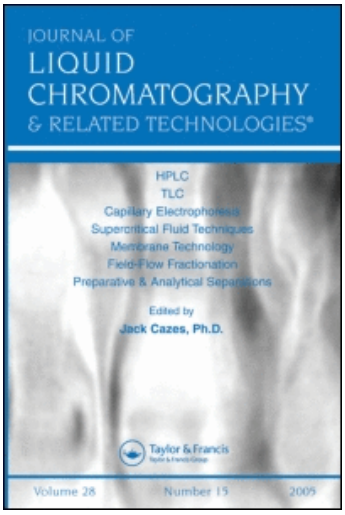


Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Use of pH-Zone-Refining Countercurrent Chromatography to Separate 2- and 6-Nitro-4-chloro-3-methoxybenzoic Acid

T. Dudding[a]; B. Mekonnen[a]; Y. Ito[b]; H. Ziffer[c]

[a] Laboratory of Neurotoxicology, National Institute of Mental Health, Bethesda, MD [b] Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, Bethesda, MD [c] Laboratory of Chemical Physics, National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD

To cite this Article Dudding, T. , Mekonnen, B. , Ito, Y. and Ziffer, H.(1998) 'Use of pH-Zone-Refining Countercurrent Chromatography to Separate 2-and 6-Nitro-4-chloro-3-methoxybenzoic Acid', Journal of Liquid Chromatography & Related Technologies, 21: 1, 195 — 201

To link to this Article: DOI: 10.1080/10826079808001948

URL: http://dx.doi.org/10.1080/10826079808001948

J. LIQ. CHROM. & REL. TECHNOL., 21(1&2), 195–201 (1998)

USE OF pH-ZONE-REFINING COUNTERCURRENT CHROMATOGRAPHY TO SEPARATE 2- AND 6-NITRO-4-CHLORO-3-METHOXYBENZOIC ACID

T. Dudding,[1] B. Mekonnen,[1] Y. Ito,[2] H. Ziffer[3]

[1] Laboratory of Neurotoxicology
National Institute of Mental Health
National Institutes of Health
Bethesda, MD 20892

[2] Laboratory of Biophysical Chemistry
National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, MD 20892

[3] Laboratory of Chemical Physics
National Institute of Diabetes,
Digestive and Kidney Diseases
National Institutes of Health
Bethesda, MD 20892

ABSTRACT

A rapid, efficient separation of multigram quantities of 2- and 6-nitro-4-chloro-3-methoxybenzoic acid was achieved by pH-zone-refining CCC.

INTRODUCTION

Quinolinic acid, **1**, is an excitotoxic metabolite of L-tryptophan that has been found in much higher than normal concentrations in humans and animals with several neurological disorders.[1-3]

Although **1** is an agonist of NMDA receptors, it may also serve as an indicator of inflamation. Heyes et al. investigated effects of 6-chlorotryptophan and 4-chloroanthranilic acid, **2**, on the metabolism of L-tryptophan to kynurenate and quinolinate in the central nervous system.[4]

4-Chloroanthranilic acid has been shown to inhibit 3-hydroxyanthranilic acid oxidase[5] and to reduce quinolinate production *in vivo*.[6,7] In order to determine if secondary damage to neurons found in animal models of stroke, spinal cord injury, and other CNS disorders can be diminished by reducing quinolinic acid production, multigram quantities of **1** were required for animal studies. A difficult separation of 2- and 6-nitro-4-chloro-3-methoxybenzoic acid was necessary in our synthesis of **1** shown in Figure 1.

