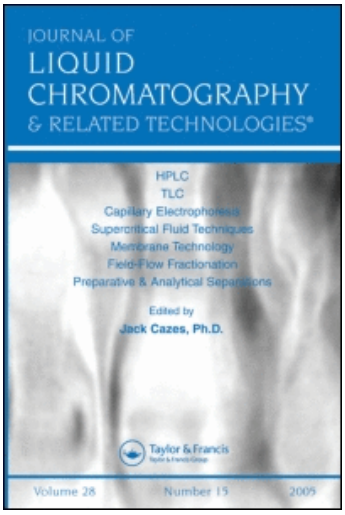

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Tore Ramstad[a]; Kent A. Mills[a]; Gustavus A. Walker[a]
[a] Department of Pharmaceutical Sciences, Pfizer Corp., Kalamazoo, Michigan, USA

To cite this Article Ramstad, Tore , Mills, Kent A. and Walker, Gustavus A.(2006) 'Determination of the Enantiomeric Purity of a CNS Agent, Sumanirole, by Electrokinetic Chromatography', Journal of Liquid Chromatography & Related Technologies, 29: 12, 1703 — 1724
To link to this Article: DOI: 10.1080/10826070600716785
URL: http://dx.doi.org/10.1080/10826070600716785

Journal of Liquid Chromatography & Related Technologies®, 29: 1703–1724, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600716785

Determination of the Enantiomeric Purity of a CNS Agent, Sumanirole, by Electrokinetic Chromatography

Tore Ramstad, Kent A. Mills, and Gustavus A. Walker
Department of Pharmaceutical Sciences, Pfizer Corp., Kalamazoo, Michigan, USA

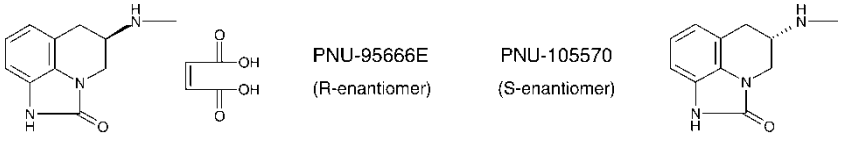
Abstract: A chiral procedure based on electrokinetic chromatography was developed and validated for sumanirole (PNU-95666E), a drug candidate that was under development for treatment of Parkinson's disease. The method underwent various iterations over a several years' period, first utilizing a neutral cyclodextrin as the chiral recognition agent, then later using a highly sulfated-beta-cyclodextrin (HS-β-CD). Separation is on a 61 cm × 50 μm ID fused silica capillary at an applied potential of −25 kV with a background electrolyte of 5% HS-β-CD in pH 2.50, 22.5 mM lithium phosphate buffer. For a sample prepared at 5 mg/mL, the limit of detection (LOD) for the undesired enantiomer (distomer) is about 0.02%. In a similar method developed for a congener of sumanirole, the LOD is approximately 0.01%, but at a sample concentration of 10 mg/mL. The separation, in both cases, takes less than 20 min. Over the range of 0.05–0.4%, the average recovery for the distomer of sumanirole was 102.1% and the linear regression correlation coefficient (r^2) 0.9999. For the congener over the range of 0.1–1.8%, a correlation coefficient of 0.9999 and an average recovery of 105.2% were obtained. The sumanirole method in its several iterations was used to collect four years of ICH stability data on the active pharmaceutical ingredient (API). No sample, either in the ICH study or any other lot manufactured over the years, yielded a value above 0.05% for the distomer. The method was additionally used to assess chiral inversion in API and in several formulations.

Keywords: Chiral analysis, Chiral capillary electrophoresis, Enantioseparation by capillary electrophoresis, CD-mediated electrokinetic chromatography, Highly-sulfated cyclodextrins, Enantiomeric excess of active pharmaceutical ingredient

Address correspondence to Tore Ramstad, c/o Donna S. Socha, Pfizer Corp., 7000 Portage Rd., Kalamazoo, Michigan, 49001, USA. E-mail: toreram@earthlink.net

INTRODUCTION

Sumanirole maleate (PNU-95666E), (5R)-5,6-Dihydro-5-(methylamino)-4H-imidazo[4,5,1-ij]quinolin-2(1H)-one (2Z)-2-butenedioate (1:1), is a selective dopamine (D2) agonist that was under development for treatment of Parkinson's disease and Restless Leg Syndrome (RLS). Sumanirole is an imidazoquinoline fused ring compound with a single chiral center at the 5-position of the quinoline ring. As the pharmacological and safety profiles of enantiomers often differ, sumanirole was being developed as a single enantiomer in the R-configuration, controlled via stereoselective synthesis. In accordance with modern pharmaceutical practice and regulatory expectations, enantiomeric purity must be controlled and monitored throughout development, hence, the need for a chiral assay.



Initial attempts (mid to late 1990s) to achieve a chiral assay by HPLC in our laboratories proved very challenging. No satisfactory separation was achieved. We attributed this, at least in part, to the high polarity of sumanirole, which made for a poor match with the normal phase solvents used. In contrast, CE often proves effective for the separation of polar, chiral compounds.

Chiral CE, particularly aqueous, chiral CE has matured and gained wide acceptance since its introduction some 15 years ago. It complements HPLC (and SFC), offering new solubility options, often outperforming HPLC. While nonaqueous, chiral CE continues to make strides,[1,2] aqueous CE dominates the practice of enantiomeric analysis by CE. The many reviews that continue to appear attest to its evolving importance in modern pharmaceutical analysis.[3–10] Chiral CE has been applied to virtually every class of drug. Also, its increasing use as a quantitative measure of enantiomeric purity (excess) is apparent from the number of reports that present validation data.[3,11–21]

Separation of enantiomers by CE relies on differential interaction with enantioselective agents in the run buffer (background electrolyte, BGE). Strictly speaking, interaction with a carrier qualifies the techniques as electrokinetic chromatography, not as CE.[22,23] Cyclodextrins (CDs) are by far the most commonly used chiral selector,[24] originally dominated by derivatized, neutral CDs, more recently by charged, principally sulfated CDs.[11,25–27] As numerous authors have proposed generic screening approaches for cyclodextrins,[19,24,28] quick assessment of the utility of CE for a given optically active compound is generally possible.

Sumanirole possesses several properties that make it a viable candidate for analysis by CE/EKC: (1) it has good aqueous solubility; (2) it is weakly

basic ($pK_a \sim 8.0$), hence fully protonated at low pH, ordinarily the region of first choice for separation; (3) although its UV maximum is at short wavelength (210 nm), this poses little challenge for CE, as the short optical path length leads to little background absorbance; and (4) the point of chirality is of a size that should fit inside the cavity of an appropriately sized CD; differential interaction between isomers would, therefore, be a possibility.

