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Semi-automated 96-well solid-phase extraction and gas chromatography–negative chemical ionization tandem mass spectrometry for the trace analysis of fluprostenol in rat plasma

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Abstract

Semi-automated 96-well plate solid-phase extraction (SPE) was used for sample preparation of fluprostenol, a prostaglandin analog, in rat plasma prior to detection by gas chromatography–negative chemical ionization tandem mass spectrometry (GC–NCI-MS–MS). A liquid handling system was utilized for all aspects of sample handling prior to SPE including transferring of samples into a 96-well format, preparation of standards as well as addition of internal standard to standards, quality control samples and study samples. SPE was performed in a 96-well plate format using octadecylsilane packing and the effluent from the SPE was dried in a custom-made 96-well apparatus. The sample residue was derivatized sequentially with pentafluorobenzylbromide followed by *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. The derivatized sample was then analyzed using GC–NCI-MS–MS. The dynamic range for the method was from 7 to 5800 pg/ml with a 0.1-ml plasma sample. The methodology was evaluated over a 4-day period and demonstrated an accuracy of 90–106% with a precision of 2.4–12.9%.

Keywords: Fluprostenol

1. Introduction

Fluprostenol (FP, see Fig. 1), a synthetic prostaglandin F2α analog, has been used in animal husbandry to regulate the estrus cycle of breeding stock [1], as well as having been evaluated in human clinical studies as a potential abortifacient [2]. Recently, it has been shown that fluprostenol exerts unique bone anabolic effects, giving rise to increased bone mass and strength without the bone resorptive effects of parathyroid hormone [3]. 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. Although FP has been widely used, the only method described in the literature for the measurement of FP was a radiochemical-based assay which requires the administration of a radioisotopic FP [4]. Highly sensitive assays for endogenous prostaglandins have historically been performed using gas chromatography–negative ion chemical ionization mass spectrometry (GC–NCI-MS) or tandem mass spectrometry (MS–MS) [5–7]. We report on the development of a rapid, semi-automated method for the

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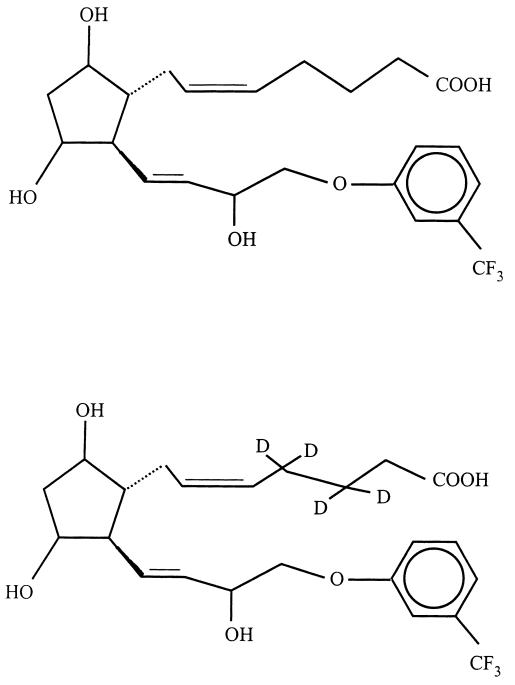

Fig. 1. The chemical structures of fluprostenol (FP, top) and 3,3,4,4-$^{2}H_4$-fluprostenol (d-FP, bottom).

analysis of fluprostenol in rat plasma samples using GC–NCI-MS–MS.

Sample preparation for bioanalytical assays is frequently a labor intensive, time-consuming process, and with the utilization of fast tandem mass spectrometry-based analysis, becomes the rate limiting factor in both method development and sample determination. The recent introduction of 96-well-based solid-phase extraction (SPE) plates and the availability of semi-automated 96-channel liquid handling devices has greatly increased the speed of sample preparation by allowing parallel processing of samples. There have been several articles describing automated SPE methods using a 96-well format [8–11]. The addition of semi-automated instrumentation for manipulating the individual samples received from animal and human studies into the standard 96-well format offers a further means of decreasing the sample preparation time.

For the fluprostenol plasma sample preparation step, an automated 12-channel liquid handling system was employed for all sample manipulation steps prior to the SPE. This included transferring the standards, quality control (QC) and samples from an individual tube format into the 96-well format for application to the SPE plate. Additionally, the liquid handling system was used to prepare working standards in a rat plasma matrix and for addition of the internal standard to standards, QC samples and unknown study samples. The plunger-in-tip design of the 12-channel pipetting system allowed the accurate and precise manipulation of small volumes (10 to 300 μl) of both aqueous and organic solvents.

