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Short communication

Determination of 2-hydroxyflutamide in human plasma by high-performance liquid chromatography and its application to pharmacokinetic studies

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Abstract

Flutamide is a potent antiandrogen used for the treatment of prostatic cancer. Flutamide undergoes extensive first-pass metabolism to the pharmacologically active metabolite 2-hydroxyflutamide. A simple, sensitive, precise, accurate and specific HPLC method, using carbamazepine as the internal standard, for the determination of 2-hydroxyflutamide in human plasma was developed and validated. After addition of the internal standard, the analytes were isolated from human plasma by liquid–liquid extraction. The method was linear in the 25 to 1000 ng/ml concentration range ($r>0.999$). Recovery for 2-hydroxyflutamide was greater than 91.4% and for internal standard was 93.6%. The limit of quantitation was 25 ng/ml. Inter-batch precision, expressed as the relative standard deviation (RSD), ranged from 4.3 to 7.9%, and accuracy was better than 93.9%. Analysis of 2-hydroxyflutamide concentrations in plasma samples from 16 healthy volunteers following oral administration of 250 mg of flutamide provided the following pharmacokinetic data (mean±SD): C_{max}, 776±400 ng/ml; $AUC_{0-\infty}$, 5368±2689 ng h/ml; AUC_{0-t}, 5005±2605 ng h/ml; T_{max}, 2.6±1.6 h; elimination half-life, 5.2±2.0 h. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Flutamide, 2-methyl-*N*-[4-nitro-3(trifluoromethyl)-phenyl] propanamide, is a potent nonsteroidal pure androgen receptor antagonist used clinically for the management of metastatic carcinoma of the prostate [1–4]. Flutamide is rapidly metabolized by extensive first-pass metabolism to the pharmacologically active metabolite 2-hydroxyflutamide. After a single 250 mg oral dose of flutamide the concentrations of the 2-hydroxylated metabolite were much higher than those of the parent drug flutamide [5–7]. Due to the low and highly variable plasma concentrations of flutamide, which may result in considerable interindividual pharmacokinetic variability, plasma concentrations of the pharmacologically active metabolite 2-hydroxyflutamide are used in pharmacokinetic studies of flutamide.

A number of different methods have been reported for the determination of 2-hydroxyflutamide in bio-

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logical fluids, including gas chromatography with electron-capture detection or high-performance liquid chromatography (HPLC) with UV detection [6–10]. However, some of these methods are poorly reproducible or are expensive or lack selectivity and are not directly applicable to the quantitation of hydroxyflutamide in human plasma.

Therefore, the purpose of this investigation was to develop and validate a method that could be used for the determination of 2-hydroxyflutamide in real human plasma samples. In the present paper a simple, rapid, sensitive, precise, accurate and specific HPLC assay is described that has been applied to pharmacokinetic studies which required high sensitivity and selectivity.

2. Experimental

2.1. Chemicals and reagents

2-Hydroxyflutamide was supplied by Mikromol (Teltow, Germany) and the internal standard carbamazepine was obtained from Sigma (St. Louis, MO, USA). Acetonitrile and methanol, both of HPLC grade were purchased from J.T. Baker (Deventer, The Netherlands). Water was Milli-Q grade and all other chemicals and solvents used were of analytical grade.

2.2. Instrumentation

Determinations were performed using a HPLC system consisting of a Shimadzu LC-600 HPLC pump (Columbia, MD, USA), a Rheodyne 7125 injector valve with a 100-μl fixed loop (Cotati, CA, USA), an ISCO V-4 variable-wavelength UV–Vis detector (Lincoln, NE, USA), a Hewlett-Packard HP3396A integrator (Avondale, PA, USA) and a Hitachi 655A-40 autosampler (Tokyo, Japan).

