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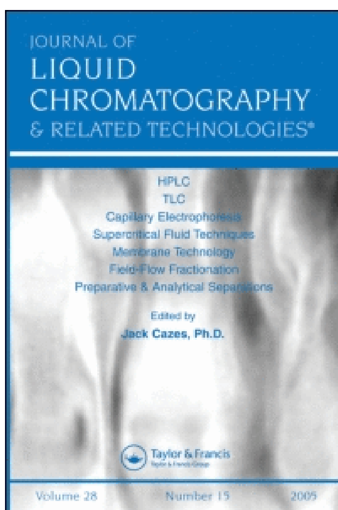

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Separation of the 2- and 6-Nitro-3-Acetamido-4-Chlorobenzoic Acid Precursors of a Potent Hydroxyanthranilic Acid Oxygenase Inhibitor by pH-Zone-Refining-Countercurrent Chromatography

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SEPARATION OF THE 2- AND 6-NITRO-3-ACETAMIDO-4-CHLOROBENZOIC ACID PRECURSORS OF A POTENT HYDROXY-ANTHRANILIC ACID OXYGENASE INHIBITOR BY pH-ZONE-REFINING-COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

Multigram quantities of 2- and 6-nitro-3-acetamido-4-chlorobenzoic acids were separated by pH-zone-refining CCC. A two-phase solvent system composed of methyl tertiary-butyl ether, acetonitrile and water was employed using trifluoroacetic acid as a displacer acid in the organic mobile phase and ammonia as a retainer base in the aqueous stationary phase.

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INTRODUCTION

Quinolinic acid, a metabolite of tryptophan in the kynurenic pathway, is present in high levels in the brain and blood of patients and primates with a broad spectrum of inflammatory neurological diseases (1). The level of quinolinic acid present was correlated with the severity of the neurological distress and prompted the suggestion that the neurological disturbances resulted from high levels of quinolinic acid. One approach to reducing the high levels of quinolinic acid was to employ inhibitors of enzymes responsible for the conversion of tryptophan to quinolinic acid, Saito et al. (2) found that 6-chlorotryptophan and its metabolite, 4-chloro-3-hydroxy-anthranilic acid were potent inhibitors of 3-hydroxy-anthranilic acid oxygenase. For animal studies large quantities of 4-chloro-3-hydroxy-anthranilic acid had to be prepared. We employed a reaction sequence developed by Glibin et al. (3) where a critical step in their sequence involves nitration of 3-acetamido-4-chlorobenzoic acid followed by separation of the isomeric mononitro-derivatives, 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid (Fig. 1). These two isomers were difficult to separate by repeated fractional crystallization of their barium salts. Our

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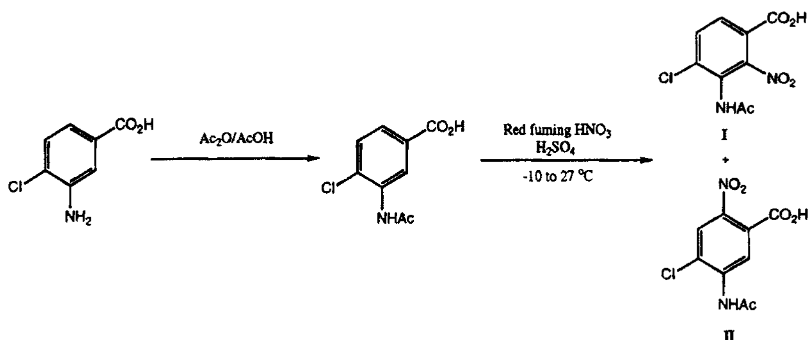

FIGURE 1. Synthesis and structures of isomers I and II: 2- and 6-nitro-3-acetamido-4-chlorobenzonic acids.

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attempts to find a solvent system for separating these isomers by column or flash chromatography were unsuccessful.

A recently developed preparative method, pH-zone-refining CCC (4-8), yields highly concentrated rectangular peaks of analytes comparative to those observed in displacement chromatography (9). The method has been applied to ionizable compounds, either acids or bases, which are eluted in the order of their pK_a's and hydrophobicities. In this paper, we describe the use of pH-zone-refining CCC (in a displacement mode) for the separation of multigram quantities of 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid. Each analyte was characterized by ^{1}H and ^{13}C NMR.