When first faced with the task of developing a chiral assay in the mid late 1990s, neutral cyclodextrins (CDs) dominated the field of chiral analysis by CE. Accordingly, we developed a method utilizing heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) as the chiral recognition agent. However, since that time, as noted above, charged CDs, principally sulfated CDs, have emerged and become the predominant chiral recognition agent. In accordance with this trend, we sought to modify our original method with a sulfated CD. As such, we developed two substantially different methods several years apart, then continued making refinements over the course of another year. As a result, several methods, each an improvement over the previous one, were used over the course of development. We report here on this evolutionary process with emphasis on the final procedure. The developed methods were used for release testing of numerous production scale batches of active pharmaceutical ingredient (API), for testing of chiral inversion in API and in several formulations, and in support of a four-year ICH (International Conference on Harmonization) stability study. A requirement throughout this work was that any method had to be capable of quantifying the undesired enantiomer (distomer) to 0.1%. We, additionally, briefly report on a related method developed for a structural analog of sumanirole, PNU-142774E.

EXPERIMENTAL

Several methods were developed over the years and on two different brands of CE instruments, a Dionex CES-1 and a Beckman P/ACE MDQ. Experimental details associated with the early work (Dionex CES-1) are mentioned as appropriate in the Results and Discussion (Method Development) Section. Details given in this Section are for the final method, validated on the Beckman MDQ.

Capillaries

Capillaries were purchased as 10 m coils of CE grade fused silica from MicroSolv (Long Branch, NJ). All capillaries were 50 μm in diameter, cut to length with a ceramic cleaving stone. Using a miniature oven (Column Stripper, MicroSolv), a window was exposed by burning off the polyimide coating. The capillaries were mounted in a liquid cooled cartridge that

automatically aligns the window with the instrument optics. Detection was in-line using a 210 nm filter mounted on a motor-driven wheel. New capillaries were conditioned using the following sequence: methanol (5 min), water (5 min), 1 N NaOH (20 min), water (5 min), 1 N HCl (15 min), and water (10 min). In general, followup conditioning, for example at the start of a new day, was not necessary. Sequences were terminated with a water wash step. The capillary ends were then stored in water.

Chemicals/Reagents

Lithium phosphate monobasic was purchased from GFS Chemicals (Powell, OH) and 85% phosphoric acid from Mallinckrodt (Phillipsburg, NJ). Beta-cyclodextrin (β-CD), gamma-cyclodextrin (γ-CD), hydroxypropyl-α-cyclodextrin (HP-α-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD), hydroxypropyl-γ-cyclodextrin (HP-γ-CD), dimethyl-β-cyclodextrin (heptakis(2,6-di-O-methyl)-β-cyclodextrin) (DM-β-CD), and trimethyl-β-cyclodextrin (heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin) (TM-β-CD) were obtained from Sigma-Aldrich (St. Louis, MO). As to sulfated cyclodextrins, sulfated β-CD ($n = 7-11$) was obtained from Aldrich, heptakis-6-sulfato-β-cyclodextrin (HpS-β-CD) ($n = 7$) from Regis (Morton Grove, IL), and highly sulfated-β-cyclodextrin (HS-β-CD) ($n = 12$) from Beckman. The HS-β-CD was a 20% (w/w) aqueous solution.

Potential internal standards scouted were purchased from several vendors. Sodium salts of benzenesulfonic acid, 4-hydroxybenzenesulfonic acid, and *p*-toluenesulfonic acid, and potassium salts of 1,2-naphthoquinonesulfonic acid and hydroquinonesulfonic acid (tech grade) were obtained from Sigma-Aldrich, 2-napthalenesulfonic acid, sodium salt, from Regis, and the sodium salts of 1-naphthol-4-sulfonic acid and 2-anthroquinonesulfonic acid from Kodak (Rochester, NY). All PNU- (and PHA-) numbered compounds were synthesized internally. Both PNU-95666E, the active ingredient, and the S-enantiomer of PNU-95666, PNU-105570, were prepared as E (maleate) salts. Similarly, PNU-142774E and its enantiomer, PHA-513550, were prepared as the maleate salts.

Buffers

The final run (separation) buffer (background electrolyte, BGE) consisted of a 5% solution of Beckman's HS-β-CD in 22.5 mM lithium phosphate adjusted to pH 2.50 with 85% phosphoric acid. During a sequence, the capillary is filled with the separation buffer from the same, dedicated vial for each run. The capillary inlet is then moved to a separate vial for the separation. The same inlet and outlet buffer vials were used for up to nine consecutive runs (the most tried) without apparent ill effect. The final separation buffer of {5% HS-β-CD in pH 2.50, 22.5 mM LiH_2PO_4} was prepared by mixing 3 parts

pH 2.50, 30 mM LiH_2PO_4 and 1 part 20% w/v HS-β-CD solution. The pH 2.50, 30 mM lithium phosphate supporting electrolyte solution was passed through a 0.2 mm filter (FP-200, Gilson Laboratory, Middleton, WI) prior to combining it with 20% HS-β-CD. It was additionally degassed by vacuum in an ultrasonic bath. The final separation buffer containing the HS-β-CD was prepared just prior to use. The 30 mM LiH_2PO_4 stock solution could be used for up to one week without refiltering, longer if refiltered. The sample buffer (SB) was pH 2.5, 6 mM LiH_2PO_4, prepared by diluting the stock 30 mM lithium phosphate solution five-fold with water. The water was purified water, obtained from a Milli-Q UV Plus water purification system (Millipore, Bedford, MA).

Search for an Internal Standard

Potential internal standards prepared at about 2.5% (w/w) were screened in the presence of 2 mg/mL PNU-95666E and ~0.6% (w/w) PNU-105570. Only compounds that are anionic at the buffer pH of 2.50 were tested. Earlier attempts at also screening cationic compounds, which could complex with the sulfated CD, proved unpredictable and, hence, was not pursued here.

Validation

Preparation of Standards

Two stock standards consisting of 1.25 mg PNU-105570E dissolved in 10 mL of SB were prepared for each experiment. A 2 mL aliquot of this solution was transferred to a 10 mL volumetric flask along with 0.5 mL of internal standard solution and diluted with SB. This results in a 0.5% standard (w/w) with respect to a 5 mg/mL preparation of sumanirole maleate.

Internal Standard Solution

For validation, a 1 mg/mL solution of the sodium salt of 2-napthalenesulfonic acid was prepared (20 mg in 20 mL SB). One-half mL of this solution was added to a 10 mL volumetric flask containing PNU-95666E and/or PNU-105570E. Subsequent to completing validation, this was modified slightly, a 1.3 mg/mL solution prepared and 0.20 mL added to 5 mL of a 5 mg/mL PNU-95666E preparation.

Linearity/Recovery of PNU-105570

The linearity/recovery of PNU-105570 relative to and in the presence of 5 mg/mL PNU-95666E was examined over the range 0.05 to 1.0% (w/w).

About 437.5 mg of PNU-95666E was dissolved in 35 mL of SB. Four mL of this solution was transferred to a 10 mL volumetric flask, thereby providing the desired 5 mg/mL (3.18 mg FBE (free base equivalents)) background for the recovery determinations. A spiking solution of PNU-105570E, nominally 2 mg/20 mL SB, was prepared, and an appropriate amount (0.25 mL for a 0.05% spike, 5.0 mL for a 1.0% spike) added to the 10 mL volumetric flask and diluted with SB to achieve the desired levels.

