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Management of nonmatrix interfering peaks in a chiral high-performance liquid chromatographic assay produced by solid-phase extraction of rat plasma

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Abstract

PD 146923, under evaluation as an alkylating radiosensitizing drug, contains one chiral center and one chemically reactive aziridine ring. A method was developed to evaluate possible in vivo enantiomeric inversion of PD 146923 in rat plasma. Normal-phase chiral HPLC was necessary to separate the enantiomers, but a typical aqueous-based solid-phase extraction (SPE) was needed to isolate the analytes from plasma. SPE at higher analyte concentrations removed all interfering peaks and gave acceptable recoveries. However, peaks (A–G) from seven new components interfering with analyte detection at lower concentrations were produced by SPE. The interfering peaks overlapped each other, so some were not observed until other, more intense interfering peaks had been managed. The low separation efficiency of the chiral column precluded management of interfering peaks by modifying chromatographic parameters. Chemical reactivity of the analytes forced the use of mild conditions for management of interfering peaks. Peaks A–F were: (A) water from the SPE cartridge; (B) SPE sorbent endcapping; (C, E and F) nonvolatile salts of the SPE elution acid reacting with bases from the injection solvent or with unidentified bases from the SPE cartridge; (D and G) analyte degradation products. This study identifies the nonmatrix peaks coeluting with the analytes, and describes how an aqueous-based SPE method was developed for isolating these very polar, highly reactive analytes in plasma for separation in a normal-phase chiral HPLC assay. Additionally, B, C, E or F probably are present in many other solid-phase extractions, but are not observed because of polarity or solubility properties.

Keywords: Enantiomer separation; Extraction methods; Interfering peaks; Nitroimidazoles

1. Introduction

CI-1010 (I, Fig. 1), a 1-substituted 2-nitroimidazole, is under development as an alkylating radiosensitizer for use as an adjuvant to irradiation treatment for cancer [1]. CI-1010 is a prodrug, and is converted rapidly in vivo to drug II (PD 146923). I and II each contain one chiral center, of *R*-configuration. A method was needed to evaluate enantiomeric inversion in vivo of II to III, the corresponding *S*-enantiomer, PD 146922. UV absorbance was preferred for detection.

Solution standards of II and III were separated only by using normal-phase chiral HPLC [2]. In contrast, the polar nature of the analyte functional groups (alcohol, amine, nitroimidazole) required an aqueous-based solid-phase extraction (SPE) to isolate the analytes from rat plasma. At high analyte concentrations, SPE removed all matrix interferences and afforded acceptable recoveries.

SPE also introduced seven nonmatrix peaks (A–G) that interfered with detecting analytes at lower

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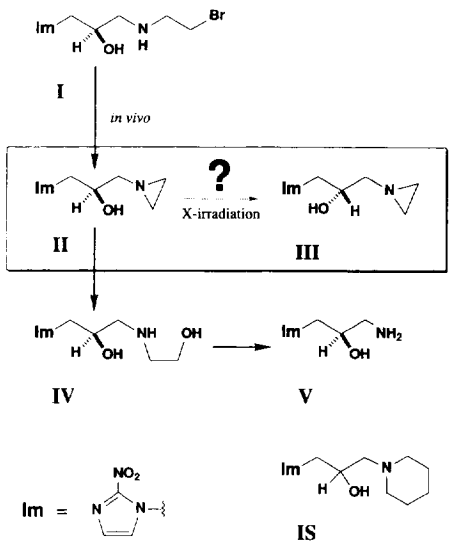

Fig. 1. Structures of the analytes and internal standard (I.S.). The possible in vivo enantiomeric inversion of concern (II to III) upon X-irradiation is highlighted in a scheme showing metabolic and/or degradation pathways.

concentrations. These interfering peaks had to be managed to achieve acceptable lower limits of quantitation. A low separating efficiency, typical of chiral HPLC, precluded management of interfering peaks by modifying chromatographic parameters. Management of peaks A–G was limited to using mild conditions because of stability concerns related to the facile acid-catalyzed attack of water or alcohols on aziridine rings [3], such as in II and III. Minimizing analyte loss was critical, given the limited volumes available for rat plasma samples (0.15 ml). Additionally, B, C, E or F probably are present in many other solid-phase extractions, but are not observed because of polarity or solubility properties.

