Determination of Flutamide and Hydroxyflutamide in Dog Plasma by a Sensitive High Performance Liquid Chromatography Method Utilizing Mid-Bore Chromatography

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INTRODUCTION

Flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl]propanamide) is a nonsteroidal antiandrogen employed in the management of prostatic carcinoma. Metabolic studies have demonstrated that flutamide is rapidly and extensively metabolized. The major plasma metabolite with pharmacological activity equal to or greater than flutamide is hydroxyflutamide. Variability in the bioavailability of flutamide has been reported (Radwanski et al., 1989); in order to study the influence of several dosage forms on overall absorption, an assay for the determination of flutamide and hydroxyflutamide in plasma was developed.

Several methods for the determination of flutamide and hydroxyflutamide have been previously reported. These methods were characterized by a variety of limiting features such as cumbersome and costly liquid–liquid extractions (Belanger et al., 1988; Schulz et al., 1988; Asade et al., 1991), complex detection equipment employing radioactivity and electron capture detection (Radwanski et al., 1989; Schulz et al., 1988), a large sample volume (2 mL) (Asade et al., 1991) or were not utilized for determination in biological samples (Snykerski, 1989). Accordingly, we have developed a simple and sensitive procedure which can be employed in the analysis of flutamide and hydroxyflutamide.

The method described demonstrates a cost-effective means of determining both components while maintaining excellent sensitivity and resolution. The utilization of mid-bore chromatography increases the method sensitivity while simultaneously conserving on the quantities of mobile phase required for analysis and subsequent disposal of as hazardous waste. A formal extraction process is not required for this process and the accuracy and precision of this method precludes the need for an internal standard. The sensitivity achieved with this method is excellent using ultraviolet (UV) detection and a sample volume of 150 µL of plasma.

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EXPERIMENTAL

Preparation and collection of samples. During a study designed to test the bioavailability of various formulations of flutamide, six male beagle dogs were dosed sequentially with one of three treatments. Treatment A was a re-encapsulated form of flutamide (Eulexin™, Schering-Plough, Bloomfield, NJ, USA) containing 60 mg, treatment B contained 60 mg of flutamide and azone (Laurocapam™, Whitby Research, Inc., Richmond, VA, USA), and treatment C contained 60 mg of flutamide and the patented excipient Gelucire (Gattefosse Corporation, Elmsford, NY, USA). Plasma samples were obtained post-dosing at predetermined time intervals and stored in polypropylene (12 × 75 mm culture tubes at −20 °C. Immediately before analysis, the samples were thawed to 20 °C (room temperature). Samples were mixed by inversion, vortexed for 15 s, and centrifuged for 10 min at 1,200 × g in an IEC-Centra-8 centrifuge.

Extraction procedure for plasma. A 150 µL plasma sample was mixed with 150 µL of acetonitrile in a polypropylene microcentrifuge tube (Fisher Scientific, Pittsburgh, PA, USA), capped and vortexed for 15 s to precipitate proteins effectively. The mixture was then centrifuged at 13,000 × g for 10 min. Subsequently, the clear supernatant was transferred to a WISP micro autosampler glass vial.

Reagents and solutions. Acetonitrile (Burdick and Jackson, Muskegon, MI, USA) and glacial acetic acid (Fisher Scientific, Pittsburgh, PA, USA) were both HPLC grade. Flutamide (C19H17F2N2O4) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and hydroxyflutamide was kindly provided by Whitby Research Inc. (Richmond, VA, USA).

Standard solutions. All solutions of flutamide were prepared from a stock solution containing flutamide 1.0 mg/mL in methanol. All solutions of hydroxyflutamide were prepared from a stock solution containing hydroxyflutamide 1.0 mg/mL in methanol. Stock solutions were stored in amber bottles at 4 °C.

Preparation of calibration standards. Using the standard solutions of flutamide and hydroxyflutamide described earlier, working solutions of 100 and 10 µg/mL were prepared by
serial dilutions with methanol. Appropriate aliquots of working standard solutions of either flutamide or hydroxyflutamide were added to blank dog plasma in order to produce concentrations in the range of 50–800 ng/mL for flutamide and 150–6000 ng/mL for hydroxyflutamide. Working standards and controls were prepared and stored in 1 mL aliquots in polypropylene culture tubes at −20°C.

