ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 1377–1381

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Short communication

New and validated high-performance liquid chromatographic method for determination of hydroxyflutamide in human plasma[1]

J. Leibinger, M. Kapás

Chemical Works of Gedeon Richter Ltd., Gyömrői út 19–21, 1103 Budapest X, Hungary

Received for review 13 September 1995

Keywords: High-performance liquid chromatography; Human plasma; Hydroxyflutamide; Solid-phase

1. Introduction

Flutamide (3-trifluoromethyl-4-nitro-2-methyl propionylaniline) is a non-steroid pure anti-androgen, inhibiting the uptake and/or binding of dihydrotestosterone to target receptors [1]. It is effective in the treatment of patients with prostatic cancer. The major plasma metabolite of flutamide is 2-hydroxyflutamide with pharmacological activity equal to/or greater than that of flutamide. Owing to extensive first-pass metabolism, the C_{max} of flutamide after a single 250 mg oral dose was 10–20 ng ml^{-1} while that of hydroxyflutamide was 1200–1300 ng ml^{-1}. The elimination half-life for flutamide was highly variable [2]. These data prompted the development of methods for monitoring hydroxyflutamide levels in human plasma to study the relative bioavailability of flutamide in various drug formulations. Gas chromatographic (with electron-capture detection) and HPLC (with UV detection) methods have been published for this purpose [2–4]. However, either these were not validated or the chromatographic resolution was poor or the wavelength of detection was not suitable or there was no internal standard. The method described here is quick and sensitive and has been validated for the concentration range of 10–1500 ng ml^{-1}.

2. Experimental

2.1. Materials

2-Hydroxy-2-methyl-*N*-[4-nitro-3-(trifluoromethyl)phenyl]propanamide (2-hydroxyflutamide) and 2-hydroxy-*N*-[4-nitro-3-(trifluoromethyl)-phenyl]-propanamide (internal standard) were manufactured by Chemical Works of Gedeon Richter (Budapest, Hungary). Methanol, 2-propanol, *n*-hexane, 1-chlorobutane and acetoni-

[1]Presented at the Fifth International Symposium on Drug Analysis, September 1995, Leuven, Belgium.

SSDI 0731-7085(95)01698-8

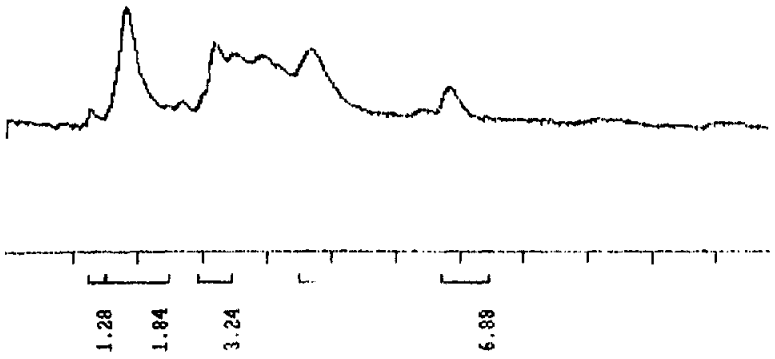

Fig. 1. Chromatogram of human blank plasma extract.

trile (all of gradient-grade quality), ethyl acetate (for residue analysis), buffer solution (pH 9.00) and columns for solid-phase extraction containing RP-18 (500 mg) were all manufactured by Merck (Darmstadt, Germany). Human ACD plasma was purchased from the National Haematological Institute (Budapest, Hungary).

2.2. Chromatographic conditions

The Gilson HPLC system consisted of a solvent delivery pump (Model 303), an autoinjector (Model 231), a UV detector (Model 116) and a Nucleosil 120-5 C18 column (250 × 2 mm i.d.) (Macherey–Nagel, Düren, Germany). The HPLC system was controlled by GME 714 software. A 20 μl sample was injected and the solute was detected at 300 nm. The eluent was acetonitrile–methanol–water (30:25:45, v/v/v). The flow rate was 0.25 ml min^{-1}.

2.3. Standard solutions

A stock standard solution was prepared in methanol and contained 1.00 mg ml^{-1} of hydroxyflutamide. Working standard solutions were prepared by dilution of the stock standard solution with methanol to concentrations of 2, 5, and 50 μg ml^{-1}.

An internal standard stock standard solution was prepared in methanol and contained 1.00 mg ml^{-1}. A working standard solution was made by dilution of the stock standard solution with methanol to 10 μg ml^{-1}.

2.4. Calibration standard samples

Calibration standard samples were freshly prepared in human ACD plasma by adding to 1.00 ml of plasma appropriate aliquots of working standard solutions to yield concentrations of 10, 20, 50, 100, 500 and 1500 ng ml^{-1}.

