinoline recovery: twenty two percent of total Pyr appeared to be in the 4:1:1 solution of fixation and washing steps (see table n° 2). These pyridinolines could be recovered by allowing the solution to decant at room temperature overnight. The aqueous phase is collected, evaporated, and can be dissolved in water for a new run in the CF1 cellulose step. Deoxypyridinoline represents about 10% of total Pyr-Dpyr in sheep bone. However, larger amounts could be obtained using human or chicken bone,

which contain about 25% and 50% of Dpyr respectively (9). In conclusion, a large scale pyridinoline production was developed by using three different purification steps in sequence. This process allows the purification of large quantities of pyridinoline and deoxypyridinoline from all types of tissues in a relatively short time.

REFERENCES

1 J. P. Brown, P. D. Delmas, L. Malaval, C. Edouard, M. C. Chapuy, P. J. M. Meunier, Lancet, 1: 1091-1093 (1984).

2 P. D. Delmas, Am. J. Med., 91(supl 5B): 59S-63S (1991).

3 A. C. Deacon, P. Hulme, R. Hesp, J. R. Green, M. Tellez, J. Reeve, Clin. Chim. Acta, 166: 297-306 (1987).

4 P. H. Weiss, L. Klein, J. Clin. Invest., 48: 1-5 (1969).

5 K. I. Kivirikko, Int. Rev. Connect. Tissue Res., 5: 93-163 (1970).

6 D. Fujimoto, Biochem. Biophys. Res. Commun., 76: 1124-1129 (1977).

7 D. Fujimoto, T. Moriguchi, T. Ishida, H. Hayashi, Biochem. Biophys. Res. Commun., 84: 52-57 (1978).

8 I. Ogawa, T. Ono, M. Tsuda, Y. Kawamishi, Biochem. Biophys. Res. Commun., 107: 1252-1257 (1982).

9 D. R. Eyre, "Collagen crosslinking amino-acids" in Methods in enzymology, L. W. Cunningham, ed., Academic Press Inc., New York, 1987, 144: 115-139.

10 D. R. Eyre, T. J. Koob, K. P. Van Ness, Anal. Biochem., 137: 380-388(1984).

11 Z. Gunja-Smith, R. J. Boucek, Biochem. J., 197: 759-762 (1981).

12 D. Uebelhart, E. Gineyts, M. C. Chapuy, P. D. Delmas, Bone Miner., 8: 87-96 (1990).

13 S. P. Robins, D. Black, C. R. Paterson, O. M. Reid, A. Duncan, M. J. Seibel, Eur. J. Clin. Invest., 21: 310-315 (1991).

14 D. Uebelhart, A. Schlemmer, J. S. Johansen, E. Gineyts, C. Christiansen, P. D. Delmas, J. Clin. Endocrinol. Metab., 72: 367-373 (1991).

15 P. D. Delmas, A. Schlemmer, E. Gineyts, B. Riis, C. Christiansen, J. Bone Miner. Res., 6: 639-644 (1991).

16 M. J. Seibel, F. Gartenberg, S. J. Silverberg, A. Ratcliffe, S. P. Robins, J.P. Bilezikian, J. Clin. Endocrinol. Metab., 74: 481-486 (1992).

17 C. R. Paterson, S. P. Robins, J. M. Horobin, P. E. Preece, A. Cushieri, Br. J. Cancer, 64: 884-886 (1991).

18 D. Black, A. Duncan, S. P. Robins, Anal. Biochem., 169: 197-203 (1988)

19 I. T. James, D. Perret, P. W. Thompson, J. Chromatogr., 525: 43-57 (1990).

Received: October 1, 1993
Accepted: December 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1995-2004 (1994)

DETERMINATION OF LIPOPHILICITY OF CHLORINATED ALICYCLIC COMPOUNDS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

JAINBO LIU[1], JANICE E. CHAMBERS[2], AND JOEL R. COATS[1]
[1]*Pesticide Toxicology Laboratory*
Department of Entomology
Iowa State University
Ames, Iowa 50011
[2]*College of Veterinary Medicine*
Mississippi State University
Mississippi State, Mississippi 39762

ABSTRACT

A RP-HPLC procedure has been developed for measuring the capacity factor (k') of a series of chlorinated alicyclic compounds. The chromatographic behavior measured on a 4.5 mm i.d. x 3.3 cm C-18 column with methanol / water as the mobile phase was related to the volume fraction of methanol (ϕ). A linear relationship was found between log k' and ϕ, showing the correlation coefficient $\gamma > 0.99$, for each of the 15 chlorinated alicyclic compounds tested. The log k_w , the capacity factor obtained by extrapolation of the retention data from binary eluents to 100 % water, was chosen as a measure of the solute lipophilicity. Since log k_w is considered as a valuble index of the lipophilicity of a compound, the determined values will be used for the quantitative structure-activity relationship studies of the chlorinated alicyclic compounds.

1995

INTRODUCTION

The lipophilicity of a bioactive molecule is one of the most important physicochemical properties which influences its capacity to move through biological compartments. It is generally defined as the tendency of a chemical to distribute between an immiscible nonpolar solvent and water. The logarithm of the partition coefficient of a chemical in the *n*-octanol/water system (log K_{ow}), which is usually measured by 'shake-flask' method, is widely used because of its simplicity and some similarity between *n*-octanol and biological membranes. The 'shake-flask' method works in most cases, but it results in large errors for chemicals with log K_{ow}'s larger than 4, and it is time-consuming and requires considerable amounts of pure stable compounds [1]. It has been proven that the retention capacity factor (k') of a compound in a reversed-phase high performance liquid chromatography (RP-HPLC) system is a reliable indirect descriptor of the lipophilicity of a compound [1-5, 10-16]. Moreover, the recent studies have shown that log k_w, the retention capacity factor which is extrapolated from a binary phase to 100% water in a RP-HPLC system, is an even better descriptor of lipophilicity than the isocratic factor [4-5,10-11].

The chlorinated alicyclic compounds, which were widely insecticides used in the past including aldrin, dieldrin, heptachlor and their structural analogs, constitute a large group of compounds which are environmentally and toxicologically important [6-7]. Their neurochemical action occurs through their binding to the γ-aminobutyric acid (GABA) receptor at the chloride channel [6]. These compounds are generally very non-polar; for example, aldrin has a log K_{ow} as high as 5.9 [9]. But unfortunately, few log K_{ow} 's have been documented for this class of compounds. Direct measurement of their *n*-octanol/water partition coefficients by the conventional 'shake-flask' method is difficult because of their highly lipophilic chracteristics and the availability of adequate amounts for the measurement. In this paper, we are reporting a

systematic study of the lipophilicity of these compounds by using a RP-HPLC method, and the measured data will be used in the on-going reseach of quantitative structure-activity relationships (QSAR) for the compounds.

MATERIAL AND METHODS

Chemicals. The purity for each of the 15 chlorinated alicyclic compounds is greater than 98%. The structures of the compounds were further confirmed by proton-NMR spectra. A stock solution of each compound was made at a concentration of 1mg/ml in methanol and stored at -20 °C. All other chemicals and solvents were of analytical reagent or of HPLC grade.

Apparatus and Chromatographic Conditions. The RP-HPLC system consisted of a Waters 6000A pump coupled with a U-6K injector, a 4.5 mm i.d. x 3.3 cm C-18 analytical column with a particle size of 3 microns, which was manufactured by Perkin-Elmer Corp., Norwalk, Connecticut, a variable-wave-length ultraviolet detector (Spectroflow 757, ABI Analytical Kratos Division, Ramsey, New Jersey), which was set at 210 nm or 220 nm, and a recorder (Cole-Parmer Instrument Company, Chicago, Illinois).

Measurement of log k'. The dead volume of the system was measured by injecting a 10% $NaNO_3$ solution. The stock solutions of the tested compounds were diluted with methanol to the final injection concentration around 100 μg/ml. A 15-μl injection was made in triplicate. According to their chromatographic behavior, the retention times were determined at five different methanol/water eluent ranges from 60% to 80% of methanol by volume. At each mobile phase composition, the capacity factor was calculated according to $k' = (t_R - t_0)/t_0$, where t_R and t_0 were the retention times of the analyte and of the non-retained compounds respectively. The log k_w values, were obtained

from y-intercept of the plots of log k' *versus* volume fraction of methanol in the mobile phase.

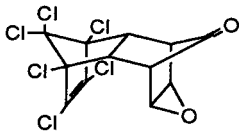
RESULTS AND DISCUSSIONS

The structural information of the 15 tested compounds is given in Figure. 1.

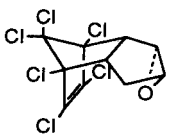
The chromatographs of all tested compounds were accomplished under a variety of conditions in which the volume fraction of methanol (ϕ) in the mobile phase varied from 0.60 to 0.80, since smaller fractions of this component led to unreliable and long retention times. Most of the chlorinated alicyclic compounds are very hydrophobic. This attribute results in unreliably long retention times and trailing of separations by using the commonly used C-18 columns (10 cm or 25 cm in length). However these obstacles were eliminated by introducing a 4.5 mm i.d. x 3.3 cm C-18 analytical column packed with 3-micron support, and this allowed the tested compounds to be eluted at a reasonable time even in the case of the most polar mobile phase. Separations were improved by adding a trace amount of phosphoric acid at a concentration of 0.01% by volume to the mobile phases, and the reproducibility of retention behavior was not affected for the tested compounds (see Figure 2.).

Retention capacity factors (k') at each methanol fraction are given in Table 1 for the 15 tested compounds. Although the monocratic log k's are possibly correlated to other lipophilic descriptors, the established log k' - lipophilicity correlation for a given class of compounds cannot be extrapolated either to different solutes or to other similar or even identical separation systems, and it may result in misleading data owing to solute-solvent interactions [4].

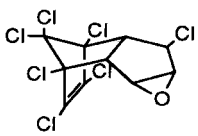
The log k_w, the retention capacity factor of a compound when 100% water is employed as mobile phase in a RP-HPLC system, was used for evaluating the lipophilicity of the compounds because it eliminated



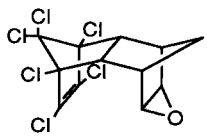
1. 12-Ketoendrin



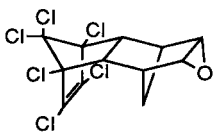
2. Chlordene epoxide



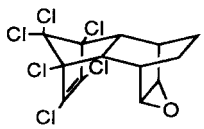
3. Heptachlor epoxide



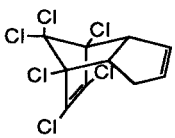
4. Endrin



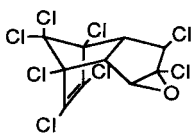
5. Dieldrin



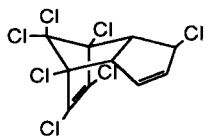
6. Epoxide of 14



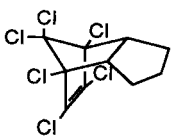
7. Chlordene



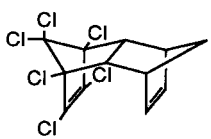
8. Oxychlordane



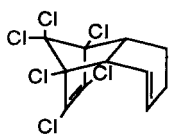
9. Heptachlor



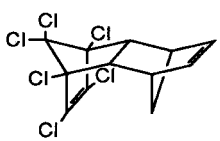
10. Dihydrochlordene



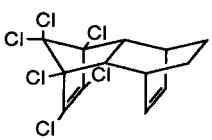
11. Isodrin



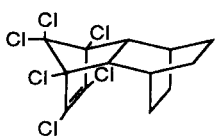
12. @



13. Aldrin



14. @@



15. Octahydro-derivative of 14

FIGURE 1. Structures of the Tested Compounds

@: Hexachlorocyclopentadiene/cyclohexa-1,3-diene adduct
@@: Hexachloronorbornadiene/cyclohexa-1,3-diene adduct

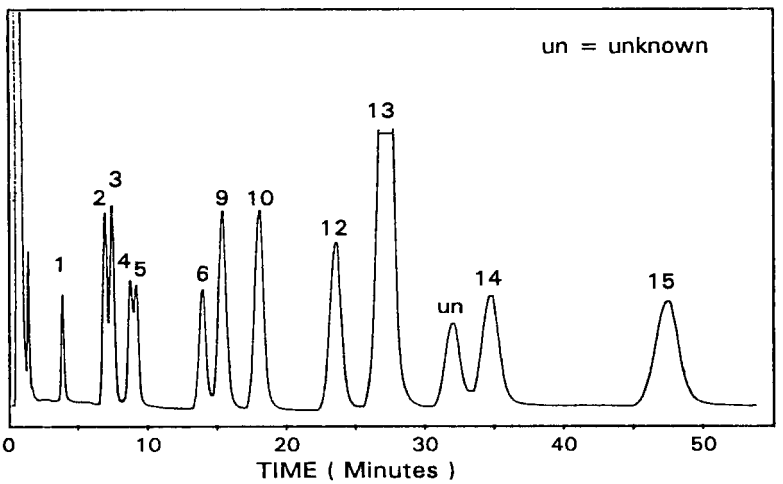


FIGURE 2. HPLC Profile of the Tested Compounds. Column: 4.5 mm i.d. x 3.3 cm C-18 cartridge pack; Mobile phase: 70/30 methanol/water + 0.01% H_3PO_4; Flow rate: 1.0 ml/min; Detector: UV-210 nm; Temp.: 25 °C.

selective solute-solvent interactions and is more closely related to log K_{ow} than isocratic capacity factors [4, 12-16].The log k_w' was determined by extrapolating the polycratic retention capacity factors (log k's) from binary eluents to 100% water. It was found that for the 15 tested compounds, the relationship between solute retention and the composition of methanol in the mobile phase can be described by the equation:

$$\log k' = \log k_w - S\,\phi \qquad (1)$$

where S refers to the slope of log k' *vs.* ϕ plots. The corrlation coefficients ($\gamma > 0.99$) showed that log k' and ϕ were highly linearly correlated for the 15 compounds. The slopes for the equations were in the scope of 4.84 to 6.93. The extrapolated log k_w's are given in Table 2.

TABLE. 1 Isocratic Retention Capacity Factors (k') of the Tested Compound

Compound	ϕ: Methanol/water (v/v)				
Number	0.60	0.65	0.70	0.75	0.80
1	22.737	11.562	7.342	3.991	2.388
2	46.286	23.366	14.081	6.887	4.253
3	53.571	26.158	15.211	7.695	4.141
4	63.395	30.921	18.363	9.296	5.285
5	71.150	33.406	19.443	9.620	5.252
6	112.514	52.522	30.467	14.946	8.019
7	120.974	54.229	28.844	14.345	7.594
8	136.601	59.478	30.662	14.495	7.215
9	136.601	60.777	33.650	14.956	7.722
10	161.867	71.660	39.641	18.238	10.103
11	220.067	87.790	50.001	20.690	11.278
12	222.032	96.616	52.047	21.391	12.224
13	267.860	113.063	60.476	24.074	13.832
14	357.519	149.764	78.426	29.466	17.502
15	517.449	210.398	108.462	43.807	20.961

TABLE 2. Linear Relationship between Log k' and ϕ:
$\log k' = \log k_w - S\,\phi$

Compound	S	$\log k_w$	γ^2 (n = 5)*
1	4.84	4.24	0.9970
2	5.21	4.78	0.9965
3	5.51	5.02	0.9987
4	5.36	5.00	0.9979
5	5.61	5.20	0.9980
6	5.68	5.44	0.9980
7	5.96	5.64	0.9983
8	6.34	5.92	0.9989
9	6.21	5.85	0.9980
10	6.01	5.79	0.9971
11	6.42	6.16	0.9945
12	6.35	6.14	0.9956
13	6.49	6.30	0.9948
14	6.70	6.55	0.9939
15	6.93	6.86	0.9989

* γ = Correlation coefficient.

The relationship between the slope S and the intercept values (log k_w) was investigated for the tested compounds. A good linear correlation was observed: correlation coefficient = 0.9937. Slope S depends on the size of the solute molecule and the structure of polar functional groups. The high linear correlation coefficient may be a reflection of the uniqueness and suitability of the methanol-water system for estimating the lipophilicity of the compounds [4].

CONCLUSION

The retention capacity factor (log k') of a compound in a RP-HPLC system can be used as a descriptor of its lipophilicity. The isocratic log k' was measured at five different compositions of the eluent, and the log k_w was extrapolated from the linear relationship between log k' and the fraction of methanol in the mobile phase for each of the 15 chlorinated alicyclic compounds. The log k_w's may be advantageous in describing the lipophilic properties of the structurally related, very nonpolar chlorinated alicyclic compounds, which for use in QSAR studies.

ACKNOWLEDGEMENTS

This study is supported by USAF Office of Scientific Research Grant No. 91-0338. It is journal article J-15622 of the Iowa Agriculture and Home Economics Experiment Station, Project No. 3187.

REFERENCES

1. Van de Waterbeemd, H. and Testa, B., The parameterization of lipophilicity and other structural properties in drug design. Adv. Drug Res. 16: 85, 1987.

2. Ellgehasen, H., D'hondt, C. and Fruerer, R., Reversed-phase chromatography as a general method for determining octan-1-ol / water partition coefficient. Pestic. Sci. 12: 219, 1981.

3. Garst, J. E. and Wilson, W. C., An accurate, wide-range, automated, high-performance liquid chromatographic method for the estimation of octanol/water partition coefficient. I. Effect of chromatographic conditions and procedure variables on accuracy and reproducibility of the method. J. Pharm. Sci. 73: 1616,1984.

4. Braumann, T., Determination of hydrophobic parameters reversed-phase liquid chromatography: theory, experimental techniques, and application in the studies on quantitative structure-activity relationships. J. Chromatogr. 373:191, 1986.

5. Calvino, R., Fruttero, R. and Gasco, A., Reversed-phase high-performance liquid chromatographic study of the lipophilicity of a series of analogues of the antibiotic "calvatic acid". J. Chromatogr. 547: 167, 1991.

6. Lawrence, L. J., and Casida, J. E. Stereospecific action of pyrethroid insecticides on the γ-aminobutyric acid receptor-ionophore complex. Science 221: 1399-1401, 1983.

7. Coats, J. R., Mechanism of Toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. Environ. Health Perspect. 87: 255, 1990.

8. Hartley, D., and Kidd, H. (Ed.), The Agrochemicals Handbook, The Royal Society of Chemistry, Nottingham, England, 1989.

9. Kenaga, E. E. and Goring, C. I. A., Relationship between water solubility, soil sorption, octanol-water partitioning, and concentration of chemicals in biota. Aquatic Toxicol., ASTM STP 707, Eaton, J. G., Parrish, P. R., and Hendricks, A. C., eds. American Society for Testing Materials, Philadephia, pp78-115, 1980.

10. Demotes-Mainard, F., Jarry, C., Thomas, and Dallet, P., RP-HPLC retention data of new 2-amino-2-oxazolines. An approach of their lipophilic properties. J. Liquid Chromatogr. 14 (4): 795, 1991.

11. Pereira, A. L., Barreiro, E. J. L., Freitas, A. C. C., Correa, C. J. C., and Gomes, L. N. L. F., Investigation of the lipophilicity of antiphlogistic pyrazole derivatives: Relationships between log Kw

and Log P values of 5-arylamino and arylhydrazono-3-methyl-4-nitro-1-phenylpyrazoles. J. Liquid Chromatogr. 14 (6): 1161, 1991.

12. Hammers, W. E., Meurs, G. J., and de Ligny, C. L., Correlation between liquid chromatographic capacity ratio data on Lichrosorb RP-18 and partition coefficient in the octanol-water system. J. Chromatogr. 247: 1, 1982.

13. El Tayar, N., Van de Waterbeemd, H. and Testa, B., The prediction of substituent interactions in the lipophilicity of disubstituted benzenes using RP-HPLC. Quant. Struc.-Act. Relat. 4: 69, 1985.

14. Harnisch, M., Möckel, H. J. and Schulze, G., Relationship between log P_{ow} shake-flask values and capacity factors derived from revesed-phase high performance liquid chromatography for n-alkylbenzenes and some OECD references. J. Chromatogr. 282: 315, 1983.

15. Thus, J. I. G. and Kraak, J. C., Comparision of phenyl- and octadecyl-modified silica gel as stationary phase for the prediction of n-octanol-water partition coefficient by high-performance liquid chromatography. J. chromatogr. 320: 271, 1985.

16. Butte, W., Fooken, C., Klussmann, R. and Schuller, D., Evaluation of lipophilic properties for a series of phenols, using reversed-phase high performance liquid chromatography ang high performance thin layer chromatography. J. Chromatogr. 214: 59, 1981.

17. Murakami, F., Retention behavior of benzene derivatives on bonded revesed-phase columns. J. Chromatogr. 178: 393, 1979.

Received: December 3, 1993
Accepted: December 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 2005-2016 (1994)

DETERMINATION OF MINOR IMPURITIES AND DIASTEREOMERS OF 6-[3-[(2-AMINO-1-OXOPENTYL)AMINO]-1-PYRROLIDINYL]-5-FLUORO-3-OXO-3H-PYRIDO[3,2,1-KL]PHENOXAZINE-2-CARBOXYLIC ACID HYDROCHLORIDE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

TIMOTHY G. GOLICH*, LEE ELROD, JR., AND JAMES A. MORLEY
PPD Physical Analytical Chemistry Department
Abbott Laboratories
North Chicago, Illinois 60064

ABSTRACT

Diastereomers of the quinobenoxazine antineoplastic drug Abbott-84441.1 and minor impurities were determined using high-performance liquid chromatography. Manufacturing impurities, degradation products, and diastereomers were separated using a reversed-phase system with gradient elution. Detector response was linear for Abbott-84441.1 to approximately 20 μg/mL which represents 4.0% of the drug concentration. The procedure provides quantitation of impurities to approximately the 0.1% level with precision (relative standard deviations) of 7.3% to 31% in typical bulk drug lots. A variety of reversed-phase columns were evaluated for the assay method with the optimum resolution achieved using a 5-μm Alltima C_{18} packing.

*Author to whom correspondence should be sent.

INTRODUCTION

The synthesis of Abbott-84441.1, a quinobenoxazine antineoplastic drug, has been previously reported [1,2]. Chemically, the drug substance is 6-[3-[(2-amino-1-oxopentyl)amino]-1-pyrrolidinyl]-5-fluoro-3-oxo-3H-pyrido[3,2,1-kl]phenoxazine-2-carboxylic acid hydrochloride. As reported recently [1], this class of compound has in vivo activity against solid tumors and leukaemias of marine origin as well as human tumor xenografts. This paper describes the use of high-performance liquid chromatography (HPLC) for quantitation of minor impurities which can occur in Abbott-84441.1 bulk drug substance. Potential manufacturing impurities and degradation products are determinable by the procedure in addition to the ratio of diastereomers in the bulk drug.

