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Use of short high-performance liquid chromatography columns and tandem-mass spectrometry for the rapid analysis of a prostaglandin analog, fluprostenol, in rat plasma

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Abstract

A short reversed-phase HPLC column and a tandem mass spectrometer were used to develop a stable-isotope-dilution assay for the rapid and sensitive analysis of fluprostenol, a prostaglandin analog, in rat plasma. A Waters Symmetry ODS column (2.1×10 mm) afforded rapid isocratic elution of fluprostenol (t_R=40 s) but still provided a relatively large k' value of 4. The use of tandem mass spectrometry allowed the interference-free detection of fluprostenol under the rapid elution conditions, with a limit of quantitation of 25 pg ml^{-1} fluprostenol, using 0.2 ml plasma sample volumes. The method was linear over three orders of magnitude, yielded accurate and precise results and allowed the pharmacokinetic profile of fluprostenol to be defined following intravenous administration in rats.

Keywords: Fluprostenol; Prostaglandin

1. Introduction

High performance liquid chromatography combined with tandem mass spectrometry (HPLC–MS–MS) has become the technique of choice for the trace analysis of drugs in biological and environmental matrices. The MS–MS detection scheme involves ionization/volatization of the analyte as it exits the HPLC column, typically by electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), isolation of a precursor ion by the 1st quadrupole region, collisionally-activated dissociation (CAD) of the precursor ion in a 2nd quadrupole region and finally isolation of a characteristic product ion in the 3rd quadrupole region. This type of detection scheme is typically referred to as selected-reaction-monitoring (SRM) and provides an extremely high degree of selectivity, often allowing essentially interference free detection of an analyte in highly complex matrices. The broad applicability of HPLC–MS–MS for trace drug analysis has been demonstrated for a wide array of compound classes.

The HPLC column is often used in the HPLC–MS–MS analyses to simply retain the analyte from the solvent front to avoid signal suppression effects during the ionization/volatilization process. Co-elution of high levels of salts and endogenous matrix components can suppress the ionization of the ana-

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lyte during the ESI. In our labs, we typically use 2 mm ID columns with lengths between 30 to 50 mm. To achieve analysis times of 2 min or less, the analytes are typically retained with k' values of 2 or less. Recently, the use of shorter HPLC columns (10 to 20 mm) for HPLC–MS–MS methods has been reported [1]. The use of columns with lengths 10 mm vs. 50 mm allows isocratic analysis times that are 5 times shorter with identical capacity factors (k'). Alternatively, the mobile phase can be modified to provide greater retention, k' values between 3 to 7, while still maintaining run times of less than 1 min. The larger capacity factors may provide more robust assays by minimizing potential suppression problems due to co-elution of high levels of salts and endogenous matrix components.

Fluprostenol (FP, see Fig. 1) is structurally related to endogenous prostaglandins and has been widely used in animal husbandry to regulate the estrus cycle of breeding stock [2], as well as having been evaluated in human clinical studies as a potential abortifacient [3]. Although FP has been widely used, no method has been described in the literature for the measurement of FP plasma levels. In order to determine prostaglandin levels, of either endogenous or synthetic analogs, high sensitivity is required due to their rapid metabolism and elimination by the body. The application of HPLC–MS–MS for the analysis of endogenous prostaglandins and leukotrienes has been reported [4–8]. We report on the development of an isotope-dilution HPLC–MS–MS method, based on the use of short (10 mm long) HPLC columns, for the trace analysis of FP in rat plasma, following intravenous (IV) administration.

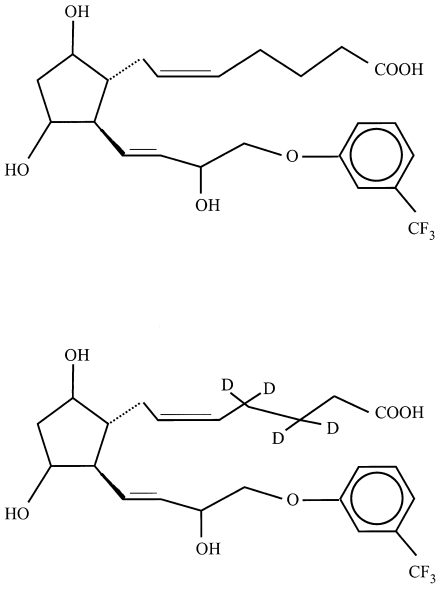

Fig. 1. The chemical structure of fluprostenol (FP, top) and 3,3,4,4-$^{2}H_{4}$-fluprostenol (d-FP, bottom).