SPE was then done using a semi-automated 96-channel liquid handling system in conjunction with octadecylsilane (ODS) packed plates. The parallel sample processing procedure allowed the semi-automated preparation of 96 samples simultaneously through a six-step SPE procedure in roughly 30 min. The effluent was removed under N_2 with heating using a laboratory-designed 96-well dryer constructed to use standard disposable plastic pipette tips to avoid potential cross-contamination between subsequent sample batches.

The dried sample residue was derivatized sequentially with pentafluorobenzylbromide (PFBBr) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) to derivatize the carboxylic acid and alcohol functional groups, respectively. The derivatized sample was then analyzed by GC–NCI-MS–MS. The combination of semi-automated sample preparation in a 96-well format with GC–NCI-MS–MS detection allowed the development and application of a rapid, selective and sensitive method for the analysis of fluprostenol in small volumes of rat plasma.

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 (Ann Arbor, MI, USA) and synthesized at Procter & Gamble Pharmaceuticals Health Care Research Center (Mason, OH, USA), respectively. Methanol (HPLC grade), acetonitrile (HPLC grade) and formic acid (SupraPur) were from J.T. Baker (Phillipsburg, NJ, USA). PFBBr (reagent grade), MSTFA (reagent grade) and diisopropylethylamine (reagent grade) were obtained from Pierce (Rockford, IL, 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. Instrumentation for sample handling and SPE

A MicroLab AT Plus 2 (Hamilton, Reno, NV, USA) was used for liquid handling prior to the SPE step. The MicroLab was used for manipulating standards, QCs and samples from a single tube format into a 96-well plate format. Additionally, the instrument was used for preparation of working standards and for the addition of internal standard to working standards, QCs and study samples. To achieve the best accuracy and precision all solutions were dispensed 0.3 mm from the bottom of the polypropylene tubes. All solutions were aspirated using liquid-level sensing with a single pre-wet mixing step before solution transfer.

The SPE steps were performed using a Quadra Tomtec 96 (TomTec, Handen, CT, USA) in conjunction with a vacuum manifold.

2.3. Preparation of FP plasma standards

FP and d-FP stock solutions were prepared in 0.1% sodium chloride–methanol (50:50, v/v) and stored at −20°C. Sodium chloride was added in order to use liquid-level sensing on the MicroLab instrument. Working plasma standards were prepared each day by first pipetting 20 μl of a 10 ng/ml d-FP solution (200 pg) into a rack of 96 empty polypropylene tubes (National Scientific Supply, Claremont, CA, USA). Then, 20 μl of the appropriate fluprostenol standard stock solutions was pipetted into individual polypropylene tubes containing the d-FP to provide FP masses ranging from 0.7 to 580 pg/tube. Finally, an aliquot (0.1 ml) of blank rat plasma was added to each tube and the contents mixed ($n=5$) providing FP standards covering a concentration range from 7 to 5800 pg/ml (7, 14, 29, 58, 145, 290, 580, 1450, 2900 and 5800 pg/ml). Replicate standards ($n=2$) were prepared for the 7, 14, 2900 and 5800 pg/ml standards on each analysis day.

2.4. Preparation of quality control samples

A 5800 pg/ml QC stock sample was prepared adding a small aliquot of the appropriate FP stock solution, prepared in 0.1% sodium chloride–methanol (50:50, v/v), into a 10-ml volumetric flask and diluting to volume with blank rat plasma. Subsequent QC stock samples were prepared at the 1200, 580, 120, 60 and 30 pg/ml level by serial dilution with blank rat plasma. Stock QC plasma samples were stored at −80°C. On each validation day, working QC samples were prepared in a 96-well plate format using the MicroLab AT. An aliquot (0.1 ml) of each QC stock solution was pipetted into a series of polypropylene tubes already containing 20 μl of 10 ng/ml d-FP solution and mixing the final contents ($n=5$). On each validation day, replicate ($n=10$) working QC samples were prepared at each level.