2. Experimental

2.1. Chemicals and reagents

CI-1010, (*R*)-1H-imidazole-1-ethanol, α-{[2-bromoethyl)amino]methyl}- 2 -nitro-monohydrobromide (I); and analogs PD 146923 (II), PD 146922 (III), PD 126675 (I.S.), PD 151745 (IV), and PD 153235 (V), were from Parke-Davis Pharmaceutical Research (Ann Arbor, MI, USA). Hexane, methanol and acetonitrile (HPLC grades) were from Mallinkrodt (Paris, KY, USA). Diethylamine (98%), and sodium phosphate (dibasic, 99.95%) were from Aldrich (Milwaukee, WI, USA). Isopropanol, phosphoric acid (85%), and toluene (reagent grade) were from EM Science (Cherry Hills, NJ, USA). Glacial acetic acid (HPLC grade) was from J.T. Baker (Phillipsburg, NJ, USA). Ethanol, 200 proof (5% methanol) was from Aaper Alcohol and Chemical (Shelbyville, KY, USA). Water was obtained using a Milli-Q System (Waters, Milford, MA, USA). Dimethydichlorolsilane was from Sigma (St. Louis, MO, USA). Rat plasma was prepared in-house by separating heparinized whole blood collected from male Wistar rats.

2.2. Solid-phase extraction equipment

Sep-Pak Plus C_{18} cartridges were from Waters. Syringes (3 ml) were from Becton-Dickinson (Franklin Lakes, NJ, USA). SPE manifold (24-Port) was from Alltech (Deerfield, IL, USA). TurboVap LV Evaporator was from Zymark (Hopkinton, MA, USA)

2.3. HPLC equipment

An HP 1090 A (upgraded to Series L) liquid chromatograph from Hewlett-Packard (Avondale, PA, USA) was fitted with a 3.2×1.5 mm silica guard column (7 μm, NewGuard from Applied Biosystems, Foster City, CA, USA), followed by two amylose-based Chiralpak AS columns in tandem (each column 250×4.6 mm, 10 μm) from J.T. Baker. A Lambda-Max Model 481 Spectrophotometer from Waters was used for detection at 325 nm, with a Labnet Data System from Spectra-Physics (San Jose, CA, USA).

2.4. Chromatographic conditions

Mobile phase A (hexane–ethanol–diethylamine, 89:11:0.2, v/v/v) and mobile phase B (ethanol) were used in a step gradient program at 1 ml/min: 0 to 35 min, isocratic 100% A; 35.0 to 35.1 min, a linear

change to 20% A and 80% B; 35.1 to 40.1 min, isocratic 20% A and 80% B; 40.1 to 40.2 min, a linear change to 100% A; and 40.2 to 60 min, isocratic 100% A.

2.5. Assay procedure

Unless otherwise indicated, SPE reagents were chilled (0–4°C) prior to use.

Sep-Pak Plus C_{18} cartridges, fitted with 3 ml syringe barrels, were conditioned with 1×3 ml each of methanol, acetonitrile, and water, all at room temperature. The cartridges then were washed with 3 ml of chilled extraction buffer (potassium phosphate, pH 6) immediately before sample addition. Plasma samples (0.3 ml) for samples or standards were added to chilled culture tubes containing 0.3 ml of extraction buffer with I.S. After 0.5 ml of extraction buffer was added, the samples were vortexed and loaded onto SPE cartridges. The cartridges were washed with water (0.5 ml), isopropanol (0.3 ml), and hexane (1.0 ml). After vacuum drying for 30 s, analytes were eluted into silanized 75×12 mm glass culture tubes[1] by using 0.5 ml of 2% acetic acid in methanol, followed by 0.5 ml methanol. The solvent was evaporated at ~20°C using a stream of nitrogen gas at 10 p.s.i. (1 p.s.i.=6894.76 Pa). Mobile phase A (0.3 ml) was added, and the tubes were centrifuged. A 0.15 ml volume was injected into the liquid chromatograph for each sample.