EXPERIMENTAL

Synthesis of 3-Acetamido-4-Chloro-2-Nitrobenzoic Acid (I) and 3-Acetamido-4-Chloro-6-Nitrobenzoic Acid (II)

To a hot (90°C) solution of 3-amino-4-chlorobenzoic acid (200 g, 1.17 mol) in glacial acetic acid (1000 ml) was added acetic anhydride (541 g, 500 ml, 5.30 mol) over 5 min. The solution was kept at 90°C for 30 min and cooled to 4°C. The resulting solids were filtered and washed with cold ether (2 X 1000 ml) to afford 3-acetamido-4-chlorobenzoic acid (212 g, 85 %, mp 206-208°C).

To a stirred solution of 3-acetamido-4-chlorobenzoic acid (200 g, 0.936 mol) at -10°C (ice/MeOH) was added a solution of red fuming nitric acid (787 g, 500 ml, 12.5 mol) in sulfuric acid (460 g, 250 ml, 4.96 mol) over 3 hr. The reaction was stirred at -10°C for 30 min then slowly warmed to RT and poured over ice (1000 g). The resulting yellow solid was filtered and washed with cold water (2 X 750 ml) to afford a mixture of nitro isomers I and II (Fig. 1).

CCC Apparatus

A commercial model (Ito Multilayer Coil Separator/Extractor, Potomac, MD, USA) of the high-speed CCC centrifuge was used throughout the present

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studies. The basic design of the apparatus was reported elsewhere (4).

The separation column was prepared in our laboratory by winding a single piece of 1.6 mm ID 160 m long PTFE (polytetrafluoroethylene) tubing around the column holder hub forming 16 coiled layers with 325ml capacity.

The revolution speed was regulated with a speed controller (Bodine Electric Company, North Chicago, IL, USA). An optimum speed of 600 - 800 rpm was used in the present studies.

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Reagents for CCC Separations

Methyl tertiary-butyl ether (HPLC grade) and ammonium hydroxide (reagent grade) were purchased from Fisher Scientific Company, Fair Lawn, NJ, USA. Acetonitrile (HPLC grade) and trifluoroacetic acid (reagent grade) were purchased from Baxter Healthcare Corporation, Muskegon, MI, USA.

Preparation of Solvent Phases and Sample Solutions

The solvent pairs were prepared as follows: Methyl tertiary-butyl ether, acetonitrile and distilled water (4:1:5, v/v/v) were thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated. The upper organic phase was

acidified with trifluoroacetic acid (0.32%) (pH 1.74) while aqueous ammonia was added to the lower aqueous phase (0.8%, pH 11.2).

The sample solution was prepared by dissolving a crude synthetic mixture of the isomers in 100 ml of a phase mixture consisting of equal volumes of each phase. The pH of the sample solution was adjusted to about 8.7 with aqueous ammonia.

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Separation Procedures

The displacement mode of pH-zone-refining CCC was performed as follows: the column was first entirely filled with the aqueous phase containing 0.8% aqueous ammonia (retainer base) followed by sample injection through the sample port. Then, the acidified organic phase containing 0.32% trifluoroacetic acid (displacer) was pumped into the inlet of the column in the tail to head elution mode while the column was rotated at 600 rpm which was later raised to 800 rpm after 10 fractions had been collected. The above manipulation of the column rotation was to minimize the carryover of the stationary phase. The effluent from the outlet of the column was continuously monitored with a uv detector (Uvicord S, LKB Instruments, Bromma/Stockholm, Sweden) at 206 nm and collected at two minute intervals (6.6ml/tube) with a fraction collector (Ultrorac, LKB Instruments).

In this elution mode, the two phases establish a reverse pressure gradient through the column where the pressure at the column inlet often plunges into a negative range causing suction of an extra volume of solvent from the reservoir through the one-way check valves of the metering pump. In order to prevent this complication, a piece of PTFE tubing (0.4mm ID x 3m long) was placed at the outlet of the detector to maintain the column pressure above an atmospheric level. This device also prevented formation of gas bubbles inside the flow cell of the uv detector, thus reducing the noise level in the recording of the elution curve.

After the desired peaks were eluted, the apparatus was stopped and the column contents were collected into a graduated cylinder by connecting the inlet of the column to a nitrogen line at 80 psi. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

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Analysis of CCC Fractions

The pH value of each fraction was manually determined with a portable pH meter (Accumet Portable Laboratory, Fisher Scientific Company, Pittsburgh, PA, USA).

