A Rapid and Sensitive HPLC Method for the Determination of 2-hydroxyflutamide in Human Plasma

Hassan Jalalizadeh, Effat Souri, Hamid Reza Javadi Ghanami, Ali Almasirad and Alireza Foroumadi
Department of Medicinal Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran (14155-6451), Iran

Abstract: In this study 2-hydroxyflutamide extracted from human plasma with dichloromethane and determined by HPLC using UV detection. The chromatographic system consisted of Nova-Pak CN cartridge, an isocratic mobile phase of acetonitrile-tetrahydrofuran-water (31.5: 2.5: 66, v/v/v) and UV detection at 300 nm. 2-Hydroxyflutamide and internal standard were eluted at about 4.2 and 5.8 min, respectively. The method was linear over the range of 10-1000 ng mL−1 of 2-hydroxyflutamide in plasma (r²=0.9998). The within-day and between-day precision values were in the range of 1.96-6.06%. The limit of quantification of the method was 10 ng mL−1. The method was successfully applied for the study of the pharmacokinetics of flutamide in healthy volunteers.

Key words: Flutamide, 2-hydroxyflutamide, reversed-phase chromatography, antiandrogen

INTRODUCTION

The non-steroidal pure antiandrogen, flutamide (3'- trifluoromethyl-4'-nitro-2-methyl-propionylamidine), is clinically used for the treatment of prostatic cancer (McLeod, 1993; Mcleod et al., 1993; Baltogiannis et al., 2004). Flutamide is rapidly metabolized to 2-hydroxyflutamide by first-pass metabolism in the liver (Schulz et al., 1988). It has been suggested that the biological activity of flutamide is associated with its metabolite, 2-hydroxyflutamide (Katchen and Buxbaum, 1975). Higher concentration of 2-hydroxyflutamide than those of parent drug is observed after oral dose of flutamide (Schulz et al., 1988; Radwanski et al., 1989). Low and highly variable plasma concentrations of flutamide, resulted in monitoring of 2-hydroxyflutamide levels in human plasma to study the relative bioavailability of flutamide in various drug formulations.

A number of different methods including gas chromatography with electron-capture detection (Schulz et al., 1988; Radwanski et al., 1989) or high performance liquid chromatography with UV detection (Asade et al., 1991; Farthing et al., 1994; Leibinger and Kapas, 1996; Niopas and Dafnisos, 2001) have been published for determination of 2-hydroxyflutamide. However, two of the reported HPLC methods need extracting steps that are time-consuming (Asade et al., 1991) or expensive (Leibinger and Kapas, 1996). The other study used mid-bore chromatography with a detection limit of 150 ng mL−1 in 150 μL of dog plasma (Farthing et al., 1994). Also another HPLC method with UV detection was reported for determination of 2-hydroxyflutamide in human plasma (Niopas and Dafnisos, 2001).

Having a sensitive, simple and reliable technique is crucial for determination of drugs in biological fluids for pharmacokinetic investigations. Therefore, the objective of the present study was to develop a simple preparation method and a relatively short chromatographic procedure suitable for determination of multiple samples in a limited amount of time for pharmacokinetic studies. This method has been applied to pharmacokinetic studies of flutamide.

MATERIALS AND METHODS

Chemicals: Flutamide was from Haumann Pharma GmbH, Germany and obtained from Mehr-Daru Pharmaceutical Company, Tehran, Iran. Internal standard and 2-hydroxyflutamide (Fig. 1) were synthesized according to a previously described method (Baker et al., 1967) and the purity of the compounds checked by mp, mass and NMR. Acetonitrile and tetrahydrofuran were HPLC grade and purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical grade and used without any further purification.
layer was discarded and the organic layer transferred to a clean test tube and 500 mg anhydrous Na₂SO₄ added. The mixture vortexed for 10 sec and centrifuged at 1500 g for 5 min. The organic layer transferred to a clean test tube and evaporated to dryness at 40°C in water bath under a stream of nitrogen. The residue was reconstituted in 100 µL of mobile phase and 50 µL were injected into the chromatographic system.

**Validation:** Six series of standard calibration solutions were prepared by spiking 50 µL of 2-hydroxyflutamide standard solutions and 50 µL of IS in 1 mL of blank human plasma to give final concentrations over the range of 10-10000 ng mL⁻¹.

The sample preparation and HPLC analysis was performed as described above. Calibration curves were constructed by plotting the measured peak area ratios of 2-hydroxyflutamide to the IS versus concentrations of standard samples and statistical analysis was performed.

To establish the within-day and between-day accuracy and precision of the method, three replicate of standard plasma solutions at three different concentrations (10, 100 and 1000 ng mL⁻¹) were assayed on one day and three separate days.