3. Results

3.1. Normal-phase chiral HPLC was required for analyte separation

The project objective was to develop a method for isolating enantiomers II and III from plasma, then separating them chromatographically for quantitation. A reversed-phase separation of II and III was favored, because it might be adapted to an aqueous-based SPE method reported for isolating II from plasma. The SPE method used C_8, C_{18} or phenyl sorbents, and gave recoveries of approximately 80% [2]. Attempts at separating II and III using reversed-phase chiral HPLC (e.g., AGP protein column or various cyclodextrin columns) or capillary electrophoresis with chiral additives (rifamycin [4], vancomycin [5], or various substituted cyclodextrins [6]) were not successful. Drug enantiomers II and III were separated only by normal-phase HPLC using a Chiralpak AS column (Fig. 2a). The elution order of III preceding II was an advantage for detecting trace levels of III in the presence of II.

3.2. Analyte isolation from rat plasma required aqueous-based SPE

Given the restriction of normal-phase HPLC, a normal-phase method for analyte isolation from plasma was preferred, but was not achieved. Liquid–liquid extraction of these very polar analytes was precluded by their high aqueous solubility. Protein precipitation was not evaluated because of concerns regarding analyte degradation during the necessary prolonged evaporations.

Attention focused on SPE using solvents compatible with normal-phase for elution. Elution of C_{18} or C_8 cartridges using 1 ml of 100% methanol, ethanol, isopropanol, acetonitrile, methylene chloride, THF or hexane gave poor (0–10%) analyte recoveries. The highest recovery occurred using methanol, but doubling its elution volume failed to give recoveries above 15%. Increasing elution volumes further was not considered, because analyte recoveries did not increase proportionally to elution volume, and there were concerns regarding analyte degradation and losses from evaporation of large eluent volumes.

The acidity of the SPE eluent was increased in an effort to improve recoveries. Adding acid to acetonitrile, methylene chloride, THF or hexane failed to improve recoveries significantly. Methanol–acid gave the best SPE recoveries, but direct injection of this solvent produced loss of resolution due to peak broadening, which also made quantitation difficult (Fig. 2b). Ethanol or isopropanol, alone or in combination with methanol, gave less peak broadening than methanol as injection solvents, but gave much lower SPE recoveries. Therefore, although methanol–acid was used for SPE elution, it was evaporated

[1] Preparation of Silanized Test Tubes. Glass culture tubes (75×12 mm) were filled with a 10% solution of dimethydichlorolsilane in toluene. After 30 min, the tubes were emptied, washed with toluene (2×) and methanol (1×), and air dried overnight.