2.5. Preparation of study samples

Plasma samples obtained from rats dosed with FP were prepared for analysis in a 96-well plate format using the MicroLab instrument. A 20-μl aliquot of a 10 ng/ml d-FP solution was added to the rack of 96 polypropylene tubes. Then, 0.1 ml of the individual rat plasma study samples was added to separate tubes and the contents of the tubes mixed ($n=5$). For samples containing levels of FP that were off the standard curve, a smaller volume of the sample was aliquoted into the polypropylene tube and diluted with blank rat plasma using the MicroLab instrument.

2.6. SPE sample preparation

Standards, QCs and study samples were prepared for analysis using a semi-automated SPE procedure with a TomTec Quadra 96 and a vacuum manifold box. Isolute (Jones Chromatography, Lakewood, CO, USA) ODS cartridges (100 mg, 1 ml) were conditioned with 400 μl of methanol followed by 400 μl of water–methanol–formic acid (97:3:0.1, v/v/v). Then 0.12 ml of the working standards, working QC samples or rat study samples were loaded onto the ODS at a flow-rate of ~1 ml/min. The plate was then washed sequentially with 400 μl of water–methanol–formic acid (97:3:0.1, v/v/v) and 400 μl

of water–methanol (75:25, v/v). The analytes were eluted from the plate using 2×350 μl aliquots of methanol and collected into a 96 well rack of polypropylene tubes contained in an custom-built aluminum block. The elution solvent was removed using a laboratory-built 96-well N_2 drying apparatus. The N_2 pressure was set at 25 p.s.i. and heat (35°C) was applied using Pierce Reacti-Therm III heating unit (1 p.s.i.=6894.76 Pa). The residue was then derivatized as described below.

Typically, a few wells per plate were found to partially plug and drain slowly with the vacuum manifold. In these cases, a laboratory-made syringe device was used to apply positive pressure on the plugged wells to increase the flow of the solutions.

2.7. Derivatization procedure for SPE residue

The SPE residues were first derivatized by adding 100 μl of an acetonitrile–diisopropylethylamine–PFBBr (70:20:10, v/v/v) solution and allowing it to remain in contact with the sample residue for 30 min at ambient temperature. The PFBBr derivatization reagent was removed under nitrogen using the custom-made 96-well drying apparatus. Then 25 μl of MSTFA was added to each tube and allowed to remain for 1 h at ambient temperature. The MSTFA solution was then transferred from the polypropylene tubes into small volume glass autosampler vials via the MicroLab instrument and capped by hand.

2.8. Relative recovery of FP from SPE

The relative recovery of FP from the SPE procedure was evaluated using blank rat plasma spiked with FP at the 290 pg/ml level. Replicate (n=3) aliquots, without the internal standard, were carried through the SPE sample preparation procedure. Before drying, 20 μl of a 10 ng/ml d-FP was added to the SPE eluent. The dried residue was then derivatized as described above.

2.9. Cross-contamination

The potential for cross-contamination of wells during the sample aliquoting and SPE procedures was examined by surrounding blank plasma samples with plasma samples containing a high concentration of fluprostenol (10 μg/ml) in a checkerboard fashion across the plate. The plate was then processed through the SPE, derivatization and analysis steps. The level of fluprostenol found in the blank samples divided by the fluprostenol level in the high concentration samples provided an indication of cross-contamination.

2.10. 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 3-h period. Aliquots (5 ml) of blank rat plasma and blank rat whole blood samples were each spiked to contain FP at the 5 ng/ml level. Aliquots of the spiked plasma and spiked whole blood were removed for processing immediately after spiking and following 3 h at ambient temperature. For the spiked plasma, replicate (n=5) aliquots (0.1 ml) were taken at each time point and added to polypropylene test tubes already containing 20 μl of a 10 ng/ml d-FP solution and mixed by vortexing. For the FP spiked whole blood samples, a 1.5-ml aliquot was removed and centrifuged for 10 min at 2700 rpm and the plasma isolated. Replicate (n=5) aliquots (0.1 ml) of the plasma isolated from the whole blood were added to polypropylene tubes already containing 20 μl of a 10 ng/ml d-FP solution and mixed by vortexing. The samples for plasma and whole blood were prepared for analysis by SPE and derivatized as described above. Stability was determined by comparing the FP concentrations obtained from the 3-h ambient incubation samples with the FP concentration obtained for the time 0 samples.