HPLC equipment. The high-performance liquid chromatograph consisted of a LKB Model 2150 HPLC pump and a LKB Model 2152 liquid chromatographic controller (Gaithersburg, MD, USA) equipped with a Shimadzu SPD-6A UV detector (Tokyo, Japan). The system was linked by a Model 712 Wisp autoinjector (Waters Assoc., Division of Millipore Corp., Milford, MA, USA). Data was collected, processed, and stored to disc utilizing the Nelson 2700 chromatography workstation Turbochrom™ (PE Nelson, Norwalk, CT, USA) on a Leading Edge 386 DX-33 personal computer system.

Chromatographic conditions. HPLC column. Sphere 3 ODS C18, 15 cm length × 3.2 mm internal diameter (Mid-bore™ Phenomenex, Torrance, CA, USA) with 3 μm packing. The guard column was a C18, 3 cm, 40–50 μm pellicular packed column dry packed with 2 μm frits. The system was also fitted with a high pressure column prefILTER employing a 0.5 μm frit (Scientific Systems Inc, State College, PA, USA). Both the guard column and the prefILTER were replaced prior to each analytical run.

Mobile phase. The mobile phase (pH 2.9) was prepared isocratically and consisted of 50% aqueous acetic acid (1% v/v) and 50% acetonitrile. It was prepared daily and degassed by purging with helium. Chromatography was performed at ambient room temperature using a flow rate of 0.5 mL/min which produced a back pressure of 163 bar (2400 p.s.i.).

Injection volume. The injection volume was 50 μL. The WISP needle rinse solution consisted of methanol and deionized water (60:40 v/v).

Ultraviolet detection. A wavelength absorbance of 300 nm was used to measure the intensity of the eluted components. A range setting of 0.001 absorbance units full scale (aUFS) was used with a response time value of 1 s. A backflow restrictor (3.4 bar) is coupled to the detector outlet to prevent outgassing within the detector.

Quantitation. The plasma concentrations of flutamide and hydroxyflutamide were calculated from a calibration curve using the equation of the line \( y = mx + b \) where \( y \) is the peak area, \( m \) is the slope of the line, \( b \) is the y-intercept, and \( x \) is the concentration. The Lotus 1-2-3™ package using normal linear regression and external standardization was utilized for validation and sample calculations.

Limit of detection. The limit of detection for the method was determined by forced integration of the straight baseline section of extracted blank plasma (\( n = 6 \)). The limit of detection for hydroxyflutamide is 5.67 ng/mL and 11.27 ng/mL for flutamide.

Limit of quantitation. The limit of quantitation for the method is 50 and 150 ng/mL for flutamide and hydroxyflutamide, respectively. Sample concentration results less than the low standard are reported as less than the lowest standard (e.g., <50 ng/mL for flutamide).
RESULTS AND DISCUSSION

Chromatography. Chromatograms from blank beagle plasma, a normal beagle plasma sample spiked with a low (50 and 150 ng/mL) and high standard (800 and 6000 ng/mL) of flutamide and hydroxyflutamide, respectively, and a plasma sample from a flutamide-treated beagle are represented in Fig. 1. The chromatograms representing the low and high standards demonstrates good detector response and baseline resolution of the flutamide and hydroxyflutamide without interferences by endogenous substances. The chromatogram representing a typical dog plasma sample (Fig. 1D) represents time point 1.0 h after dosing and demonstrates the rapid metabolism of flutamide to hydroxyflutamide. Under the method conditions described, the retention time of flutamide was 7.4 min and hydroxyflutamide, 4.3 min.

Recovery, selectivity, and stability. Absolute recovery for the method was determined by evaluating extracted plasma standards versus unextracted deionized water standards throughout the concentration range for each analyte. The mean recovery (n = 2) for flutamide and hydroxyflutamide was 99.6% and 99.8%, respectively. Method selectivity was demonstrated by the absence of endogenous interferences at flutamide and hydroxyflutamide retention times from the biological drug-free blank (Fig. 1, blank beagle plasma). A comedication study was not performed since it was not relevant to this study conducted in beagle dogs. Regression analysis of the standard curves over time showed no significant differences in the slope, y-intercept and correlation coefficient thus demonstrating the reproducibility of the method from inter-day analytical runs. Control samples undergoing repetitive freeze–thaw cycles (n = 3) demonstrated no significant degradation to flutamide or hydroxyflutamide. Control samples stored frozen (−20 °C) were evaluated over the time period of