Various methods using HPLC have been reported for the determination of fluoroquinolones and their metabolites[3-7]. The HPLC technique allows the direct determination of these materials without the derivatization which is necessary in gas chromatographic procedures [8-9]. Previously this laboratory has reported the quantitation of impurities in other quinolones using reverse-phase chromatography and gradient elution, but those compounds did not contain the amino acid substituent [10-11]. For Abbott-84441.1 the presence of the L-norvalyl-substituent provides a pair of diastereomers (Abbott-79775.1, (2'S,3S)-6-[3-[(2-amino-1-oxopentyl)amino]-1-pyrrolidinyl]-5-fluoro-3-oxo-3H-pyrido[3,2,1-kl]phenoxazine-2-carboxylic acid hydrochloride and Abbott-79583.1, (2'S,3R)-6-[3-[(2-amino-1-oxopentyl)amino]-1-pyrrolidinyl]-5-fluoro-3-oxo-3H-pyrido[3,2,1-kl]phenoxazine-2-carboxylic acid hydrochloride) which must be controlled. In this work a separation was pursued to adequately resolve the diastereomers and in the same chromatographic run quantitate minor impurities and degradates which can show marked differences in retention times using reversed-phase systems.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Model SP-8800 ternary pump and Chromjet data handling system (Spectra-Physics, Santa Clara, CA, USA). A Model 757 variable-wavelength UV detector (ABI Analytical Kratos Division, Ramsey, NJ, USA) and a Model WISP-710B (Waters Associates, Milford, MA) autosampler were used. Chromatographic separations described in this method were made using Alltima C_{18} columns (5 μm, 100Å) measuring 15 cm x 4.6 mm I.D. (Alltech Associates, Deerfield, IL, USA). The following columns were also evaluated for their suitability: Nucleosil C_{18} 4.0 mm x 125 mm, 5 μm, Macherey-Nagel (Bodman Chemicals, Aton PA); CSC-Nucleosil C_{18}, 4.6 mm x 150 mm, 5 μm (Chromatographic Sciences Company (CSC), Resolution Systems, Wilmette, IL); Zorbax SB-C_{18}, 4.6 mm x 250 mm (Mac Mod Analytical, Chadds Ford, PA), 5 μm; Lichrosorb RP-8, 4.6 mm x 250 mm, 5 μm (Alltech); Inertsil C_{18}, 4.6 mm x 250 mm, 5 μm (Metachem Technologies, Redondo Beach, CA). Prior to use, the components of the eluent were filtered through 0.45 μm nylon membranes (Alltech).

Reagents

Acetonitrile and methanol were HPLC grade from EM Sciences (Cherry Hill, NJ, USA). Both sodium citrate dihydrate and citrate acid were reagent grade and were from Fisher Scientific (Fair Lawn, NJ, USA) and Mallincrodkt (St. Louis, MO), respectively. Disodium EDTA and dimethylsulfoxide (DMSO) were reagent grade and were from Aldrich Chemical Co.(Milwaukee, WI, USA). A citrate buffer solution containing 0.01 M citrate and 0.001 M EDTA was prepared by dissolving 2.93 g of sodium citrate·$2H_2O$, 1.96 g citric acid, and

0.75 g EDTA in 2 liters of deionized water and adjusted to pH 3.0 using perchloric acid (Fisher Scientific). All bulk drugs and related impurities were synthesized at Abbott Labs (North Chicago, IL, USA). Isolated compounds were characterized by ^{1}H, ^{13}C, ^{19}F NMR and mass spectrometry. The organic modifier was prepared by mixing 1 liter of acetonitrile and 1 liter of methanol. The diluent was prepared by mixing 1 liter of citrate buffer solution and 1 liter of organic modifier.

Chromatographic Conditions

A linear gradient was used, mixed with the ternary pump as shown in Table I. Other conditions were: flow-rate, 1.0 mL/min; pressure, approximately 1500 psi; detector, 326 nm at 0.10 a.u.f.s., attenuation at 32, and injection volume, 20 μL. All separations were performed at ambient temperature.

Analytical Procedure

Abbott-84441.1 bulk drug samples were prepared by initially dissolving approximately 50 mg of drug substance in 50 mL of DMSO. The solution was diluted with diluent to 100 mL for a sample concentration of approximately 0.5 mg/mL. A 1% standard was prepared by serially diluting the above stock solution 5 mL to 50 mL then 5 mL to 50 mL with diluent. The amounts of impurities were estimated in the sample by comparing the corresponding peak areas in the sample and standard preparations. Impurity content was calculated on the anhydrous basis by correcting the sample concentration for the amount of water contained in the drug substance. The drug substance typically contains approximately 2-4% water, as measured by Karl Fischer titration.

Table I
Linear Gradient for HPLC Eluent

Time (min)	Citrate Buffer (%)	Organic Modifier (%)
0	55	45
30	55	45
60	10	90
75	10	90
76[a]	55	45
90[a]	55	45

[a] Used to re-equilibrate the column to the initial conditions.

RESULTS AND DISCUSSION

Since Abbott-84441.1 has both acid and base functional groups, the chromatographic behavior on reverse-phase columns is dependent on pH as well as the organic modifier of the eluent. The most symmetrical peak shapes for the drug substance (diastereomers) and impurities were obtained in eluents containing aqueous buffers at pH values of approximately 2 to 4. The amount of retention of the drug substance (diastereomers) and impurities did not vary significantly with the type of buffer. The citrate system used has proven rugged in the analysis of a variety of quinolones. Single isocratic eluents failed to adequately resolve the drug from early eluting impurities while still eluting the more strongly retained impurities within a reasonable time. For this reason, a gradient elution system was developed. Single organic modifiers of tetrahydrofuran, methanol and acetonitrile were used in this approach. Again, no single modifier provided optimum resolution of the diastereomers in combination with resolution of both early and late eluting impurities. Acceptable results were obtained using the ternary solvent system described in the text where acetonitrile/methanol (1:1) was

Table II
COMPARISON OF COLUMN PACKING ON THE RESOLUTION OF ABBOTT-84441.1

Packing	Conditions Citrate Buffer/Acetonitrile	Resolution Factor[a]
Lichrosorb RP-8 4.6 mm x 250 mm, 5 μm	60/40	[b]
Inertsil C-8 4.6 mm x 250 mm, 5 μm	70/30	2.2
Zorbax SB-C-8 4.6 mm x 250 mm, 5 μm	65/35	1.6
Nucleosil C_{18} 4.0 mm x 125 mm, 5 μm (Macherey-Nagel)	70/30	4.1
Nucleosil C_{18} 44.6 mm x 150 mm, 5 μm (CSC)	65/35	2.8
Alltima C_{18} 4.6 mm x 150 mm, 5 μm	67/33	5.8[c]

[a]U. S. Pharmacopia XXII, p 1867.
[b]unacceptably Poor Peak Shape.
[c]Typical resolution factors are 7-8 for the method described in the text.

used as the modifier, thereby producing the needed resolution for early and late eluting impurities, as well as for impurities retained close to the diastereomers of Abbott-84441.1. A detection wavelength of 326 nm provides a very similar response for the impurities and drug substance, providing an accurate estimation of unknowns quantitated versus the drug substance. In this procedure EDTA was included as an additive in the eluent to minimize the adsorption of the drug to metal surfaces and to sharpen the peak shape due to trace metals present. Quinolones can form strong complexes with metals [12].

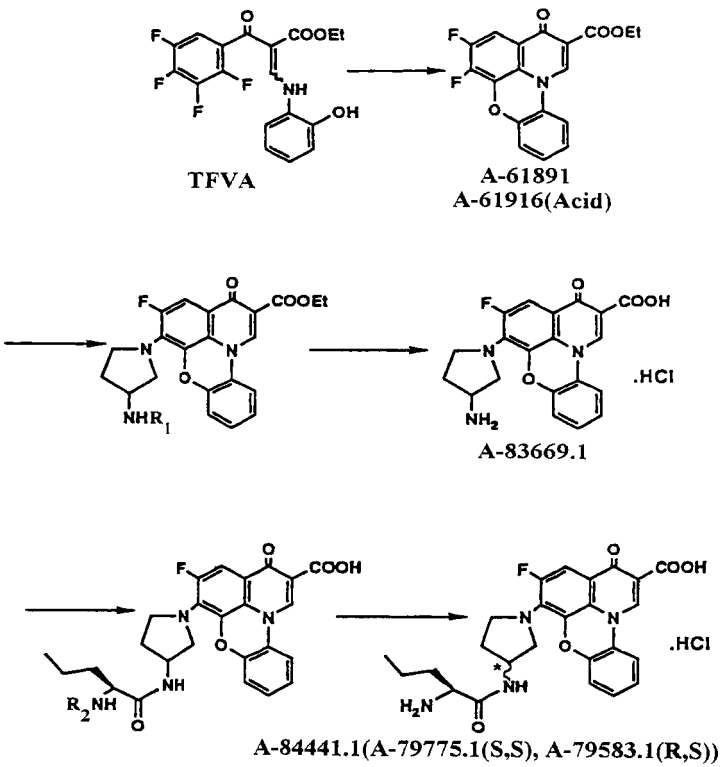


Figure 1. **Synthetic scheme for preparation of Abbott-84441.1**

Route 1: R_1=H, R_2=carbobenzoxycarbonyl (CBZ)

Route 2: R_1=R_2=t-butyloxycarbonyl (Boc)

Several C-8 and C-18 reversed-phase packings were evaluated for the determination of Abbott-84441.1 impurities. For this evaluation, similar isocratic profiles were used as described in the text. However, the initial amount of organic solvents in the starting conditions was adjusted to produce similar retention times for the diastereomers of Abbott-84441.1. The results are

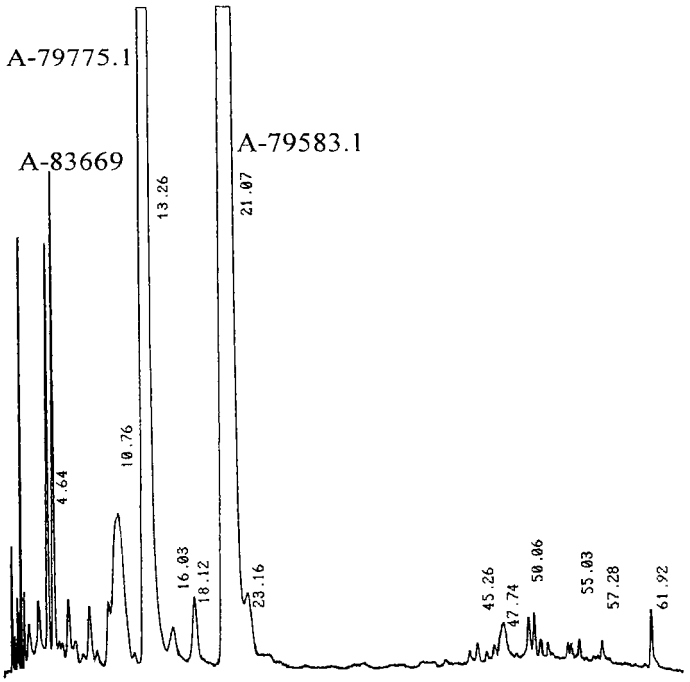


Figure 2. Representative Chromatogram of Abbott-84441.1 Prepared from Route 1

summarized in Table II. The comparision of the resolution factors calculated between the diastereomers of Abbott-84441.1, which is a critical separation in our application, demonstrates that the separation is largely a function of the type of reversed-phase column used. The system used in the procedure is also more useful than others evaluated in the resolving more impurities.

The HPLC conditions described in the text were developed to resolve the drug substance (diastereomers), manufacturing impurities and possible degradation

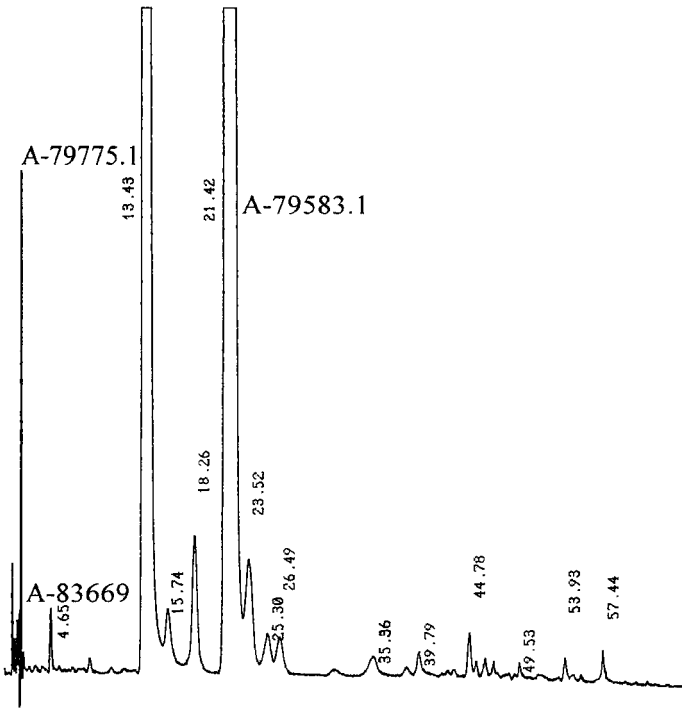


Figure 3. Representative Chromatogram of Abbott-84441.1 Prepared from Route 2

products by different synthetic routes. Shown in Figure 1 are schemes for two synthetic routes used to produce the bulk drug. The routes differ in the method of protection of the L-norvaline amino acid portion and the primary amine on the pyrrolidine ring. Different manufacturing impurities arise from the different synthetic routes.

Shown in Figures 2 and 3, are typical chromatograms of representative lots of Abbott-84441.1 prepared as illustrated. Figure 4 shows an identity

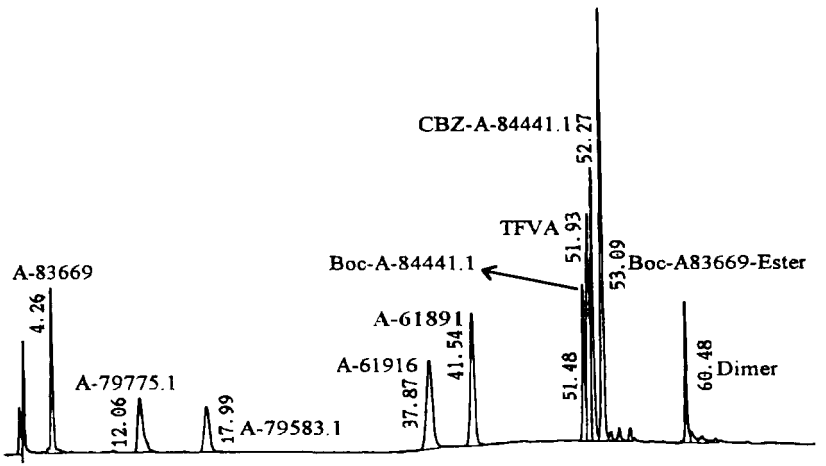


Figure 4. Synthetic Mixture of Typical Impurities Seen in Abbott-84441.1

mixture of various impurities. Comparison of the two chromatograms shows the presence of similar and dissimilar impurities arising from the two synthetic routes. The precursor A-83669 (RT = 4.5 minutes) is present in both samples, although route 1 contains significantly more. Other unknown impurities (RT= 16, 18, and 23 minutes) are formed in both routes. These appear to be isomers of Abbott-84441.1 from the limited LC-MS data obtained. Figure 2 also shows the presence of a dimerized impurity which forms by coupling across the 3-aminopyrrolidine moiety (RT= 61 minutes) and isomers at RT=10.8 minutes. These impurities were identified by LC-MS and NMR techniques. These types of impurities have been observed previously in other quinolones. N-acetal impurities can be detected at RT=30 and 35 minutes. The region between 40 and 58 minutes typically contains peaks at 0.2% or less and is the region where the protected intermediates elute (Figures 2 and 3). These components have not been identified.

Detector response for Abbott-84441.1 was linear to at least 20 μg/mL (correlation coefficient $\geq$ 0.9999). Linearity curve of concentration versus detector response essentially intersected the origin, allowing the use of one-point calibration for quantitation of impurities. Assay precision was assessed by performing the procedure on one lot of Abbott-84441.1. Two analysts performed the determinations on different days. The assay precision (relative standard deviation values) ranged from 31% to 7.3% for impurities having mean values of 0.1% to 1.3%.

ACKNOWLEDGEMENTS

The authors thank Dr. Jeff Wang, Mr. Steve Cepa and Mr. Mike Cirovic for their assistance in the spectral identification of related minor impurities, Mr. Grant Esser, Mr. Marzban Vandrevala, Dr. Padam Sharma and Mr. Lee Reif for synthesis support, and Ms. Diane Horgen for her assistance in preparation of the manuscript.

REFERENCES

1. D.T.W. Chu, R. Hallas, J. J. Clement, J. Alder, E. Mc Donald, J. J. Plattner, Drugs Exptl. Clin. Res. XVIII, 7 (1992) 275.
2. D.T.W. Chu, R.E. Maleczka, J. Heterocyclic Chem., 24 (1987) 453.
3. C. Y. Chan, A. W. Lam, G. L. French, J. Antimicrob. Chemother., 23 (1989) 597.
4. A. LeCoquic, R. Bidault, R. Farinotti, A. Dauphin, J. Chromatogr., 434 (1988) 320.

5. M. C. Myers, J. L. Blumer, J. Chromatogr., 422 (1987) 153.

6. S. Horil, C. Yasuoka, M. Matsumoto, J. Chromatogr., 388 (1987) 459.

7. G. R. Granneman, L. T. Sennello, J. Chromatogr., 413 (1987) 199.

8. K. J. Wang, S. H. Chen, S. J. Lin, H. L. Wu, J. Chromatogr., 308 (1986) 443.

9. H. L. Wa, T. Nakagawa, T. Uno, J. Chromatogr., 157 (1978) 297.

10. L. Elrod, Jr., T.G. Golich, D. I. Shaffer, B. P. Shelat, J. Liquid Chrom., 15 (1992) 451.

11. L. Elrod, Jr., C. L. Linton, B. P. Shelat, C. F. Wong, J. Chromatogr., 519, (1990) 125.

12. K. Timmers and R. Sternglanz, Bioinorganic Chem., 9 (1978) 145.

Received: November 5, 1993
Accepted: December 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 2017-2029 (1994)

DETERMINATION OF PENTAMIDINE IN *LEISHMANIA* INFANTUM PROMASTIGOTES BY ION-PAIRED LIQUID CHROMATOGRAPHY

B. RABANAL[1], R. G. DE ARRIBA[1], M. J. GARZÓN[1],
R. M. REGUERA[2], R. BALAÑA-FOUCE[2], AND A. NEGRO[1]*
[1]*Departamento de Bioquímica y Biología Molecular*
[2]*Departamento de Fisiología, Farmacología y Toxicología*
Universidad de León
E-24071, León, Spain

ABSTRACT

Pentamidine is an aromatic diamidine, which have recently been shown to be non-competitive inhibitors of putrescine uptake in parasitic protozoa. In order to understand the mechanism involved in Pentamidine uptake by parasitic protozoa cells, a HPLC analytical method for determination of pentamidine in *Leishmania* infantum promastigote cultures was developed.

The influence on the capacity factor of various characteristic parameters in the mobile phase such as pH, percentage of methanol and temperature of column is studied, taking into account that pentamidine can form ion-pairs with sulphonic compounds, the influence of the length of the lateral chain and concentration of various compounds of this type are also studied.

The results obtained in this study allow us to know the best chomatographic conditions for the determination of pentamidine in *Leishmania* infantum promastigotes and they can also be used for the optimization of analytical methods in order to determine pentamidine in other biological media or other complex mixtures.

The chromatographic procedure uses a reversed-phase column Nucleosil C_{18} 5μm, the column effluent was monitored by ultraviolet-visible spectrophotometry at 261 nm. The procedure involves a simple method of the cleaning-up *Leishmania* promastigote extract samples cultured with pentamidine

by ultrafiltration through a polysulfone membrane with 100,000 relative molecular mass cut-off.The method shows good recovery, precision and accuracy. The limit of detection for pentamidine is 5.70 ng/ml in *Leishmania* cells culture.

INTRODUCTION

Polyamine (putrescine, spermidine and spermine) metabolism in lower eukaryotes is a highly regulated biosynthetic pathway involved in proliferative proccesses which have been widely used for therapeutic purposes in parasitic infection diseases (1). The enzymes involved in polyamine biosynthesis have been targeted with a large number of compounds either specifically synthesized to inhibit these enzymes or those whose chemical structure resembles higher polyamines (2). Aromatic diamidines belong to this last kind of compounds. These drugs were designed as antiparasitic compounds, being successfully used against infections produced by species of *Trypanosoma, Leishmania* and *Babesia* genus both in human and veterinary medicine (3). Pentamidine is one of the most important drugs of this family, which is micromollar inhibitors of S-adenosyl-L-methionine decarboxylase (SAMDC), the enzyme involved in spermidine biosynthesis, and Diamine Oxidase, the key enzyme of polyamine terminal catabolism in mammals and lower eukaryotic cultures (4,5). Recently, aromatic diamidines have been shown to be non-competitive inhibitors of putrescine uptake in parasitic protozoa (6).

Several chromatographic methods have been developed in order to determine pentamidine in serum and urine (7,8,9). The aim of this work was to develop a HPLC analytical method specific with regard to retention times and sample preparation for the determination of pentamidine in *Leishmania* infantum promastigotes cultures so as to study the mechanisms implied in pentamidine accumulation by parasitic protozoa cells. In order to determine the optimal chromatographic conditions we carried out a wide study on the influence which it has on the capacity factor of various parameters related to the mobile phase such as, pH variation, methanol variation percentage, temperature of column and the influence of the chain length and concentration of different sulphonate salts which form ion-pairs with the pentamidine guanidinio groups.

MATERIALS AND METHODS.