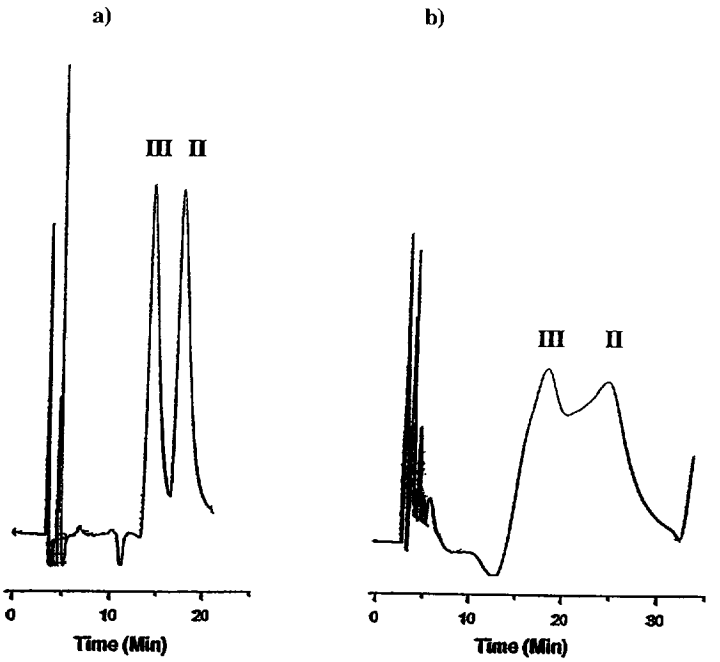

Fig. 2. Chromatograms showing the separation of enantiomers II and III from 10 000 ng/ml solution standards. Injection solution: (a) 100% mobile phase A; (b) methanol–mobile phase A (90:10, v/v).

prior to reconstitution in a normal-phase solvent. Adding the eluent evaporation step limited acid selection for SPE to those with sufficient volatility to avoid being concentrated during methanol evaporation.

3.3. Nonmatrix interfering peaks

3.3.1. A: Water in the SPE eluent

In early SPE studies, a brief vacuum drying (−10 p.s.i.) was used prior to elution with methanol–trifluoroacetic acid (TFA), and isopropanol was used for reconstitution. Chiral HPLC of the residue gave an interfering peak (A) not present after evaporation of methanol–TFA; if analytes also were extracted, the enantiomer peaks were much broader than normal. Peak A and broadening effects were inversely proportional to the completeness of vacuum drying or eluent evaporation, and were increased by adding traces of water to the injection solvent. They were attributed to the presence of water in the injection solution, displaced from the SPE cartridge during elution, but not removed by evaporation.

One explanation for peak A and peak broadening is that the solution of isopropanol, water and analytes undergoes gradual phase separation (aqueous and organic) after injection due to dilution with mobile phase during the chromatographic run. The aqueous phase, and analytes dissolved within it, can travel rapidly through the column due to minimal stationary phase interactions, and will elute early as an interfering peak A. Additionally, if analytes partition between the injection solution and the organic component (mobile phase), they could experience differing degrees of interactions with the stationary phase before they elute, resulting in peak broadening.

Evaporation of the SPE eluent to complete dryness eliminated peak A. However, analyte recoveries were reduced relative to those from the more facile evaporation of analytes spiked into anhydrous methanol–TFA, suggesting instability. Therefore, it was better to eliminate water from the SPE cartridge prior to elution, rather than by evaporation.

Extended vacuum drying was considered, but raised concerns of analyte stability. Extraction disc cartridges were evaluated for their reduced elution volume, but gave low recoveries using conditions developed for the standard SPE cartridges. The

difference between disc and cartridge recoveries demonstrated the importance of ion-exchange relative to partitioning in the mixed-mode retention [7] occurring between the analytes and the cartridge sorbent. This interaction is not prominent in extraction disc cartridges, with their reduced volume of sorbent. The mixed-mode mechanism proposed also was consistent with the observation that analyte recoveries from C_8, C_{18} and phenyl SPE cartridges were similar.

Peak A was managed effectively by removing most of the water from the SPE cartridges using sequential isopropanol and hexane washes prior to elution. Any water in the eluant could be removed during its facile (20 min at 20°C) evaporation, which minimized analyte degradation.

3.3.2. B: SPE endcapping

After peak A was eliminated, a hexane soluble, non-volatile residue was observed after SPE using C_{18}, C_8, and phenyl cartridges from three of four manufacturers. Upon chiral HPLC at 325 nm, the residue produced a large broad peak overlapping II and III, increasing dramatically at lower wavelengths, and decreasing only slightly above 325 nm. The mass spectrum of the residue indicated trimethylsilyl ethers, suggesting that this was probably material from SPE cartridges. Prewashing the cartridges with 10 ml of methanol–TFA reduced the residue by approximately half. However, this interference was managed best by using Waters Sep-Pak Plus C_{18} SPE cartridges, which did not produce peak B.