<table>
<thead>
<tr>
<th>Theoretical concentration (ng/mL)</th>
<th>Measured concentration (ng/mL)</th>
<th>Relative standard deviation (%)</th>
<th>Per cent error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flutamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.0</td>
<td>149.4 (n = 21)</td>
<td>5.3</td>
<td>−0.4</td>
</tr>
<tr>
<td>300.0</td>
<td>302.0 (n = 19)</td>
<td>3.1</td>
<td>0.7</td>
</tr>
<tr>
<td>600.0</td>
<td>607.1 (n = 14)</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Hydroxyflutamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200.0</td>
<td>196.8 (n = 21)</td>
<td>6.4</td>
<td>−1.6</td>
</tr>
<tr>
<td>500.0</td>
<td>497.2 (n = 19)</td>
<td>2.9</td>
<td>−0.8</td>
</tr>
<tr>
<td>2000.0</td>
<td>2013.7 (n = 14)</td>
<td>1.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>
2 months demonstrated no significant analyte degradation, thus demonstrating stability of flutamide and hydroxyflutamide for at least 2 months.

**Linearity, accuracy, precision, and sensitivity.** Standard curves were constructed for flutamide and hydroxyflutamide by plotting integrated peak areas versus standard concentrations. For the analysis of plasma, the method demonstrated good linearity over a dynamic range 50–800 ng/mL for flutamide and 150–6000 ng/mL for hydroxyflutamide with mean correlation coefficients \( (n = 8) \) of 0.999365 and 0.999879, respectively.

The accuracy of the method was evaluated by calculating the per cent error of calculated concentrations of either flutamide or hydroxyflutamide spiked control samples versus the theoretical concentrations. The values listed in Table 1 reflect combined intra-day and inter-day statistics. The method is accurate, as indicated by the observation that all the per cent standard errors were less than 1.2 and 1.6% for flutamide and hydroxyflutamide, respectively. The precision of the method was determined by calculating the per cent relative standard deviation at each control concentration of either flutamide or hydroxyflutamide added to blank beagle plasma samples (Table 1). Excellent precision for the method is indicated by relative standard deviations of less than 5.3 and 6.4% for flutamide and hydroxyflutamide, respectively.

**Pharmacokinetics study.** Figures 2 and 3 show the mean plasma concentration versus time data for flutamide and hydroxyflutamide in six beagle dogs for each of the three dosage forms. The pharmacokinetic parameters \( T_{\text{max}}, C_{\text{max}} \), and \( AUC_{\text{last}} \) were calculated for hydroxyflutamide and reported from PCNONLIN and were 3.3 ± 1.0 h, 3.7 ± 0.5 h, 3.5 ± 1.4 h; 5062.9 ± 1604.3 ng/mL, 6099.3 ± 869.3 ng/mL, 4573 ± 1062 ng/mL; and 63.92 ± 25.27 ng-h/L, 80.01 ± 20.82 ng-h/L, 57.98 ± 21.74 ng-h/L for treatments A, B, and C, respectively.

The \( C_{\text{max}} \) and \( AUC_{\text{last}} \) values for treatment B were greater than those resulting from either treatment A or C. None of these values were statistically significant. \( C_{\text{max}} \) for treatment B was 20% greater than treatment A and 33% greater than treatment C. Due to the rapid metabolism of flutamide, most data for flutamide resulted in one or two measurable peaks for most dogs. No attempt was made to calculate the pharmacokinetic data for flutamide.

**CONCLUSION**

We have developed an extremely simple and sensitive procedure which can be employed for the analysis of flutamide and hydroxyflutamide. The method is a cost-effective means of determining both components while maintaining excellent sensitivity and resolution. The utilization of mid-bore chromatography increases the method sensitivity greater than 90%, conserves on the quantities of mobile phase utilized, and requires no changes in conventional chromatography settings (e.g. tubing size, injection volume, etc.) to employ. A formal extraction process is not required for this procedure and the accuracy, precision, and recovery of the method demonstrates that internal standardization is not required. The sensitivity achieved with this method is excellent using UV detection and requires a plasma sample volume of only 150 \( \mu \)L. This method can be modified, if necessary, to be employed for human plasma by using a different mobile phase mixture. The composition of this mixture would be deionized water, methanol and acetonitrile (50:20:30 v/v/v), which would allow for a different column selectivity. All other parameters (e.g. column, sample preparation, etc.) would be identical to the dog plasma method, including analytical run time.

**REFERENCES**