Chemicals and materials

Pentamidine Isethionate salt is (1,5-bis [p-amidinophenoxy] pentane bis [2-hydroxyethanesulfonate salt] was supplied by Sigma Chemical Co. (St. Louis. USA). HPLC-grade methanol was supplied by Farmitalia Carlo Erba (Milan. Italy). The ion-pairing agents pentane, hexane, heptane, octane, and decane sulphonates sodium salts were supplied by Sigma Chemical Co. Medium 199 and gentamicin sulphate (150 μg/ml) were purchased from Sigma Chemical Co. (St Louis. USA). The foetal calf serum came from Boehringer Manhein. The water was purified using a Milli-Q II water purification system purchased from Millipore (Bedford MA. USA). All other chemicals were of analytical grade. The ultrafiltration system with a 100 000 relative molecular mass cut-off polysulfone membrane, (Ultrafree MC UFC3THK 25), was purchased from Millipore.

Chromatographic System and Conditions

The HPLC system consisted of a Beckman 116 programable solvent pump. Beckman 116 absorbance detector with "System Gold" chromatography software and a Reodyne 7125 20 μl loop injector. Analyses were carried out on a reversed-phase Nucleosil C_{18} column (5 μm particle size, 20 cm x 0,2 cm I.D) purchased from Teknokroma (Barcelona. Spain). A guard column (2 cm x 2 mm I.D) packed with Spherisorb RP-18 (30-40 μm pellicular) was supplied by Upchurch Scientific (Oak Harbor. WA. USA). The most adequate mobile phase and the one which was used in the analysis of real samples of Leishmania cultures is made up of 5.0 mM of citric acid-methanol (50 : 50 v/v) and pentane sulphonate sodium salt 5.0 mM adjusted to pH = 4.0 with NaOH. The flow-rate was 0.5 ml/min. The detection wavelength was 261 nm All injection volumes for HPLC analysis were 40 μl (a 20 μl loop being used). The whole system was kept at room temperature ($22^{\circ} \pm 3^{\circ}$ C)

Standard Solutions

Standard solutions of pentamidine were prepared by dissolving pentamidine isethionate salt in Milli Q II purified water in order to obtain

solutions of the concentrations in pentamidine (172.0 ; 17.20 ; 8.60 ; 4.30 ; 1.72 and 0.43 μg/ml). All the standard solutions were stored at 4.0° C.

Solutions for the calibration curve

Solutions for the calibration curve were pentamidine-free *Leishmania* extracts mixed with the appropiate quantity of an aqueous 17.2 μg/ml standard solution of pentamidine and with 10 μl of ortophosphoric acid - water 10 % (p/p) to obtain 400 μl of final mixture of acidulated extracts with pentamidine concentrations of 8.60 ; 4.30 ; 1.72 ; 0.43 and 0.0129 μg/ml to be obtained. The resultant mixture was vortex-mixed and transfered to an Ultrafree-MC ultrafiltration system with a 100,000 relative molecular mass cut-off polysulphone membrane and centrifuged for 5 min. at 5 000 g. The clear filtrate was used directly for chromatographic analysis.

Samples of Leishmania treated with pentamidine

Extracts of *Leishmania* infantum promastigotes cultured with pentamidine in concentrations 0.03 ; 0.06 ; 0.14 ; 0.29 and 0.57 mM were treated using the same procedure as the solutions for the calibration curve and were without the addition of pentamidine.

Cell culture

Leishmania infantum promastigotes obtained by thermic transformation of amastigotes (PEP1G11 line) from lymph nodes of a naturally infected dog, were kindly supplied by Dr. J.M. Alunda and L.Carrera , Dpto. Patología Animal I , Universidad Complutense de Madrid. Cells were routinally grown at 26.5 °C in medium 199 supplemented with 10% heat inactivated foetal calf serum and gentamicin sulphate (150 μg/ml). Promastigotes were subcultured once a week and used with a low number of subpassages. Cell growth was estimated by counting in improved Neubauer chambers.Pentamidine isethionate was dissolved in water purified by Milli Q II and sterilised prior to its addition to the cultures. In order to determine the inhibitory effect of pentamidine, this compound was added at several concentrations to the cultures, and after four days subpassage, cell viability was estimated. In order to evaluate the pentamidine internalized by

cells, late lag phase promastigotes (3rd day post inoculation) were pulsed with 0.03 ; 0.06 ; 0.14 ; 0.29 and 0.59 mM pentamidine. Twenty four hours afterwards, cells were harvested, washed in phosphate saline-glucose (99:1 w/v) buffer, and resuspended in the same buffer up to a soluble protein concentration of 1.00 mg/ml.

RESULTS AND DISCUSION

Pentamidine (1,5-bis [p-amidinophenoxy] pentane) (Fig.1) is an aromatic diamidine, making it possible to introduce long-chain ionic alkyl sulphonates into the mobile phase in order to form ion- pairs (10) (11). That formation of ion-pairs allows the capacity factor (k') to be easily altered while making it adaptable for use with different biological systems (12) (13) (14). In order to obtain an efficient method for the determination of pentamidine in *Leishmania,* we studied the effect on the capacity factor (k') by varying some chromatographic parameters: concentration and chain length of the ion-pairing forming agent, pH, percentage of methanol in the mobile phase and temperature of column. The influence of the length and concentration of the side chain of the ion-pair forming agent is shown in Fig. 2. The effect of sulphonates salt concentrations on (k') was studied by measuring capacity factor using mobile phase consisting of 5.0 mM citric acid - methanol (50:50 v/v) containing pentane, hexane, heptane and octane sulphonate sodium salts at concentrations of 0.0 ; 5.0 ; 10.0 ; 20.0 and 30.0 mM respectively and adjusting to pH = 4.0 with NaOH. A lineal increase in the capacity factor on adding different sulphonic salts formed from ion-pairs studied is observed. This increase is pronounced as hydrocarbonated chain length increases, which is to be expected.

The effect of pH on k' is shown in Fig.3 and was studied using a mobile phase consisting of 5.0 mM citric acid - methanol (50:50 v/v) and 1.0 mM hexane sulphonate sodium salt., adjusted with NaOH to pH = (3.5 ; 4.5 ; 5.5 and 6.5. A decrease in k' when pH varied from 3.5 to 4.8 is observed; from this value an increase in k' is produced.

The effect of methanol percentage in the mobile phase is shown in Fig.4 and was studied using mobile phase consisting of 5.0 mM citric acid methanol 40 ; 50 ; 60 and 70 %, 1.0 mM hexane sulphonate sodiun salt, adjusted with NaOH to pH = 3.5 . The results obtained show a notable decrease of k'

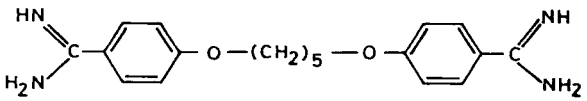


Fig. 1. Structure of pentamidine.

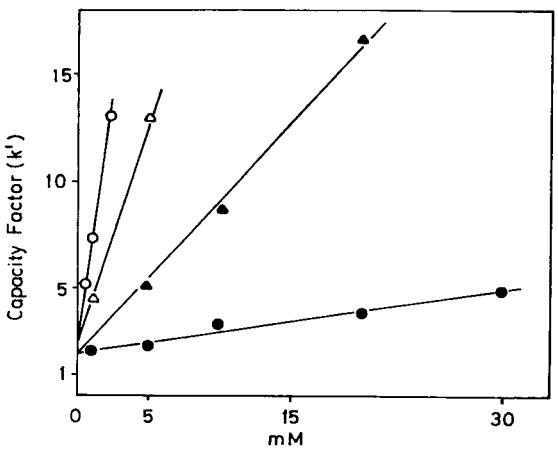


Fig. 2. Influence of length and concentration of the side chain in the capacity factor (k'). This effect was studied using a mobile phase consisting of 5.0 mM citric acid - methanol (50:50 v/v) containing 0.0 ; 5.0 ; 10.0 ; 20.0 and 30.0 mM of pentane (●) , hexane (▲) , heptane (△) and octane (○) sulphonate sodium salts and adjusting to pH = 4.0 with NaOH.

when the percentage of methanol in the mobile phase drops from 30 % to 50% , preactically maintaining a constant k' value when the quantity of methanol increases from 50 % to 70 %.

Figure 5 shows the variation of k' when the temperature of the column varies between 25 and 65° C. The mobile phase used to carry out this test was made up of 5.0 mM citric acid - methanol (50 :50 v/v) and 5.0 mM pentane sulphonate sodiun salt, adjusting to pH = 4.0 with NaOH. The results obtained show that the increase in temperature from 25 to 65°C makes the values of k'

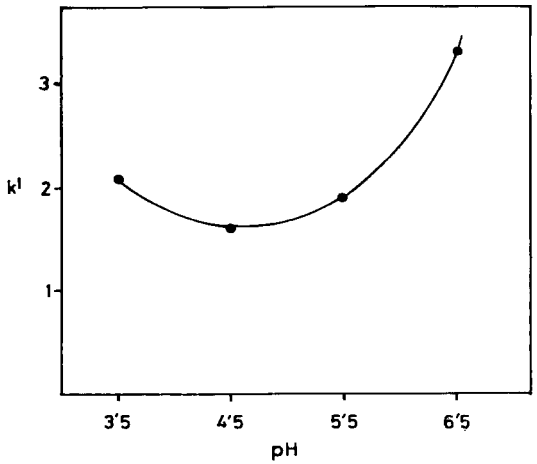


Fig. 3. Effect of variation of pH in mobile phase in the capacity factor of pentamidine. This effect was studied using a mobile phase consisting of 5.0 mM citric acid - methanol (50 : 50 v/v) and ajusting with NaOH to pH = (3.5 ; 4.5 ; 5.5 and 6.5) and 1.0 mM hexane sulphonate sodiun salt.

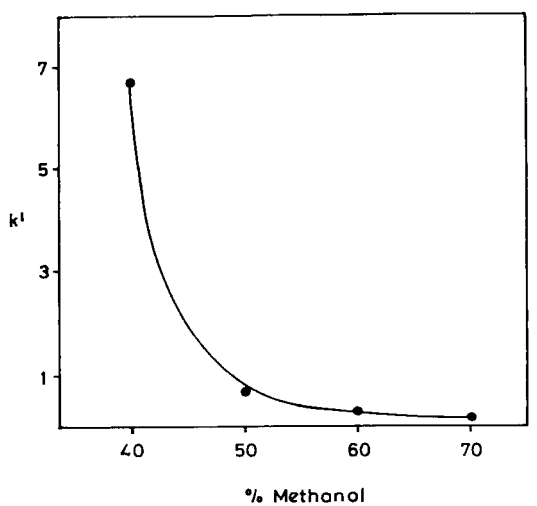


Fig.4 Effect of variation of percentage of methanol content in the mobile phase on the capacity factor of pentamidine. Mobile phase consisting of 5.0 citric acid - methanol 40 ; 50 ; 60 and 70 % and 1.0 mM hexane sulphonate sodium salt adjusting with NaOH to pH = 4.0

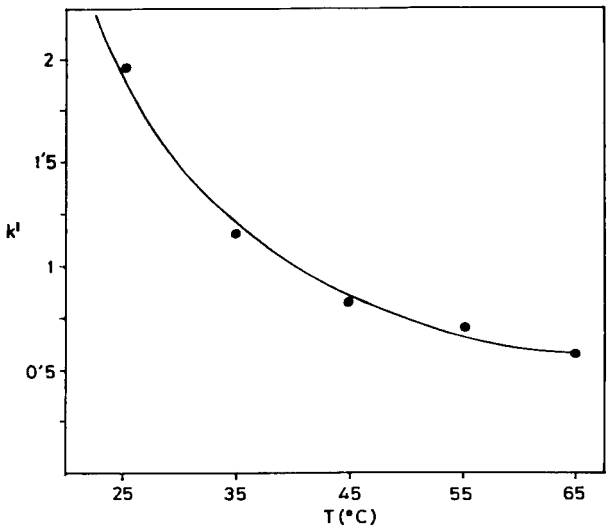


Fig. 5 Effect of column temperature on the capacity factor. Mobile phase consisting in 5.0 mM citric acid - methanol (50 :50 v/v) and 5.0 mM pentane sulphonate sodium salt, adjusting with NaOH to pH = 4.0.

decrease in only 1.5 units. The use of this parameter does not turn out to be of interest in this case as greather values of k' can be obtained on varying other chromatographic factors which are easier to modify. However, the data may be of interest in other cases.

The results of the study allows us to select the optimum conditions for the HPLC determination of pentamidine in *Leishmania* extracts, a mobile phase consisting of 5.0 mM of citric acid - methanol (50:50 v/v) containing 5.0 mM of pentane sulfonate sodium salt, adjusting to pH = 4.0 with NaOH and temperature of column from ($22\pm3^{\circ}$ C)

The chromatograms obtained for a blank extract of *Leishmania* and *for* a extract of *Leishmania* cultured with pentamidine are shown in Fig.6 .

Linearity, Precision and Detection Limit

Linearity, accuracy and precision were determined with blank extracts of *Leishmania* treated, as previously described, with known amounts of

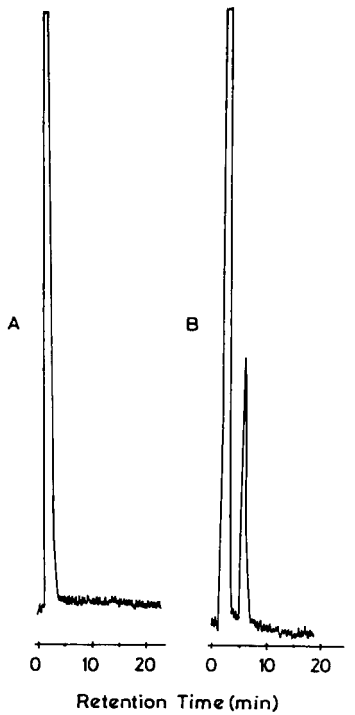


Fig. 6. Chromatograms. (A) Extract of *Leishmania* culture. (B) Extract of *Leishmania* culture with 6.02 ng/ml of pentamidine. The mobile phase used for analysis is 5.0 mM of citric acid - methanol (50:50 v/v) and pentane sulphonate sodium salt 5.0 mM final pH = 4.0. Flow-rate 0.5 ml/min. Column Nucleosil C_{18} (5 μm particle size, 20 cm x 0.2 cm I.D)

pentamidine in order to obtain concentrations of pentamidine in the range 0.0 - 4.30 μg/ml. A linear relationship was observed between the pentamidine peak areas and the concentrations of pentamidine in the extracts. The linear relationship can be expressed by the equation $Y = 170419\ X - 8798$ where Y and X are, respectively, the peak area and the amount of pentamidine in μg/ml. The correlation coefficient was 0.9998.

The accuracy and precision (C.V) of the method (Table I) were determined in extracts with pentamidine concentrations at 8.60 ; 4.30 ; 1.72 and

TABLE I

Accuracy and precission of determination of pentamidine in *Leishmania* culture samples.

Spiked concentration (μg/ml)	n	Observed concentration (μg/ml)	CV (%)	Accuracy (%)
8.60	6	8.94±0.51	4.5	104.0
4.30	6	4.64±0.02	0.7	108.0
1.72	6	1.71±0.20	3.8	99.4
0.43	6	0.37±0.02	7.7	86.7

0.43 μg/ml. The coefficient of variation (C.V) was determined from the equation C.V = (Stardard deviation / Mean value) x 100 and the accuracy of the assay was estimated using the following equation:

Accuracy (%) = (Observed concentration/Spiked concentration) x 100

Accuracy ranged from 87 % to 108 % with a coefficient of variation never exceeding ± 7.7 The detection limit of analytical method in *Leishmania* samples (protein concentration of 1.00 mg/ml) is 5.70 ng/ml with a signal to noise ratio 5:1.

With the aim of proving the interest and validity of the analytical method in real samples, a study of the inhibition of pentamidine in *Leishmania* growth was carried out and the results are shown in Fig. 7. The quantity of pentamidine internalized by *Leishmania infantum* promastigotes grow cultured with pentamidine concentrations 0.03 ; 0.06 ; 0.14 ; 0.29 and 0.57 mM was determined by this analitical method. The results are shown in Table II.

As a system of sample preparation ultrafiltration was used. The advantages of ultrafiltration are that no compounds that might interfere with the HPLC analysis are added to the sample, proteins and macromolecules are removed and the method is quick and efficient. In order to facilitate the passing of all the pentamidine contained in the *Leishmania* sample through the filter and obtain

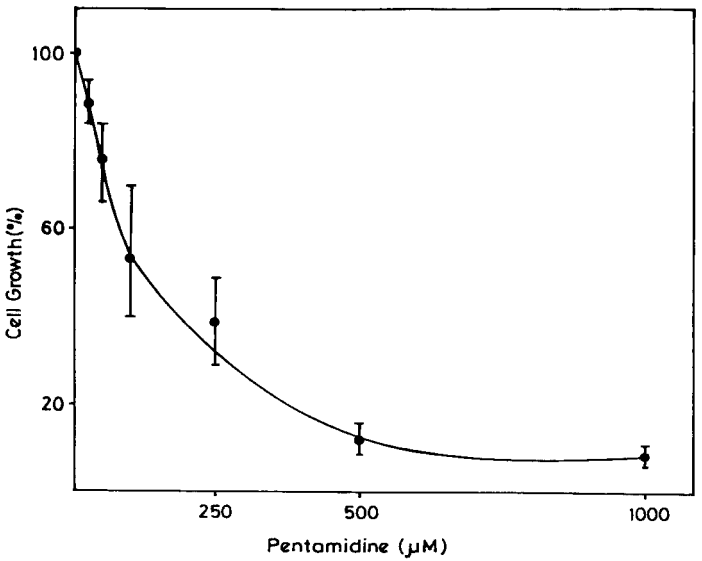


Fig.7. Inhibitory effect of pentamidine in *Leishmania* growth.

TABLE II

Analysis of Pentamidine internalized by cells of *Leishmania*.

Sample	Pentamidine added to the culture (mM)	Protein concentration of the extract (mg/ml)	Pentamidine observed concentration (μg/ml)	Pentamidine internalized*
1	0.03	1.34	0.12	0.09
2	0.06	1.39	0.15	0.11
3	0.14	1.36	0.17	0.12
4	0.29	1.19	0.16	0.14
5	0.57	0.89	0.66	0.74

*μg/mg Protein.

good recoveries, we have proved that it is necessary to acidify the medium. In order to do so we added small amounts of phosphoric acid-water as has been previously described.

To sum up, we used a wide study of various chromatographic parameters which affect pentamidine analysis using ion-pairs. the data obtained allow us predict which are the most adequate chromatographic conditions to carry out quantitative analysis of pentamidine in *Leishmania* infantum promastigotes. These data can also be used to create an analitical method which would allow us to analyze pentamidine in any other biological matrix or complex mixture in which it is found.

Acknowledgements.

The autors wish to thank Mr. E. Viñas and Mr. E. Casas for their technical assistance. This work was carried out using the equipment of el Laboratorio de Técnicas Instrumentales (Universidad de León) and was supported by the " Comisión Mixta Diputación-Universidad de León".

REFERENCES

1. A.S. Tyms, J.D. Williamson, and C.J. Bacchi, J. Antimicrob. Chemother. , 22 : 403 (1988)
2. P.P. McCann, and A.E. Pegg, Pharm. Ther., 54 : 195 (1992)
3. W.C. Van Voorish, Drugs., 40 : 176 (1990)
4. R. Balaña-Fouce, T.Garzón, A.Garrido and D. Ordoñez, Biochem. Pharmac., 35 .1597 (1986)
5. J.C.C ubría, R. Balaña-Fouce, M.L. Alvarez-Bujidos, A. Negro, A.I.Ortiz and D. Ordoñez, Biochem. Pharmac., 45(6) : 1355 (1993)
6. R. Balaña-Fouce, D. Ordoñez and J.M. Alunda, Mol. Biochem. Parasitol., 35 : 43 (1989)
7. L.J. Dusci, L.P. Hackett, A.M. Forbes and K.F Ilett, Ther. Drug. Monit.,9 (4) : 422-5 (1987)
8. B.J. Berger, J.E. Hall and R.R. Tidwell, J. Chromatg., 494 : 191-200 (1989)
9. B. Vinet, R. Comtois, A. Gervais and C.Lemieux, Clin. Biochem., 25(2) : 93-7 (1992)
10. B.A.Bidlingmeyer, J. Chromatogr. Sci., 18 : 525 (1980)
11. A.P. Goldberg, E. Nowakowska, P.E. Antle and L.R. Snyder, J. Chromatogr., 316: 241 (1984)
12. A.Negro, M.L. Alvarez-Bujidos, A.I Ortiz, J.C Cubría, R. Mendez, and D.Ordoñez, J.Chromatgr. Biomed. Applic., 576 : 135 (1992)

13. B.J. Berger, J.E. Hall and R.R. Tidwell, J.Chromatgr. Biomed. Applic., 494 : 191 (1989)
14. C.M. Dickinson, T.R. Navin and F.C.Churchill, J.Chromatgr. Biomed. Applic., 345 : 91(1985)

Received: October 30, 1993
Accepted: December 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 2031-2044 (1994)

HPLC INVESTIGATION OF 11-AMINO UNDECANOIC ACID'S ION PAIRING ABILITY ON FLUOROQUINOLONE GYRASE INHIBITORS

ZS. BUDVÁRI BÁRÁNY[1], A. LŐRE[1], GY. SZÁSZ[1], K. TAKÁCS-NOVÁK[1], AND I. HERMECZ[2]
[1]*Institute of Pharmaceutical Chemistry*
Semmelweis University of Medicine
Budapest, Hungary
[2]*Chinoin Pharmaceutical Works*
Budapest, Hungary

ABSTRACT

The bonding of 11-aminoundecanoic (11-AA), 8-amino octanoic and 6-aminohexanoic acids as representatives of Zwitterionic ion-pairing agents, was determined on C_{18} column in the pH range 3-8. The break through curve of 11-AA has shown minimum in the vicinity of the isoelectric point. In the same pH range the chromatographic behaviour of fluoroquinolone gyrase inhibitor derivatives was studied in 0.002 M 11-AA containing methanol-phosphate buffer (1:1) eluent. The retention of the fluoroquinolones has shown a maximum close to the pH of the isoelectric points. As explanation the adsorption of the neutral form is suggested by the authors. The reverse chromatographic behaviour of 11-AA and the fluoroquinolone lomefloxacin harmonizes well with the results of pH dependent species distribution.