Precision

Precision was determined at two levels, 0.1% and 0.4% (w/w) PNU-105570 relative to PNU-95666. Measurements were made on separate days on the same capillary. (Our experience has been that capillaries from the same supplier subjected to identical preconditioning behave the same, hence precluding a need to prepare separate capillaries.) Fortification was necessary even at the 0.1% level, as the amount of indigenous PNU-105570 in PNU-95666E was negligible.

Final, Optimized Conditions

The final CE conditions were as follows: capillary, 61 cm (50 cm to detector) × 50 μm ID; detection, 210 nm; applied potential, −25 kV (reverse polarity); injection, 0.5 psi for 5 s; separation buffer, 5% HS-β-CD in pH 2.50, 22.5 mM lithium phosphate; sample buffer, pH 2.5, 6 mM lithium phosphate; sample preparation, 5.0 mg/mL of PNU-95666E in sample buffer; data acquisition, 10 pt s^{-1}. Quantitation was accomplished using adjusted areas (A/t_m) vs. an external standard with an internal standard added. DIAMIR (Varian, Palo Alto, CA) was used for data acquisition.

RESULTS AND DISCUSSION

Method Development

Dionex CES-1. The initial method was developed on a Dionex CES-1, which utilized gravity injection. The cyclodextrins explored, all at pH 2.5, were β-CD, γ-CD, HP-α-CD, HP-β-CD, HP-γ-CD, DM-β-CD, and TM-β-CD. Of these, only HP-β-CD and DM-β-CD were effective, with the latter selected. As part of method development and optimization, we investigated the nature of the run buffer (background electrolyte, BGE) counter ion (Li^+ vs. Na^+ vs. K^+), run buffer and cyclodextrin concentration, capillary diameter, and injection parameters. All known, achiral impurities were separated both from the major component, sumanirole, and from the distomer, PNU-105570. Because PNU-105570 eluted shortly after the major

component, a highly concentrated sample was precluded. For a concentration greater than about 0.3 mg/mL, baseline resolution of PNU-95666, PNU-95512 (the *N,N*-dimethyl analog of PNU-95666), and PNU-105570 was impaired. The separation achieved with PNU-105570 spiked in at the 0.1% level is shown in Figure 1. Assay conditions are given in the legend. Quantitation was by percent (adjusted) area, preferable from the standpoint that no reference standard was required. A concentration of about 0.1% represented the quantitation limit for the assay. The RSD obtained in a precision study at 0.1% was 18.8%.

Although we eventually abandoned this method in favor of newer instrumentation (*vide infra*), we used it to assess chiral inversion of PNU-95666 in bulk drug, in an injectable (1 mg/mL), in a 5 mg immediate release tablet, and in 2.5 mg extended release tablets composed of HPMC (hydroxypropylmethylcellulose)/lactose and HPMC/starch. Samples had been stressed for up to 2 years at 40°C. As attested to by the electropherograms of Figure 2, in no case was there evidence for the formation of any of the undesired enantiomer. Hence, we were able to conclude that no chiral inversion took place. While not detailed here, the methodology used to extract the drug followed standard protocols, although none of the procedures used were validated. However, comparisons with HPLC confirmed the presence of the process

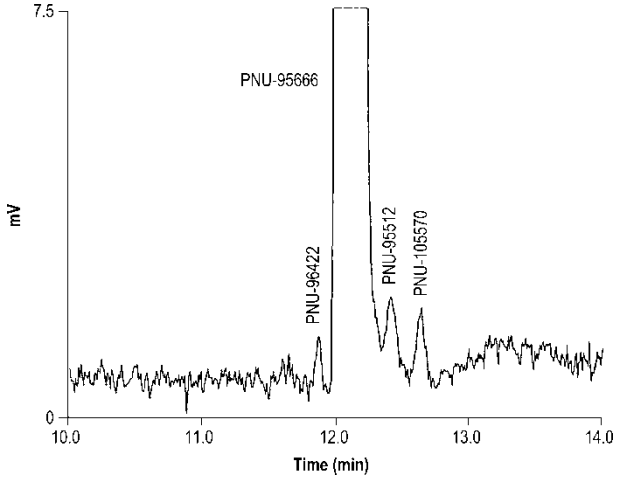


Figure 1. Example electropherogram (expanded time scale) obtained on the Dionex CES-1 of a PNU-95666 solution fortified with 0.1% w/w PNU-105570. Conditions: capillary, 60 cm × 50 μm ID; run buffer (BGE), pH 2.50, 50 mM LiH_2PO_4 + 100 mM DM-β-CD; sample buffer, pH 2.5, 5 mM LiH_2PO_4; sample concentration, 0.3 mg/mL; injection, by gravity, 20 s × 100 mm; detection, 210 nm; applied potential, 25 kV (normal polarity); resultant current, ~30 μA. PNU-96422, not mentioned in the text, is the corresponding *des*-methyl analog of PNU-95666. The peaks for PNU-96422 and PNU-95512 correspond to indigenous amounts of these two substances.

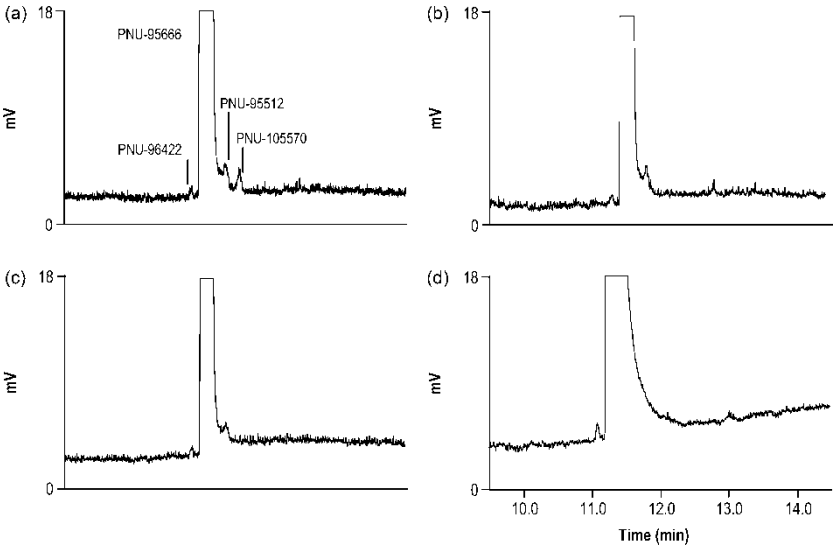


Figure 2. Study of chiral inversion using the Dionex CES-1: (a) API showing the position of process impurities and the sought-after enantiomer, PNU-105570; (b) a1 mg/mL injectable; (c) a 5 mg immediate release tablet; and (d) a 2.5 mg extended release HPMC/starch tablet. Note, that the bulk drug used in (d), different from that used in (b) and (c), contained none of impurity PNU-95512.

impurities at comparable levels. As will be seen below, we assessed the chiral stability of API for up to four years in an ICH study, starting with the Dionex CES-1 procedure, then advancing through a progression of methods.