3.3.3. C: Diethylammonium trifluoroacetate

After elimination of peak B from the chromatogram, a new peak (C) coeluting with II and III after SPE elution using methanol–TFA was identified. Initially, this was thought to be water (A), but studies demonstrated that it was not volatile. Peak C was collected, and its positive- and negative-ion mass spectra were used to assign it as diethylammonium trifluoroacetate, a salt produced by diethylamine from the reconstitution solution (and mobile phase) reacting with TFA from the SPE eluent. Peak C was eliminated by removing TFA from the SPE eluent, using nitrogen gas to facilitate complete evaporation. A high flow of nitrogen produced a rapid evaporation that avoided high temperatures, and minimized analyte degradation.

3.3.4. D: Some analyte degradation products produced by SPE and evaporation

After peak C had been eliminated, it was apparent that two new interfering peaks (D, Fig. 3) had been produced by extracting/evaporating each aziridine enantiomer. For each pair of D peaks, one eluted immediately before, and one after the corresponding aziridine peak. This was of most concern in standards with high concentrations of III, where the earlier eluting D overlapped II, appearing as if enantiomeric inversion of III to II had occurred. Because the intensities of D were proportional (5–10%) to the corresponding enantiomer peak intensities, and their formation was facilitated by heat, peaks D were assigned as enantiomer degradation products. Peak D might be related to an opening of the aziridine ring by methanol–TFA, because using isopropanol–TFA for SPE elution did not produce D.

The management of peak D included reducing analyte exposure to conditions favoring acid-catalyzed attack. First, reduced temperatures were used for handling samples, buffers, SPE and eluent evaporation. Second, the amount of acid in the elution

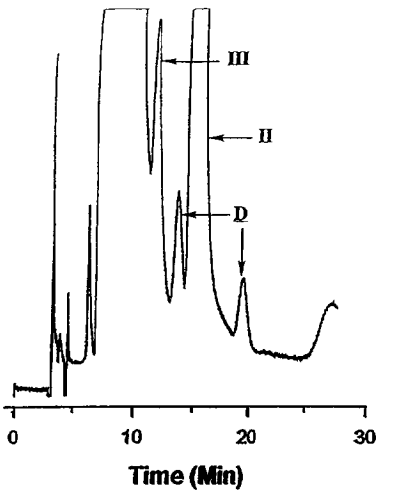

Fig. 3. Chromatogram showing interfering peaks D produced from degradation of II during SPE/evaporation of rat plasma spiked with 10 000 ng/ml of II and 1000 ng/ml of III. The corresponding peaks (D) from degradation of III can not be seen in this chromatogram.

solvent was reduced. Finally, collection tubes used for SPE were silanized to block catalytic silanol sites. Additionally, two chiral HPLC columns were connected in tandem to improve separation between analytes and interfering peaks.

3.3.5. E: Salt of TFA reacting with an undefined base from the SPE cartridge

Management of peak C also revealed a new, broad, smaller interfering peak (E, Fig. 4), overlapping III. Evidence suggested that E was a nonvolatile salt of TFA and an undefined base from SPE cartridges dissolved in methanol. Peak E was not present after evaporation of methanol, TFA or methanol–TFA that had not been passed through SPE cartridges. However, peak E was produced when TFA was added to methanol that had previously been passed through SPE cartridges.

Decreasing the TFA concentration from 1–0.001% in the SPE eluent reduced the intensity of E, but reduced analyte recoveries proportionally. Because it appeared that peak E could not be managed successfully using TFA, different volatile acids were evaluated for SPE elution. Peak E was not present after using methanol–hydrochloric acid (1–0.000001%) for SPE elution, but neither were II or III. Analyte degradation was suspected when using the higher concentrations of HCl, and analytes were not eluted using the lowest HCl concentrations. This suggested that the minimum concentration of HCl necessary for analyte elution also caused analyte degradation.