2.11. Accuracy and precision

QC samples were analyzed on 4 separate days to determine the accuracy and precision of the overall methodology. For each validation day, replicate (n=10) working QC samples were prepared by adding 0.1 ml of each QC level (30, 60, 120, 120, 580, 1200 and 5800 pg/ml FP) sample to test tubes already containing 20 μl of a 10 ng/ml d-FP solution, using the MicroLab AT, mixed and prepared along with the standards, by SPE and derivatization as described above. The derivatized QC samples and standards were analyzed by GC–NCI-MS–MS as detailed below.

2.12. GC–NCI-MS–MS conditions

The GC system was a Hewlett-Packard (Palo Alto, CA, USA) 5890 Series II gas chromatograph equipped with a Leap A200S autosampler (Carrboro, NC, USA). Detection was performed with a Finnigan MAT TSQ-700 (San Jose, CA, USA) triple quadrupole mass spectrometer with a TSQ-7000 source upgrade. Derivatized standards, QCs and unknown samples were analyzed using a 1.0 min splitless injection (2 μl) performed at 300°C into a 4 mm single gooseneck liner lightly packed with deactivated glass wool. The samples were injected onto a Restek (Bellefonte, PA, USA) Rtx-5MS column (30 m×0.25 mm I.D., 0.10 μm film thickness) using helium (AGA Gas, Cincinnati, OH, USA) as the carrier gas. The analytes were eluted using both a thermal and a pressure program. The thermal program consisted of an initial temperature of 225°C for 1 min, followed by a linear thermal ramp at 25°C/min to 325°C with a hold at 325°C for 4 min. The pressure program consisted of an initial head pressure of 207 kPa for 1 min, followed by a 676 kPa/min ramp to 69 kPa and then a final 8 kPa/min ramp to 138 kPa. The GC column was inserted directly into the mass spectrometer source and the transfer line was heated to 275°C.

The mass spectrometer was operated in the negative ion ionization mode using ultrahigh purity methane (AGA Gas) as the chemical ionization gas at a pressure of 9500 mTorr (1 Torr=133.322 Pa). Product ions were produced in the second quadrupole by collisionally activated dissociation (CAD) with 1.2 mTorr argon (AGA Gas) and an applied collision energy of 31 eV. A filament current of 600 μA was used along with a source temperature of 220°C. The selective reaction monitoring (SRM) transitions, m/z 673→m/z 161 and m/z 677→m/z 161, were sequentially monitored for detection of FP and d-FP. Dwell time for each transition was 100 ms.

2.13. 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 ($1/x$) quadratic regression line, using the vendor's LC-Quan software package. Drug concentrations in the QC and unknown samples were then interpolated from the weighted quadratic regression curve.

2.14. Animal study

A male Sprague–Dawley rat, approximately 500 g, was obtained from Harlan Industries (Indianapolis, IN, USA). The animal was 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''. The rat was housed individually in suspended, stainless steel cage and acclimated to standard laboratory conditions of 12 h light/dark cycle for at least 3 days prior to dosing. The rat was allowed free access to food and water throughout the study. It received a subcutaneous dose of 100 μg/kg FP in a 0.03 *M* sodium citrate buffer which was pH adjusted to 6.0 with citric acid. Serial plasma samples (~300 μl) were obtained from the animal over the 8-h period following dosing. Samples were stored frozen at −70°C until the day of analysis.

3. Results

3.1. Negative chemical ionization mass spectra

The NCI mass spectra obtained for FP and d-FP derivatives were dominated by intense ions at m/z 673 and m/z 677, respectively, resulting from the loss of the pentafluorobenzyl group from the $[M-H]^-$ ion of the derivative (data not shown). The product ion MS–MS spectra obtained from CAD of those ions are shown in Fig. 2. Those product ion spectra show a base peak at m/z 161. Therefore, SRM transitions chosen for the analysis were m/z 673→m/z 161 and m/z 677→m/z 161 for FP and d-FP, respectively.

