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A validated high-performance liquid chromatographic method for the determination of glibenclamide in human plasma and its application to pharmacokinetic studies

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Abstract

Glibenclamide is a potent second generation oral sulfonylurea antidiabetic agent widely used for the treatment of type II diabetes melitus. A rapid, sensitive, precise, accurate and specific HPLC assay for the determination of glibenclamide in human plasma was developed and validated. After addition of flufenamic acid as internal standard, the analytes were isolated from human plasma by liquid-liquid extraction. The method was linear in the 10-400 ng/ml concentration range ($r > 0.999$). Recovery for glibenclamide was greater than 91.5% and for internal standard was 93.5%. Within-day and between-day precision, expressed as the relative standard deviation (RSD%), ranged from 1.4 to 5.9% and 5.8 to 6.6%, respectively. Assay accuracy was better than 93.4%. The assay was used to estimate the pharmacokinetics of glibenclamide after oral administration of a 5 mg tablet of glibenclamide to 18 healthy volunteers.

Keywords: Glibenclamide; HPLC determination; Pharmacokinetics

1. Introduction

Glibenclamide (glyburide) is a potent, second generation oral sulfonylurea antidiabetic agent widely used to lower blood glucose levels in patients with type II non-insulin-dependent diabetes mellitus. It acts mainly by stimulating endogenous insulin release from beta cells of pancreas [1]. Glibenclamide is rapidly and completely absorbed from the gastrointestinal tract. As there is no significant first pass metabolism, 100% of the oral dose is bioavailable [2]. Glibenclamide concentration–time curves in plasma exhibit biphasic elimination [1] with a terminal elimination rate of 1.4–5 h [3].

Different HPLC methods coupled with UV detection [4–11], fluorescence detection [12] or mass spectrometry [13–15] have been developed for the determination of glibenclamide in biological fluids. However, some of these methods were not sufficiently specific and sensitive, some were not validated and some were time-consuming and ex-

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pensive and not directly applicable for the determination of glibenclamide in human plasma.

Therefore, the objective of this report was to develop and validate a HPLC method in order to study the pharmacokinetics of an oral formulation of glibenclamide. The method reported was successfully used to determine glibenclamide in human plasma samples and was proved to be suitable for pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Glibenclamide was obtained from the European Pharmacopoeia (Strasbourg, France). Flufenamic acid, used as internal standard, was supplied by Sigma (St. Louis, MO). Acetonitrile, dichloromethane and hexane, all of HPLC grade, were purchased from J.T. Baker (Deventer, Netherlands). All other chemicals and solvents used were of analytical grade and water was milli-Q grade.

2.2. Chromatographic conditions

The development and validation work was carried out on a HPLC system consisting of an ISCO 2300 HPLC pumb, an ISCO V-4 variable wavelength UV–Vis detector (Lincoln, NE), a Hewlett-Packard HP3396A integrator (Avondale, PA) and a Hitachi 655A-40 autosampler (Tokyo, Japan). The column used was Hypersil MOS C8 analytical column (3 μm particle size 100×30.2 mm i.d.) purchased from Aldrich (St. Louis, MO).

The HPLC system was equilibrated with the mobile phase consisting of acetonitrile/water/acetic acid (500:500:0.3, by volume), at a flow-rate of 0.5 ml/min. The injection volume was 100 μl and the chromatographic peaks were detected at 325 nm. The integrator attenuation was 8 and the chart speed was 0.2 cm/min.

2.3. Standard solutions

A stock standard solution was prepared in acetonitrile/water 1:1 and contained 20 μg/ml of glibenclamide. This solution was further diluted with acetonitrile/water 1:1 to prepare the calibration solutions containing 0.2, 0.5, 1, 2, 4 and 8 μg/ml.

An internal standard stock solution was prepared in acetonitrile/water 1:1 and contained 400 μg/ml. This solution was further diluted with acetonitrile/water 1:1 to prepare the working standard solution contained 20 μg/ml of flufenamic acid.

Calibration standard samples were freshly prepared in 0.5 ml of human plasma by adding 25 μl of the glibenclamide calibration solutions and of the internal standard working standard solution to yield concentrations corresponding to 10, 25, 50, 100, 200 and 400 ng/ml of plasma.