INTRODUCTION

The idea of Zwitterion-pair chromatography has been emerged in earlier works of Knox and Jurand (1,2), when they used 11-amino undecanoic acid (11-AA) as Zwitterionic pairing agent in a reversed phase HPLC (RP HPLC) system. The examined purine-, pyridine- and pyrimidine-nucleotides have shown

a characteristic, pH and ion-pairing agent concentration dependent behaviour. Beside of the usually occuring bipolar ion-pair products Knox and Jurand suggest the formation of "quadrupolar" ionpair species in a reaction between the amphoteric solute (S) and pairing agent (P)

$$S^{\pm} + P^{-}_{+} \rightleftarrows [S^{\pm}_{-}{}_{+}P].$$

Although, according to the mentioned authors the method ("Zwitterionic pairing HPLC) seemed to promise a great versatility and flexibility we found no data about the further application. Therefore, a more detailed study of binding and ion pairing properties of 11-AA in RP HPLC systems appeared reasonable. The investigation was extended to 8-amino octanoic- and 6-amino hexanoic acid. Eight fluoroquinolone gyrase inhibitor derivatives were applied as model substances in a RP HPLC system containing Zwitterionic pairing agent.

EXPERIMENTAL

Chromatography

The HPLC apparatus was comprised in an ISCO pump, Model 2350 (USA) combined with a Valco injector unit. An ISCO variable wavelength (230-800 nm) absorbance detector was used. In case of fluoroquinolone derivatives the effluent was monitored at 254 nm.

For plotting of the break through curves of the alkanecarboxylic acids a Waters Differential Refractometer, Model R 401 was employed. For the break through determination in case of lomefloxacin the UV detector was used. These equipment units subsequent to the pump were thermostatted at 25° ±0.1°. (Ultrathermostat MLW Type U2C, Freital, Germany). The chromatograms were recorded and the retention data were collected by a Hewlett-Packard integrator, Model 3396 ser.II. The sorbent Chromsil-6 C_{18} (6 μm particle size, Labor MIM Budapest) and LiChrosorb RP-18 (5 μm, Merck) were packed in stainless steel columns (250x4.6 mm I.D.). As mobile phase mixtures of methanol and aqueous phosphate buffer solutions (pH 3-8) containing 0.002 M of amino alkanecarboxylic acid were applied. Each retention data was calculated as an

average of three parallel runs. The column void time was signalled by the solvent peak of methanol. Following testing, the columns, were brought to their initial state by two hours elution with methanol-water (1:1) mixture and finally with methanol. Flow rate 1.0 ml/min.

Adsorption isotherms of the three alkanecarboxylic acids were measured by using of the break through method. The equipment used for the break through measurements (Fig. 1) is a modified version of the one was described in a previous work (3).

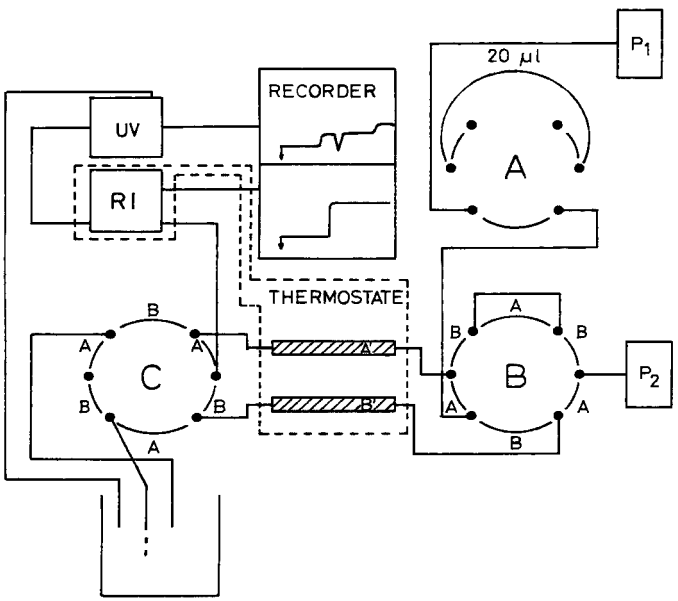
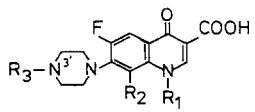


Fig. 1
Diagram of the equipment for plotting of the break through curves.

P_1 : pump (eluent delivery to the detector)
P_2 : pump (for washing)
A,B,C : injectors
A', B' : columns
UV : UV detector
RI : RI detector



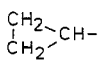
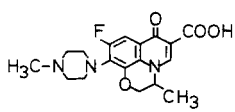
		R_1	R_2	R_3
1.	Norfloxacin	$-C_2H_5$	-H	-H
2.	8-F-Norfloxacin	$-C_2H_5$	-F	-H
3.	Pefloxacin	$-C_2H_5$	-H	$-CH_3$
4.	8-F-Pefloxacin	$-C_2H_5$	-F	$-CH_3$
5.	Lomefloxacin	$-C_2H_5$	-F	-H ($3'-CH_3$)
6.	Ciprofloxacin		-H	-H
7.	Amifloxacin	$-NHCH_3$	-H	$-CH_3$
8.	Ofloxacin			

Fig. 2
Structures for the investigated fluoroquinolones

Materials

The fluoroquinolone model substances (**Fig. 2**) were synthetized at Chinoin Pharmaceutical Works (Budapest) and used without further purification.

11-amino undecanoic acid 99%, 8-amino octanoic acid, 6-amino hexanoic acid 98% (Aldrich).

Buffer solutions in the pH range 3-8 were prepared by mixing the proper volumes of 0.067 M aqueous solutions of potassium dihydrogenphosphate and disodium hydrogenphosphate (KH_2-PO_4, $Na_2HPO_4.2H_2O$, anal. grade, Reanal, Budapest). The pH of the solutions was tested by potentiometry with an accuracy ±0.02 unit.

Methanol, HPLC grade (Chemolab, Budapest).

RESULTS AND DISCUSSION

The binding of 11-amino undecanoic acid.

Figure 3 demonstrates the effect of temperature and sorbent quality on the binding of 11-amino undecanoic acid (11-AA) was calculated by the evaluation of the break through curves. Since the pH of the mobile phase must have a great influence on the measure of binding, a more detailed study seemed reasonable. **Figure 4** shows the pH dependence of 11-AA adsorption in the range 3-8. As it can be seen, the adsorption process may be described by a minimum curve. The amount of 11-AA was adsorbed from the eluent containing-30% of methanol, is comparable with the finding of Knox et al. (**1**) working with an eluent with 10% methanol content. As it is expected, the amount of adsorbed 11-AA strongly decreases with the increase of methanol content in the eluent. The pH of minimum binding coincides rather well with the isoelectric point of 11-AA : 7.65 (pK_{a1} : 10.74, pK_{a2} : 4.56)*. This experience indicates the highly polar character of 11-AA Zwitterion, since the C_{10}-chain allows no interaction between the Zwitterionic poles..

8-amino octanoic acid (8-AA) was adsorbed by the C_{18} column, in a very small extent even from a 30% methanol containing eluent:

pH	3	5	6	7	8
adsorbed 8-AA μM/g	2.2	1.1	0.8	0.4	0.5

6-amino hexanoic acid practically was not binded by the C_{18} sorbent.

The behaviour of fluoroquinolones.

Table 1 and 2 involve the retention data of the investigated fluoroquinolone derivatives in eluents with different pH, 50% and 30% methanol and 0.002 M 11-AA content.

---

* The macroscopic dissociation constants of 11/AA were determined by the potentiometric method was described previously (4).

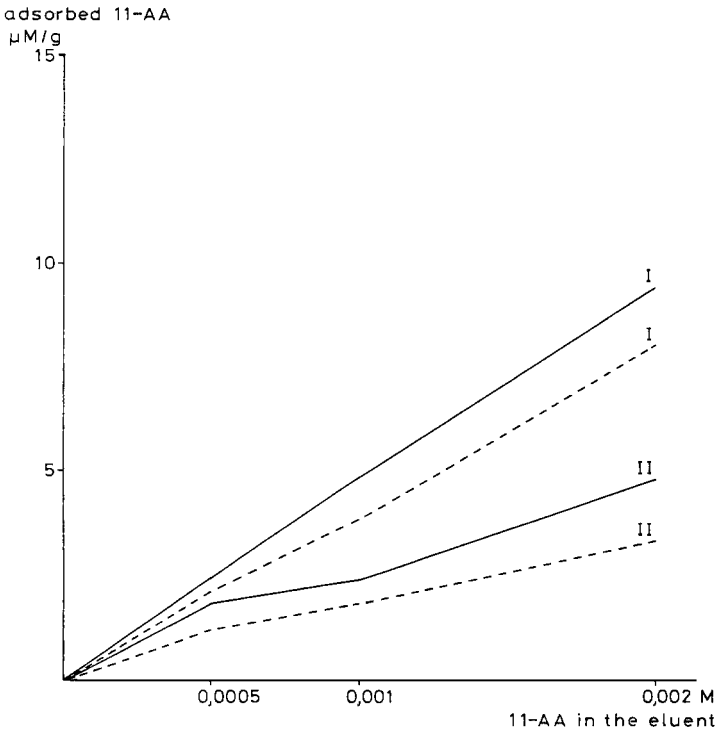


Fig. 3
The effect of temperature and sorbent quality on the adsorption of 11-AA.

Column: I. Chromsil C_{18} (6 μm), II. Lichrosorb RP-18 (5 μm)
Mobile phase: Methanol-phosphate buffer 50:50 (pH = 6)
——— 25 °C
--------- 40 °C

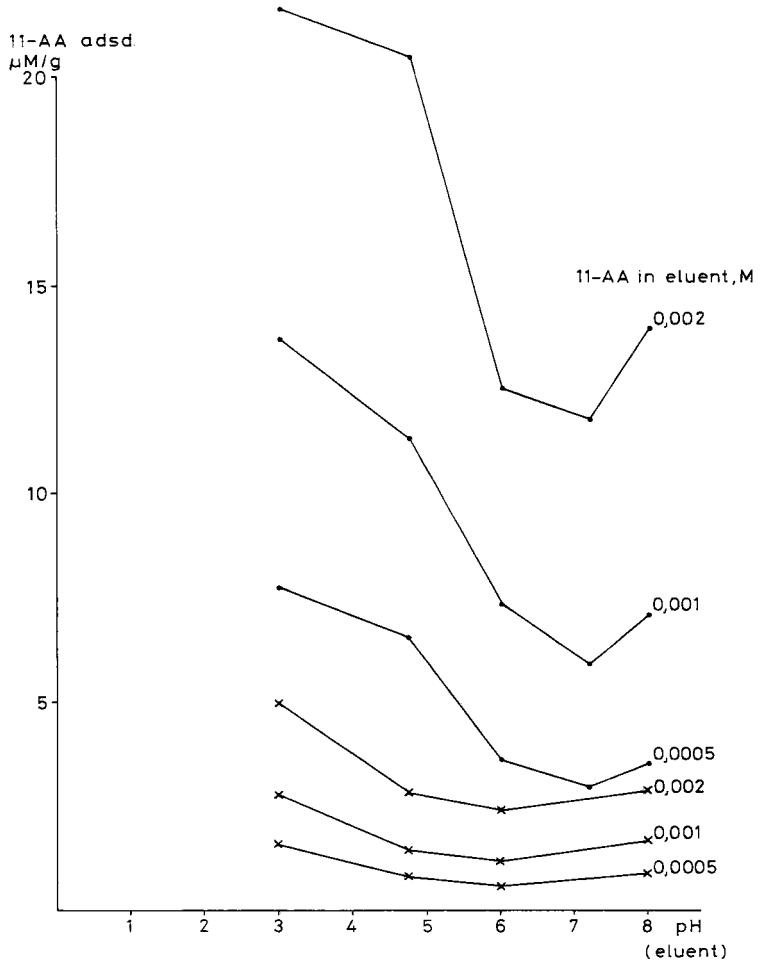


Fig. 4
The pH-influence on the binding of 11-AA.

Column: Chromsil-C^{18} (6 μm)
Mobile phase: methanol-phosphate buffer in ratio 50:50, or 30:70 v/v
• MeOH 30%
x MeOH 50%

Table 1.

pH dependence of the retention

COMPOUND	RETENTION DATA						
	pH*						
	3.0	5.0	5.5	6.0	6.5	7.0	7.5
(i. e. p.)	k'_0 k'_{AA}	k'_0 k'_{AA}	k'_0 k'_{AA}	k'_0 k'_{AA}	k'_0 k'_{AA}	k'_0 k'_{AA}	k'_0 k'_{AA}
1. (7.37)	0.76 1.46	2.69 4.23	3.75 4.60	4.11 4.63	3.77 4.14	2.28 3.22	1.17 1.66
2. (7.44)	0.52 1.13	1.86 2.54	1.99 2.70	1.95 2.17	1.44 1.59	0.99 1.15	0.70 0.90
3. (6.91)	1.17 3.56	9.40 13.90	13.26 15.42	15.43 15.08	11.49 12.15	5.76 7.90	2.40 3.23
4. (6.73)	0.69 2.54	6.50 7.62	7.81 9.02	8.38 8.97	6.73 6.73	3.75 3.76	1.81 2.14
5. (7.14)	0.61 1.15	1.88 2.47	2.11 2.79	2.24 2.54	1.76 1.87	1.21 1.81	0.93 1.25
6. (7.50)	0.82 1.55	2.87 3.95	3.78 4.78	3.88 4.35	3.29 3.55	2.01 2.25	1.01 1.33
7. (6.50)	0.83 2.18	6.92 9.05	9.24 10.34	10.48 10.54	7.05 7.48	3.23 4.17	1.42 1.79
8. (7.00)	0.81 2.17	4.67 6.83	6.72 8.07	7.06 8.37	5.91 8.07	3.33 4.31	1.74 2.08

k'_0 mobile phase: methanol - phosphate buffer (0.067 M) 50 : 50
k'_{AA} mobile phase: methanol - phosphate buffer (0.067 M) 50 : 50 + 0.002 M 11-AA

* pH values measured in aqueous buffer solutions increased with approximately 1 pH unit after the mixing with methanol

Table 2.

pH dependence of the retention

COMPOUND	RETENTION DATA									
	pH*									
	3.0		5.0		6.0		7.0		8.0	
	k'_0	k'_{AA}	k'_0	k'_{AA}	k'_0	k'_{AA}	k'_0	k'_{AA}	k'_0	k'_{AA}
1.	8.97	3.20	13.58	7.69	15.26	12.30	7.40	9.10	4.69	4.18
2.	6.90	2.35	10.99	5.63	6.58	6.18	3.10	3.57	3.77	3.31
3.	14.42	5.02	34.18	26.85	∞		25.37	39.30	12.06	11.87
4.	11.04	4.80	24.15	15.99	∞		12.21	19.10	11.57	12.49
5.	8.45	3.097	12.79	7.34	7.70	8.23	4.50	5.33	5.34	5.02
6.	9.84	3.35	14.81	8.37	14.52	12.72	5.92	8.39	4.58	4.30
7.	9.67	3.41	23.88	16.69	∞		11.96	22.24	6.64	6.85
8.	11.71	3.84	19.57	15.09	26.00	25.20	14.69	19.43	8.28	8.59

k'_0 mobile phase: methanol - phosphate buffer (0.067 M) 30 : 70
k'_{AA} mobile phase: methanol - phosphate buffer (0.067 M) 30 : 70 + 0.002 M 11-AA

* for explanation see Table 1.

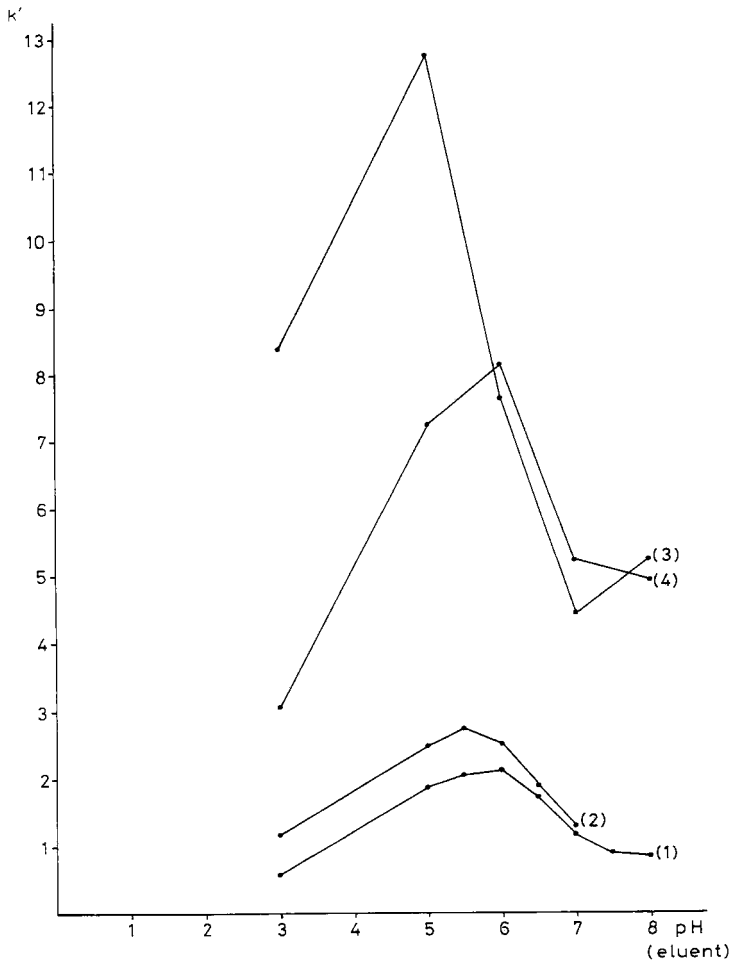


Fig. 5
The effect of 11-AA on the retention of lomefloxacin in eluent with 30% and 50% methanol content.

(1) Methanol 50%-phosphate buffer solution (0.067 M) 50%
(2) As (1), 0.002 M of 11-AA is added
(3) Methanol 30%-phosphate buffer solution (0.067 M) 70%
(4) As (3), 0.002 M of 11-AA is added
Column: Chromsil-6 C_{18}

Table 3

The effect of phosphate, hexansulfonic acid, cetrimide and 11-AA on the retention of floxacins

Compound	1	2	3	4	5	6	7
Norfloxacin	0.43	0.76	1.46	1.46	1.17	1.19	1.66
8-F-Norfloxacin	0.38	0.52	1.15	1.13	0.70	0.96	0.90
Pefloxacin	0.46	1.17	1.85	3.56	2.40	2.42	3.23
Ciprofloxacin	0.49	0.82	1.54	1.55	1.01	1.21	1.33

1. MeOH - Ph (0.025 M pH = 3) 50 : 50
2. MeOH - Ph (0.067 M pH = 3) 50 : 50
3. MeOH - Ph (0.067 M pH = 3) 50 : 50 + 0.01 M hexansulfonate
4. MeOH - Ph (0.067 M pH = 3) 50 : 50 + 0.002 M 11- AA
5. MeOH - Ph (0.067 M pH = 7.5) 50 : 50
6. MeOH - Ph (0.067 M pH = 7.5) 50 : 50 + 0.01 M cetrimide
7. MeOH - Ph (0.067 M pH = 7.5) 50 : 50 + 0.002 M 11-AA

(Ph: phosphate buffer solution)

As it is shown the retention reaches maximum at a pH value close to the isoelectric point of the compounds (the pH-shift by methanol is to be considered, see **ref 5**). **Figure 5** clearly illustrates the similarities and differences between the retention curve-pairs gained in 11-AA containing and noncontaining eluents. The similar shape of the curve-pairs indicates the ion pair forming effect of the phosphate ion itself. The quite different effect of 11-AA on the retention in 30% and 50% methanol containing systems may be explained by the different contribution of ion pair formation and substitution in the development of the final retention values. The dominancy of the substitution effect appears to result in great retention depression by 11-AA in the case of eluent with 30% methanol content.

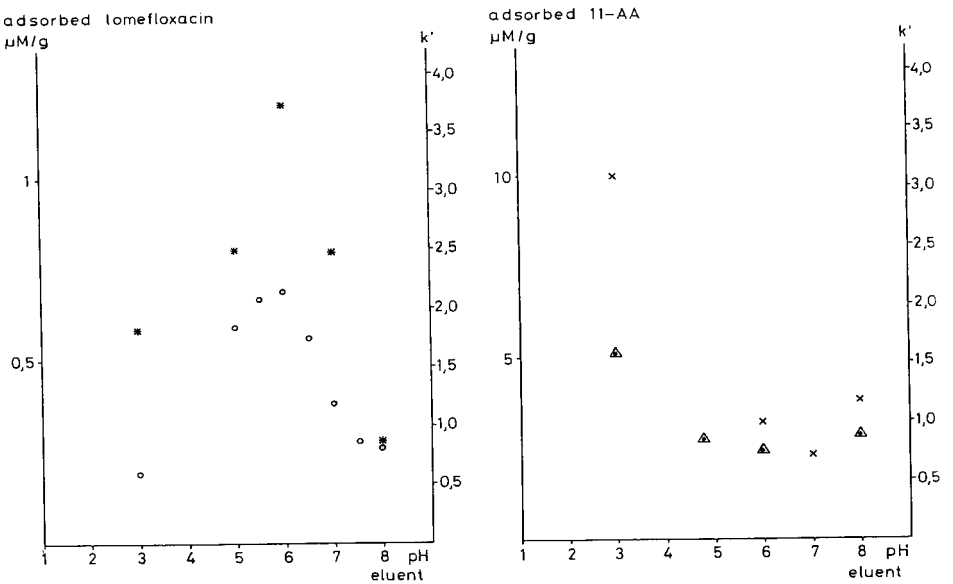


Fig. 6
Comparing of adsorption and retention curves of lomefloxacin and 11-AA

lomefloxacin: o Retention (k') (MeOH-Ph 50:50)
* Adsorption (MeOH-Ph 50:50 + 0,0005 M lomefloxacin)

11-AA: x Retention (k') (MeOH-Ph 50:50)
Δ Adsorption (MeOH-Ph 50:50 + 0.002 M 11-AA)

Ph: phosphate buffer solution (0.067 M), pH = 3-8,
Column: Chromsil C_{18} (6 μm)

Table 3 shows the effect of phosphate, hexanesulfonate, cetrimide and 11-AA on the retention of fluoroquinolone derivatives. A comparison of the respective data suggests that phosphate ion functions as an anionic ion pairing agent, while 11-AA, depending upon the pH of the medium can act as a dual (cationic or anionic) ion pairing reagent. To the formation of "quadrupolar ion pairs" (**1**) can not be concluded in case of the investigated fluoroquinolones.