3.2. Chromatographic profiles of blank and FP spiked rat plasma

GC–NCI-MS–MS chromatographic profiles, obtained using the selected SRM schemes, for blank rat

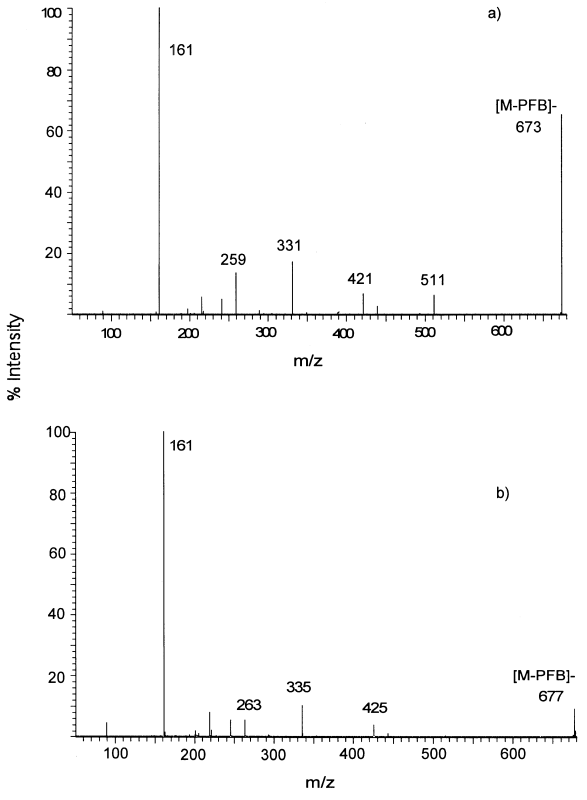

Fig. 2. Negative ion chemical ionization tandem mass spectra of: fluprostenol (top) and 3,3,4,4-$^{2}H_{4}$-fluprostenol (bottom).

plasma and blank rat plasma spiked with 10 pg/ml of FP and 2,000 pg/ml of d-FP are shown in Fig. 3a and b respectively. Typically, FP eluted as a symmetrical peak with a retention time around 5.4 min. Using the chosen SRM schemes, blank rat plasma was free of interferences in the FP and d-FP retention time regions (Fig. 3a).

3.3. Calibration curves

The calibration curve for FP was fitted over three-orders of magnitude using a weighted ($1/x$) quadratic regression, with correlation coefficients for the regressions being 0.996 or greater. Typically, the back-calculated concentrations for the standards were 100±15% of expected across the range. Replicate ($n=4$) injections of the 580 and 5800 pg/ml calibration standards resulted in relative standard deviation (RSD) values for the peak area ratios of 6.8% and 5.6%, respectively.

3.4. Relative recovery of FP from SPE

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 sample preparation in order to obtain a robust and sensitive method. The relative recovery of FP during sample preparation was examined by processing plasma samples ($n=3$) that were spiked with only FP (290 pg/ml), through the SPE elution step. The sample SPE eluent was spiked with d-FP, dried and derivatized. The relative recovery of FP following the SPE, drying and derivatization steps was determined to be 89% with an RSD of 5.6%.

3.5. Cross-contamination

The potential for cross-contamination due to the close proximity of the wells in the 96-well plate format was examined using checkerboard approach. On a single plate, blank wells were surrounded on all sides by high concentrations (10 μg/ml) of fluprostenol plasma samples. The entire plate was then taken through the sample preparation, derivatization and analysis procedures. Cross-contamination of the blank wells were found to be insignificant (0.01% <). To avoid cross-contamination between wells attention needs to be given to the aspirating and dispensing heights to insure optimum performance.

3.6. Stability of FP in whole blood and plasma

The stability of FP in whole blood and plasma was evaluated over a 3-h period using samples spiked with 5000 pg/ml FP. The concentration of FP in the whole blood and plasma at 3 h was compared with the value obtained from the same whole blood and plasma samples with no incubation. The recoveries for FP after 3 h incubation in whole blood and plasma, relative to the zero time point samples, were 105±10.3% and 101±7.9%, respectively. The results indicate that FP is stable in plasma and whole blood over a 3-h period.