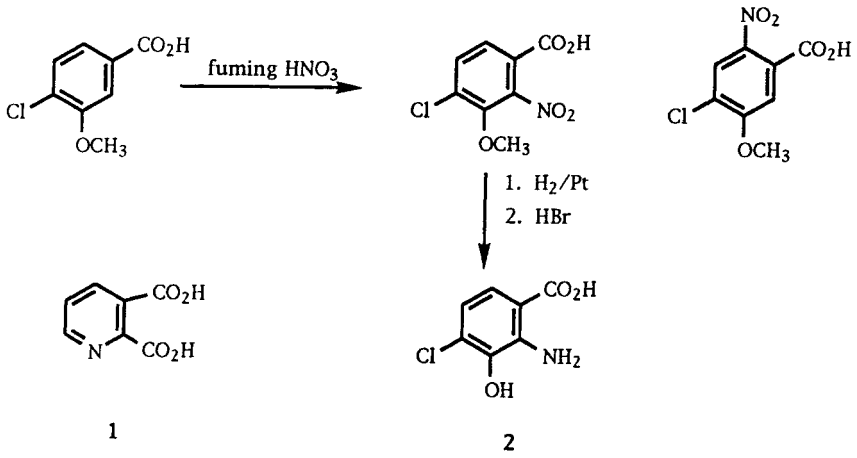
Although 10 g or less of the isomeric mixture could be separated by column chromatography on silica gel, the large quantities of solvent, and silica gel required prompted us to examine the use of pH-zone-refining Countercurrent Chromatography (CCC).

pH-Zone-refining CCC is a preparative method for purification recently developed in our laboratory.[8-10] Compounds elute as highly concentrated rectangular peaks. Multigram quantities of ionic compounds have been separated using CCC according to differences in their pK_a and hydrophobicity.

EXPERIMENTAL

Synthesis of 2- and 6-Nitro-4-Chloro-3-Methoxybenzoic acid

An ice-cold solution of red fuming nitric acid (151 mL, 3.6 mol) was added dropwise to similarly cooled acetic anhydride (59.5 mL, 0.6 mol). Slow addition of this cold mixture to solid 4-chloro-3-methoxybenzoic acid (42.3 g, 0.227 mol) over 30 min with vigorous stirring at 0°C afforded a mixture which, on gradual warming to room temperature, became a reddish-brown solution. The solution was poured onto 400 g of ice and the mixture stored at 0°C overnight. The resulting precipitate was collected by filtration, washed with



1 2

Figure 1. Synthesis of 2-amino-3-hydroxy-4-chlorobenzoic acid.

cold water, and dried under high vacuum to yield 43.76 g (83%) of a white solid. The crude reaction product contains a 2:1 mixture of the 2- and 6-nitro isomers, as determined by integration of the peaks at δ = 7.82 and 8.10 ppm in a ^{1}H NMR spectrum of the mixture, and other materials.

Reagents for CCC Separations

The organic solvents employed were methyl t-butyl ether, acetonitrile, and trifluoroacetic acid, glass-distilled chromatographic grades purchased from Fisher Scientific, and reagent grade ammonium hydroxide (28%) from Mallinckrodt.

CCC Apparatus

The separation was performed using a commercial model of the high-speed CCC centrifuge (Ito Multilayer Coil Separator/Extractor, P.C. Inc., Potomac, MD, USA). The detailed design of the apparatus is described elsewhere.[9] A 160-m long, 1.6 mm i.d. Tefzel tube (Zeus Industrial Products, Raritan, NJ, USA) was wound onto a spool-shaped holder, making multiple coiled layers with a total capacity of 310 mL. Each end of the column was connected to a flow tube of 0.85 mm i.d. The speed of the apparatus was regulated at 800 rpm using a speed controller (Bodine Electric Co., Boston, MA, USA).

Preparation of Solvent Phases and Sample Solutions

A two-phase solvent system, composed of methyl t-butyl ether, acetonitrile and water, at a volume ratio of 4:1:5, was prepared. The solvent mixture was thoroughly equilibrated in a separatory funnel and the phases were separated shortly before use. Trifluoroacetic acid (TFA) was added to the upper organic phase at 0.3% (12 mM, pH 2.2) which was employed as the stationary phase. Ammonia 0.8% (100 mM, pH 10.6) was added to the lower aqueous phase. The mixture of isomers (10 g) was dissolved in the stationary phase (40 mL). Prior to injecting this solution onto the column, 1 mL of the lower aqueous phase was added to verify formation of two phases (cloudy appearance) in the sample solution.