2. Experimental

2.1. Chemicals and reagents

FP (see Fig. 1) and 3,3,4,4-$^{2}H_{4}$-fluprostenol (d-FP, see Fig. 1) were purchased from Cayman Chemical (Ann Arbor, MI, USA) and synthesized at Procter & Gamble Pharmaceuticals Health Care Research Center (Mason, OH, USA), respectively. Methanol (HPLC grade), ammonium acetate (reagent grade) and formic acid (SupraPur) were from J.T. Baker (Phillipsburg, NJ, USA). Rat plasma and whole blood were obtained from Pel-Freez Biologicals (Rogers, AR, USA). Distilled-deionized water was obtained from a Barnstead NanoPure II system (Dubuque, IA, USA).

2.2. Preparation of FP plasma standards

FP and d-FP stock solutions were prepared in water–methanol (50:50; v/v) and stored at −20°C. Working plasma standards, covering a FP concentration range from 10 to 2500 pg ml^{-1}, were prepared on the day of analysis by pipeting 10 μl of the appropriate fluprostenol stock solution into individual glass test tubes already containing 200 pg of d-FP and then adding 0.2 ml of blank rat plasma to each tube. Each working standard was then vortexed for 30 s and prepared for analysis as described below.

2.3. Preparation of quality control (QC) samples

QC samples were prepared at 25, 100, 500 and 10 000 pg ml^{-1} FP levels by adding an appropriate aliquot of FP stock solution, prepared in water–methanol (50:50; v/v), into a 5 ml volumetric flask and diluting to volume with rat plasma. Replicate

(n=5) aliquots (0.2 ml) of each QC sample were then added to test tubes already containing 200 pg of d-FP, mixed by vortexing for 30 s and prepared for analysis as described below.

2.4. Preparation of study samples

Unknown plasma samples obtained from rats dosed with FP were prepared for analysis by aliquoting 0.2 ml of the unknown sample into a test tube already containing 200 pg of d-FP and vortexing to mix. For samples containing levels of FP that were off the standard curve, an aliquot of the sample was first diluted with blank rat plasma and then 0.2 ml of the diluted sample was added to a test tube containing 200 pg of d-FP and mixed by vortexing.

2.5. Solid phase extraction (SPE) sample preparation.

Standards, QC and study samples were prepared for analysis manually by SPE using octadecylsilane (ODS) cartridges and a vacuum manifold. IST Isolute (Jones Chromatography, Lakewood, CO, USA) ODS cartridges (1 ml, 100 mg) were conditioned with 1.0 ml of methanol followed by 1.0 ml of water–methanol–formic acid (97:3:0.1, v/v/v). Then 0.2 ml of standard, QC or unknown rat samples were loaded onto individual cartridges at a flow-rate of ~1 ml min^{-1}. The cartridges were washed sequentially with 1.0 ml of water–methanol–formic acid (97:3:0.1, v/v/v) and 1.0 ml of water–methanol (75:25, v/v). The analytes were eluted from the columns using 1.0 ml of water–methanol (10:90, v/v) and collected in polypropylene tubes. The elution solvent was removed in a TurboVap™ evaporator (Zymark, Hopkinton, MA, USA) using 172 kPa nitrogen and an evaporation temperature of 35°C. The residue was then reconstituted in 120 μl of water–methanol (70:30, v/v) and placed in a polypropylene autosampler vial prior to analysis by HPLC–MS–MS as described below.

2.6. Relative recovery of FP from SPE

The relative recovery of FP from the SPE procedure was evaluated using the 500 and 10 000 pg ml^{-1} QC samples. For each QC level, replicate (n=5) aliquots, without the internal standard, were carried through the SPE sample preparation procedure, dried and reconstituted in 120 μl of water–methanol (70:30, v/v) containing 200 pg of internal standard, d-FP.