2.5. Quality control (QC) samples

Volumes of 50 ml of human ACD plasma were spiked with 2-hydroxyflutamide at concentrations of 10, 20, 50, 100, 500 and 1000 ng ml^{-1}. The spiked plasma was divided into 1 ml portions and was kept at −20°C until analysis.

2.6. Sample clean-up

A volume of 10 μl of the internal standard working solution (100 ng) was added to each sample (1 ml). For the sample clean-up a new solid-phase extraction (SPE) method was developed. A vacuum manifold for 12 columns (Macherey–Nagel) was used to maintain a sufficient vacuum. After conditioning of the cartridges, the sample was sucked through the columns and washed once with 2.0 ml of buffer (pH 9.00)–water (1:1, v/v) with 2.5 ml of water–2-propanol (85:15, v/v) and with 2.0 ml of *n*-hexane. The vacuum was set between −1.5 and −2.5 psi, after rinsing with *n*-hexane at −5 psi for 15 min. After elution

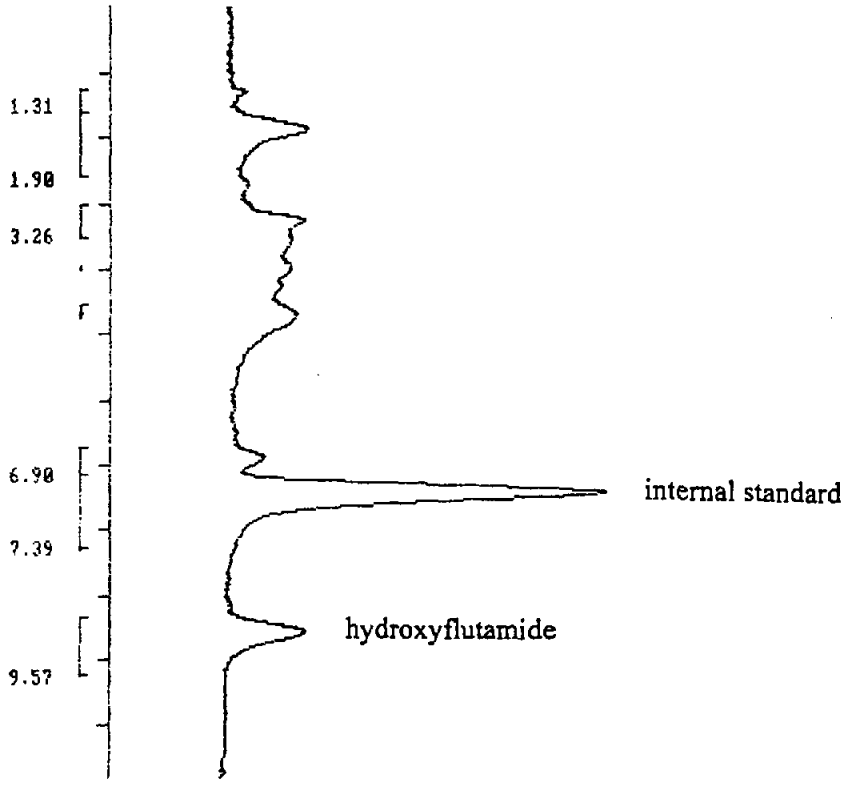

Fig. 2. Chromatogram of extracted human plasma containing 20 ng of hydroxyflutamide and 100 ng of internal standard.

with 2.0 ml of ethyl acetate–1-chlorobutane (1:1, v/v), the organic solvent was evaporated to complete dryness at 40°C under nitrogen. The residue was dissolved in 150 μl of HPLC eluent and 20 μl were injected into the chromatograph.

Table 1
Inter-day precision and accuracy for QC samples[a]

Hydroxyflutamide concentration added to plasma (ng ml^{-1})	Hydroxyflutamide concentration found (ng ml^{-1}) ($\bar{X} \pm$ SD)	RSD (%)	Accuracy (%)
10	9.84 ± 1.21	12.26	−1.56
20	21.31 ± 0.84	3.92	6.55
50	47.75 ± 2.76	5.78	−4.50
100	102.14 ± 4.30	4.21	2.14
500	486.96 ± 22.60	4.64	−2.60
1000	1018.14 ± 62.68	6.16	1.81

[a] $n = 5$.