Beckman MDQ. Because Dionex withdrew from the CE market, we transferred and adapted the original method to the Beckman MDQ, a newer instrument. As the MDQ is liquid cooled, whereas the CES-1 had no forced cooling, we were able to increase the concentration of the lithium phosphate separation buffer from 50 mM to 75 mM, thereby producing slightly sharper peaks. Because at low pH the EOF is negligible, the capillary could be filled from a dedicated fill vial that contained the full separation buffer, 100 mM DM-β-CD in pH 2.5 lithium phosphate, and then inserted into inlet and outlet separation reservoirs that contained only lithium phosphate. Over time (i.e., with repeated use of the assay), this could result in considerable savings due to the cost of the derivatized CD. This approach would not be possible for a charged cyclodextrin, as the capillary would become depleted of the CD with time. The resolution between PNU-95666 and PNU-105570 was improved on the MDQ relative to the CES-1, but suffered from a dip in the baseline that placed an upper limit on the concentration of the sample preparation. (The dip, not fully understood, became less prevalent with time.) Quantitation was versus authentic PNU-105570, a change from the percent area measurement used in the original method. As it is difficult to achieve a constant injection

volume in CE, the method was modified to utilize PNU-95666, a component of every sample, and added to reference standard preparations, as an internal standard. (This was possible because of a well controlled synthetic process. Every batch of sumanirole was of high purity, devoid of significant achiral or chiral impurities.) When fortified with PNU-105570 at the 0.2% level, the RSD for precision (n = 6) was 8.4%, an improvement over the original 18.8% (*vide supra*). Electropherograms of sumanirole alone (a) and sumanirole fortified with 0.2% distomer (b) are shown in Figure 3. For a 1.0 mg/mL sample preparation, the detection limit, based on a 3:1 signal-to-noise (S/N) ratio (peak-to-peak), was 0.1%.

Switch to Sulfated Cyclodextrin

Based on experience with a structurally related candidate (PNU-142774E, *vide infra*), several sulfated beta-cyclodextrins were scouted and compared. (In later, more comprehensive screening of sulfated cyclodextrins, the beta-cyclodextrin chosen here still proved to be the most effective cyclodextrin.): sulfated β-CD from Aldrich (n = 7–11), HpS-β-CD (n = 7) from Regis, and highly sulfated beta-cyclodextrin, HS-β-CD, from Beckman ($n_{avg} = 12 \rightarrow 1.7$ degree of substitution per glucopyranose unit). The Aldrich material, presumed to be of variable composition, could prove

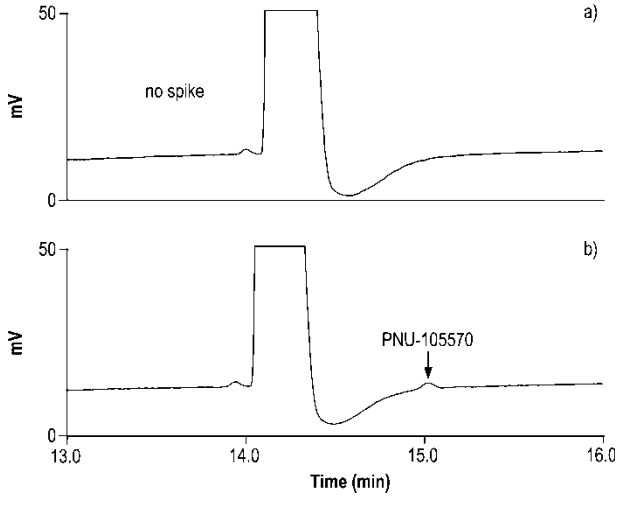


Figure 3. Electropherograms obtained on the Beckman MDQ of (a) sumanirole alone; and (b) sumanirole fortified with 0.2% distomer. Conditions: capillary, 61 cm (50 cm to detector) × 50 μm ID; run buffer, pH 2.50, 75 mM LiH_2PO_4 + 100 mM DM-β-CD; sample buffer, pH 2.5, 7.5 mM LiH_2PO_4; sample concentration, 1.0 mg/mL; injection, 0.5 psi for 5 s; detection, 210 nm; applied potential, 30 kV (normal polarity); resultant current, ~40 μA.

problematical for long term ruggedness. On the other hand, given its far lower cost, it was worth investigating. The second, a single isomer substituted at the 6-position developed in the laboratory of Prof. G. Vigh of Texas A&M University,[29] has been available from various suppliers over the years, but is currently available exclusively from Elpho Tech (College Station, TX). The third is a proprietary product from Beckman-Coulter, and although a mixture, often referred to as randomly substituted (somewhat pejoratively), is purportedly made under highly controlled conditions, and, in fact, has a relatively narrow distribution of sulfated structures.[27,30] In our experience, Beckman's product has proven to be highly reproducible over many batches over a five-year period. Since *a priori* one cannot know the binding constants for the analyte-CD complexes, screening should be conducted at more than one concentration of cyclodextrin in order to harness a range of interactions. Hence, the Aldrich- and Regis-sourced CDs, sold as solids, were compared at 10 and 25 mM in pH 2.50 LiH_2PO_4. The Beckman HS-β-CD reagent was run exclusively as a 5% w/v solution in pH 2.50, 22.5 mM LiH_2PO_4, this in conformance with Beckman's recommendation. For screening, a 2.0 mg/mL solution of PNU-95666E in sample buffer was fortified with 2.5% of PNU-105570E. Both normal and reverse polarity were tested at 25 kV, although the more highly sulfated the cyclodextrin, the less likely it is that normal polarity will yield a separation or result in detection. Nevertheless, because we were uncertain of the behavior of the Aldrich-sourced CD, both reverse and normal polarity were tested.

HS-β-CD (Beckman) under reverse polarity and sulfated β-CD (Aldrich) under normal polarity both showed promise. Example electropherograms from this screening are shown in Figure 4. Based on these results, followup experiments were conducted on the Aldrich and Beckman sulfated beta-cyclodextrins. The concentration of PNU-95666E was increased from 2 mg/mL (1.27 mg/mL FBE) to 5 mg/mL (3.18 mg/mL FBE) with the aim of lowering the detection limit. No deleterious effects resulted from the increased drug concentration; the PNU-95666 peak continued to be sharp. This was in contrast to DM-β-CD (*vide supra*), where a dip in the baseline following the PNU-95666 peak and preceding the PNU-105570 peak was made worse the higher the drug concentration. Although ordinarily it is an advantage to have the targeted analyte (the undesired enantiomer here) elute first, the resolution was so large for both the Beckman and Aldrich sulfated CDs, that it would make little difference. A disadvantage to the Aldrich sulfated β-CD was the high current it produced. At 25 mM at a potential of 28 kV (could not reach 30 kV) the current was 295 μA, right at the maximum allowed by the MDQ, thereby inviting heating problems. On the other hand, the current induced by Beckman's HS-β-CD at 5%, which is equivalent to about 25 mM (assuming $n = 12$), was $\sim$180 μA. We selected the HS-β-CD from Beckman for further development. No known impurities coeluted with PNU-105570 (or with the major component, PNU-95666). PNU-96422, the *des*-methyl analog of PNU-95666, eluted just before