2.4. Quality control samples

Volumes of 50 ml of human plasma were spiked with 125, 250 and 500 μl of 20 μg/ml glibenclamide solution to obtain quality control samples containing 50, 100 and 200 ng/ml glibenclamide, respectively. These samples were divided into 3 ml portions and kept at -20 °C until analysis.

2.5. Sample preparation

To plasma sample (0.5-ml) calibration standard (25 μl) and internal standard (25 μl) were added. To control blanks and to quality control standards 50 and 25 μl of acetonitrile/water 1:1 were added, respectively. The contents were shaken with 2 ml of dichloromethane/hexane 1:1 for 10 min. They were then centrifuged at $2000 \times g$ for 10 min and the organic phase was transferred to a clean test tube and evaporated to dryness at 30 °C with the aid of a gentle stream of air. One millilitre of mobile phase and 2 ml of isooctane were added to the residue and after shaking for 10 min and centrifugation for 5 min, isooctane was aspirated off. The residue was transferred to an autosampler vial and a 100-μl volume was injected into the HPLC system for quantitation.

2.6. Pharmacokinetic study

The HPLC method developed was used to investigate the plasma profile of glibenclamide after single 5-mg oral dose of a glibenclamide oral formulation. A clinical study on 18 healthy volunteers (8 female, 10 male) was conducted under fasting conditions. Mean age $\pm$ SD was 25.0 ± 5.7 years (range 18–44 years), mean body weight was 72.6 ± 15.3 kg (range 47–100 kg) and mean body height was 177.0 ± 11.8 cm (range 158–197 cm). Following written informed consent, volunteers received a 5-mg oral dose of a micronised glibenclamide tablet with 240 ml of 20% glucose solution in water. Each volunteer was given 60 ml of 20% glucose solution in water, post-dose, every 15 min for 4 h, in order to prevent hypoglycemia. Blood samples were collected in heparinized tubes pre-dose (0 h) and at 20, 40, 60, 80, 100 min and 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 16 and 24 h post-dose. Plasma was immediately separated by centrifugation at $3000 \times g$ and stored in polypropylene tubes at -20 °C until analysis.

3. Results and discussion

3.1. Chromatography

Symmetrical peaks were observed for glibenclamide and internal standard. Typical chromatograms obtained with a drug-free plasma and a plasma sample containing 98 ng/ml of glibenclamide obtained from a volunteer 5 h post-dose, after a single oral dose of 5 mg glibenclamide, are illustrated in Fig. 1. There were no peaks interfering with glibenclamide or internal standard. The retention times of glibenclamide and internal standard were 4.3 and 6.3 min, respectively. The overall chromatographic run time was 8 min.

3.2. Sample extraction

A number of the previously published HPLC methods for the determination of glibenclamide in human plasma using solid-phase or liquid–liquid extraction were tested without success. Solid-phase extraction using C18 cartridges [11] was very inconsistent, while other methods using liquid–liquid extraction co-extracted endogenous plasma components that interfere with glibenclamide and internal standard, causing problems with quantification [4,5,10,12]. These results led us to investigate other liquid–liquid extractions for sample pretreatment. The method developed finally gave clean chromatograms without interfering peaks at the retention time of glibenclamide or internal standard.

3.3. Validation

The assay was validated by assaying six calibration standards and three quality control samples in triplicate on three separate occasions. Data were obtained through linear regression analysis of peak height ratios of glibenclamide/internal standard (y) versus glibenclamide concentrations

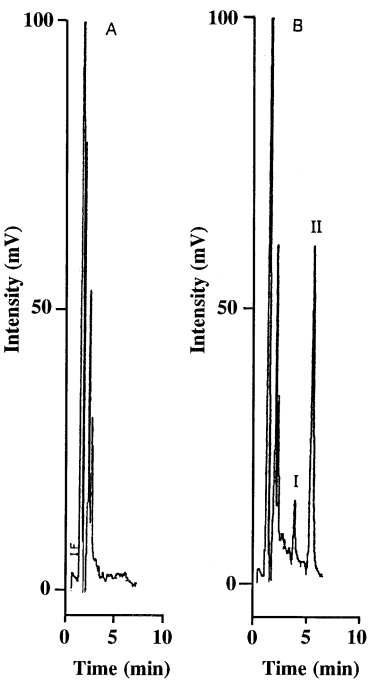

Fig. 1. Examples of chromatograms: (A) extract of 0.5 ml drug-free plasma; (B) plasma sample obtained from a volunteer 5 h after a single oral dose of 5 mg of glibenclamide containing 98 ng/ml of glibenclamide. Peaks: I = glibenclamide; II = internal standard.