**Extraction yield:** Aliquots of 50 µL of 2-hydroxyflutamide standard solutions (0.2, 10 and 20 µg mL⁻¹) and 50 µg of IS solution were added to two sets of three test tubes. To one set, 1 mL plasma was added and extracted according to the sample preparation method. The other set was adjusted to the same volume by mobile phase. Fifty microliter of each solution was injected into the HPLC system. The peak area ratios of the extracted samples and unextracted samples were compared. The experiment was repeated on three consecutive days.

**Sample collection:** To test the applicability of the analytical method to pharmacokinetic studies, the plasma concentration of 2-hydroxyflutamide was measured in human plasma after administration of a single oral dose of 250 mg Flutamide 250 mg (Orion Pharma, UK, Bach No: ZAF27A). This study was performed in Faculty of Pharmacy, Tehran University of Medical Sciences. Twelve healthy adult male volunteers (age: 29±5.3 years, height: 173±7.9 cm, body mass: 75.3±6.3 kg) selected and participated in the study based on acceptable physical examination, medical history and clinical laboratory test results. After an overnight fast, each subject received a single 250 mg oral dose of flutamide. All subjects gave written consent to their participation after having been informed verbally by the medical supervisor about the experimental procedures. Blood samples (5 mL) were

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**Fig. 1.** Chemical structure of 2-hydroxyflutamide and internal standard

**Instrumentation:** The HPLC system consisted of a 600 pump, 710 plus Autosampler and a variable 480 UV detector all from Waters (Milford, MA, USA). The data processing system was a multi-channel Chrom and Spec software for chromatography, version 1.5 x.

**Chromatographic conditions:** Separation was achieved using a Nova-pack® CN HP 4 µm cartridge (4.6×250 mm, Waters, Milford, MA, USA). The isocratic mobile phase pumped at a flow-rate of 1 mL/min consisted of acetonitrile-tetrahydrofuran-water (31.5: 2.5: 66, v/v/v) prepared daily and degassed by passing through a 0.45 µm filter. All separations were performed at room temperature. Detection was performed at 300 nm.

**Solutions:** Stock standard solution of 2-hydroxyflutamide was prepared by dissolving appropriate amount of the compound in methanol to give a final concentration of 200 µg mL⁻¹. Standard solutions of 2-hydroxyflutamide (0.2, 0.4, 1, 2, 5, 10 and 20 µg mL⁻¹) was prepared by subsequent dilution. A solution of IS was prepared by dissolving IS in methanol to a final concentration of 5 µg mL⁻¹. All these solutions were stored at 4°C.

Fifty microliter of these standard solutions were used to spike 1 mL plasma samples for calibration curves to reach to concentrations of 10, 20, 50, 100, 250, 500 and 1000 ng mL⁻¹ 2-hydroxyflutamide.

**Sample preparation:** To one mL of plasma sample, in a test tube, 50 µL of standard solution of 2-hydroxyflutamide and 50 µL of IS (5 µg mL⁻¹) were added and vortex-mixed for 5 sec. Then, 50 µL of Na₂CO₃, 1M and 4 mL of dichloromethane were added. The test tubes were vortex-mixed for 1 min and centrifuged at 1500 g for 10 min. Upper
drawn into heparinized test tubes immediately before (0) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 24, and 36 h following drug administration. Blood samples were centrifuged at 2000 g for 10 min and plasma samples were separated and stored at -20°C until analysis. Before analysis, the plasma samples were thawed at 18°C.

**RESULTS**

**Chromatographic condition:** Optimization was achieved by monitoring varying reversed-phase columns, mobile systems, flow-rate and wavelength. Using CN column instead of C18 columns created sharper peak which resulted in an increased sensitivity of the method. Typical chromatograms obtained from blank and plasma samples spiked with 2-hydroxyflutamide and a plasma sample obtained from a volunteer 6 h after a single oral dose of 250 mg flutamide are presented in Fig. 2. Under the chromatographic conditions described, 2-hydroxyflutamide and IS were well resolved in plasma samples and eluted at 4.2 and 5.8 min, respectively. No interfering peaks of endogenous plasma components were found at the retention time of 2-hydroxyflutamide or internal standard in blank plasma.

**Linearity:** Calibration curves were constructed using six series of 2-hydroxyflutamide solutions in the range of 10-1000 ng mL⁻¹ (Table 1). The linearity of the calibration curve is validated by the high value of the correlation coefficient.