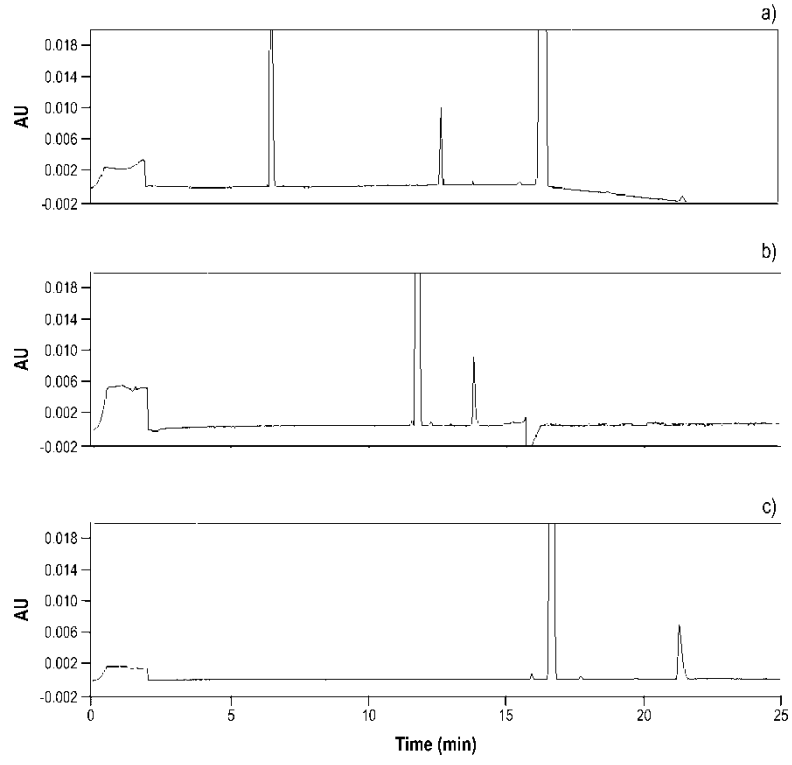


Figure 4. Results of scouting sulfated CDs: (a) separation with 5% HS-β-CD (Beckman) under reverse polarity. The peak at 6.5 min is maleate, the peak at about 12.5 min PNU-105570, and the peak at about 16 min PNU-95666; (b) separation with sulfated-β-CD (Aldrich) under normal polarity with 10 mM and (c) with 25 mM of the cyclodextrin. The larger, first peak is PNU-95666, the second, PNU-105570. Maleate does not elute under normal polarity.

PNU-95666. PNU-107994, often the primary (process) impurity in sumanirole, eluted well after PNU-95666 (at a relative elution time of $\sim$1.3).

Method Optimization

Figure 4a reveals that the 61 cm (total length) capillary used affords excess resolution, hence resulting in wasted time. Although shorter capillaries resulted in shorter elution times, the capillary was easily overloaded, leading to distorted peaks. In order to achieve the requisite detection and quantitation limits, a sample concentration of about 5 mg/mL was needed. Additionally, unless the voltage was reduced, currents were excessive. Hence, no gain was realized in going to a shorter capillary. The final, optimized conditions are given above at the end of the Experimental Section.

Preliminary Validation (No Internal Standard)

Validation was conducted twice and on two different MDQs for the system utilizing HS-β-CD, first with no internal standard, then later with an internal standard. The final method utilizes an internal standard. Because quantitation by percent relative area is most convenient, since no standard is required, we assessed the linearity of the major component over the range 0.10–110% relative to 5 mg/mL. A plot of PNU-95666 area response vs. concentration proved non linear, displaying convex curvature. Although not the preferred result, this did not automatically preclude quantitation by area percent, as long as the recoveries for PNU-105570 over the applicable range (0.10–2.0%) proved linear. Nevertheless, because authentic PNU-105570 was available to us, we elected to quantitate PNU-105570 vs. PNU-105570 as an external standard.

A combined linearity/recovery experiment, where PNU-105570 was added to a 5 mg/mL preparation of sumanirole maleate, resulted in a correlation coefficient (r^2) of 0.9992 and a mean recovery of 97.5% with a relative standard deviation (RSD) (coefficient of variation) of 5.0%. The lower and upper 95% confidence intervals for the intercept bracketed 0 and for the slope 1. Hence, there was no statistical bias for either the intercept or the slope. Precision was tested on two capillaries on two days. Although the second capillary yielded longer elution times, the migration times on each capillary were quite constant. For $n = 19$ on the first capillary on day 1, an RSD of 0.97% was obtained for migration time, while on the second capillary on day 2 ($n = 19$), it was 0.47%. Precision testing on two capillaries on two days at 0.2 and 0.5% PNU-105570 relative to API, resulted in RSDs of 2.04% ($\bar{x} = 101.8\%$) and 5.5% ($\bar{x} = 106.0\%$) at the 0.2% level over the two days, and 1.61% ($\bar{x} = 97.2\%$) and 1.53% ($\bar{x} = 104.8\%$) at the 0.5% level. Although this was not a recovery experiment *per se*, the recoveries (denoted by $\bar{x}$) are seen to be acceptable at these levels. The grand average for absolute recovery over the two days was 0.21% with an RSD of 4.5% at the 0.20% level and 0.50% with an RSD of 4.2% at the 0.50% level. The detection limit for the 5 mg/mL preparation based on a 3:1 S/N ratio using peak-to-peak baseline noise was 0.01–0.02%.

As the chirality of sumanirole was well controlled in the synthetic process, all batches, no matter where produced, proved to be devoid of the undesired enantiomer at 0.1% or above. The analyst, in a position to anticipate this, was not required to prepare standards for quantitation purposes, but rather only needed to prepare a resolution material (i.e., a performance check) to demonstrate that the enantiomer could be detected at 0.1% or above, if present. An example of this approach is shown in Figure 5.

Search for an Internal Standard

Six potential internal standards were screened, all aromatic sulfonic acids, each negatively charged at pH 2.5. Under reverse polarity, they migrate toward the

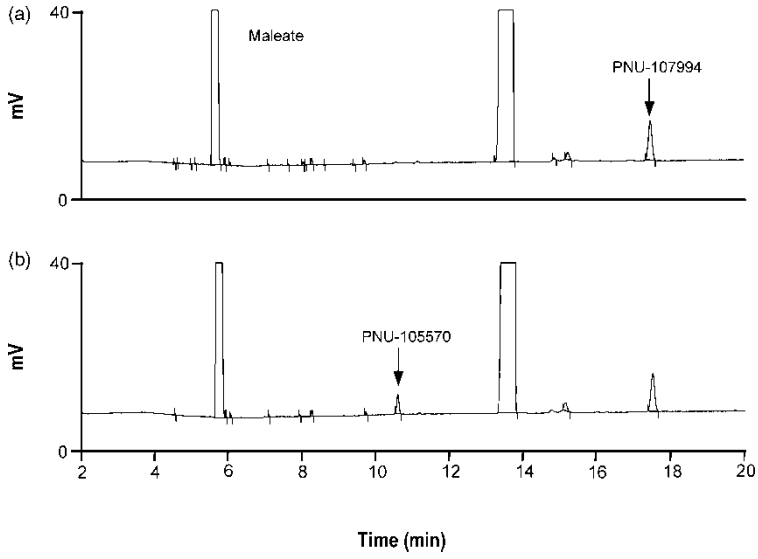


Figure 5. Electropherograms of (a) a profiled lot of sumanirole; and (b) the same lot fortified at the 0.2% level with PNU-105570. Clearly, there was no detectable PNU-105570 in the original sample. As explained in the text, the analyst, anticipating this, was not required to prepare standards. PNU-107994, denoted in (a), is an achiral impurity.