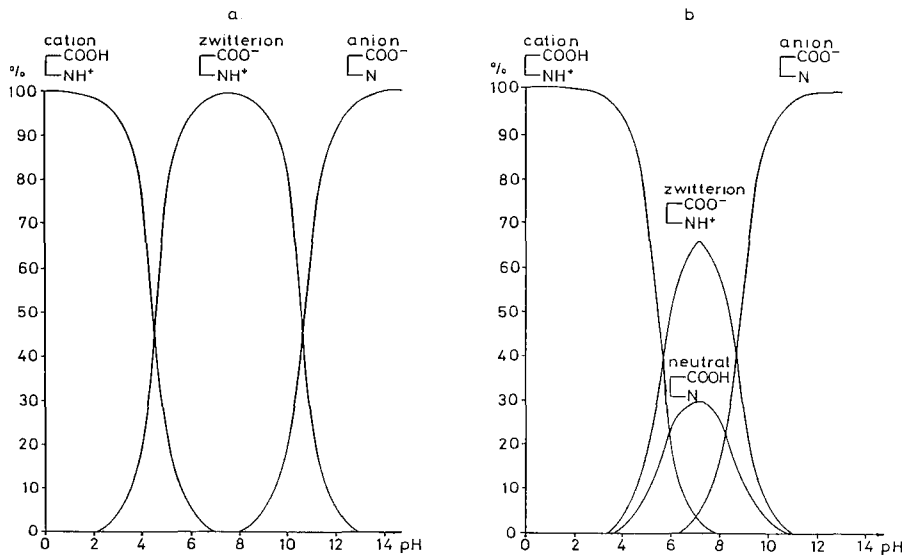


Fig. 7
The microspeciation diagram for 11-AA (a) and lomefloxacin (b)

The quite reversed (i.e. the minimum-curve and the maximum curve character of the adsorption and the retention curves of 11-AA and lomefloxacin) reflects the presence of the most polar and the most apolar species at the isoelectric point respectively (**Fig. 6**). This finding becomes understandable by **Fig. 7**. At the pH of the isoelectric point the 11-AA solution contains exclusively Zwitterionic species, while a significant amount of the neutral form is present in lomefloxacin solution. It appears, that in the retention of lomefloxacin the adsorption of the neutral species plays a dominant role. This assumption seems to confirm the conclusion was drawn by Takács-Novák et al. on the pH dependence of log P among fluoroquinolones (**4**).

ACKNOWLEDGEMENT

The authors are greatful to I. Kovács-Derzsi and E. Asztalos-Túróczy for the excellent technical assistance.

REFERENCES

1. Knox, J.H., Jurand, J.: J. Chromatogr. 203, 85 (1981)
2. ibid: 186, 763 (1979)
3. Lőre, A., Budvári-Bárány, Zs., Szász, Gy., Shalaby, A.: J. Liq. Chrom. 14, 2065 (1991)
4. Takács-Novák, K., Noszál, B., Hermecz, I., Keresztúri, G., Podányi, B., Szász, Gy.: J. Pharm. Sci. 79, 1023 (1990)
5. Budvári-Bárány, Zs., Szász, Gy., Takács-Novák, K., Hermecz, I., Lőre, A.: J. Liq. Chrom. 14, 3411 (1991)

Received: December 3, 1993
Accepted: December 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 2045-2056 (1994)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION AND ANALYSIS OF METALLOTETRA(PENTAFLUORO-PHENYL)PORPHOLACTONE

J. RIVER WAN, MARTIN GOUTERMAN, EDMOND GREEN, AND GAMAL-EDDIN KHALIL
Department of Chemistry
University of Washington
Seattle, Washington 98195

ABSTRACT

The analytical separation and analysis of silver, nickel, zinc and palladium complexes of tetra(pentafluorophenyl)porpholactone (TFPL) from free bases is described. An isocratic mobile phase of n-hexane and diethyl ether was developed to separate these complexes using a normal phase μPorasil HPLC column (150 x 3.9 mm I. D.) at a flow rate of 1 mL/min. In addition, two porphodilactone isomers were also isolated from the reaction products, a result which we were unable to achieve with flash chromatography nor with a reversed phase C18 Ultrasphere HPLC column (250 x 4.6 mm I. D.). The optical absorption spectra of the metal derivatives show two bands in the visible region, compared to the four bands shown by the free base. The retention of these metal complexes on a normal phase column was found to be increasing in the order Pd(TFPL) < Ag(TFPL) < Zn(TFPL) < Ni(TFPL).

INTRODUCTION

Porphyrin and metalloporphyrins are a class of important biologically important molecules. Much work have been reported on HPLC separation of these biological porphyrins (1-8). Reversed phase columns were usually used to separate these porphyrins due to their hydrophillic nature. In some cases, when satisfactory separation can not be achieved on reversed phase column, these biologically active porphyrins can be derivatized to water insoluble derivatives so that normal phase column can be deployed to give better separation (9-12).

However, relatively few studies have been reported on HPLC separation of water insoluble free base porphyrins, and even less work has been carried out on metalloporphyrins, despite extensive study on almost every other aspects of porphyrins and metalloporphyrin (13). Eglinton et al. (14) used a silica column to separate some free base porphyrins in crude oil. Rowlands and coworkers (15) separated some metalloporphyrins successfully on a Hypersil column, using 9:1 hexane and ethyl acetate as mobile phase. Suzuki et al (16) developed a 40:60 acetone-acetonitrile mobile phase to separate Mg(II), V(IV), Fe(III), Ni(II), Cu(II), Zn(II), Pd(II) and Cd(II) bonded *meso*-tetraphenylporphyrin on a reversed phase C18 column. Freeman et al. (4) successfully separated a mixture of the octaethylporphyrins of zinc(II), nickel(II), copper(II) and cobalt(II) derivatives also on a reversed phase column, using acetonitrile-ethanol amine (10:1) as mobile phase.

Recently, a new type of porphyrin compound called tetra(pentafluorophenyl)porpholactone (H_2TFPL) was synthesized from tetra(pentafluorophenyl)porphyrin (H_2TFPP) via a simple reaction in our lab (17) (Steps 1 and 2, Figure 1.). This new compound has an unusually intense farthest red band is usually intense (Figure 2). We further synthesized some of its metal derivatives and studied their spectra (17, 18).

However, this seemingly simple one step reaction not only gave complicated products but the products also varies from batch to batch, as shown by our own work and others (17, 19). As a result, we were unable to resolve all the components with flash chromatography. According to our calculation, tetra(pentafluorophenyl)porphodilactone should be a

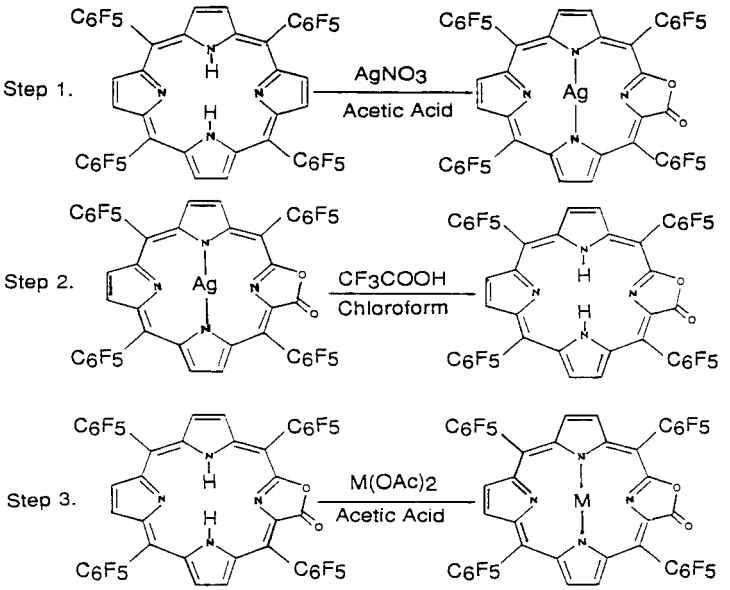


FIGURE 1. Schematic of the synthesis of metallotetra(pentafluorophenyl)porpholactone and its metal derivatives.

stable compound, and we predicated that this molecule may be produced during the reaction (17). Yet so far, we haven't isolated this species. In order to fully characterize the products and understand the mechanism, we need to resolve all the components in the products. Therefore, we decided to pursue the separation of these complexes on HPLC. Since porphyrins have very characteristic absorption in the range from 350nm-700nm, and furthermore, the metal derivatives show a two banded spectra while free bases show a four banded spectra (Figure 2), a UV/VIS detector can be used for both easy detection and analysis of free base porphyrins and metalloporphyrins.

In this paper, we wish to report the HPLC separation and analysis of these metal complexes from their free bases, using a μPorasil column with a hexane and diethyl ether mobile phase.

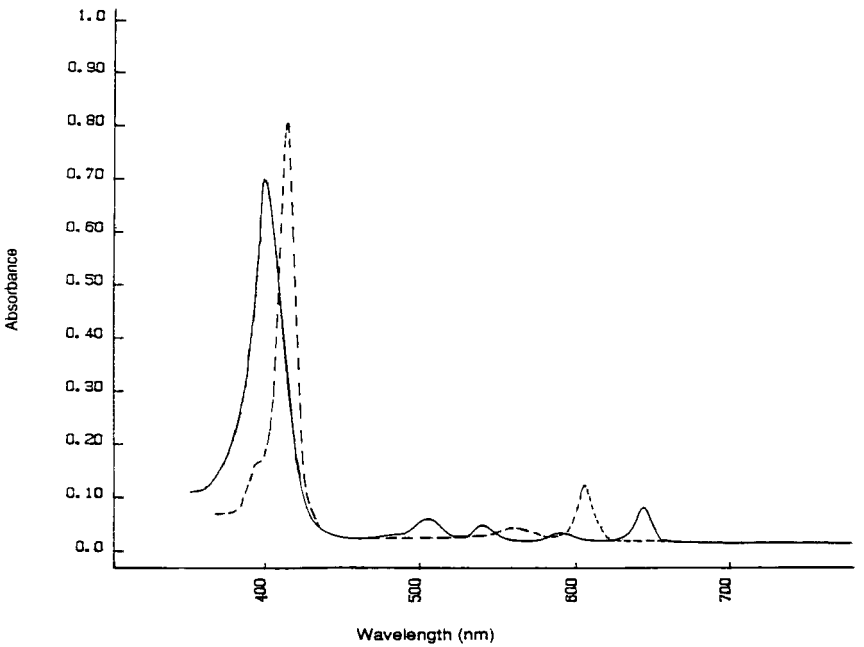


FIGURE 2. Optical absorption spectra of free base H_2TFPL (solid line) and Zn(TFPL) (dotted line) in methylene chloride.

EXPERIMENTAL

Materials

All solvents but diethyl ether were HPLC reagent grade from J. T. Baker and used without further purification. Anhydrous diethyl ether was supplied by J. T. Baker as reagent grade. Flash chromatography grade Silica Gel (100-200 mesh or 70-140 μm with average pore diameter 25 Å) was purchased from Sigma Chemical Company. Tetra(pentafluorophenyl)porphyrin was purchased from Aldrich Chemical Company and purified according to our procedure (18). Silver, zinc, nickel and palladium derivatives of tetrakis(pentafluorophenyl)porpholactone were synthesized according to a procedure described elsewhere (18).

HPLC systems

Metalloporpholactones were separated on a system consisting of two Altex Model 100A pumps with a solvent programmer, a Varian VARI-CHROM variable UV/VIS detector with a plotter and a Hewlett Packard 8452A diode array spectrophotometer. The diode array detector was controlled by an IBM Nec/Multisync 2A computer using a HP-UV program for data acquisition and a coloPro plotter for plotting spectra.

The second system was from Hitachi, which included a Hitachi single L-6200A intelligent pump, a Hitachi L-4250 UV-VIS detector (170 nm - 700 nm) and a Hitachi D-2500 CHROMATO-Integrator. The UV-VIS detector has a stopped flow scanning function.

Columns

The normal phase column was a μPorasil column (150 x 3.9 mm) purchased from Waters. The reversed phase column was a Ultrasphere ODS 5 micro (250 x 4.6 mm) supplied by Beckman. A Direct-Connect ™ cartridge guard column system consisting of a direct connect universal guard column cartridge holder and a guard column cartridge was from Alltech Associates. Adsorbsphere Silica 5μ and adsorbsphere C18 5μ guard column cartridges were connected to μPorasil column and Ultrasphere ODS column respectively.

Procedure

Mobile phase was obtained by volume to volume mixing of hexane and diethyl ether. Samples were dissolved in a small amount of mobile phase whenever possible. If the solubility of the sample is poor in the mobile phase, methylene chloride was added to enhance its solubility. All samples were prepurified by flash chromatography using Silica Gel (Sigma) as packing and 9:1 ratio of hexane and methylene chloride as eluents. Sample solution was filtered through a 0.4 μm microfilter. Detector was set at 400nm. A 5 μL sample loop was used for sample injection. Flow rate was set at 1 mL/min.

RESULTS AND DISCUSSION

Silver tetra(pentafluorophenyl)porpholactone (Ag(TFPL)) was synthesized in a procedure as shown in Step 1 of Figure 1 (18). After demetallation, Ag(TFPL) was converted to H_2TFPL (Step 2). Zinc, nickel and palladium were subsequently inserted into the porpholactone ring (Step 3).

HPLC separation of these metalloporpholactones was first tried on reversed phase C18 Ultrasphere ODS column, using 40:60 acetone-acetonitrile as mobile phase according to a procedure described by Suzuki et al (1). However, we were unable to separate these complexes with this mobile phase, not even with a gradient of acetone and acetonitrile. Many other mobile phases have also been tried on this column without good separation.

We then investigated the separation on a normal phase μPorasil (3.9 x 150 mm I. D) column. We eventually came up with an isocratic mobile phase of diethyl ether and hexane. Silver, nickel, palladium and zinc complexes of tetra(pentafluorophenyl)porpholactone were successfully separated from other components with this mobile phase.

HPLC Separation of Ag(TFPL)

A HPLC chromatogram of Ag(TFPL) is shown in Figure 3. The chromatogram showed a total of eight components in the reaction (Step 1). The two major components were identified as free base H_2TFPL and Ag(TFPL). Identifications of peaks were based on two features: (a) optical spectra in each case and (b) FAB-mass spectra in some cases (17, 18). In each of the chromatograms, boldface labels were used if both (a) and (b) are available and standard labels were used if just (a) is available. The optical absorption spectra of Ag(TFPL) is not shown here since its two banded spectra is very similar to that of Zn(TFPL) (Figure 2). The optical absorption spectra of Zn(TFPL) is shown in Figure 2 as an example for all the metal complexes of H_2TFPL. The two minor peaks in the chromatogram were identified as silver tetra(pentafluorophenyl)-porphyrin (Ag(TFPP)) and a tetra(pentafluorophenyl)porphodilactone

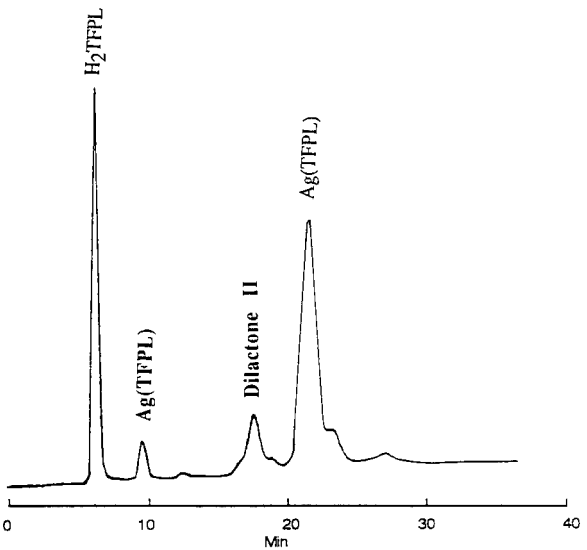


FIGURE 3. HPLC separation of Ag(TFPL) at ambient temperature. Principal peaks are Ag(TFPP), Dilactone II and Ag(TFPL) (see text). Peaks labelled boldface are identified by both optical absorption spectra and FAB-mass spectra.
Mobile Phase: Diethyl ether - hexane 1.5:98.5v/v. Column: Waters μPorasil (3.9 x 150mm I. D.). Detection wavelength: 400nm. Sample loop: 5μl. Flow rate: 1ml/min. * : Not identified.

isomer. We shall call this isomer Dilactone II since we were unable to determine which one of the five isomers it is (17). The other four minor peaks were not identified, although a Soret band at about 400nm for each of them indicates that they are still porphyrin species. The retention time of Ag(TFPL) is 22 minutes with a mobile phase of 98.5% hexane and 1.5% diethyl ether.

HPLC chromatogram of Pd(TFPL)

A HPLC chromatogram of Pd(TFPL) is shown in Figure 4. The chromatogram showed four principal peaks, two of which were identified

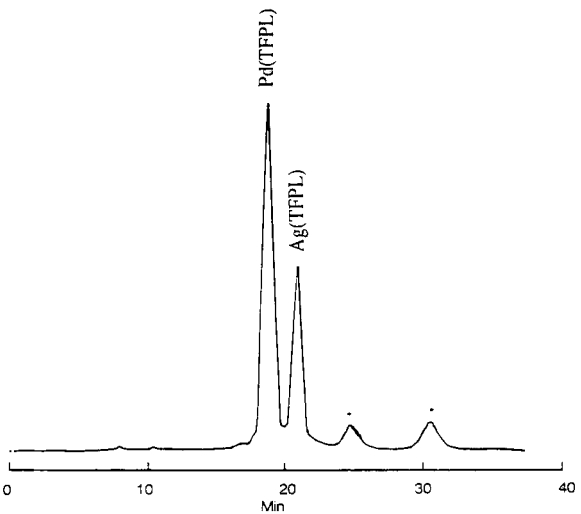


FIGURE 4. HPLC separation of Pd(TFPL) at ambient temperature. All conditions but mobile phase were same as described in Figure 3. Principal peaks are Pd(TFPL), Ag(TFPL) (see text). Mobile Phase: Diethyl ether - Hexane 1:99 v/v. * : Not identified.

as Pd(TFPL) and Ag(TFPL). The two minor peaks were not identified, although they are believed to be porphyrin species as indicated by their Soret bands. No H_2TFPL was detected from this sample, which may indicate that H_2TFPL was totally converted to Pd(TFPL) during the reaction. Again, Pd(TFPL) showed a two banded spectra, similar to that of Zn(TFPL). Pd(TFPL) has a retention time of 18 minutes with a mobile phase of 99% n-hexane and 1% diethyl ether.

HPLC chromatography of Ni(TFPL)

The HPLC chromatogram of Ni(TFPL) showed very complicated products (Figure 5). A total of ten principal peaks and seven minor peaks were detected. Among the ten principal components, four were identified

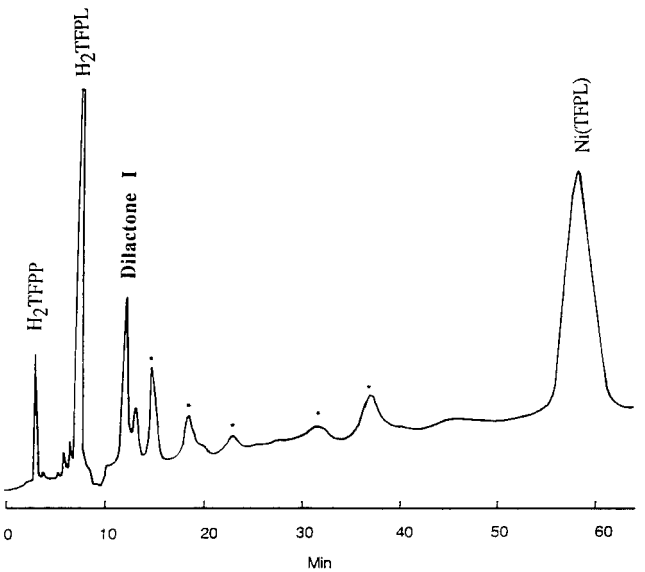


FIGURE 5. HPLC separation of Ni(TFPL) at ambient temperature. All conditions were same as described in Figure 3. Principal peaks are H_2TFPP, H_2TFPL, Dilactone I and Ni(TFPL) (see text).
* : Not identified.

as H_2TFPP, H_2TFPL, a porphodilactone isomer and Ni(TFPL). The other principal peaks were not identified. Again, their optical absorption spectra indicated that all were porphyrin compounds. We were unable to obtain spectra for other minor peaks. The porphodilactone isomer isolated has different absorption spectra from Dilcatone II, and we shall call this isomer Dilactone I. Both isomers were confirmed by their excitation spectra and FAB-mass spectra (18). A fairly large peak of H_2TFPL appeared in the chromatogrm, which indicates that only part of H_2TFPL was converted to Ni(TFPL) in the metal insertion reaction (Step 3, Figure 1). Again, Ni(TFPL) showed a typical two banded spectra, similar to that of Zn(TFPL). The retention time of Ni(TFPL) is very long at 58 min, using 98.5% n-hexane and 1.5% diethyl ether as mobile phase.

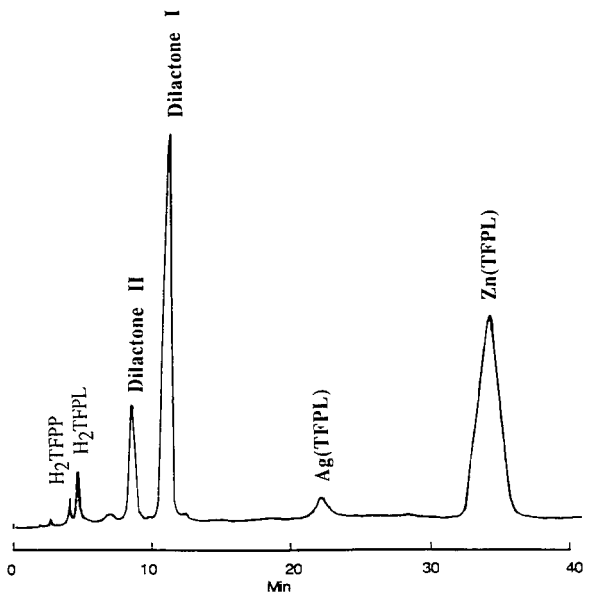


FIGURE 6. HPLC separation of Zn(TFPL) at ambient temperature. All conditions but mobile phase were same as described in Figure 3. Principal peaks are H_2TFPP, H_2TFPL, Dilactone II, Dilactone I, Ag(TFPL) and Zn(TFPL) (see text).
Mobile phase: Diethyl ether - Hexane 2.5:97.5 v/v.