2.3. Chromatographic conditions

Chromatography was conducted using a mobile phase of methanol–acetonitrile–Milli-Q grade-water (45:30:25, v/v) pumped at a flow-rate of 1 ml/min through a Brownlee Spheri-5 reversed-phase C_{18} analytical column (220×4.6 mm I.D.), particle size 5 μm (Alltech, Deerfield, IL, USA). The injection volume was 100 μl and peaks were detected at 300 nm. The integrator attenuation was 8 and the chart speed was 0.2 cm/min. The total run time for an assay was approximately 8 min.

2.4. Standard stock solutions

The standard stock solutions of 2-hydroxyflutamide and the internal standard carbamazepine were prepared daily by dissolving appropriate amounts of the compounds in acetonitrile–water (1:1) to give final concentrations of 20 μg/ml and 40 μg/ml for each compound, respectively. The stock solution of 2-hydroxyflutamide was then successively diluted with acetonitrile–water (1:1) to achieve concentrations of 0.5, 1, 2, 5, 10 and 20 μg/ml. The stock solution of the internal standard was diluted with acetonitrile–water (1:1) to prepare the working internal standard solution containing 10 μg/ml carbamazepine. Volumes (25 μl) of these standard solutions were used to spike 0.5-ml plasma samples for calibration curves containing 25, 50, 100, 250, 500, and 1000 ng/ml 2-hydroxyflutamide.

2.5. Quality control samples

Volumes of 50 ml of human plasma were spiked with 25, 62.5 and 125 μl of 200 μg/ml 2-hydroxyflutamide solution in acetonitrile–water (1:1) to obtain quality control samples containing 100, 250, and 500 ng/ml 2-hydroxyflutamide, respectively. These samples were divided into aliquots of about 3 ml into one-dram vials capped tightly, and placed at −20°C pending analysis. These samples were used in the analysis of plasma samples as quality controls for the purpose of checking recovery of analyte in the daily analyses of plasma samples.

2.6. Extraction from plasma

To 0.5 ml plasma sample in a 100×13 mm glass test tube, 25 μl of calibration standard and 25 μl internal standard were added. To control blanks and to quality control standards 50 and 25 μl of acetonitrile–water (1:1) were added, respectively. Following addition of 250 μl of 0.1 *M* sodium carbonate and 2 ml ethyl acetate the samples were shaken for 10 min.

They were then centrifuged at 2000 *g* for 10 min. The organic phase was transferred to a clean 100×13 mm test tube and evaporated to dryness at 45°C with the aid of a gentle stream of air. The residue was dissolved in 1 ml of mobile phase, transferred to an autosampler vial for analysis and a 100 μl volume was injected into the chromatographic system for quantitation.

2.7. Assay validation

Linearity of the assay was demonstrated over the concentration range of 25 to 1000 ng/ml 2-hydroxyflutamide by assaying plasma standards in triplicate at six separate concentrations on three separate occasions. Data were obtained through linear regression analysis of peak height ratios versus concentrations of added 2-hydroxyflutamide. A weighting factor of 1/concentration was employed.

Assay precision and accuracy were determined in conjunction with the linearity studies by assaying on three separate occasions using three quality control samples at each of three concentrations (100, 250 and 500 ng/ml). Concentrations of 2-hydroxyflutamide in quality control samples were determined by application of the appropriate standard curve obtained on that occasion.

Recovery of 2-hydroxyflutamide and internal standard was assessed by direct comparison of peak heights from extracted versus non-extracted samples by using six replicate plasma samples at each of three 2-hydroxyflutamide concentrations 100, 250, and 500 ng/ml plus the appropriate amount of internal standard. Individual specificity in relation to endogenous plasma components was demonstrated by analysis of a series of randomly selected drug-free samples ($n=10$).