3. Results and discussion

3.1. Chromatography

Chromatograms of blank plasma extract and

Table 2
Intra-day precision and accuracy for QC samples[a]

Hydroxyflutamide concentration added to plasma (ng ml^{-1})	Hydroxyflutamide concentration found (ng ml^{-1}) (X ± SD)	RSD (%)	Accuracy (%)
10	8.92 ± 0.44	4.95	−10.76
20	20.76 ± 0.26	1.26	3.82
50	45.93 ± 1.44	3.13	−8.13
100	103.15 ± 1.69	1.64	3.15
500	493.91 ± 7.14	1.44	−1.22
1000	1041.57 ± 44.67	4.29	4.16

[a] $n = 5$.

plasma spiked with hydroxyflutamide and internal standard are presented in Figs. 1 and 2. There were no peaks interfering with the solutes.

3.2. Linearity

The linearity of the calibration curve was examined at concentrations of 10, 20, 50, 100, 500 and 1500 ng ml^{-1}. The calibration curve was derived on the basis of the least-squares method using a weighting factor of $1/y^2$. The mean and RSD values of the correlation coefficient (r), slope and intercept were calculated after the analysis of five sets of samples: correlation coefficient, mean = 0.9996 and RSD = 0.057%; slope, mean = 0.0099 and RSD = 4.12%; intercept, mean = −0.0106 and RSD = 45.90%.

3.3. Inter-day precision

The results obtained from analysis of a single set of QC samples on five different days are summarized in Table 1. The precision and accuracy of the method were characterized by calculation of RSD and relative error, which ranged from 3.92 to 12.26% and from −4.5 to 6.55%, respectively.

3.4. Intra-day precision and accuracy

For the determinaion of the repeatibility of the method, a set of replicate QC samples ($n = 5$) was analysed within a run. The data on intra-day precision and accuracy are shown in Table 2. In the concentration range of 10–1000 ng ml^{-1} the RSD and relative error values ranged from 1.26 to 4.95% and from −10.76 to 4.16%, respectively.

3.5. Lower limit of quantitation

The lower limit of quantitation (LQ) of the method was set at 10 ng ml^{-1}, which showed acceptable precision and accuracy (see Tables 1 and 2).

3.6. Stability tests

The stability of hydroxyflutamide in human plasma was studied at two concentrations after 0, 3, 6 and 9 weeks by comparison of the peak-height ratios of freshly prepared and stored (−20°C) QC samples. The data were analysed by a computer program in accordance with the theory of Timm et al., [5]. The results are summarized in Table 3. The data indicate that there was no

Table 3
Results of plasma stability tests[a]

Concentration (ng ml^{-1})	Samples	Parameter	Start	3 weeks	6 weeks	9 weeks
20	Freshly prepared samples	GMx[b]	0.196	0.205	0.230*	0.205
	Quality control samples stored at −20°C	GMy[c]	0.195	0.198	0.214	0.207
		LL (%)[d]	−3.46	−7.88	−11.42	−4.65
		UL (%)[e]	2.60	1.30	−1.73	6.21
		D (%)[f]	−0.48	−3.40	−6.70	0.63
1000	Freshly prepared samples	GMx[b]	10.57	10.11	10.98	9.26
	Quality control samples stored at −20°C	GMy[c]	10.75	9.96	10.45	9.21
		LL (%)[d]	−0.82	−2.58	−9.83	−4.65
		UL (%)[e]	4.27	−0.22	2.61	6.21
		D(%)[f]	1.69	−1.41	−3.81	0.63

[a] $n = 5$.
[b] GMx = geometric mean of detector responses for freshly prepared samples.
[c] GMy = geometric mean of detector responses for quality control samples.
[d] LL = lower limit.
[e] UL = upper limit.
[f] D = difference.

significant or relevant decrease in hydroxyflutamide concentration after storage for 9 weeks at −20°C.

The effect of freezing and thawing cycles was studied using five parallel samples at two concentrations (50 and 1000 ng ml^{-1}). The evaluation of the data was the same as for the long-term stability test. The results indicate that there was no significant or relevant change in concentration of hydroxyflutamide during two cycles of freezing and thawing.

4. Conclusions

The acceptable values of the correlation coefficients, the low variability of the slope of the calibration curve and the inter-day and intra-day precision and accuracy of QC results demonstrate that the method fulfils the requirements of validation and is suitable for bioequivalence studies.

References

[1] B.R. Goldspiel and D.R. Kohler, Ann. Pharmacother. 24 (1990) 616–622.
[2] E. Radwansky, G. Perentesis, S. Symchowicz and N. Zampaglione, J. Clin. Pharmacol. 29 (1989) 554–558.
[3] M. Schulz, A. Schmoldt, F. Donn and H. Becker, Eur. J. Clin. Pharmacol. 34 (1988) 633–636.
[4] R.H. Asade, L. Prizont, G. Ponce Muino and J. Tessler, Cancer Chemother. Pharmacol., 27 (1991) 401–405.
[5] U. Timm, M. Wall and D. Dell, J. Pharma. Sci., 74 (1985) 972–977.