2.7. Stability of FP in plasma and whole blood

The stability of FP in rat whole blood and plasma was examined at ambient temperature over a 2 h period. Replicate (n=6) aliquots (1.0 ml) of whole rat whole blood or plasma were added to polypropylene test tubes already containing 2500 pg of FP and mixed by gentle swirling. Three of the blood samples were centrifuged immediately to obtain the time 0 samples. Then 200 μl of plasma obtained from the time 0 samples, as well as from three of the original plasma samples, were spiked with 200 pg of internal standard and prepared for analysis by SPE as described above. The other three plasma and whole blood samples were processed in a similar fashion after sitting 2 h at ambient temperature. Stability was determined by comparing the FP concentrations obtained from the 2 h ambient incubation samples with the FP concentration obtained for the time 0 samples.

2.8. Stability of prepared samples

The stability of the prepared samples was determined by injecting a set of standards and spiked samples immediately after preparation and then again injecting the same samples after 24 h at ambient temperature and 5 days refrigerated storage.

2.9. Accuracy and precision

QC samples were analyzed on 4 separate days to determine the accuracy and precision of the method. Replicate (n=7) QC samples were prepared on each day by adding 0.2 ml of each QC sample to test tubes already containing 200 pg of d-FP. The samples were mixed by vortexing for 30 s and then prepared for analysis, along with the standards, by SPE as described above. The reconstituted QC samples and standards were analyzed by HPLC–MS–MS as detailed below.

2.10. HPLC–MS–MS conditions

The HPLC system was a Gilson (Middletown, WI, USA) model 305 master pump, two model 306 piston pumps and a model 234 autoinjectior, a model 805 manometric module and a model 811C dynamic mixer. Detection was done with a PE-Sciex (Concord, Ontario, Canada) API III+ triple quadrupole mass spectrometer. Reconstituted standards, QC and unknown samples were injected (25 μl) into a Waters (Milford, MA, USA) Symmetry Sentry Guard C_{18} column (2.1×10 mm, 3.5 μm) and eluted using a mobile phase of water–methanol–formic acid (62:38:0.1, v/v/v) modified with 70 mg l^{-1} ammonium acetate, and a flow-rate of 0.40 ml min^{-1}. The entire chromatographic effluent was passed into the mass spectrometer interface for subsequent detection.

The mass spectrometer was operated in the TurboIonSpray configuration, consisting of the articulated IonSpray inlet used in conjunction with the heated TurboProbe desolvation unit. The TurboProbe temperature and nitrogen gas flow-rate were 475°C and 8 l min^{-1}, respectively, and the nebulizer gas pressure was 62 p.s.i. (nitrogen). The ammoniated analyte ions detection was optimized using ESI and orifice potentials of 3800 and 40 V, respectively. The MS–MS detection scheme utilized CAD and SRM. CAD was accomplished using argon as the collision gas, at a thickness of 250×10^{13} molecules cm^{-2}, and an ion energy of 21 eV. The SRM transitions, m/z 476→m/z 279 and m/z 480→m/z 283, were sequentially monitored for detection of FP and d-FP, respectively. Dwell time for each transition was 100 ms. Peak area ratios from the SRM chromatographic peaks were determined using the PE-Sciex software package, MacQuan Version 1.4.

2.11. Quantitation of FP

Calibration curves were constructed by plotting the peak area ratios (FP/d-FP) for standards versus FP concentration and fitting these data to a weighted ($1/x^2$) linear regression line, within the MacQuan software package. Drug concentrations in the QC and unknown samples were then interpolated from this line.

2.12. Dosing solutions

Fluprostenol was dissolved in a 0.03 *M* sodium citrate buffer. The pH was adjusted to 6.0 with citric acid, sodium chloride was added to control osmolality and 0.1% methyl paraben was added as a bactericide. The final concentration was 100 μg ml^{-1} and final osmolality was 280 mOsm.