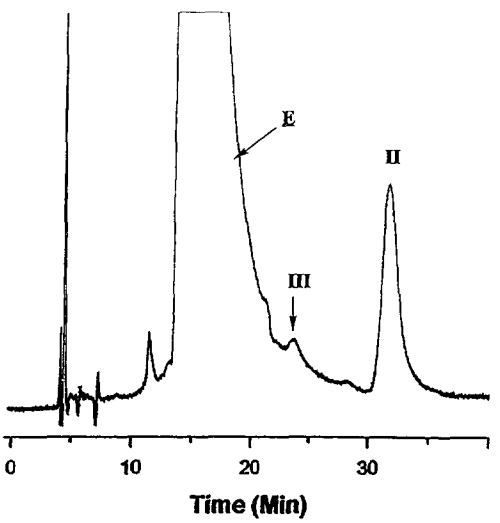

Fig. 4. Chromatogram of an extract of rat plasma spiked with 5000 ng/ml of II and 500 ng/ml of III. Interfering peak E was assigned as a salt produced from TFA reacting with an undefined base eluted by methanol from the SPE cartridge.

3.3.6. F: Salt of acetic acid reacting with an undefined base from the SPE cartridge

Acetic acid–methanol, rather than TFA–methanol, was used for SPE elution in an effort to eliminate peak E. Peak E was not observed, but a new, broad, tailing peak (F, Fig. 5a) eluting near II was present. This peak (F) was assigned as the acetic acid analog of the TFA peak E, i.e., a non-volatile salt of acetic

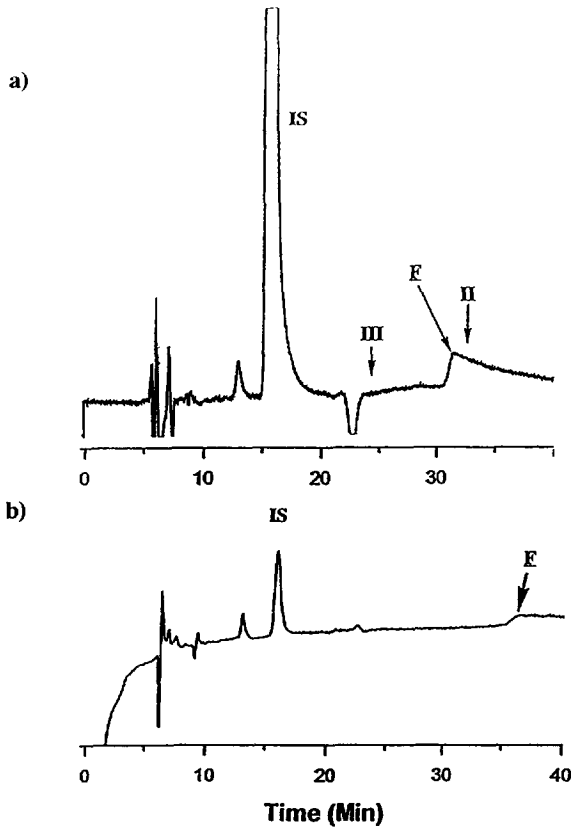

Fig. 5. Chromatograms of extracts of rat plasma spiked with I.S. Interfering peak F, was assigned as a salt produced from acetic acid reacting with an undefined base eluted by methanol from the SPE cartridge. The positions for elution of II and III (if present) are indicated. (a) SPE elution using increased amount of acetic acid; (b) SPE elution using reduced amount of acetic acid (see text).

acid with an undefined base extracted by methanol from the SPE cartridge. In contrast to peak E, peak F was manageable because it was separated chromatographically from II and III. SPE elution using formic acid–methanol produced a peak similar to F, but with lower analyte recoveries.

Mixtures of acetic acid with different alcohols were evaluated for SPE elution in efforts to minimize extraction of the undefined base from the cartridge. Reducing alcohol polarity (isopropanol<ethanol< methanol) reduced interfering peak F intensity, but also reduced analyte recovery proportionally. Thus, acetic acid–methanol was optimal for SPE elution. The best ratio for analyte recovery vs. peak F intensity occurred using 0.5 ml of 2% acetic acid in methanol, followed by 0.5 ml of methanol. This sequence eluted all analytes while reducing the acid in the drydown by approximately 50%, thereby improving analyte stability and reducing peak F intensity. Reducing the intensity of peak F also produced a shift in its peak apex toward a longer retention time and further from III (Fig. 5b). A rationale for this shift was not investigated, but may be related to the ion-pair nature of the salt and its interactions with the stationary phase.