detector (anode). Because they are of the same charge as the sulfated cyclodextrins, they are repelled and, therefore, presumed not to complex with the CDs. Hence, the mechanism of transport is not the same as for the analytes, PNU-105570 and PNU-95666, which form guest host inclusion complexes with the CDs. However, this is immaterial to their use as an internal standard. The compounds screened were (all sodium salts) benzenesulfonic acid, 4-hydroxybenzenesulfonic acid, *p*-toluenesulfonic acid, 2-napthalenesulfonic acid, 1-naphthol-4-sulfonic acid, 2-anthroquinonesulfonic acid, 1,2-naphthoquinone-4-sulfonic acid, and hydroquinonesulfonic acid. It was desired that the internal standard elute after the maleate peak, at about 5.5 min (with −30 kV applied), and before the sumanirole peak, which eluted at about 13.5 min. PNU-105570 eluted at about 10.5 min.

Two promising candidates based on their elution position, 2-anthroquinonesulfonic acid and 1,2-napthoquinone-4-sulfonic acid, formed intense yellow complexes in the presence of 5 mg/mL PNU-95666, and hence were rejected. Invoking all the usual criteria in choosing an internal standard (purity, availability, cost, electrophoretic behavior, etc.), 2-napthalenesulfonic acid was selected. It was mentioned earlier, that no known impurities coeluted with PNU-105570. Although there were tiny peaks indigenous to PNU-95666 in the vicinity of 2-napthalenesulfonic acid, they were too small to have an

effect on quantitation even if resolution was not attained. Normally, when assaying samples, the API batch was run both with and without the internal standard as a check on interference. No interferences were seen in any lot of PNU-95666.

Validation

Reproducibility of Migration Time

Repeatable migration times can be a challenge in CE due, principally, to changes in the electroosmotic flow (EOF). Fortunately, at pH 2.5 the EOF is small. Hence, it is possible to dispense with a capillary rinse cycle between samples and simply replenish the capillary with fresh run buffer prior to each run. In order to save on expensive cyclodextrin reagent, a given pair of buffer vials is generally used as many times as possible. In nine separate experiments, where the buffer vial pairs were used at least six times, the RSD for migration time was under 1.0% in every case. Actually, a slight shift in migration time poses little, if any, difficulty for peak identification for modern data acquisition systems. Also, quantification by area is unaffected by (slight) drift since adjusted area (A/t_m), not raw area, is used in the calculations.

Linearity/Recovery of PNU-105570

Combined linearity/recovery results from a 5 mg/mL PNU-95666E solution for PNU-105570 over the range 0.05–1.0% are given in Table 1. Quantitation

Table 1. Linearity/recovery results for PNU-105570

Nominal % spiked	Exact amount spiked (mg)	Exact % spiked	Found (%)	Recovered (%)
0	0	0	0.0153	
0.05	0.0273	0.0473	0.0493[a]	104.2
0.1	0.0473	0.0943	0.0967	102.6
0.2	0.0946	0.189	0.192	101.9
0.4	0.189	0.377	0.376	99.6
0.6	0.284	0.566	0.488	86.3
0.8	0.378	0.755	0.718	95.1
1.0	0.473	0.943	0.881	93.4
			Mean recovery = 97.6%	
			s = 6.36%	
			RSD = 6.5%	

[a]Corrected for the indigenous amount of PNU-105570, calculated as 0.015%.

was versus PNU-105570 authentic reference standard (n = 8), with 2-napthalenesulfonic acid added as an internal standard. One questionable data point (low recovery) at the 0.6% fortification level detracted from the data set. Had this point registered 100% recovery, the overall recovery would have been 99.5% with an RSD of 4.0%. With the apparent outlier removed, the RSD was 4.5%. Linear regression analysis of the full data set yielded a correlation coefficient (r^2) of 0.996. The y-intercept of 0.0104 was not different from zero at the 95% confidence level, as the lower and upper 95% confidence levels encompassed the range −0.025 to 0.046. The slope was statistically different from one, as the upper 95% confidence level reached only to 0.986. As noted, the apparent outlier at the 0.6% spike level is the cause of the nonideal behavior. Linear regression through only the first four points (up through the 0.4% level) yielded a correlation coefficient of 0.9999 and a mean recovery of 102.1%. The intercept was not different from zero nor the slope different from one at the 95% confidence level. As no sample of sumanirole has ever registered higher than 0.05% for the undesired enantiomer, the reduced range is relevant to actual samples. Representative electropherograms are shown in Figure 6 of a standard, of native API, and for fortification at the 0.1 and 0.4% levels.

Precision

Precision data had been previously collected at the 0.2% and 0.5% levels of the distomer PNU-105570 for the method without internal standard (*vide supra*, Preliminary Validation Section). Here, precision was tested at the 0.1% and 0.4% levels (0.1% taken to be the reporting limit for the assay) with inclusion of an internal standard. Because sumanirole is essentially devoid of measurable enantiomer, fortification was required even at the 0.1% level. The type of precision testing normally done in validation, where both sample preparation variability and instrument variability are separately tested, did not apply here, as the sample preparation used (addition of enantiomer to a 5 mg/mL preparation of sumanirole) is trivial and introduces no variability. The same solution was placed in each of two vials, with each vial sampled three times.

At the 0.4% level (n = 6) the results were similar to those reported above at the 0.5% level, but where no internal standard was used. Based on the amount added, the recovery at 0.4% was 94.0%, although, as above, this was not a recovery experiment *per se*. The RSD was 2.4%. In three experiments conducted at 0.1%, RSDs of slightly more than 10%, 8.8%, and 3.7% were obtained, hence, a fair amount of variability. This may have been due, in part, to having to subtract a small, indigenous amount that contributed to the overall signal. Subsequent to the validation, an important change was made to the injection protocol whereby the inlet (and outlet) of the capillary is quickly dipped into water to wash off any residual sample remaining on the outside. In using the procedure subsequent to this change,