HPLC chromatography of Zn(TFPL)

The HPLC chromatogram of Zn(TFPL) is shown in Figure 6. The chromatogram showed a total of six principal peaks, and all of which were identified as H_2TFPP, H_2TFPL, Dilactone II, Dilactone 1, Ag(TFPL) and Zn(TFPL). Identification of Zn(TFPL) was also confirmed by its FAB-mass spectrum (18). Both dilactone isomers isolated from Ag(TFPL) and Ni(TFPL) samples respectively were found in Zn(TFPL) sample. Only a very small peak of H_2TFPL was detected, which indicates that most free base H_2TFPL was metallated during the reaction (Step 3, Figure 1).

Zn(TFPL) has a retention time of 33 minutes, using a mobile phase of 97.5% n-hexane and 2.5% diethyl ether.

The mobile phases we developed has enabled us to separate Zn(TFPL), Ag(TFPL), Ni(TFPL) and Pd(TFPL) from their free bases successfully. In addition, we also isolated two porphodilactone isomers, a result we were unable to achieve with flash chromatography. This demonstrated the power of HPLC to the separation and characterization of these complicated compounds. Unfortunately, we could not obtain enough sample for the dilactone isomers on a analytical HPLC column to study their NMR.

SAFETY NOTES

Diethyl ether is potentially explosive, and therefore should be handled with great caution. HPLC instrument should be operated in a cool and well ventillated room. Waste mobile phase should be properly disposed.

ACKNOWLEDGEMENTS

We would like to thank Jim Roe and Professor James Callis at the department for the arrangement of using the Altex HPLC system.

REFERENCES

1. V. Miller, L. Malina, J. Chromatogr., 145, 290 (1978).
2. R. M. Smith, D. Doran, M. Mazur, B. Bush, J. Chromatogr., 191, 319 (1980).
3. C. K. Lim, J. M. Rideout, T. J. Peters, J. Chromatogr., 317, 333 (1984).

4. J. S. Flynn, D. H. Freeman, J. Chromatogr., 386, 111, (1987).

5. M. Takayama, J. Chromatogr., 423, 313 (1987).

6. A. A. Lamola, M. Joselos, T. Yamane, Clin. Chem. 21 (1), 98 (1975).

7. G. R. Gotelli, J. H. Wall, P. M. Kabra, L. J. Marton, Clin. Chem., 26 (2), 205 (1980).

8. C. K. Lim, F. Li, T. J. Peters, J. Chromatogr., 429, 123 (1988).

9. N. Evans, D. E. Games, A. H. Jackson, S. A. Matlin, J. Chromatogr.,115, 325 (1975).

10. C. H. Gary, C. K. Lim, D. C. Nicholson, Clin. Chim. Acta, 77, 167 (1977).

11. J. G. Straka, Methods Enzymol., 123, 352 (1986).

12. E. Rossi, D. H. Curnow, HPLC of Small Molecules, A Practical Approach, C. K. Lim, Eds., IRL Press, Oxford, 1986, Ch. 10, pp.261-303.

13. The Porphyrins, Vol. I-VII, D. Dolphin, eds., Academic Press, Inc., New York, 1978.

14. S. K. Hajibrahim, P. J. C. Tibbetts, C. D. Watts, J. R. Maxwell, G. Eglinton, Anal. Chem., 50 (40), 549 (1978).

15. J. C. Evans, A. H. Jackson, K. R. N. Rao, C. C Rowlands, J. High Resolution Chromatogr. and Chromatogr. Commun., 269 (1985).

16. K. Saitoh, M. Kobayashi and N. Suziki, J. Chromtogr., 243, 291 (1982).

17. M. Gouterman, R. J. Hall, G. Khalil, P. Martin, E. G. Shankland, R. L. Cerny, J. Am. Chem. Soc., 111, 3702 (1989).

18. J. Wan, M. Gouterman, E. Green and G. Khalil, Inorg. Chem., submitted.

19. Assad Salehi, PhD Thesis, Chapter V, Michigan State University, 1988.

Received: December 1, 1993
Accepted: December 9, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 2057-2067 (1994)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AROMATIC POLY-AMIDINES: FORMULATORY AND PRECLINICAL APPLICATIONS

C. NASTRUZZI[1]*, C. PASTESINI[1], E. MENEGATTI[1], AND S. SCALIA[2]

[1]*Department of Pharmaceutical Sciences*
University of Ferrara
I-44100 Ferrara, Italy

[2]*Institute of Pharmaceutical Chemistry*
University of Catania
I-95100 Catania, Italy

ABSTRACT

A high-performance liquid chromatographic procedure has been developed for the simultaneous determination of aromatic poly-amidines (with 2, 3 or 4 benzamidine residues or different 2'-halogen substitutions). A Supelcosil LC-18-DB column with UV detection at 261 nm and an eluent consisting of phosphate buffer (200 mM, pH 3) - methanol - tetrahydrofuran - triethylamine (75:25:3:0.5, v/v) were employed. The proposed method was found suitable both in cellular biology/pharmacology studies, for the quantification of aromatic poly-amidines in serum and serum containing cell culture medium, as well as in preformulatory studies for the determination of drug release kinetic from delivery systems as in the case of microspheres.

INTRODUCTION

Poly-amidines are a class of compounds of great pharmaceutical interest since, as recently published, they can be proposed in the experimental and clinical

treatment of a large variety of pathologies (1-8). In this respect, pentamidine, an aromatic di-amidine derivative with antiprotozoal activity, has been demonstrated to be effective in the treatment of *Pneumocystic carinii* pneumonia (PCP) in patients with acquired immunodeficiency syndrome (AIDS) (1-3). Moreover other poly-amidinic compounds, with 2, 3 or 4 benzamidine residues in their chemical structure and their 2' halo-derivatives, possess interesting biological activities. These compounds were found (a) to inhibit the activity of serine proteinases involved in thrombosis and hemostasis, such as factor Xa and thrombin (4, 5) and (b) to be potent antitumor agents, both "in vitro" (6) and "in vivo" (7) on a variety of cell lines, including breast, kidney and colon carcinoma cells. In addition, these polyamidines were reported (c) to inhibit the "in vitro" metastatic activity of a highly tumorigenic cell lines (8) and (d) to specifically bind to DNA molecules interfering with DNA-protein recognition (9-11).

On the other hand, despite the large spectrum of biological activities, polyamidines, due to their peculiar chemico-physical properties (i.e. solubility, charge density, affinity for protein and nucleic acid), represent a class of compounds difficult to analyse. For these adverse properties, reliable methods for their determination in formulatory and preclinical studies are still lacking.

The development of an accurate and precise analytical protocol is a prerequisite both for pharmacological and clinical trials (e.g. determination of drug pharmacokinetic, tissue distribution and bioavailability) and for pharmaceutical studies aimed to the design of efficient drug delivery systems such as liposomes and microspheres.

To this purpose, in this paper we describe a high-performance liquid chromatographic (HPLC) procedure based on the use of a ternary solvent system containing a high ionic strength buffer and an organic competing base. The analytical method, here presented, is suitable for the simultaneous determination of different aromatic polyamidines with two (1,3-di-(*p*-amidinophenoxy)-propane, shortly, propamidine or DAPP), three (1-(*p*-amidinophenoxy)-2,2-bis-(*p*-amidinophenoxy-methyl)-butane, shortly TAPB) and four (1,3-di-(*p*-amidinophenoxy)-2,2-bis-(*p*-amidinophenoxymethyl)-propane, shortly TAPP) Benzamidine residues as well as for 2'-halo derivatives (TAPP-Cl, TAPP-Br and TAPP-I) of the tetra-benzamidine TAPP (see chemical structures in Fig.1) (12-14). The application of the method to the determination of poly-amidines in human serum and in cell culture media is also described. In addition, this HPLC based analytical method can be efficiently utilized in preformulatory studies such as the

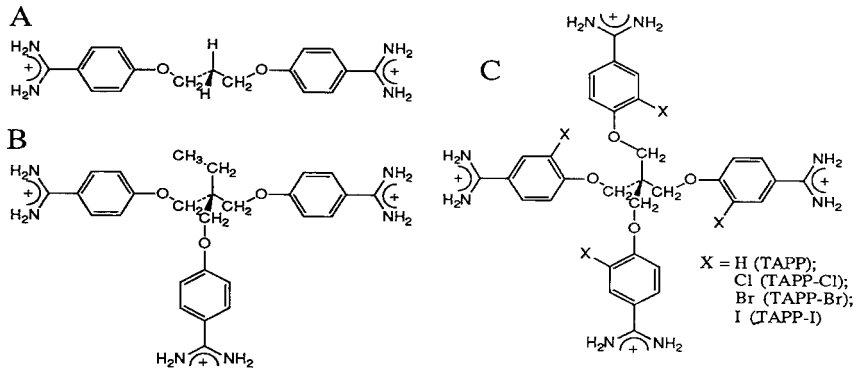


FIGURE 1. Chemical structures of the poly-benzamidines. A: 1,3-di-(p-amidinophenoxy)-propane (DAPP); B: (1-(*p*-amidinophenoxy)-2,2-bis-(*p*-amidinophenoxy-methyl)-butane (TAPB); C: 1,3-di-(p-amidinophenoxy)-2,2-bis-(p-amidinophenoxymethyl)-propane (TAPP) and its 2'-halo derivatives (TAPP-Cl, TAPP-Br and TAPP-I).

determination of TAPP-Br release kinetic from gelatine-microsphere, especially designed for the controlled delivery of this drug (15).

MATERIALS AND METHODS

Chemicals

Synthesis, melting points, yields, crystallization solvents and analytical data of the aromatic polyamidines (see Fig.1 for chemical structures) have been reported elsewhere (12-14). Methanol, tetrahydrofuran and water were all HPLC-grade as supplied by J.T.Baker (Phillisburg, USA). Cell culture medium was α-medium (GIBCO, Grand Island, New York, USA), 50 mg/l streptomycin, 300 mg/l penicillin, supplemented with 10% fetal calf serum (Flow Laboratories, Inc., McLean, USA). All other chemicals were of analytical grade (Farmitalia Carlo Erba, Milan, Italy)

Chromatographic Instrumentation

The RP-HPLC analyses were performed utilizing a HPLC system consisting of a Bruker LC21-C chromatographic pump (Bruker, Bremen, Germany), a

Rheodyne 7125 sample injection valve, equipped with a 50-µl loop (Rheodyne, Cotati, USA), and a Chrom-A-Scope rapid scan UV detector (Carlo Erba Strumentazione, Milan, Italy) able to measure and store 10 spectra/min.

Chromatographic Conditions

Samples were chromatographed on a 150 x 4.6 mm stainless steel column packed with 5 µm particles (Supelcosil LC-18-DB, Supelco, Bellefonte, USA) and eluted with phosphate buffer (200 mM, pH 3) - methanol - tetrahydrofuran (75:25:3, v/v) containing 0.5% triethylamine. Chromatography was performed at room temperature, at a flow rate of 1 ml/min.

The polyamidine content of sample solutions was obtained from calibration curves constructed from polyamidine standard solutions. The detection was carried out at 261 nm and the wavelengths scanned between 220 and 340 nm.

Determination of TAPP and TAPP-Br in Culture Medium and Serum Samples

Sample were prepared by adding 10 µl aliquots of tetra-amidine standard solutions in water (10 µg/µl) to 0.5 ml of α-medium or human serum and mixing them thoroughly. These solutions were passed through a pre-conditioned (1 ml of methanol and then 1 ml of water) C_{18}-cartridge (sorbent weight, 200 mg; Baker) which was eluted successively with 1 ml of water, 1 ml of methanol and 3 ml of phosphate buffer (300 mM, pH 3) - methanol - tetrahydrofuran (60:30:5, v/v) containing 3% triethylamine. The last fraction was directly analysed by HPLC. The percentage recovery was determined by comparing the peak areas of TAPP and TAPP-Br extracted from samples with those obtained by direct injections of equivalent amounts of tetra-amidines.

Determination of TAPP-Br Release Kinetic from Microspheres

The determination of the TAPP-Br "in vitro" release kinetic from microspheres was carried out by horizontal shaker method as previously described (15). Typically, 20-25 mg of microspheres were poured into a dialysis tube (molecular weight cut off 10,000-12,000; Medi Cell International, England) placed into 100 ml of 50 mM borate buffer, pH 7.5 and then in a horizontal shaker. Afterwards, samples were withdrawn at regular time intervals from the receiving buffer. The

amount of released drug was determined by reversed-phase HPLC using the analytical method described above.

RESULTS AND DISCUSSION

Until now the aromatic poly-benzamidines DAPP, TAPB and TAPP, have been determined by spectrophotometric techniques (16), which have been proved to be unsatisfactory due to interference problems caused by the intrinsic lack of specificity of the method especially at the low absorption wavelength (260 nm) used for determination of poly-benzamidines.

The present investigation describes an isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the simultaneous identification and quantification of different aromatic polyamidines (see Fig. 1 for chemical structures). The analytical procedure here presented is suitable for the determination of poly-amidines both in cell culture medium or in human plasma as well as for preformulatory pharmaceutical studies.

Chromatographic Separation

Preliminary experiments revealed that the foregoing poly-amidine compounds were not eluted from a cation exchanger column even when a mobile phase buffer concentration up to 1 M was employed. A previously published report indicated that optimal retention for pentamidine, which is structurally similar to the poly-amidines examined in this study, is achieved on a C_{18} column with an eluent consisting of acetonitrile - water and containing tetramethylammonium chloride and phosphoric acid (17).

However, under these conditions, unsatisfactory separation of DAPP, TAPB and TAPP was obtained. In addition, severe peak tailing for the compounds with three and four benzamidine residues was observed. This was probably due to the higher number of positive charges present in their structure as compared to pentamidine. In order to overcome these problems, other mobile phase systems, in combination with a Supelcosil LC-18-DB column, were examined to find conditions were efficient and baseline separations of the above polyamidines could be obtained.

Concerning the choice of the mobile phase pH, we utilized a pH 3 buffer since at acidic pH, interactions between positively charged compounds and silanolic groups are reduced.

In order to diminish residual silanophilic interactions that were observed even at low pH, a high molarity phosphate buffer (200 mM) was used in conjunction with an organic competing base (triethylamine). The influence of a third mobile phase component was also evaluated. When tetrahydrofuran was added to the eluent with the methanol - phosphate buffer ratio kept constant, a decrease in retention for all poly-amidines was observed due to an increase of the eluotropic strength of the mobile phase. Furthermore an enhanced resolution of the above compounds was obtained with this ternary solvent conditions.This finding is in agreement with other reports in the literature showing that mobile phases containing more than two solvents are required for the maximum exploitation of selectivity in RP-HPLC (18).

Figure 2 shows a typical chromatogram of the separation of poly-amidines with 2, 3 or 4 benzamidine residues (DAPP, TAPB and TAPP) obtained with the optimized mobile phase described above. Under the same chromatographic conditions, the 2' halo derivatives of TAPP (TAPP-Cl, TAPP-Br and TAPP-I respectively) are also resolved (see Fig. 3). In addition, Figures 2 and 3 report the spectra of the compounds recorded by the rapid scan UV detector. All spectra obtained in the mobile phase showed an absorption band of different intensity localized at 261 nm and were respectively identical to the spectra of the pure compounds recorded (off line) with a double beam spectrophotometer (data not shown).

Determination of TAPP-Br in Cell Culture Medium and Serum

Since tetra-benzamidine were found the most active poly-benzamidines in both in vitro and in vivo studies (6-9) they were chosen as model compounds to evaluate the applicability of the proposed method to the determination of poly-benzaminides in cell culture media and human serum.

Prior to RP-HPLC assay of TAPP and TAPP-Br, a sample clean-up step was introduced to remove matrix peaks which were found to interfere with the analyte determination. Because of the strong affinity of poly-benzamidines for proteins (in particular, serine proteinase) (4, 5), and their scarce solubility in organic solvents, classical sample pretreatment methods, were found uneffective. For instance, protein precipitation with ethanol or trichloroacetic acid produced unsatisfactory recoveries (less than 20%). For this reason, purification procedures based on solid-phase extraction were examined. Tetra-benzamidines were quantitatively extracted from α-medium or human serum on a octadecyl-bonded (C_{18}) silica cartridge. In

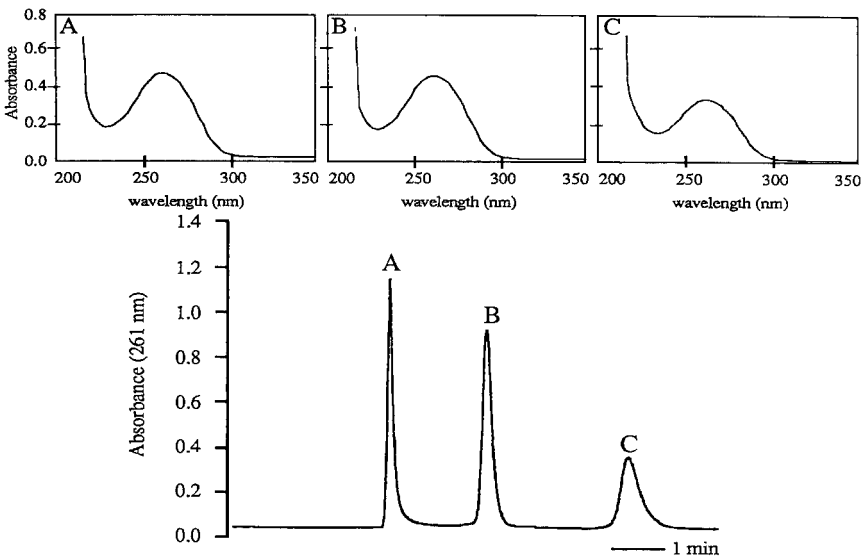


FIGURE 2. Chromatogram and spectra of aromatic poly-amidines. A: 1,3-di-(p-amidinophenoxy)-2,2-bis-(p-amidinophenoxymethyl)-propane (TAPP); B: 1,3-di-(p-amidinophenoxy)-propane (DAPP); C: 1-(p-amidinophenoxy)-2,2-bis-(p-amidinophenoxy-methyl)-butane (TAPB). Operating conditions as described under Materials and Methods.

order to eliminate interfering substances, the C_{18} sorbent was flushed with water and methanol. This latter washing step could be used because of the high affinity of poly-benzamidines for the stationary phase. Complete desorption of the tetra-amidines from the cartridge was then obtained with phosphate buffer (300 mM, pH 3) - methanol - tetrahydrofuran (60:30:5, v/v) containing 3% triethylamine. The recovery of TAPP and TAPP-Br from cell culture medium and serum, are reported in Table 1. Representative HPLC traces are shown in Figure 4.

Determination of TAPP-Br Release from Microspheres

Starting from the consideration that the therapeutic effect of a microencapsulated drug is function of the free drug concentration at the site of action, the analysis of

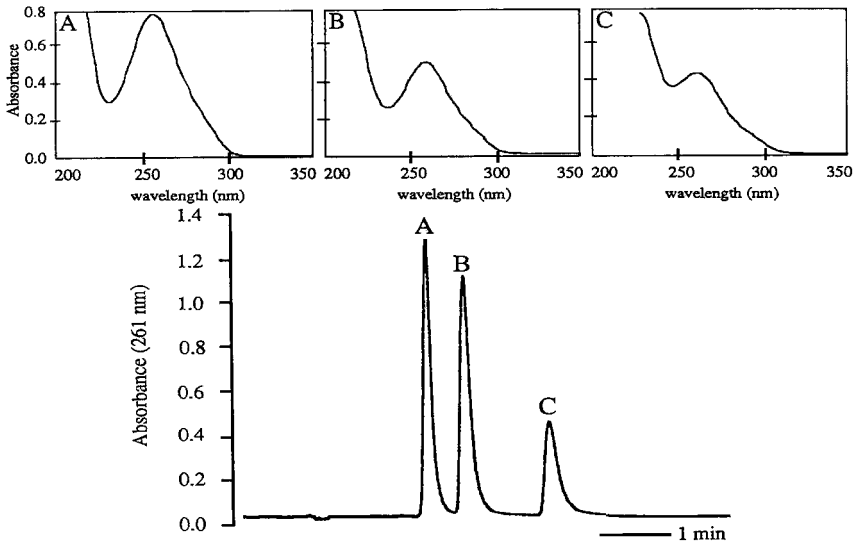


FIGURE 3. Chromatogram and spectra of 2' halo derivatives of the aromatic tetra-amidine 1,3-di-(p-amidinophenoxy)-2,2-bis-(p-amidinophenoxymethyl)-propane (TAPP). A: TAPP-Cl; B: TAPP-Br; TAPP-I. Operating conditions as in Fig. 2.

TABLE 1
Tetra-amidines Recovery from Cell Culture Medium and Human Serum

	α-medium	Human serum
TAPP	91.2±3.2	78.4±7.0
TAPP-Br	88.6±2.1	81.0±5.6

Data represent the average of percentage recovery (n=5) ± SD.

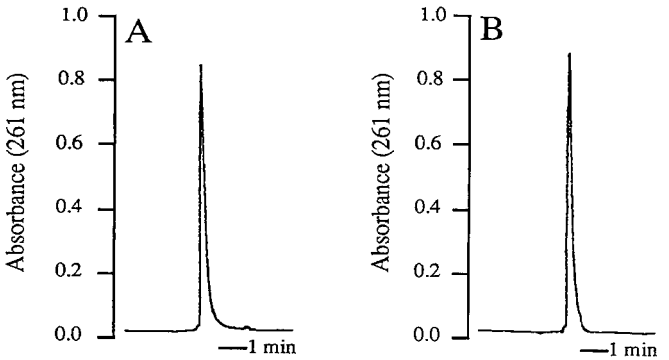


FIGURE 4. Representative HPLC traces of TAPP (A) and TAPP-Br (B) from human serum. Operating conditions as in Fig. 2. Control serum did not show any chromatographic interference in the areas of interest.

both amount and mechanism(s) of drug release from microparticulate systems, represents a fundamental step for the rationale design of nano- and microsphere based delivery systems (19).

In order to obtain reproducible and reliable quantitations of TAPP-Br during release experiments, the above reported HPLC method was employed. Figure 5 shows typical chromatograms of TAPP-Br containing solution from release experiments and the relative release profile obtained.

It is important to underline that the comparison of the TAPP-Br spectra collected by the rapid scan UV detector with that of pure compound suggests that interferences due to TAPP-Br degradation products or microsphere impurities, possibly contained in the release buffer are not present.

In this respect, the use of rapid scan UV detectors to identify and quantitate drugs by both retention time and absorption spectrum is more reliable than the use of HPLC with a conventional UV detector or simply UV determinations. This is especially true in the case of release experiments where drug degradation products and/or components released from microsphere matrix could (a) interfere with the analytical determination or (b) represent bioactive or toxic compounds playing important pharmacological roles after in vivo administration.