3.3.7. G: Very late eluting metabolites/degradation products

After interfering peaks A–F had been managed, interfering peaks G could be seen as random, broad bumps in the baseline of some chromatograms. They interfered with the evaluation of some blanks, or standards and samples containing low concentrations of analytes. Expanding the time of the isocratic run from 40 min to 180 min for extracted high standards of II revealed that G was caused by several very late (125 min) eluting peaks (Fig. 6). The retention times of G corresponded to IV and V, known metabolites/

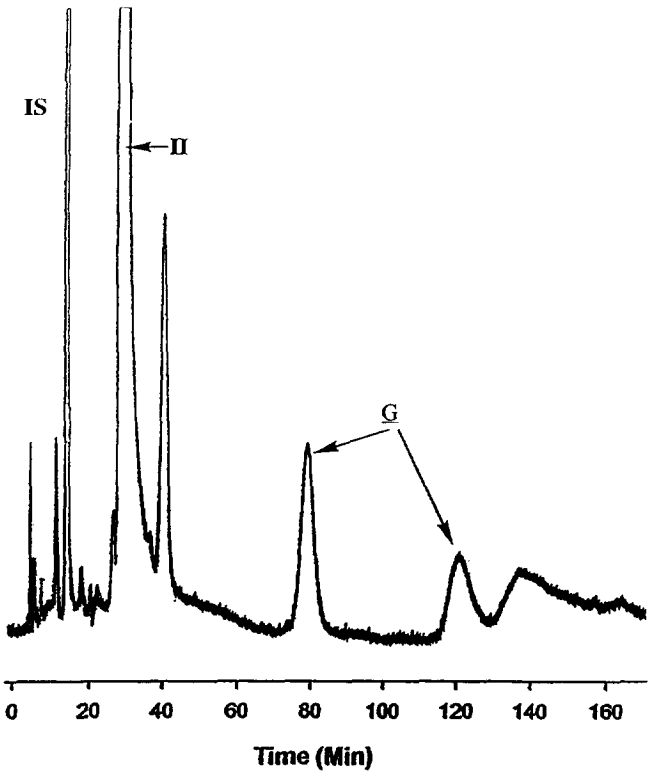

Fig. 6. Chromatogram from an extended HPLC isocratic run to show IV and V in an extract of rat plasma spiked with I.S. and 10 000 ng/ml of II. Analytes IV and V, produced by degradation of II during SPE/evaporation, would have produced interfering peaks G in subsequent chromatograms had the normal run time of 40 min been used.

degradation products of prodrug (I) or drug (II) [8]. Comparable peaks also were present after injecting high standards of III, and were assigned as enantiomers of IV and V, although these enantiomers were not available to confirm identity based on retention times. Therefore, analyte degradation during SPE and evaporation produced G. Because all reasonable steps to minimize analyte degradation had been incorporated into the method, these very late eluting interfering peaks were managed by adding a step gradient after elution of drug enantiomer II. This flushed the columns of very late eluting peaks prior to the next injection.

3.4. Summary

Separation of drug enantiomers II and III required normal-phase chiral HPLC. The incorporation of SPE, necessary for analyte isolation from plasma, removed all matrix interfering peaks but produced seven new nonmatrix interfering peaks (A–G). Peaks A–G interfered with baseline stability and quantitation of analytes at low concentrations.

These peaks were managed by using: (A) an isopropanol prewash immediately before SPE elution, (B) SPE cartridges with minimal loss of endcapping, (C) careful, yet complete, evaporation of the SPE elution acid during drydown, (D) SPE at reduced temperatures, silanized tubes for evaporating SPE eluent, and tandem chiral columns, (E and F) a reduced amount of acetic acid for SPE elution, and (G) a column wash after each HPLC run.