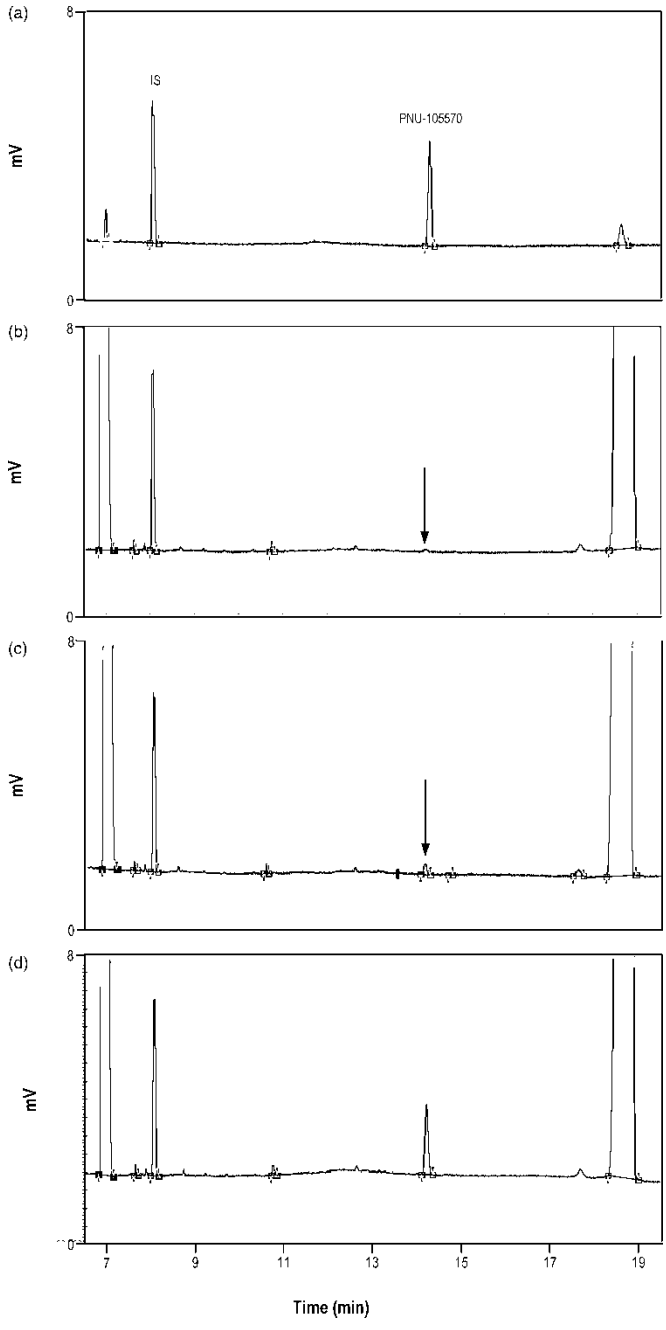


Figure 6. Electropherograms of (a) a standard; (b) of native API; (c) of a 0.1% spike; and (d) of a 0.4% spike. The internal standard, 2-napthalenesulfonic acid, is seen at ~8.2 min.

RSDs of 4.2, 3.3, and 1.9% were obtained for standards (n = 8), hence, attesting to system reproducibility. In a similar assay for a different drug candidate, one run more frequently than the sumanirole assay, RSDs ranging from 1.7–4.1% (n = 7, i.e., 7 different experiments), with an average of 2.8%, have been obtained using this revised protocol, hence, attesting to the effectiveness of the change. With this change, we can expect RSDs under 5.0% on a routine basis. Note that, although the highest precision possible is sought, the effect of somewhat higher RSDs on accuracy at the 0.1% level is slight, as can be seen from the following table:

RSD	Nominal concentration	95% Confidence
5%	0.10%	0.10% ± 0.01%
7.5%	0.10%	0.10% ± 0.02%

A difference of ±0.02% has little meaning at this level. Although 0.1% is above the quantitation limit of the assay based on a 10:1 peak-to-peak S/N, the assay is somewhat taxed to yield reliable values at this low concentration, hence, the designation of 0.1% as the reporting limit. Note that in practical terms, PNU-95666 was consistently produced with nondetectable or negligible (≪0.1%) levels of undesired enantiomer. Clearly, the stereoselective synthesis of sumanirole was very well controlled. The LOD for the assay is ~0.02% (based on S/N = 3).

Quantitation of PNU-105570 by HPLC

Although initial attempts at HPLC (in the mid 1990s, *vide supra*) proved unsuccessful, a new effort was undertaken to develop an HPLC method at the conclusion of the CE developmental cycle. Applying new, improved screens, conditions were identified that led to an HPLC procedure. Illustrative chromatograms are shown in Figure 7 for native material and for spikes at the 0.05, 0.1, and 0.2% levels. With an LOQ of 0.2%, the method would be usable, but does not match the performance of the CE procedure. Assay conditions are given in the legend.

CE Method for a Related Compound

A similar procedure to that described above for sumanirole was developed for a related compound, PNU-142774E, also a selective dopamine (D2) receptor agonist that was under development for treatment of sexual dysfunction. PNU-142774 is of identical structure to PNU-95666, except that the oxygen of the quinoline group is replaced by a sulfur atom. Like PNU-95666E,

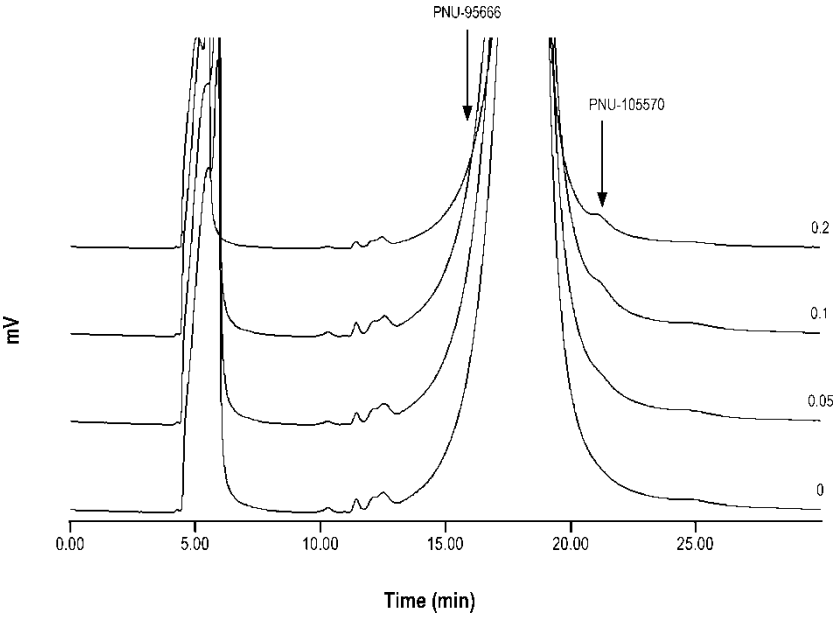


Figure 7. HPLC chromatograms of native PNU-95666E and of 0.05, 0.1, and 0.2% spikes of enantiomer (PNU-105570). The PNU-95666 arrow points to the front side of the massive peak. Conditions: instrument, Agilent 1100; column, Chirobiotic TAG, 5 μm, 4.6 × 250 mm; mobile phase, methanol with 0.1% TEA; flow rate, 0.4 mL/min; column temperature, 40°C; detection, 280 nm; injection volume, 10 μL.

PNU-142774E was being developed as the pure 5R-enantiomer and, hence, needed a sensitive method for determination of trace amounts of the S-enantiomer, PHA-513550.