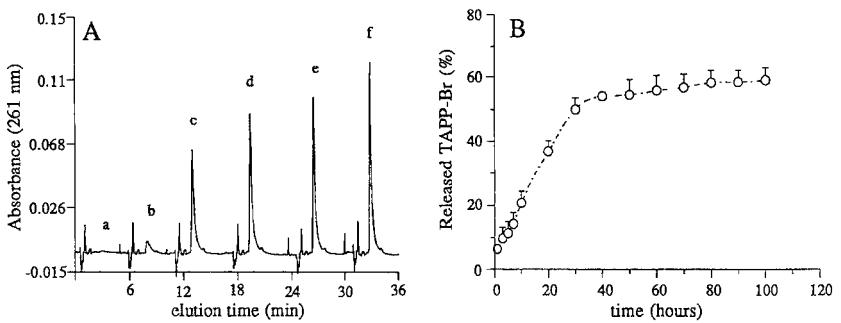


FIGURE 5. A: Typical chromatograms obtained from release experiments. Receiving buffer from gelatin microspheres was analysed for TAPP-Br content after 5 (a) 10 (b), 20 (c), 30 (d), 60 (e) and 100 (f) minutes. B: Resulting TAPP-Br release profile; the reported values represent the average of 6 independent experiments, bars = SD.

Concluding Remarks

In conclusion, a relatively simple and sensitive HPLC method for the simultaneous analysis of different poly-benzamidines has been developed. In addition a sample clean-up procedure is described for the determination of the compounds in plasma, suitable for pharmacokinetic studies. Further work is in progress to validate the method here proposed for the assay of poly-amidines in pharmaceutical formulations, in order to ensure batch to batch homogeneity in quality control tests.

Ackowledgements

This work was supported by AIRC and Regione Emilia-Romagna. We thank Prof. Ferroni of the Department of Pharmaceutical Sciences, University of Ferrara, for the generous gift of poly-benzamidines.

REFERENCES

1. K.L. Goa, D.M. Campori-Richards, Drugs, 33: 242-258 (1987).
2. J.P. Monk, P. Benfield, Drugs, 39: 741-756 (1990).
3. D. Smith, B. Gazzard, Drugs, 42: 628-639 (1991).

4. E. Menegatti, M. Guarneri, R. Ferroni, M. Bolognesi, P. Ascenzi, E. Antonini, FEBS Letters, 141: 33-36 (1982).

5. E. Menegatti, R. Ferroni, C. Nastruzzi, F. Bortolotti, S. Scalia, G. Amiconi, M. Bolognesi, M. Coletta, S. Onesti, R. Fruttero, P. Ascenzi, Il Farmaco, 46: 1297-1310 (1991).

6. C. Nastruzzi, G. Feriotto, D.A. Spandidos, R. Ferroni, M. Guarneri, R. Barbieri, R. Gambari, Clin. Exp. Metastasis, 7: 25-41 (1989).

7. A. Bartolazzi, R. Barbieri, C. Nastruzzi, P.G. Natali, R. Gambari, In Vivo, 3: 383-388 (1989).

8. C. Nastruzzi, R. Barbieri, R. Ferroni, M. Guarneri, D.A. Spandidos, R. Gambari, Cancer Letters, 50: 93-102 (1990).

9. R. Gambari, V. Chiorboli, G. Feriotto, C. Nastruzzi, Int. J. Pharmaceutics, 72: 251-258 (1991).

10. C. Nastruzzi, E. Menegatti, C. Pastesini, R. Cortesi, E. Esposito, M. Spanò, R. Biagini, E. Cordelli, G. Feriotto, R. Gambari, Bioch. Pharmacol., 44: 1985-1994 (1992).

11. R. Gambari, C. Nastruzzi, Biochem. Pharmacology, in press.

12. R. Ferroni, E. Menegatti, M. Guarneri, U. Taddeo, M. Bolognesi, P. Ascenzi and G. Amiconi, Il Farmaco, 39: 901-909 (1984).

13. D. G. Parsons, Brit. Pat., 1 288 376; C.A., 78: 398Or (1972).

14. S. S. Berg, G. Newbery, J. Chem. Soc., 28: 642-648 (1949).

15. C. Nastruzzi, C. Pastesini, R. Cortesi, E. Esposito, R. Gambari, E. Menegatti, J. Microencapsulation, in press.

16. C. Nastruzzi, R. Gambari, E. Menegatti, P. Walde and P.L. Luisi. J. Pharm. Sci., 79: 672-677 (1990).

17. J. Min-Hwa Lin, R.J. Shi and E.T. Lin. J. Liq. Chromatogr., 9: 2035-2046 (1986).

18. J. L. Glaich, J.J. Kirkland, K.M. Squire, J.M. Minor. J. Chromatogr., 199: 57-79 (1980).

19. B. Magenheim, S. Benita, STP Pharma Sciences, 1: 221-241 (1991).

Received: December 1, 1993
Accepted: December 10, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 2069-2083 (1994)

VALIDATION OF A HPLC METHOD FOR THE DETERMINATION OF PROPYLTHIOURACIL IN PLASMA

L. KABANDA[1], C. DE MUYNCK[1], R. A. LEFEBVRE[2], AND J. P. REMON[1]
[1]*Laboratory of Pharmaceutical Technology*
University of Gent
Harelbekestraat 72
B-9000 Gent, Belgium
[2]*Heymans Institute of Pharmacology*
University of Gent Medical School
De Pintelaan 185
B-9000 Gent, Belgium

ABSTRACT

A high-performance liquid chromatography method for the analysis of propylthiouracil in plasma was validated. Methylthiouracil was used as the internal standard. A one step extraction procedure and an isocratic HPLC method with UV detection were used. No plasma components were found to interfere in the assay. Linear calibration ($r^2 > 0.99$) curves using water and plasma as matrices in the range of 0.05 to 15 $\mu g.mL^{-1}$ were obtained. Good recoveries for propylthiouracil (> 85%) and methylthiouracil (> 65%) were seen both in water and in plasma. The coefficient of variation for repeatability was < 7%, for reproducibility < 8% and for accuracy <6%. The limit of detection was 2 $ng.mL^{-1}$ in water and 5 $ng.mL^{-1}$ in plasma. A poor stability of propylthiouracil was observed even at -20°C and it is recommended to perform the analyze shortly after sampling.

INTRODUCTION.

Colorimetry, the first method reported for propylthiouracil (PTU) determination in serum or plasma [1], was lacking both specificity and sensitivity. Gas chromatographic, high-performance liquid chromatographic (HPLC) and radioimmunoassay methods were developed [2-11] to overcome this problem. Because gas chromatography can only be carried out after conversion of PTU into a salt, this method is difficult and time consuming [2]. Several radioimmunoassay methods have been published [10,11] but they all require extensive sample pretreatment and overnight incubation.

Several HPLC methods were developed, using ion exchange [3] or reversed-phase systems [4-9] with UV detection. Some of these methods do not use an internal standard [4] or add the internal standard only after extraction [5]. Other methods require a deproteinisation step before PTU quantization [7]. Extraction recoveries for the internal standard [8] or for both the internal standard and PTU [9] are often low.

The chromatographic method and the extraction procedure in this paper were adapted from Rosseel M.T. and Lefebvre R.A. [8] in order to obtain a better specificity and sensitivity with low intra and inter day variability. This optimized method is used to analyze plasma samples from dogs and humans in the development of a sustained release formulation (data not included).

MATERIALS AND METHODS.

Chemicals.

The products used were: PTU (USP reference standard, Rockville, MD, USA), PTU and methylthiouracil (MTU) (Sigma, St. Louis, MO, USA), methyl

alcohol (HPLC grade, Janssen Chimica, Geel, Belgium), dichloromethane (HPLC grade, Fluka AG, Buchs, Switzerland), acetone (pro analyze, UCB, Leuven, Belgium) tetrahydrofuran (HPLC grade, LAB-SCAN, Brussels, Belgium), sodium hydroxide (pro analyze, Novolab, Gent, Belgium), potassium dihydrogen orthophosphate (highest purity, UCB, Leuven, Belgium), hydrochloric acid (pro analyze, Novolab, Gent, Belgium), N,N-dimethylacetamide (E.Merck, Darmstadt, F.R.Germany) and distilled water. Stock solutions of PTU (1 $mg.mL^{-1}$) and MTU (0.5 $mg.mL^{-1}$) were prepared in methanol. These solutions were stored at 8°C and were stable for at least 20 days. Working solutions were made by diluting the stock solutions in distilled water.

Instrumentation.

The HPLC system consisted of an Iso-Chrom LC pump (Spectra Physics, San Jose, California, USA) and a Spectra SYSTEM UV 2000 variable wavelength detector (Spectra Physics, San Jose, California, USA) set at 275 nm (λ max of PTU and MTU). Compound separation was performed at ambient temperature on a reversed-phase column (5 μm particles Lichrospher RP-18; 125 mm x 4 mm, E.Merck, Darmstadt, F.R.Germany) equipped with a precolumn (5 μm particles Lichrospher RP-18; E.Merck, Darmstadt, F.R.Germany). A 0.9% tetrahydrofuran solution in phosphate buffer pH 6.0 $\pm$ 0.1 was used as the mobile phase. The composition of the buffer was 34 g of potassium dihydrogen orthophosphate, 170 mL of NaOH 0.2 N and enough distilled water to make 5 L [17]. The mobile phase was degassed before use. The flow rate was 1.5 $mL.min^{-1}$. The samples were injected via a septumless syringe-loaded injector loop of 20 μL (Valco

Instruments Corporation, Houston, USA). Peak area response was calculated using the Spectra Station software (Spectra Physics, San Jose, California, USA).

Sample preparation.

The stock solutions of PTU (1 $mg.mL^{-1}$) and MTU (0.5 $mg.mL^{-1}$) were prepared in methanol. Dilutions in distilled water were prepared in borosilicate volumetric flask and extractions were performed in 15-mL borosilicate glass tubes (16 x 100 mm, Corning, NY, USA). Next, 450 μL of water or plasma (from dogs) were pipetted into a 15-mL borosilicate glass followed by the addition of 60 μL of hydrochloric acid 0.1 N and 50 μL of the internal standard solution (25 $\mu g.mL^{-1}$ in water). The mixture was vortexed for 10 s. followed by the addition of 6 mL of organic phase (dichloromethane-acetone: 75-25, v/v). After vortexing during 2 min. and centrifugation at 1500 g for 5 min., the organic phase was transferred to another tube and evaporated to dryness at 60°C under nitrogen. The samples were redissolved in 250 μL of water and vortexed for 20 s. Twenty μL were injected and analyzed using the chromatographic conditions described above.

Calibration Curves (in water and in plasma).

The solutions used to prepare the calibration curves contained PTU in the following concentrations: 0.5, 5, 10, 30, 50, 100 and 150 $\mu g.mL^{-1}$. MTU was used in a concentration of 25 $\mu g.mL^{-1}$.

The calibration graphs were prepared by adding 50 μL of PTU and internal standard (MTU) to blank samples (450 μL) in order to provide concentrations in

the range of 0.05 to 15 $\mu g.mL^{-1}$ (0.05, 0.5, 1, 3, 5, 10 and 15 $\mu g.mL^{-1}$) for PTU and 2.5 $\mu g.mL^{-1}$ for MTU.

Stability of PTU in plasma.

Known amounts of PTU were added to blank plasma in order to obtain concentrations of 1 $\mu g.mL^{-1}$ and 10 $\mu g.mL^{-1}$. These samples were stored at -20°C and analyzed after various storage times (0, 15, 90, 150 and 180 days). The stability of the samples was expressed as the amount recovered with the coefficient of variation (n=4) and related to the freshly prepared samples.

Calculations.

The calibration graphs were obtained by plotting the peak-area ratio of PTU to the internal standard versus the PTU concentration calculating the regression line parameters. Unknown concentrations were determined from the regression equation.

RESULTS AND DISCUSSION.

Optimisation of mobile phase.

PTU is a very weak acid (pKa of 7.5) [16]. An extraction in the unionised form was performed after acidification of the medium. During the optimisation of the extraction procedure it was observed that PTU showed, even in the unionised form, a better solubility in polar solvents (acetone, methyl alcohol, N,N-dimethylacetamide, etc.). Optimal extraction yield was obtained with a dichloromethane-acetone mixture (75:25; v/v). The volume of the extraction

liquid was increased up to 6 mL. Using MTU as the internal standard, a better separation was obtained from PTU than when 5-PTU was used. Two absorption maxima were found for PTU and MTU at 214 and 275 nm. At 214 nm interferences of endogenous compounds with PTU and the internal standard were observed while at 275 nm no such interferences occurred. A detection at 300 nm as proposed by Rosseel and Lefebvre [8] caused a sensitivity loss of about 50%.

Selectivity.

Figure 1 shows chromatograms from extracted plasma (A), extracted plasma spiked with PTU and MTU (B), and extracted plasma after I.V. administration of 300 mg of PTU (C). No interference of endogenous compounds or anticoagulant (Heparin Novo 5000 U.I.mL^{-1}, Novo Nordisk A/S, Bagsvaerd, Denmark) used during sample collection were detected. Under the chromatographic conditions used, there was a very good separation between PTU and the internal standard with retention times of 15.20 ($\pm$ 10%) and
2.60 ($\pm$ 10%) min., respectively. It can be concluded that the proposed method is selective for PTU [12].

Stability of PTU in plasma.

In plasma, drugs may be degraded due to storage conditions such as temperature, light, air and enzymes [13]. Plasma samples were stored over a six month period at -20°C. Two different concentrations were used: 1 $\mu g.mL^{-1}$ and 10 $\mu g.mL^{-1}$. For 1 $\mu g.mL^{-1}$ the measured concentrations were 0.87 $\mu g.mL^{-1}$

(CV: 5.4), 0.77 $\mu g.mL^{-1}$ (CV: 2.6), 0.46 $\mu g.mL^{-1}$ (CV: 5.5), 0.39 $\mu g.mL^{-1}$

(CV: 1.1), 0.25 $\mu g.mL^{-1}$ (CV: 2.2) respectively for 0, 15, 90, 150 and 180 days of storage and 9.47 $\mu g.mL^{-1}$ (CV: 3.13), 8.44 $\mu g.mL^{-1}$ (CV: 1.6), 6.58 $\mu g.mL^{-1}$ (CV: 0.7), 5.98 $\mu g.mL^{-1}$ (CV: 3.9) and 4.5 $\mu g.mL^{-1}$ (CV: 1.7) respectively for 10 $\mu g.mL^{-1}$. These results showed the poor stability of PTU in plasma when stored at -20°C indicating that analysis should be performed shortly after sampling.

Reference standard.

The standard curves were made using PTU obtained from Sigma (Sigma, St. Louis, MO, USA). A comparison of the purity of this PTU source to the USP reference standard was performed. From both PTU sources, a solution of 2 $\mu g.mL^{-1}$ in distilled water was prepared. After analysis (n=6) peak areas were compared. The mean area of PTU obtained from Sigma was 100.22% (CV: 1.31) in comparison to the USP reference standard.

Calibration and linearity.

Linear calibration curves were obtained in water and in plasma and were forced through the origin. In Table 1, the x coefficient and correlation coefficient are shown for the plasma calibration curves. The response was linear over the entire concentration range (0.05 to 15 $\mu g.mL^{-1}$).

Run-by-run variability in plasma was determined from calibration curves by calculating the coefficient of variation on the x coefficient of different calibration curves obtained on ten different days over a 3 month period. An average value of 1.11 (CV: 3.21%) was calculated.

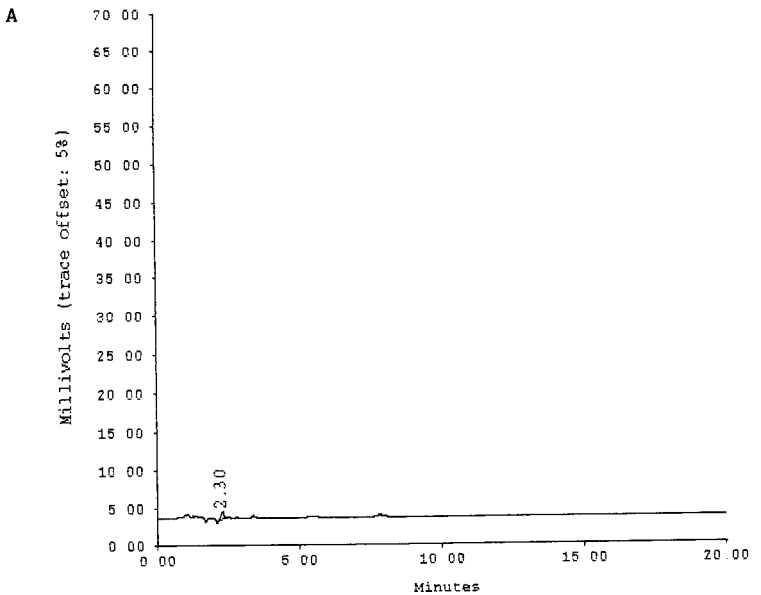


FIGURE 1.

A-Representative Chromatogram for Extracted Blank Plasma.

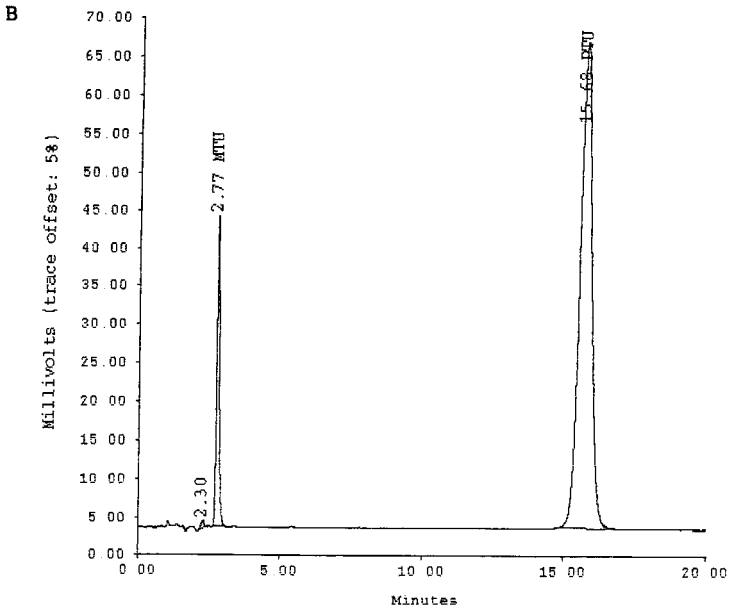
B-Representative Chromatogram for Extracted Plasma spiked with MTU (2.5 $\mu g.mL^{-1}$) and PTU (15 $\mu g.mL^{-1}$).

C-Representative Chromatogram for Extracted Plasma after I.V. Administration of 300 mg of PTU.

For within-run variability an average value of 1.09 (CV: 0.67%) was calculated (n=5).

Precision, repeatability and reproducibility.

Precision of a test procedure is defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same



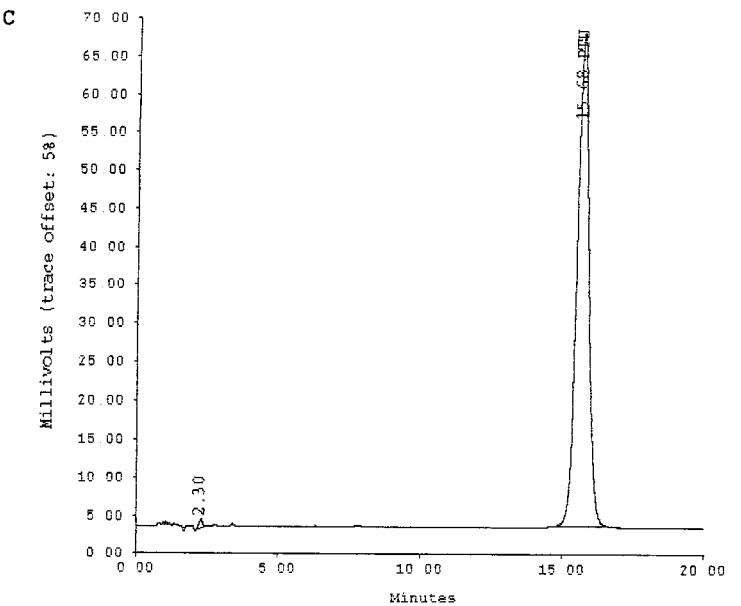


FIGURE 1 (continued)

TABLE 1.

X Coefficients and Correlation Coefficients for Calibration Curves (Mean ± C.V.) in Plasma for both within-Run (n=5) and Run-by-Run (n=10) Experiments. Calibration Curves were made for PTU Concentrations between 0.05 and 15 $\mu g.mL^{-1}$. The Concentration of the Internal Standard was 2.5 $\mu g.mL^{-1}$.

Within-run variability	
X coefficient	1.09 (± 0.67)
R squared	0.999 (± 0.03)
Run-by-run variability	
X coefficient	1.11 (± 3.21)
R squared	0.998 (± 0.29)

homogeneous sample under prescribed conditions (within-run variability: repeatability) or under different conditions (run-by-run variability: reproducibility) [12].

Repeatability and reproducibility were calculated for the different PTU concentrations in plasma (n=6). Table 2 shows the results obtained for different concentrations. Coefficients of variation for the repeatability test was within a 2.86-6.84% range of the expected concentration. For the reproducibility test it was within a 2.08-7.17% range. As can be seen in Table 2, higher variations were found only at the lowest concentration (0.5 $\mu g.mL^{-1}$). The acceptance criteria for precision is that the CV should not be greater than 15% [15].

Accuracy.

Accuracy is the closeness of agreement between the value accepted as the conventional true value (USP reference standard) and the values obtained by

TABLE 2.

Accuracy and Precision for Plasma containing PTU (n=6; Mean ± C.V.)

Concentration $\mu g.mL^{-1}$	Accuracy	Precision	
		Reproducibility (C.V)	Repeatability (C.V)
0.50	0.63 (±5.45)	0.52 (±7.17)	0.51 (±6.84)
1.00	1.07 (±3.51)	1.05 (±3.72)	1.07 (±2.86)
7.50	7.52 (±1.20)	7.62 (±2.08)	7.68 (±3.57)
9.00	8.66 (±4.74)	9.48 (±4.24)	9.29 (±3.88)
15.00	14.97 (±3.75)	15.12 (±2.67)	15.06 (±4.72)

applying the test procedure a number of times [12].