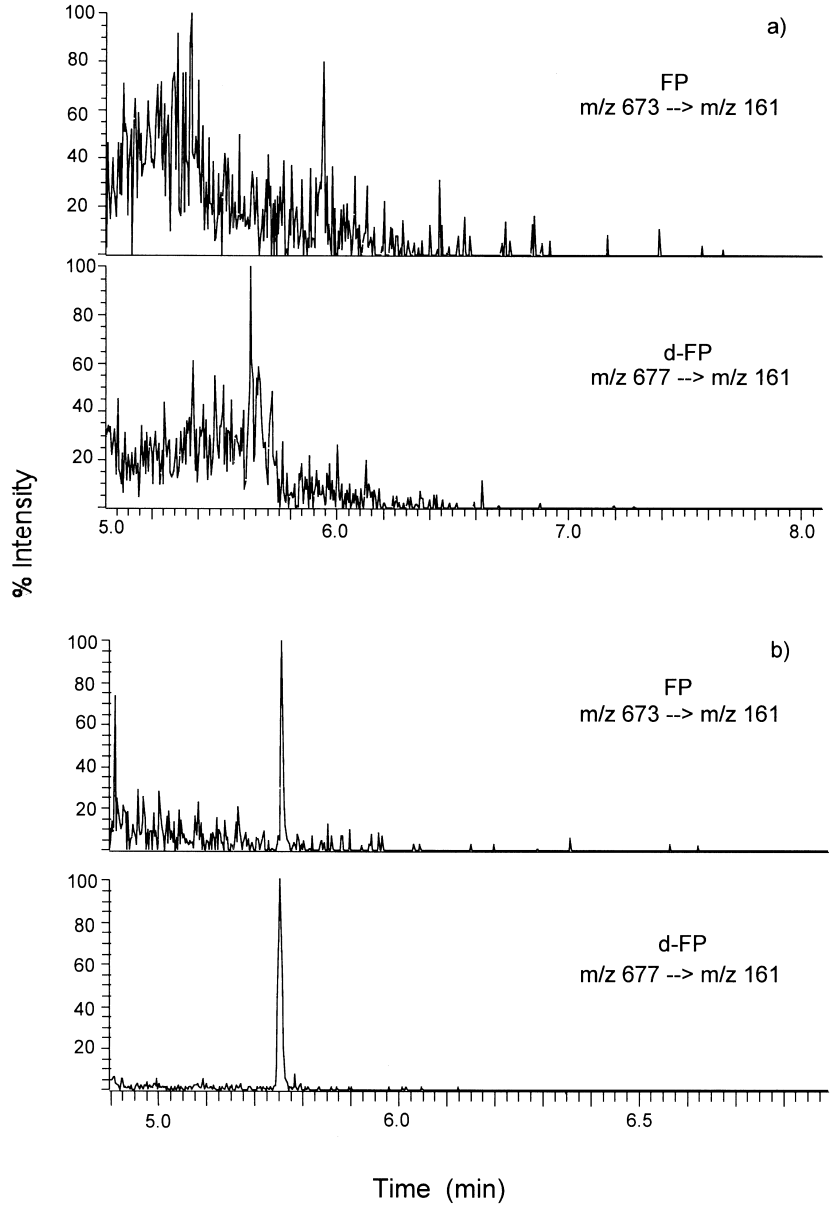

Fig. 3. GC–NCI-MS–MS chromatographic profiles corresponding to fluprostenol (top, SRM scheme: m/z 673/161) and 3,3,4,4-$^{2}H_{4}$-fluprostenol (bottom, SRM scheme: m/z 377/161) for 0.2 ml plasma samples containing: (a) blank and (b) 10 pg/ml fluprostenol and 2000 pg/ml 3,3,4,4-$^{2}H_{4}$-fluprostenol (bottom).

3.7. Accuracy and precision – analysis of QC samples

The accuracy and precision data for the GC–NCI-MS–MS analysis of blank rat plasma spiked with FP at various levels analyzed on 4 separate days are presented in Table 1. The lower limit of quantitation was 30 pg/ml, where the accuracies ranged from 90 to 101% across 4 days, with RSD values between 8.3 and 12.9%. The accuracies from 60 to 5800 pg/ml,

Table 1
Accuracy and precision of FP analysis in rat plasma[a]

Spiked [FP] (pg/ml)	Percent accuracy (% RSD)			
	Day 1	Day 2	Day 3	Day 4
30	98 (11.3)	101 (12.9)	90 (8.3)	96 (9.2)
60	100 (8.1)	105 (9.0)	93 (9.1)	92 (9.2)
120	102 (3.5)	104 (7.6)	98 (7.4)	92 (10.6)
580	100 (3.9)	106 (6.5)	102 (4.6)	94 (3.6)
1200	97 (4.6)	106 (3.8)	102 (4.5)	93 (2.5)
5800	99 (2.6)	95 (2.4)	92 (6.8)	94 (3.0)

[a] $n=10$ for each day at each concentration level.

across the 4 analysis days, ranged from 92 to 106% of target, with RSD values between 2.4 and 10.6%.