2.8. Pharmacokinetic study

The HPLC method developed was used to investigate the plasma profile of 2-hydroxyflutamide, a major pharmacologically active metabolite of flutamide, after a single oral dose of flutamide (Flucinom; Schering-Plough, 250 mg tablets). Sixteen healthy adult volunteers (six females, 10 males) age: 25.6±2.0 years, body mass: 72.4±9.5 kg, height: 172.8±7.2 cm, participated in the study. Following written informed consent, each subject received a single 250 mg oral dose of flutamide under fasting conditions. Blood samples (5 ml) were drawn into heparinized test tubes immediately before (0) and at 20, 35, 50, 65, 80, 100 min and 2, 2.5, 3, 4, 5, 6, 7, 8, 10, 12, 16, 24, and 36 h following drug administration. Blood samples were immediately placed on ice after collection and centrifuged at 3000 *g* for 10 min at 4°C, and the plasma fraction was separated and stored in polypropylene tubes at −20°C until analysis. Before analysis, the plasma samples were thawed at 18°C.

3. Results and discussion

3.1. Specificity

Typical chromatograms obtained from extracts of a drug-free plasma and a plasma sample obtained from a volunteer 12 h after a single oral dose of 250 mg flutamide containing 248 ng/ml of 2-hydroxyflutamide are presented in Fig. 1. No endogenous

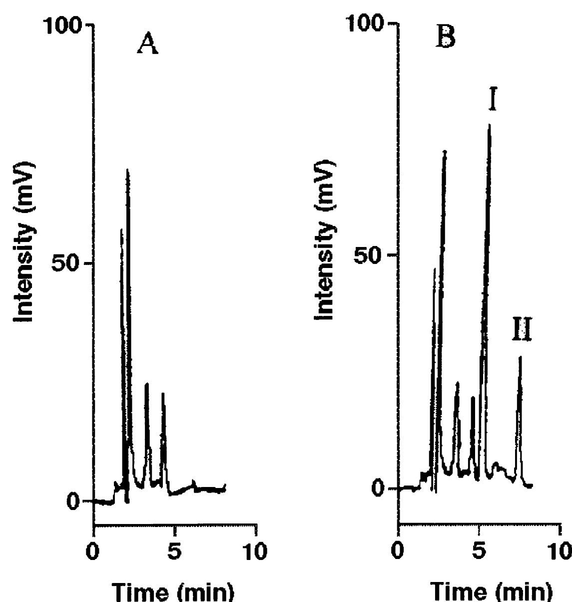

Fig. 1. Examples of chromatograms: (A) extract of 0.5 ml drug-free plasma; (B) plasma sample obtained from a volunteer 12 h after a single oral dose of 250 mg flutamide containing 248 ng/ml of 2-hydroxyflutamide. Peaks: I=carbamazepine (internal standard); II=2-hydroxyflutamide.

plasma components elute at the retention time of 2-hydroxyflutamide or internal standard. Internal standard and 2-hydroxyflutamide were eluted in 5.2 and 7.6 min, respectively.

3.2. Linearity

The linear regression analysis of 2-hydroxyflutamide was constructed by plotting the peak height ratio of 2-hydroxyflutamide to the internal standard (y) versus analyte concentration (ng/ml) in spiked plasma samples (x). The calibration curves were linear for concentrations ranging from 25 to 1000 ng/ml. A typical calibration curve had the regression equation of $y=0.01576+0.15307x$ with a correlation coefficient (r) of 0.999. Calibration curves were established on each day of analysis.

3.3. Accuracy and precision

Assay precision and accuracy were assessed by assaying three quality control samples in triplicate on three separate occasions. The following validation criteria for accuracy and precision were used to assess the suitability of the method: accuracy should be within 85 to 115% except at the limit of quantitation where it should be within 80 to 120%; RSD should not exceed 15% except at the limit of quantitation where it should not exceed 20% [11]. Assay precision for 2-hydroxyflutamide was 7.9% based on RSD values of 7.9, 4.3 and 5.5% for samples containing 100, 250 and 500 ng/ml, respectively. Assay accuracy, assessed by calculating the estimated concentrations as a percent of the nominal concentrations, was better than 93.9% (Table 1).

3.4. Limit of quantitation

The limit of quantitation, defined as the lowest concentration on the calibration curve at which both accuracy and precision should be within 20%, was deemed to be 25 ng/ml, whose precision and accuracy were well within the proposed criteria (Table 1).