2.13. Animal dosing

Male Sprague-Dawley rats, approximately 500–550 g, were obtained from Harlan Industries, Indianapolis, Indiana. All animals were housed, fed and handled in compliance with the standards set forth by the Animal Welfare Act as amended or with recommendations on HHS Publications (NIH) No. 85–23, ''Guide for the Care and Use of Laboratory Animals,''. They were housed individually in suspended, stainless steel cages and acclimated to standard laboratory conditions of 12 h light/dark cycle for at least 3 days prior to dosing. Animals were allowed free access to food and water throughout the study. Rats ($n=3$) received an intravenous injection of 25 μg kg^{-1} fluprostenol. Serial plasma samples of ~300 μl were obtained from the animals over the 6 h period following dosing. Samples were stored frozen at −70°C until the day of analysis.

3. Results

3.1. ESI mass spectra

The ESI mass spectra obtained for FP and d-FP were dominated by intense ammoniated molecular ions with m/z of 476 and m/z 480, respectively, as well as significant fragments at m/z 240 and m/z 244, respectively (data not shown). The product spectra obtained for FP and d-FP, following CAD of their respective ammoniated molecular ions are shown in Fig. 2. The product spectra of both FP an d-FP showed a base peak at m/z 279 and m/z 283, respectively. The SRM transitions chosen for the analysis were m/z 476→m/z 279 and m/z 480→m/z 283 for the FP and d-FP, respectively.

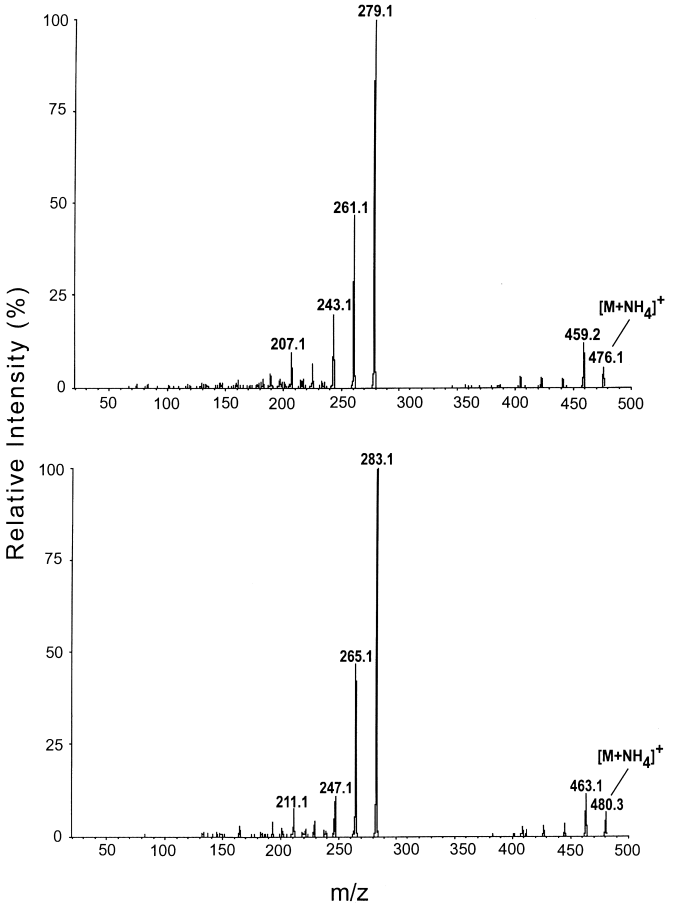

Fig. 2. Electrospray ionization tandem mass spectra (products-of-MNH_4^+) of: fluprostenol (top) and 3,3,4,4-$^2H_4$-fluprostenol (bottom).

3.2. Chromatographic profiles of blank and FP spiked rat plasma

HPLC–MS–MS chromatographic profiles, obtained using the selected SRM schemes, for blank plasma and blank plasma spiked with 100 pg ml^{-1} of FP and 1000 pg ml^{-1} of d-FP are shown in Fig. 3a–b, respectively. Typically, FP eluted as a symmetrical peak with a retention time around 40 s and a k' value of 4. Using the chosen SRM schemes, blank rat plasma was free of interferences in the FP and d-FP retention time regions (Fig. 3a). The combination of a short (10 mm) HPLC column and MS–MS detection allowed the rapid and selective analysis of FP in rat plasma (Fig. 3b), with run-to-run times of 1.2 min. The short HPLC columns typically tolerated at least 100 injections before column performance began to deteriorate.