Different concentrations were tested and the results are shown in Table 2. The accuracy is within 6% (n=6) of the theoretical value at the concentrations tested. The acceptance criteria for accuracy is that the CV should not be greater than 15% [15].

Analytical recovery.

Sample preparation procedures are often causing loss of drug substance. Recovery is the percentage of reference material measured to the amount which has been added to water without performing an extraction [14].

The experiments were performed at different concentrations of PTU and at one concentration for the internal standard. At the different concentrations tested (n=10) the extraction was above 85% for PTU and around 66% for MTU in both media (Table 3). Extraction recovery reported in others HPLC methods were 59.4% [9], 71.1% to 81.6% [8], 74% [7] for PTU and 52.1% [8] for MTU. The

TABLE 3.

Recovery of PTU and MTU from Water and Plasma (n=10; Mean ± C.V.)

		Concentration ($\mu g.mL^{-1}$)	Recovery expressed as the percentage of the area of PTU or MTU obtained after extraction in water or in plasma to the area which has been measured in water without performing an extraction.
PTU	Plasma	0.5	94 (± 10.64)
		1	89 (± 4.83)
		7.5	90 (± 9.89)
		9	92 (± 3.69)
		15	95 (± 5.16)
	Water	1	85 (± 4.59)
		7.5	90 (± 4.89)
		15	92 (± 2.61)
MTU	Plasma	2.5	66 (± 4.09)
	Water	2.5	77 (± 3.24)

extraction recovery was improved for both PTU and MTU by the proposed procedure.

Limit of detection and limit of quantification.

The limit of detection is the lowest concentration of an analyte that the analytical process can reliably differentiate from background levels [15].

Using a bunching factor (BF) of 7 and a peak threshold (PT) of 291, the limit of detection was 2 and 5 ng.mL^{-1} in water and plasma, respectively. In other HPLC methods, limits of detection in plasma were 40 ng.mL^{-1} [9] and 50 ng.mL^{-1} [8]. The limit of quantification is the lowest concentration of an analyte that can be measured with a stated level of confidence [15]. With the same conditions (BF: 7 and PT: 291) and taking 15% CV as the level of acceptance, the limit of quantification was 20 and 40 ng.mL^{-1} in water and in plasma respectively.

Quality control.

Over a twelve month period, more than 500 plasma samples containing PTU were analyzed. During these experiments a quality control of the method was performed. Using two concentrations (2 and 10 μg.mL^{-1}) in plasma, one spiked sample was analyzed every 25 samples. The acceptability was evaluated using the bracket approach [14] not allowing a larger coefficient of variation than 15% for more than 10% of the spiked samples. This requirement was fulfilled over the period the analyses were carried out.

REFERENCES

1.C.R.Ratliff, P.F.Gilliland and F.F.Hall, Serum propylthiouracil: determination by a direct colorimetric procedure. Clin. Chem., 18 1373-1375 (1972).

2.D.Schuppan, S.Riegelman, B.V.Lehmann, A.Pilbrant and C.Becker, Preliminary pharmacokinetic studies of propylthiouracil in humans. J.Pharmacokinet.Biopharm., 1 307-318 (1973).

3.D.S.Sitar and D.B.Hunninghake, Pharmacokinetics of propylthiouracil in man after a single oral dose. J.Clin.Endocrinol.Metab., 40 26-29 (1975).

4.H.P.Ringhand and W.A.Ritschel, High-pressure liquid chromatographic determination of propylthiouracil in human plasma. J.Pharm.Sci., 68 1461-1462 (1979).

5. H.G.Giles, R.Miller and E.M.Sellers, High-performance liquid chromatographic determination of plasma propylthiouracil. J.Pharm.Sci., 68 1459-1460 (1979).

6.B.MacArthur and J.N.Miceli, Micro high-performance liquid chromatographic procedure for the quantization of serum propylthiouracil. J.Chromatogr., 278 464-468 (1983).

7.C.Kim, Simple and sensitive method for the determination of propylthiouracil in blood by high-performance liquid chromatography. J.Chromatogr. 272 376-379 (1983).

8.M.T.Rosseel and R.A.Lefebvre, High-performance liquid chromatographic determination of propylthiouracil in plasma. J.Chromatogr., 507 247-251 (1990).

9.G.R.Cannell and J.P.Williams, Selective liquid chromatographic assay for propylthiouracil in plasma. J.Chromatogr. 564 310-314 (1991).

10.D.S.Cooper, V.C.Saxe, F.Maloof and E.C.Ridgway, Studies of propylthiouracil using a newly developed radioimmunoassay. J.Clin.Endocrinol.Metab., 52 204-213 (1981).

11.R.Halperen, D.S.Cooper, J.D.Kieffer, V.Saxe, H.Mover, F.Maloof and E.C.Ridgway, Propylthiouracil (PTU) pharmacology in the rat. I.Serum and thyroid PTU measurements by radioimmunoassay. Endocrinology, 113 915-920 (1983).

12.Commission of the European Communities. GMP working party on quality of medicinal products. Note for guidance for analytical validation (August, 1989)

13.W.G.Peng and W.L.Chiou, Analysis of drugs and other toxic substances in biological samples for pharmacokinetic studies. J.Chromatogr., 531 3-50 (1990).

14.H.T.Karnes, G.Shiu and V.P.Shah, Validation of bioanalytical methods. Pharm.Res., 8 421-426 (1991).

15.V.P.Shah, K.K.Midha, S.Dighe, I.J.McGilveray, J.P.Skelly, A.Yacobi, T.Layloff, C.T.Viswanathan, C.E.Cook, R.D.McDowall, K.A.Pittman and S.Spector, Analytical methods validation: Bioavailability, bioequivalence and pharmacokinetic studies. Int.J.Pharm., 82 1-7 (1992).

16.J.P.Kampmann and J.M.Hansen, Clinical pharmacokinetics of antithyroid drugs. Clin.Pharmacokinet., 6 401-428 (1981).

17.U.S.Pharmacopoeial Convention, Inc., Rockville, USA., XXI (1985) p.1420.

Received: December 3, 1993
Accepted: December 11, 1993

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 2085-2092 (1994)

LIQUID CHROMATOGRAPHY CALENDAR

1994

MAY 8 - 13: HPLC '94: Eighteenth International Symposium on High Performance Liquid Chromatography, Minneapolis Convention Center, Minneapolis, Minnesota. Contact: Mrs. Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, USA.

MAY 23 - 25: International Symposium on Polymer Analysis and Characterization (ISPAC-7), Les Diablerets, Switzerland. Contact: Howard G. Barth, ISPAC Chairman, DuPont Company, Central Research & Development, P. O. Box 80228, Wilmington, DE 19880-0228, USA or Dr. M. Rinaudo, CERMAV-CNRS, B. P. 53X, 38041 Grenoble Cedex France.

JUNE 1 - 3: Joint 26th Central Regional/27th Great Lakes Regional Meeting, ACS, Kalamazoo and Huron Valley Sections. Contact: H. Griffin, Univ of Michigan, Ann Arbor, MI 48109, USA.

JUNE 1 - 3: International Symposium on Hormone & Veterinary Drug Residue Analysis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. C. Van Peteghem, University of Ghent, L:aboratory of Food Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 5 - 7: VIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques & Applications in Chromatography and Capillary Electrophoresis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. Dr. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Dept. of Pharmaceutical Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 6 - 9: 8th International LIMS Conference, The Westin William Penn Hotel, Pittsburgh, PA. Contact: Richard R. Mahaffey, 8th Int'l LIMS Conference, c/o Eastman Chemical Co., P. O. Box 1973/Bldg. 284, Kingsport, TN 37662, USA.

JUNE 12 - 15: 1994 Prep Symposium & Exhibit, sponsored by the Washington Chromatography Discussion Group, at the Georgetown University Conference Center by Marriott, Washington, DC. Contact: Janet E. Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 15: Fourth International Symposium on Field-Flow Fractionation (FFF'94), Lund, Sweden. Contact: Dr. Agneta Sjogren, The Swedish Chemical Society, Wallingatan 24, 2 tr, S-111 24 Stockholm, Sweden.

JUNE 17 - 19: 49th Northwest Regional ACS Meeting, Anchorage, Alaska. Contact: G. L. Trigiano, University of Alaska, 3890 University Lake Drive, Anchorage, AK 99508, USA.

JUNE 19 - 22: 68th Colloid & Surface Science Symposium, Stanford University, Stanford, California. Contact: Dept. of Chem. Engineering, Stanford, University, Stanford, CA 94305-5025, USA.

JUNE 19 - 22: 24th Northeast Regional ACS Meeting, Burlington, Vermont. Contact: W. R. Leenstra, Dept. of Chem., Iniversity of Vermont, Burlington, VT 05405, USA.

JUNE 19 - 23: 24th National Medicinal Chem. Symposium, Little America Hotel & Towers, Salt Lake City, Utah. Contact: M. A. Jensen & A. D. Broom, Dept. of Medicinal Chem, 308 Skaggs Hall, Univ of Utah, Salt Lake City, UT 84112, USA.

JUNE 19 - 24: 20th International Symposium on Chromatography, Bournemouth International Centre, Bournemouth, UK. Contact: Mrs. Jennifer A. Challis, The Chromatography Society, Suite 4, Clarendon Chambers, 32 Clarendon Street, Nottingham, NG1 5JD, UK.

JUNE 24 - 27: BOC Priestly Conference, Bucknell University, Lewisburg, PA, co-sponsored with the Royal Soc of Chem. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

JULY 31 - AUGUST 5: American Society of Pharmacognosy Annual Meeting, International Research Congress on Natural Products, joint meeting with the Assoc. Français pour l'Enseignement et la Recherche en Pharmacognosie, and the Gesellschaft fur Arzneipflanzenforschung, and the Phytochemical Society of Europe, Halifax, Nova Scotia, Canada. Contact: D. J. Slatkin, P. O. Box 13145, Pittsburgh, PA 15243, USA.

AUGUST 14 - 17: Summer National Meeting & Particle Technology Forum, AIChE, Denver, Colorado. Contact: AIChE Express Service Center, 345 East 47 Street, New York, NY 10017, USA.

AUGUST 21 - 23: Australasian Plastics & Rubber Inst. 7th Technology Convention, Melbourne, Australia. Contact: APRI, P. O. Box 241, Mont Albert 3127, Australia.

AUGUST 21 - 26: 208th ACS National Meeting, Washington, DC. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 29 - SEPTEMBER 2: Synthetic Membranes in Science & Industry, University of Tubingen, Germany. Contact: Dechema e.V., Exhibitions & Congresses, Theodor-Heuss-Allee 25, P. O. Box 150104, D-60486 Frankfurt am Main, Germany.

SEPTEMBER 4 - 9: 4th European Rheology Conference, Seville, Spain. Contact: C. Gallegos, Dept. de Ingenieria Quimica, Universidad de Seville, 41071 Seville, Spain.

SEPTEMBER 5 - 7: 25th International Symposium on Essential Oils, Grasse, France. Contact: Assoc. des Ingenieurs et Techniques de la Parfumerie (A.I.T.P.), 48 ave. Riou-Blanquet, 06130 Grasse, France.

SEPTEMBER 8 - 10: Surfaces in Biomaterials 1994, Scottsdale, Arizona. Contact: Surfaces in Biomaterials Foundation, c/o Ardel Management, P. O. Box 26111, Minneapolis, Minn, USA.

SEPTEMBER 12 - 15: 3rd International Conference on Separations in Biotechnology, University of Reading, U.K. Contact: SCI, Conference Office, 14/15 Belgrave Square, London SW1X 8PS, U.K.

SEPTEMBER 25 - 28: Future of Spectroscopy: From Astronomy to Biology, Ste.-Adele, Que., Canada. Contact: Doris Ruest, Conference Mgr., NRC Canada, Ottawa, Ont., Canada K1A 0R6.

SEPTEMBER 27 - 29: CCPC International Conference on Chemical Process Automation, Houston, Texas. Contact: AIChE Express Service Center, 345 East 47th Street, New York, NY 10017, USA.

OCTOBER 9 - 14: 186th Meeting of the Electrochemical Society. Contact: Electrochem. Soc., 10 S. Main St., Pennington, NJ 08534-2896, USA.

OCTOBER 16 - 19: 46th Southeastern Regional Meeting, ACS, Birmingham, Alabama. Contact: L. Kispert, Chem Dept, Univ of Alabama, Box 870336, Tuscaloosa, AL 35115, USA.

OCTOBER 17 - 19: 3rd Int'l Symposium on Supercritical Fluids, Strasbourg, France. Contact: Int'l Soc for the Advancement of Supercritical Fluids, 1 rue Grandville, B. P. 451, F-54001 Nancy Cedex France.

OCTOBER 18 - 22: 30th ACS Western Regional Meeting, Sacramento, California. Contact: S. Shoemaker, Calif. Inst of Food & Agricultural Res., Univ. of Calif-Davis, 258 Cruss Hall, Davis, CA 95616-8598, USA.

OCTOBER 23 - 27: Annual Meeting of the American Association of Cereal Chemists, Nashville, Tennessee. Contact: J. M. Schimml, AACC, 3340 Pilot Knob Road, St. Paul, MN 55121-2097, USA.

OCTOBER 24 - 26: 2nd Internat'l Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: SAICSC-ACS, P. O. Box 1723, Dhahrain 31311, saudi Arabia.

NOVEMBER 2 - 5: 29th Midwestern Regional Meeting, ACS, Kansas City, Kansas. Contact: M. Wickham-St. Germain, Midwest Res. Inst, 425 Volker Blvd, Kansas City, MO 64110, USA.

NOVEMBER 7 - 10: 13th Int'l Conference on the Application opf Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan, Univ. of N. Texas, Dept. of Physics, P. O. Box 5368, Denton, TX 76203, USA.

NOVEMBER 10 - 11: 17th Int'l Conference on Chemistry, Biosciences, Pharmacy & Environmental Pollution, New Delhi, India. Contact: V. M. Bhatnagar, Alena Chem of Canada, P. O. Box 1779, Cornwall, Ont., Canada K6H 5V7.

NOVEMBER 11 - 12: 3rd Conference on Current Trends in Computational Chemistry, Jackson, Mississippi. Contact: J. Leszczynski, Jackson State University, Dept. of Chem., 1400 J. R. Lynch St., Jackson, MS 39217, USA.

NOVEMBER 13 - 16: 50th Southwest Regional Meeting, ACS, Fort Worth, Texas. Contact: H. C. Kelly, Texas Christian Univ, Chem Dept, Ft. Worth, TX 76129, USA.

NOVEMBER 13 - 18: Annual Meeting of the A.I.Ch.E., San Francisco, California. Contact: AIChE Express Service Center, 345 East 47th St., New York, NY 10017, USA.

NOVEMBER 14 - 17: 33rd Eastern Analytical Symposium, Somerset, New Jersey. Contact: Sheri Gold, EAS, P. O. Box 633, Montchanin, DE 19710, USA.

NOVEMBER 16 - 18: Envirosoft'94, 5th Int'l Conference on Development and Application of Computer Techniques to Environmental Studies, San Francisco, California. Contact: Computational Mechanics, Inc., 25 Bridge St., Billerica, MA 01821, USA.

DECEMBER 4 - 6: IBEX'94, International Biotechnology Expo & Scientific Conference, Moscone Center, San Francisco, California. Contact: Cartlidge & Associates, Inc., 1070 Sixth Avenue, Suite 307, Belmont, CA 94002, USA.

1995

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 31 - JUNE 2: 27th Central regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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

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

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

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

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

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

1997

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

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

1998

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

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

1999

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

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

2000

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

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

2001

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

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

2002

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

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

The Journal of Liquid Chromatography will publish, **at no charge**, announcements of interest to liquid chromatographers in every issue of the Journal. To be listed in Meetings & Symposia, we will need to know: Name of the meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P.O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

Keep abreast of the **latest** *theoretical and applied research developments in the field with...*

Contains over 340 equations, tables, drawings, and photographs!

Advances in Chromatography

Volume 34

edited by
PHYLLIS R. BROWN, *University of Rhode Island, Kingston*
ELI GRUSHKA, *The Hebrew University of Jerusalem, Israel*

May, 1994 / 456 pages, illustrated / $165.00

Reviewer praise for this valuable series...

"...the articles [are] of high scientific standard, up to date, very well written, and interesting to read... well presented and edited."
—*Journal of Chromatography*

"...a valuable contribution to the chromatography literature...belongs in every library used by chromatographers." —*Liquid Chromatography*

"...maintains the high quality that chromatographers have come to expect from this invaluable series." —*Journal of Pharmaceutical Sciences*

The rapidly expanding growth of the literature on chromatography and capillary electrophoresis makes it difficult for any single individual to maintain a coherent view of progress in the field. Rather than attempt to read the avalanche of original research papers, investigators trying to preserve even a modest awareness of advances must rely upon **authoritative** surveys.

Featuring reliable, **up-to-the-minute** reviews of major developments in chromatography, this critically praised series separates the most important advances from an overabundance of supplementary materials.

Internationally acclaimed experts analyze the most* current *innovations in their areas of specialization!

Furnishing more than **800** literature citations, allowing for further, in-depth study of recent trends in research, **Volume 34** examines timely subjects such as

- high performance capillary electrophoresis—*of particular interest to engineers, chemists, biotechnologists, and environmental scientists*
- gas chromatography, matrix isolation, and infrared spectrometry—*geared toward the needs of food scientists, medical professionals, and agriculturalists*
- statistical theories of peak overlap in chromatography—*especially applicable to quality control engineers*
- capillary electrophoresis of carbohydrates and proteins—*expressly for pharmacologists, biochemists, biologists, and medical professionals*
- supercritical fluid chromatography—*essential for chemists, environmental scientists, engineers, and biologists*
- and much more!

Advances in Chromatography is an indispensable resource for all researchers who need to use separation methods effectively—especially analytical, organic, inorganic, clinical, and physical chemists; chromatographers; biochemists and biological chemists; agrochemists; chemical, materials, and quality control engineers; biotechnologists; toxicologists; pharmacologists; pharmacists; physiologists; zoologists; botanists; food, cosmetic, polymer, and environmental scientists; oceanographers; research and quality control scientists in academia, government, hospitals, and industry; and upper-level undergraduate and graduate students in these disciplines.

Contents

ISBN: 0—8247—9087—1

This book is printed on acid-free paper.

Marcel Dekker, Inc.

270 Madison Avenue, New York, NY 10016
(212) 696-9000

Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
Tel 061-261-8482

ELECTRONIC MANUSCRIPT SUBMISSION

Effective immediately, manuscripts will be accepted on computer diskettes. A printed manuscript must accompany the diskette. For approximately one year, the diskettes will be used, on an experimental basis, to produce typeset-quality papers for publication in the Journal of Liquid Chromatography. Diskettes must be in an IBM-compatible format with MS-DOS Version 3.0 or greater. The following word processing formats can be accommodated:

ASCII	DisplayWrite Native
EBCDIC	Enable 1.0, 2.0, 2.15
Framework III 1.0, 1.1	IBM Writing Assistant
Microsoft Word 3.0, 3.1, 4.0, 5.0	Multimate 3.3
Multimate Advantage 3.6	Multimate Advantage II 3.7
Navy DIF	Office Writer 4.0, 5.0, 6.0, 6.1
PeachText 5000 2.12	PFS:First Choice 1.0, 2.0
PFS:Write Ver C	Professional Write 1.0, 2.0, 2.1
Q&A Write 3.0	RapidFile (Memo Writer) 1.2
Samna Word IV & IV+ 1.0, 2.0	Total Word 1.2, 1.3
Volkswriter 3, 4	Volkswriter Deluxe 2.2
Wang PC Ver 3	WordPerfect 4.1, 4.2, 5.0, 5.1*
WordStar 3.3, 3.31, 3.45, 4.0, 5.0, 5.5, 6.0	XyWrite III XyWrite III+

* The **preferred** word processor is **WordPerfect 5.1.**

Manuscripts and diskettes should be prepared in accordance with the **Instructions for Authors** given at the back of this issue of the Journal. They should be sent to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 2180
Cherry Hill, NJ 08034

INSTRUCTIONS TO AUTHORS

Journal of Liquid Chromatography is published in the English language for the rapid communication of research in liquid chromatography and its rela' ' sciences and technologies.

Directions for Submission

One typewritten manuscript, suitable for direct reproduction, and two (2) clear copies with figures must be submitted. Since the Journal is produced by direct photography of the manuscripts, typing and format instructions must be strictly followed. Non-compliance will result in return of the manuscript to the author and will delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards before mailing.

Manuscripts may also be submitted on **computer diskettes**. A printed manuscript must also be submitted with diskettes because, at the present time, we are experimenting with manuscripts on diskettes. Diskettes must be readable with an IBM-compatible computer (Macintosh or other type not acceptable) and must be formatted with MS-DOS 3.1 or greater. Be sure to indicate the word processing software that was used to prepare the manuscript diskette.

Manuscripts and computer diskettes should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 2180
Cherry Hill, NJ 08034

Reprints

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

Format of the Manuscript

1. The general format of the manuscript should be:
   Title
   Author(s)' names and full addresses
   Abstract
   Text Discussion
   References

2. **Title & Authors:** The entire title should be in capital letters and centered within the width of the typing area, located at least 2 inches (5.1 cm) from the top of the page. This should be followed by 3 lines of space, then by the names and addresses of the authors, also centered, in the following manner:

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

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

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

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

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGEMENTS
REFERENCES

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

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

5. The first word of each **paragraph** within the body of the text should be indented five spaces.

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

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

Following are acceptable reference formats:

Journal:

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

Book:

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

2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used.

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

Additional Typing Instructions

1. The manuscript must be prepared on good quality **white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). The typing area of the first page, including the title and authors, should be 5½ inches wide by 7 inches high (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches high (14 cm x 21.6 cm).

2. The **title, abstract, tables and references** are typed single-spaced. All other text should be typed 1½-line spaced or double line spaced.

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

4. **Tables** should be typed on separate pages, one table to a page. A table may not be longer than one page. If a table is larger than one page, it should be divided into more than one table. The word **TABLE** (capitalized and followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

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

Captions for figures should be typed single-spaced on a separate sheet of white paper, along the full width of the type page, and should be preceded with the word FIGURE and an Arabic numeral. All figures and lettering must be of a size that will remain legible after a 20% reduction from the original size. Figure numbers, name of senior author and an arrow indicating "top" should be written in light blue pencil on the back of the figure. Indicate the approximate placement for each figure in the text with a note written with a light blue pencil in the margin of the manuscript page.

6. The **reference list** should be typed single-spaced. A single line space should be inserted after each reference. The format for references should be as given above.