It turned out that the adsorptive sulfur atom in PNU-142774 necessitated some adjustments to the assay, notably use of *glass* autosampler vials (nominal 2 mL size, 1.5 mL fill), each used only once, and incorporation of a 0.1 N NaOH → H_2O → 0.1 N HCl → H_2O rinse cycle for the capillary between every sample. Even though the sawtoothed PNU-142774 peak was nonlinear up to the sample prep concentration of 10 mg/mL, recovery of the PHA-513550 distomer proved highly linear over the range 0.01–1.8% ($r^2 = 0.9999$). Similarly excellent performance was obtained for precision at the 0.1% level, where the relative standard deviation for migration time was 0.5%, for relative migration time ($t_{PHA\text{-}513550}/t_{PNU\text{-}142774}$) was 0.1%, for (adjusted) area, A_{adj} (A/t_m), was 3.3%, and for area composition ($A_{adjPHA\text{-}513550}/(A_{adjPHA\text{-}513550} + A_{adjPNU\text{-}142774}$) was 3.8%. This degree of assay repeatability was achieved, presumably, in part, by programming into each run capillary preconditioning that utilized both alkaline and acidic rinses. A full scale electropherogram of a 0.1% spike into a 10 mg/mL (6.54 mg/mL FBE) preparation of PNU-142774E is shown in Figure 8a,

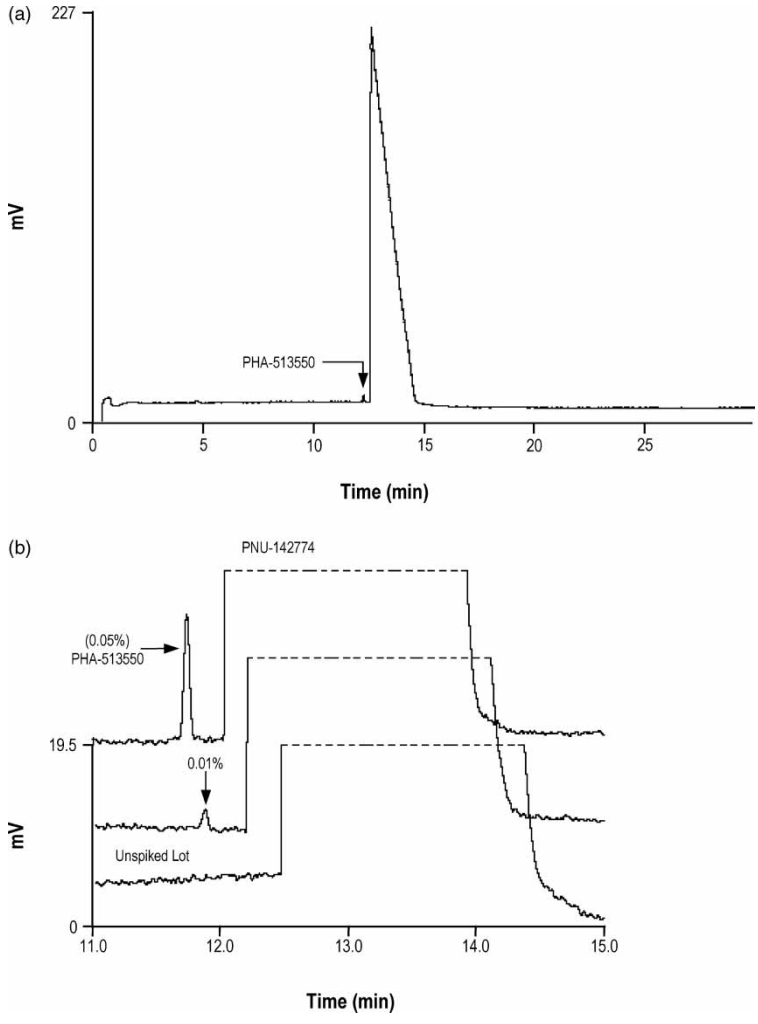


Figure 8. (a) Full scale electropherogram of a 0.1% (w/w) spike of PHA-513550E into PNU-142774E; (b) a batch of native PNU-142774E, a 0.01% spike, and a 0.05% spike. Conditions: capillary, 61 cm (50 cm to detector) × 50 μm ID; BGE, 5% (w/v) HS-β-CD in pH 2.50, 22.5 mM lithium phosphate; capillary temperature, 25°C; applied potential, −30 kV; detection, 300 nm; sample preparation, 10 mg/mL in pH 2.5, 30 mM lithium phosphate.

and expanded views of native PNU-142774E and of 0.01% and 0.05% spikes in Figure 8b. The 10 mg/mL sample preparation allows for an impressively low detection limit for CE. Although the limit of quantitation was about 0.02%, a reporting limit of 0.1% was assigned, the usual expectation for chiral analysis. Consistent with the bottom electropherogram in Figure 8b,

for five lots of PNU-142774E examined, all yielded either nondetectable or levels below 0.1% of the undesired enantiomer.

CONCLUSIONS

A chiral capillary electrophoresis procedure for sumanirole (PNU-95666) utilizing highly sulfated beta-cyclodextrin (HS-β-CD) as the enantioselective agent was developed and validated on the Beckman P/ACE MDQ Capillary Electrophoresis System. Separation is effected on a 61 cm × 50 μm ID fused silica capillary at −25 kV with a separation buffer of 5% (w/v) HS-β-CD in pH 2.50, 22.5 mM lithium phosphate. The resultant current is about 150 μA. The sample buffer consists of pH 2.5, 6 mM LiH_2PO_4. A 5 mg/mL PNU-95666E sample preparation is injected for 5 s at 0.5 psi. Quantitation is via authentic enantiomer as external standard with 2-napthalenesulfonic acid added as an internal standard. The internal standard elutes at about 8 min, the undesired enantiomer PNU-105570 at about 14 min, and PNU-95666 at about 18 min; the run time is nominally 20 min. A similar method was also validated for the structurally related compound, PNU-142774E, and its enantiomer, PHA-513550.

The detection limit for PNU-105570 in PNU-95666 is about 0.02%, whereas for PHA-513550 in a 10 mg/mL preparation of PNU-142774E, it is about 0.01%. Average recovery for PNU-105570 over the range 0.05–1.0% was 97.6% with $r^2 = 0.996$. Over the truncated, and more relevant range of 0.05–0.4%, the average recovery was 102.1% with a correlation coefficient of 0.9999. Note, that an amount of the distomer above 0.05% was never detected in any lot of sumanirole. For PHA-513550 in PNU-142774, a correlation coefficient of 0.9999 and an average recovery of 105.2% was obtained over the range 0.1–1.8%. PNU-142774E fortified with 0.1% PHA-513550E proved highly reproducible, whereas somewhat greater variability was observed for PNU-105570 in sumanirole at the same level. At the higher fortification level of 0.4%, the RSD was 2.4% for sumanirole. Subsequent to validation, improved, more reliable performance was obtained as the result of a change in the injection sequence, namely inclusion of a water dip for the capillary immediately following injection. Four years of ICH stability data were acquired for sumanirole using several iterations of the method. By comparison, PNU-142774E was in development for a shorter time. Both sumanirole and PNU-142774E registered $<0.1\%$ of the undesired enantiomer in every lot of API examined.

ACKNOWLEDGMENTS

J.M. Vasas is recognized for having contributed lab work to this project. Also, we acknowledge T.R. Alcumbrack, P.A. Hartman, A.K. Bose, and P.A. Irish for the HPLC Section. The enantiomers were synthesized by P. Gm. Wuts.

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Received February 12, 2006
Accepted March 8, 2006
Manuscript 6822