3.3. Calibration curves

The calibration curve for FP was linear over four orders of magnitude, with correlation coefficients for the regressions being typically 0.999 or greater. Typically, the read backs for the standards were 100±10% across the range. Replicate (n=5) injections of the 500 and 10,000 pg ml^{-1} calibration standards resulted in RSD values for the peak area ratios of 2.9 and 4.6%, respectively.

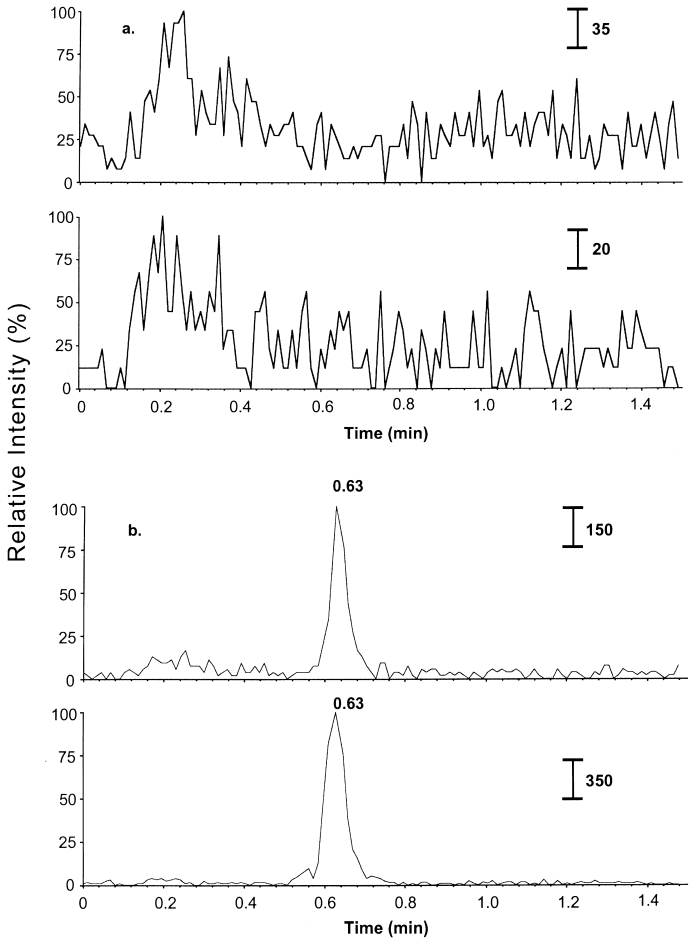

Fig. 3. HPLC–MS–MS chromatographic profiles corresponding to fluprostenol (top, SRM scheme: m/z 476/279) and and 3,3,4,4-$^2H_4$-fluprostenol (bottom, SRM scheme: m/z 480/283) for 0.2 ml plasma samples containing: (a) blank and (b) 100 pg ml^{-1} fluprostenol and 1000 pg ml^{-1} 3,3,4,4-$^2H_4$-fluprostenol (bottom).

3.4. Relative recovery of FP from SSPE

Although the use of the stable-isotope-labeled internal-standard, d-FP, would correct for any loss of FP during the sample preparation procedure, it is still important to measure and optimize the recovery of the drug during this step in order to obtain a robust and sensitive method. The relative recovery of FP during sample preparation was examined by processing plasma samples (n=5) that were spiked with only FP, at the 500 and 10 000 pg ml^{-1} levels, through the SPE and drying steps. The sample residues were reconstituted, spiked with d-FP and analyzed. The relative recovery of FP following the SPE, drying and reconstitution steps was found to be 85% with an RSD of 2.0% and 87% with an RSD of 8.1% for plasma samples containing FP at the 500 and 10 000 pg ml^{-1} levels, respectively.