Separation Procedure

The column was filled with the organic stationary phase, followed by injection of the sample solution through the sample port. The aqueous mobile phase was then pumped through the head-end of the column at a flow-rate of 3 mL/min while the apparatus was rotated at 800 rpm. The effluent from the tail end of the column was continuously monitored with a UV detector (Uvicord S, LKB Instruments, Stockholm, Sweden) at 280 nm. The solution was passed through a pH flow cell (Sensorex, Stanton, CA, USA),[12] and collected as 6 mL portions using a fraction collector (Ultrorac, LKB Instruments). Alternatively, the pH of the fractions was measured using a portable pH meter (Accumet 1001). The separation of 11.7 g of crude mixture of 2- and 6-nitro-4-chloro-3-methoxybenzoic acid was accomplished with 711 mL of a 4:1:5 mixture of methyl t-butyl ether : acetonitrile: water.

Analysis of CCC Fractions

The fractions were analyzed by thin layer chromatography on silica gel using 98% methylene chloride and 2% acetic acid. The pure 6-nitro isomer (3.11 g) eluted in fractions 33-43, while the pure 2-nitro isomer (5.93 g) eluted in fractions 53-89. Fractions 44-52 (0.78 g) contained a mixture of the two isomers.

RESULTS AND DISCUSSION

A typical separation of 2- and 6-nitro-4-chloro-3-methoxybenzoic acid by pH-zone refining CCC is shown in Fig. 2. The two isomers were eluted as

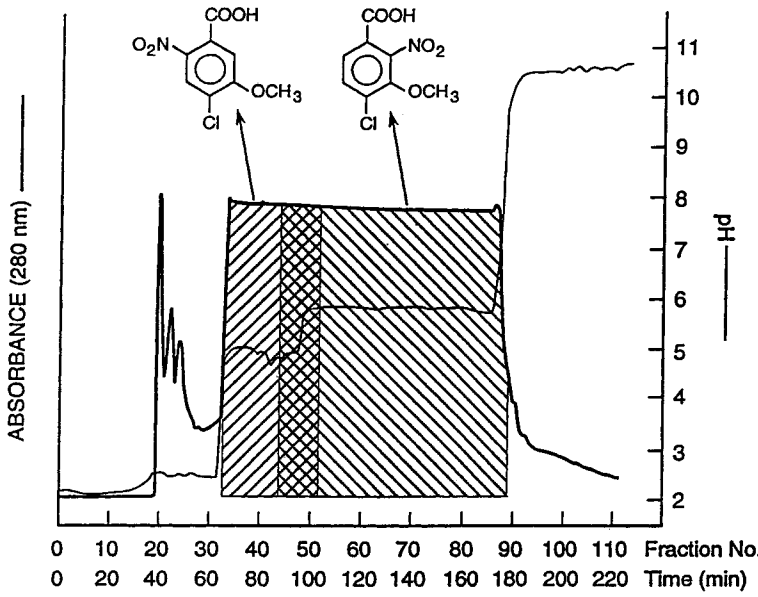


Figure 2. Separation of 2- and 6-nitro-4-chloro-3-metyhoxybenzoic acids by pH-zone-refining countercurrent chromatography.

fused rectangular peaks (thick curve) each corresponding to the specific pH zone (thin curve). Utilizing this procedure, an 11.7 g sample of the mixture was separated to yield 5.93 g of 2-nitro-4-chloro-3-methoxybenzoic acid, 3.11 g. of 6-nitro-4-chloro-3-methoxybenzoic acid, and 0.7 g of a mixture of isomers. With a sample size of 20 g the separation yielded 9 g of the pure 2-nitro compound, 5.3 g of the 6-nitro isomer and 2 g of the mixture of isomers. In an earlier separation of 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid by pH-zone-refining CCC the upper organic phase was employed as the mobile phase (displacement mode).[12] In that separation approximately 40% of the material was present as a mixture, i.e. there was a relatively large overlapping zone. The results of these studies demonstrate that pH-zone-refining CCC can be efficiently used for the purification of multigram quantities of structural isomers from a crude reaction mixture. The separation took less than four hours and required much less solvent than what would have been used in conventional column chromatography. Furthermore, the methodology does not require the use of expensive silica gel.