CCC fractions were analyzed by reversed phase high performance liquid chromatography (HPLC) with a Shimadzu HPLC consisting of a Model LC-6A pump, a manual injector kit, a Model SDP-6A detector, and a Model C-R5A recording data processor (Shimadzu Corporation, Kyoto, Japan) and a Mos-hypersil-1 RPC-8 column, 5 μm, 250 x 4.6 mm (Keystone Scientific Co., Bellefonte, PA, USA). The mobile phase, composed of 0.1M aqueous NH_4OAc and methanol at a volume ratio 4:1, was isocratically eluted at a flow rate of 0.9 ml/min, and the effluent was monitored at 280nm.

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Two structural isomers, 3-acetamido-4-chloro-2-nitrobenzoic acid (I) and 3-acetamido-4-chloro-6-nitrobenzoic acid (II) were identified by ^1H and ^{13}C-NMR (MeOH-d_4).

I: ^1H-NMR(MeOH-d_4): 2.11(s,3H), 7.88(d,J=8.14 Hz,1H), 8.03(d,J=8.14Hz,1H), 9.13(bs,1H); ^{13}C-NMR(DMSO-d_6): 22.5, 123.8, 128.9, 130.6, 132.0, 138.5, 149.4, 163.8, 169.6.

II: ^1H-NMR(MeOH-d_4): 2.29(s,3H), 8.14(s,1H), 8.84(s,1H), 9.12(bs,1H); ^{13}C-NMR(DMSO-d_6): 23.9, 123.6, 125.5, 126.4, 127.2, 139.5,143.2, 165.4, 169.8.

RESULTS AND DISCUSSION

Figure 2 shows the chromatogram obtained from the separation of 15 grams of crude nitro-isomers with the

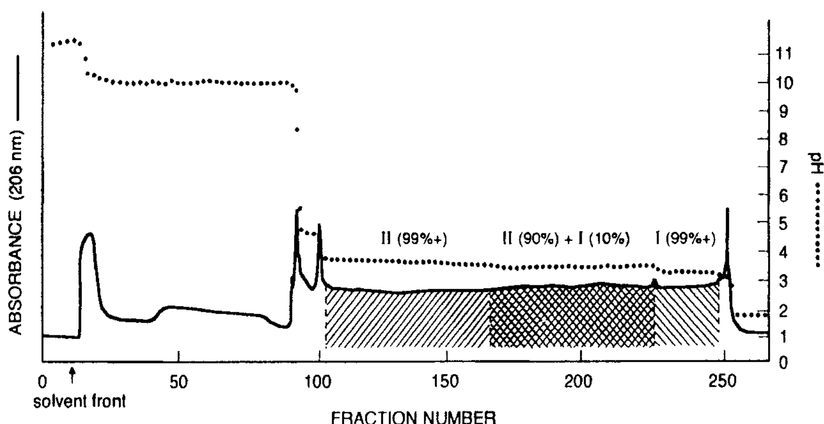

FIGURE 2. Chromatogram of isomeric mononitro-derivatives of 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid by displacement mode of pH-zone-refining CCC. Experimental conditions were as follows: Apparatus: High-speed CCC centrifuge equipped with a multilayer coil of 1.6 mm ID and 325 ml capacity; Solvent system: Methyl-tertiary-butyl ether/acetonitrile/water (4:1:5); Stationary phase: Lower phase (0.8% aqueous ammonia); Mobile phase: Upper phase (0.32% trifluoroacetic acid); Flow rate: 3.3ml/min; Sample: Nitration product of 3-acetamido-4-chlorobenzoic acid (15 grams) dissolved in 100ml in equal volumes of the upper and lower phases; Revolution: 800rpm (600 rpm until 66ml of mobile phase was eluted).

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displacement mode of pH-zone-refining CCC. The two isomeric mononitro-derivatives, II and I (see Fig. 1), were eluted in successive rectangular peaks associated with sharp impurity peaks at their boundaries. The separation required slightly over 8 hours. The pH measurement of the fractions revealed that the pH curve (dotted line) formed a characteristic downward staircase pattern where each plateau of the analyte corresponds to the distinct pH zone.

The first peak represents elution of isomer II and the second peak, that of isomer I. HPLC and NMR analyses of peak fractions showed that the early portion of the first peak (fractions 103-165, 5.4 g) and the entire portion of the second peak (fractions 229-254, 2.2 g) were essentially pure, while fractions at the later portion of the first peak (fractions 166-225, 5.3 g) were contaminated with about 10% of isomer I as indicated in the chromatogram. If desired, this fraction may be rechromatographed under the identical condition to improve the yield of pure fractions.

The overall results of our studies demonstrate that pH-zone-refining CCC can be efficiently used for purification of multigram quantities of structural isomers from a crude reaction mixture in 8 hours.

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