**Accuracy and Precision:** The accuracy and precision were determined by assaying three samples of 2-hydroxyflutamide at 10, 100 and 1000 ng mL⁻¹ in plasma.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>2-hydroxyflutamide</th>
</tr>
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<tbody>
<tr>
<td>Linearity</td>
<td>10-1000 ng mL⁻¹</td>
</tr>
<tr>
<td>Regression equation</td>
<td>( Y = 0.0055 X - 0.0086 )</td>
</tr>
<tr>
<td>SD of slope (%)</td>
<td>1.03 x 10⁻⁴</td>
</tr>
<tr>
<td>RSD of slope (%)</td>
<td>1.87</td>
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<tr>
<td>SD of intercept</td>
<td>0.0076</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9998</td>
</tr>
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</table>

Table 1: Statistical data of calibration curves of 2-hydroxyflutamide in spiked plasma

<table>
<thead>
<tr>
<th>Concentration added (ng mL⁻¹)</th>
<th>Concentration found (mean±SD) (ng mL⁻¹)</th>
<th>CV (%)</th>
<th>Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-day (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10.06±0.61</td>
<td>6.06</td>
<td>+0.60</td>
</tr>
<tr>
<td>100</td>
<td>99.46±3.70</td>
<td>3.72</td>
<td>-0.54</td>
</tr>
<tr>
<td>1000</td>
<td>1008.25±24.25</td>
<td>2.41</td>
<td>+0.83</td>
</tr>
<tr>
<td>Between-day (n = 9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10.31±0.61</td>
<td>5.92</td>
<td>+3.10</td>
</tr>
<tr>
<td>100</td>
<td>98.60±3.21</td>
<td>3.22</td>
<td>-0.40</td>
</tr>
<tr>
<td>1000</td>
<td>1014.16±19.92</td>
<td>1.96</td>
<td>+1.42</td>
</tr>
</tbody>
</table>

Table 2: Precision and accuracy of method for determination of 2-hydroxyflutamide in spiked plasma (n = 9; three sets for 3 days)

Fig. 2: HPLC chromatogram of 2-hydroxyflutamide and IS. (a) drug free plasma; (b) plasma sample spiked with 2-hydroxyflutamide and IS; (c) human plasma sample, 6 h after oral administration of 250 mg flutamide. Peaks: 1, 2-hydroxyflutamide; 2, IS.

on three separate days. Concentrations were determined using calibration standard curve prepared for 2-hydroxyflutamide in the range of 10-1000 ng mL⁻¹ for each day (Table 2). Good accuracy and precision were observed over the entire concentration range. The within-day and between-day variability showed CV values less than 6.06% in all three selected concentrations.

**Sensitivity:** The limit of quantification with CV<6.06% was found to be 10 ng mL⁻¹ for 2-hydroxyflutamide. The limit of detection that can be reliably detected with a S/N ratio of 3 was found to be 3 ng mL⁻¹.
Fig. 3: Mean±SD of plasma concentration-time curve of 2-hydroxyflutamide following a single oral dose of 250 mg flutamide to 12 healthy volunteers

**Extraction yield:** The recovery of 2-hydroxyflutamide and internal standard was determined. The mean recoveries for 2-hydroxyflutamide at concentrations of 10, 500 and 1000 ng mL\(^{-1}\) were 74.20, 78.35 and 81.35%, respectively. Mean recoveries of internal standard was 85.2%.

**Application to pharmacokinetic study:** The plasma concentration-time profile of 2-hydroxyflutamide after a single oral administration of 250 mg flutamide to 12 volunteers was determined (Fig. 3). The following pharmacokinetic parameters (mean±SD) were provided for 2-hydroxyflutamide: \(C_{\text{max}} = 734.75±88.14 \text{ ng mL}^{-1}\), \(AUC_{0-\infty} = 4357.41±870.74 \text{ ng h mL}^{-1}\), \(AUC_{0-24} = 4079.04±814.59 \text{ ng h mL}^{-1}\), \(T_{\text{max}} = 2.42±0.29 \text{ h}\). Elimination half-life = 9.39±0.63 h. The observed values of pharmacokinetic parameters were comparable to those reported in previous studies (Doser et al., 1997).

**DISCUSSION**

A few HPLC methods were reported for the determination of 2-hydroxyflutamide. The method reported by Asade et al. (1991) involved a time consuming liquid-liquid extraction method and need a large sample volume (2 mL). Farthing et al. introduced a method using mid-bore chromatography with UV detection for analysis of 2-hydroxyflutamide in the range of 150-6000 ng mL\(^{-1}\) in 150 μL of dog plasma. A sensitive HPLC method with a quantitation limit of 10 ng mL\(^{-1}\) was reported by Leibinger and Kapas (1996) using solid-phase extraction for sample preparation. Another HPLC method with UV detection was reported by Niopas and Daftsis with a detection limit of 25 ng mL\(^{-1}\) in 0.5 mL of human plasma. The run time of the last method is about 8 min. The main features of our method were an improved sensitivity to 10 ng mL\(^{-1}\) with a CV of about 6%, relatively simple pretreatment method with no interfering peaks and shorter run time (about 6 min) which makes this method suitable for routine monitoring of 2-hydroxyflutamide in human plasma. The sensitivity of this assay method is sufficient for determination of 2-hydroxyflutamide 36 h after administration of a single oral dose of 250 mg flutamide to human.

**CONCLUSIONS**

The present method is comparatively rapid, simple, reliable and sensitive, allowing the processing of multiple determinations in a short time. Using UV detection with a low limit of quantification in low volume of samples makes this method very suitable for pharmacokinetic studies.

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**REFERENCES**