3.5. Recovery

The recovery of 2-hydroxyflutamide and internal standard was determined by direct comparison of absolute peak heights from plasma samples and those found by direct injection of standards of the same concentration prepared in acetonitrile–water (1:1). The mean recoveries for 2-hydroxyflutamide were 91.5±4.4, 93.7±2.5 and 91.4±2.0% at the 100, 250 and 500 ng/ml concentrations, respectively ($n=6$). Mean recovery of internal standard was 93.6±2.0% ($n=18$).

3.6. Application to pharmacokinetic study

The present method was used to determine the plasma concentrations of 2-hydroxyflutamide. Fig. 2 shows the mean±standard error of the mean (SEM) plasma concentration–time profile of 2-hydroxyflutamide. Pharmacokinetic analysis was performed using standard noncompartmental methods [12]. Analysis of 2-hydroxyflutamide concentrations in plasma samples from 16 healthy volunteers following oral administration of 250 mg of flutamide provided the following pharmacokinetic parameters (mean±SD): C_{max}, 776±400 ng/ml; area under the curve (AUC) $AUC_{0-\infty}$, 5368±2689 ng h/ml; AUC_{0-t}, 5005±2605 ng h/ml; T_{max}, 2.6±1.6 h; elimination half-life, 5.2±2.0 h (Table 2). The

Table 1
Inter-batch accuracy and precision for 2-hydroxyflutamide in quality control samples in human plasma

Nominal concentration (ng/ml)	Mean found concentration (n=9, ng/ml)	Accuracy[a] (%)	Precision[b] (RSD, %)
100	100.3	100.3	7.9
250	234.8	93.9	4.3
500	489.8	98.0	5.5

[a] Accuracy: found concentration expressed in % of the nominal concentration.
[b] RSD, Relative standard deviation.

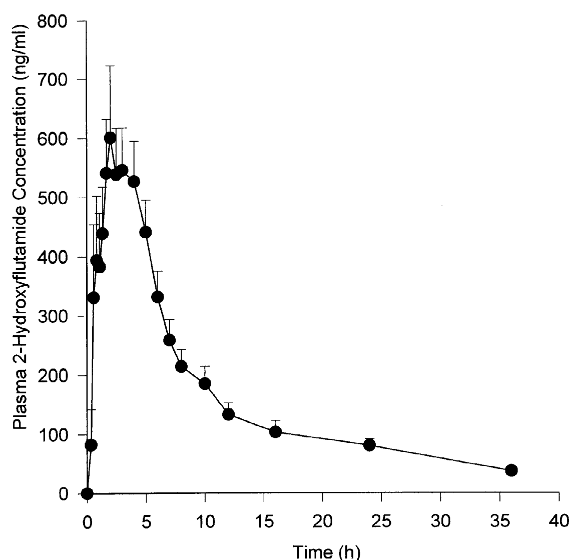

Fig. 2. Mean±SEM of plasma 2-hydroxyflutamide concentration–time curve following a single oral dose of 250 mg flutamide to 16 volunteers.

observed values of the pharmacokinetic parameters were comparable to those reported for 2-hydroxyflutamide in previous studies [5].

Table 2
Pharmacokinetic parameters for 2-hydroxyflutamide after a single oral administration of 250 mg flutamide to 16 volunteers

Pharmacokinetic parameter	Mean±SD	Range
C_{max} (ng/ml)	776±400	333–1874
$AUC_{0-\infty}$ (ng h/ml)	5368±2689	1768–9855
AUC_{0-t} (ng h/ml)	5005±2605	1594–9329
T_{max} (h)	2.6±1.6	0.8–6
$t_{1/2}$ (h)	5.2±2.0	2.6–8.5

4. Conclusion

This paper describes a simple, rapid, sensitive, specific, accurate and precise procedure for the determination of 2-hydroxyflutamide, suitable for the analysis of large numbers of human plasma samples. The assay was validated to meet the requirements of pharmacokinetic or bioequivalence studies.

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