ACKNOWLEDGMENT

The authors thank Drs. H. M. Fales and M. Garraffo for their valuable suggestions and editing the manuscript.

REFERENCES

1. T. W. Stone, "Neuropharmacology of Quinolinic and Kynurenic Acids," Pharm. Revs., **45**, 309 (1993).

2. M. P. Heyes, B. J. Brew, R. W. Price, A. M. Salazar, J. J. Sidtis, J. A. Yergey, M. M. Mouradian, A. E. Sadler, J. Keilip, D. Rubinow, S. P. Markey, "Quinolinic Acid in Cerebrospinal Fluid and Serum in HIV-1 Infection: Relationship to Clinical and Neurological Status," Ann. Neurol., **29**, 202 (1991).

3. Y. Sei, I. A. Paul, K. Saito, R. Layer, J. W. Hartley, H. C. Morse, P. Skolnick, M. P. Heyes, "Quinolinic Acid Levels in a Murine Retrovirus-Induced Immunodeficiency Syndrome," J. Neurochem., **66**, 296 (1996).

4. D. B. Naristin, K. Saito, S. P. Markey, C. Y. Chen, M. P. Heyes, "Metabolism of L-Tryptophan to Kynurenate and Quinolinate in the Central Nervous System: Effects of 6-Chlorotryptophan and 4-Chloro-3-Hydroxyanthranilate," J. Neurochem., **65**, 2217 (1995).

5. M. P. Heyes, B. Hutto, S. P. Markey, "4-Chloro-3-hydroxyanthranilate Inhibits Brain 3-Hydroxyanthranilate Oxidase," Neurochem. Int., **13**, 405 (1988).

6. W. P. Todd, B. K. Carpenter, R. Schwarcz, "Preparation of 4-Halo-3-Hydroxyanthranilates and Demonstration of their Inhibition of 3-Hydroxyanthranilate Oxygenase Activity in Rat and Human Brain Tissue," Prep. Biochem., **19**, 155 (1989).

7. J. L. Walsh, H.-Q. Wu, U. Ungerstedt, R. Schwarcz, "4-Chloro-3-Hydroxyanthranilate Inhibits Quinolinate Production in the Rat Hippocampus *in Vivo*," Brain Res. Bull., **35**, 513 (1994).

8. A. Weisz, A. L. Scher, K. Shinomiya, H. M. Fales, Y. Ito, "A New Preparative-Scale Purification Technique: pH-Zone-Refining Countercurrent Chromatography," J. Am. Chem. Soc., **116**, 704 (1994).

9. Y. Ito, **High-Speed Countercurrent Chromatography**, Wiley & Sons, New York, 1996, pp. 121-175.

10. Y. Ito, Y. Ma, "pH-Zone-Refining Countercurrent Chromatography," J. Chromatogr. A, **753**, 1 (1996).

11. A. Weisz, A. L. Scher, Y. Ito, "Isolation of 4'-Bromo-4,5,6,7-tetrachloro-fluorescein From a Synthetic Mixture by pH-Zone-Refining Countercurrent Chromatorgaphy From a Synthetic Mixture with Continuous pH Monitoring," J. Chromatogr., A, **732**, 283 (1996).

12. Y. Ma, Y. Ito, D. S. Torok, H. Ziffer, "Separation of the 2- and 6-Nitro-3-acetamide-4-chlorobenzoic Acid Precursors of a Potent Hydroxy-anthranilic Acid Oxygenase Inhibitor by pH-Zone Refining-Counter-Current Chromatography," J. Liq. Chromatogr., **17**, 3507 (1994).

Received March 20, 1997
Accepted May 13, 1997
Manuscript 4477