3.8. Pharmacokinetic profile of FP following subcutaneous dosing in rats

A plot of measured FP plasma levels vs. post-dose time interval for one rat subcutaneously dosed with 100 μg/kg FP is shown in Fig. 4. The plasma concentrations of FP declined rapidly and quickly fell below the detectable levels suggesting that fluprostenol is rapidly cleared from the body. The peak plasma concentrations were reached fairly rapidly after the subcutaneous administration (T_{max}~ 10 min). Fluprostenol was detected over an 8-h period and had a half-life of 1.4 h and the mean residence time was about 1.8 h.

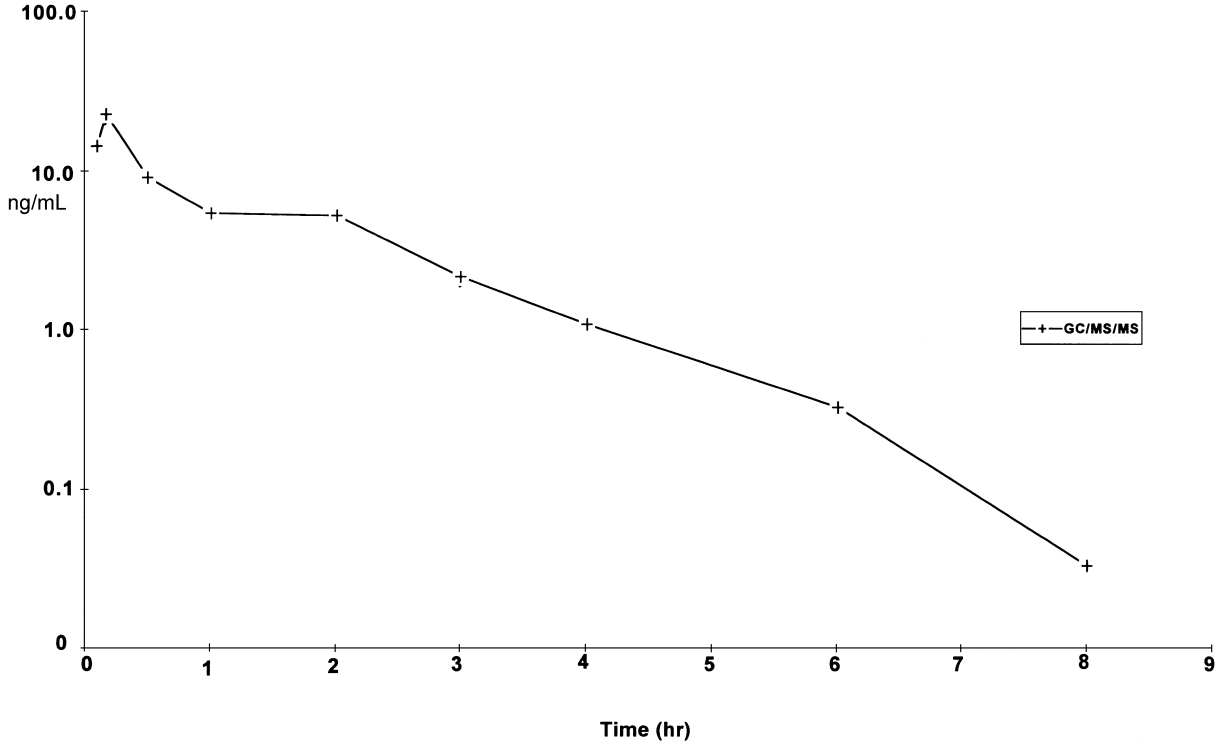

Fig. 4. Profile of fluprostenol plasma concentration versus post-dose sampling time for one rat dosed subcutaneously with 100 μg/kg of fluprostenol.

4. Conclusions

A sensitive and selective semi-automated GC–NCI-MS–MS method was developed for the analysis of fluprostenol in rat plasma. Overall, the methodology was demonstrated to be accurate and precise, with a limit of quantitation for FP of 30 pg/ml using a sample volume of 100 μl. The use of semi-automated methods for liquid handling in a 96-well plate format and the use of 96-well parallel SPE greatly decreased the time required for the sample preparation. Traditional serial processing of 96 samples by SPE can take from 4 to 6 h depending on the degree of serial automation of the SPE procedure. However, parallel SPE processing of the same number of samples in a 96-well plate format took only 0.5 h, resulting in a total reduction of overall sample preparation time by a factor of three.

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