Successful management of nonmatrix interfering peaks allowed a lower limit of quantitation of 200 ng/ml for enantiomers II or III from 0.3 ml of rat plasma. Resolution was sufficient in standards to detect 200 ng/ml of III in the presence of 10 000 ng/ml of II, the equivalent of 2% enantiomeric inversion Fig. 7. Average recoveries of each analyte were approximately 50% at all concentrations used for the standard curve. A representative chromatogram (Fig. 8) demonstrates that the method can be used to demonstrate lack of in vivo enantiomeric inversion in a rat dosed intravenously with 40 mg/kg IV of prodrug I (sampled 15 min after X-irradiation, 45 min after dosing). This chromatogram shows that the peak from enantiomer II (4700 ng/ml) is separated completely from the region where enantiomer

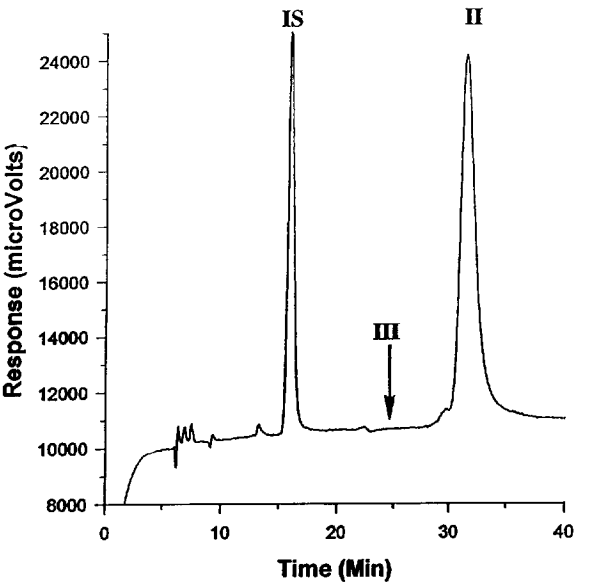

Fig. 7. Chromatogram of an extract of rat plasma spiked with I.S. and 10 000 ng/ml of II. The position for elution of III (if present) is indicated. All interfering peaks have been managed.

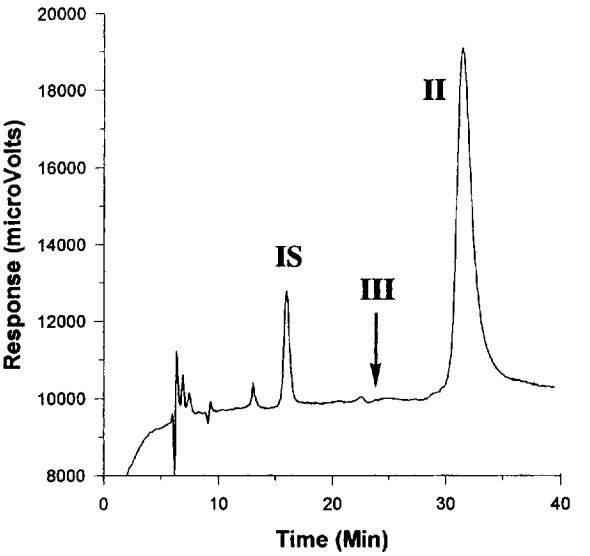

Fig. 8. Chromatogram of an extract of plasma from a rat dosed intravenously with 40 mg/kg of prodrug I, exposed to X-irradiation and then sampled. This chromatogram shows a peak for only one enantiomer (4700 ng/ml of II) and no interfering peak near the position for elution of III (if present).

III would have eluted, and that the latter does not contain any interfering peaks.

4. Conclusions

Appropriate management of interferences A–G introduced by SPE gave an SPE-chiral HPLC method for quantitating enantiomers II and III in rat plasma. The method also required addressing issues of analyte reactivity to achieve a lower limit of quantitation of 200 ng/ml for each drug enantiomer, and a detection limit of 2% of the inversion product, III, in the presence of the upper limit of quantitation of the drug II, 10 000 ng/ml. The method can be applied to evaluate plasma samples from dosed rats.

Acknowledgments

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