3.5. Stability of FP in whole blood and plasma

The stability of FP in whole blood and plasma was evaluated over a 2 h period using samples spiked with 2000 pg ml^{-1} FP. The concentration of FP in the whole blood and plasma at 2 h were compared with the values obtained from the same whole blood and plasma samples with no incubation. The recovery for FP after 2 h incubation in whole blood

Table 1
Accuracy and precision of FP analysis in rat plasma

Spiked [FP] (pg ml^{-1})	Per cent accuracy (% RSD)[a]			
	Day 1	Day 2	Day 3	Day 4
25	131 (13.6)	92 (12.1)	123 (13.8)	–
100	105 (4.1)	100 (9.1)	98 (6.6)	99 (6.1)
500	88 (7.1)	84 (3.8)	90 (4.4)	91 (6.2)
10 000	100 (6.0)	95 (9.5)	96 (4.4)	103 (3.8)

[a] $n=7$ for each day at each concentration level.

and plasma, relative to the zero time point samples, were 95±1.6% and 103±4.4%, respectively. The results indicate that FP is stable in plasma and whole blood over a 2 h period.

3.6. Stability of prepared samples

The prepared samples were found to be stable sitting at ambient temperature for at least 24 h and for at least 5 days when stored refrigerated.

3.7. Accuracy and precision-analysis of QC samples

The accuracy and precision data for the HPLC–MS–MS analysis of blank rat plasma spiked with FP at various levels and analyzed on 4 separate days are presented in Table 1. The lower limit of quantitation was 25 pg ml^{-1}, where the recoveries ranged from 92 to 131% across 3 days, with RSD values typcially around 13%. The recoveries from 100 to 10 000 pg ml^{-1} across the 4 analysis days were within 3 to 15% of target, with RSD values all below 10%.

3.8. Pharmacokinetic profile of FP following intravenous dosing in rats

A plot of FP plasma levels vs. post-dose time interval is shown for three individual rats following a intravenous injection of 25 μg kg^{-1} of FP is shown in Fig. 4. The average terminal half-life calculated from the results for the three animals was 0.44±0.11 h.

4. Conclusion

A sensitive and selective HPLC–MS–MS method was developed for the analysis of fluprostenol in rat plasma. The use of a short (10 mm) HPLC column, in conjunction with MS–MS detection, allowed the development of a rapid, selective and rugged method for the analysis of trace levels of FP in rat plasma samples. FP was eluted with a retention time around 40 s, a k' value of 4 and injection-to-injection run

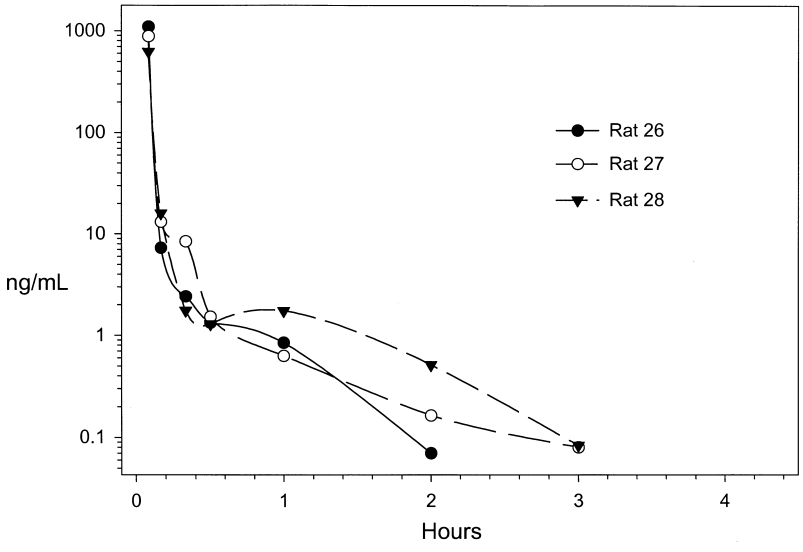

Fig. 4. Profile of fluprostenol plasma concentration versus post-dose sampling time for three rats dosed intravenously with 25 μg kg^{-1} of fluprostenol.

times of 1.2 min. The short HPLC columns proved to be fairly rugged, typically allowing at least 100 injections before column performance degraded. The methodology compares favorably, in terms of detection limits and speed, with previously reported methods for the analysis of prostaglandins and leukotrienes [4–9]. The previous methodologies reported detection limits in the range of 10 pg using sample volumes of 0.5 to 1 ml and analysis times from 1.5 to 15 min. Additionally, the methodology is comparable, in terms of speed, detection limits, accuracy and precision, with LC–MS–MS methods reported for the analysis of typical small drug molecules [10,11,12].

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