Table 1
Within-day and between-day accuracy and precision for glibenclamide in quality control samples in human plasma

Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	Accuracy[a] (%)	Precision[b] (RSD, %)
Within-day			
50	51.4	102.8	5.9
100	95.0	95.0	4.2
200	192	96.0	1.4
Between-day			
50	51.4	102.8	6.6
100	93.4	93.4	6.1
200	194	97.0	5.8

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

(ng/ml) in spiked plasma samples (x). A weighting factor of 1/concentration was employed. The linearity of the method was confirmed over the concentration range of 10–400 ng/ml using a 0.5-ml plasma sample. A typical calibration curve had the regression equation of $y = 0.01681 + 0.01808x$ ($r = 0.999$).

The absolute recovery of glibenclamide and internal standard was determined by direct comparison of peak heights from extracts versus non-extracted samples. The mean recoveries for glibenclamide were $91.5 \pm 4.3\%$, $93.7 \pm 2.5\%$, and $93.8 \pm 2.0\%$ at the 50, 100, and 200 ng/ml concentration, respectively ($n = 6$). The mean recovery of internal standard was found to be $93.5 \pm 2.1\%$ ($n = 18$).

Within-day precision (repeatability) was determined by calculating the relative standard deviation (RSD) for five determinations at each concentration of three quality control samples and was found to be less than 6%. Between-day precision and accuracy were assessed by assaying three quality control samples in triplicate on three separate occasions. The assay precision was 6.6% based on RSD values (RSD%) of 6.6, 6.1, and 5.8% for samples containing 50, 100, and 200 ng/ml, respectively. Assay accuracy, assessed by calculating the estimated concentrations as a percent of the nominal concentrations, was better than 93.4% (Table 1). The lowest quantifiable concentration was deemed to be 10 ng/ml based on the maximum tolerable CV of 15%.

The method was successfully used to perform the determination of plasma concentrations of glibenclamide, after oral administration of a 5 mg dose of a micronised glibenclamide formulation to 18 healthy volunteers. Fig. 2 shows mean $\pm$ SD

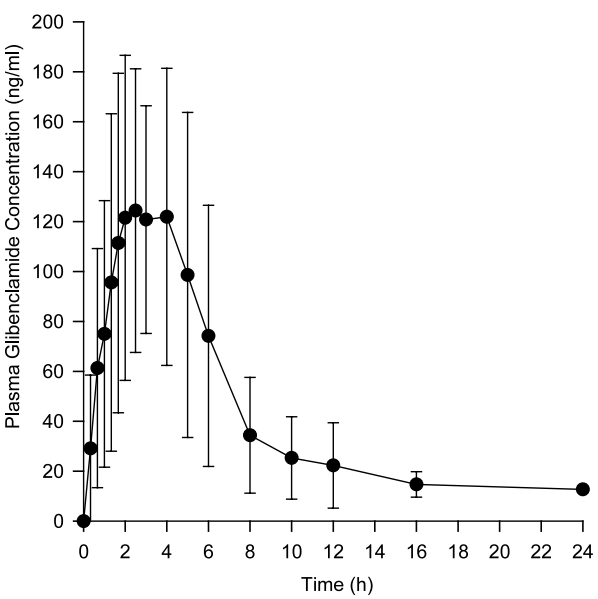

Fig. 2. Plasma concentration–time profile (mean $\pm$ SD) for glibenclamide following a single 5-mg oral dose in 18 volunteers.

Table 2
Pharmacokinetic parameters for glibenclamide after a single oral administration of 5 mg glibenclamide to 18 volunteers

Pharmacokinetic parameter	Mean ± SD	Range
C_{max} (ng/ml)	167.7 ± 63.4	(71.3–282)
$AUC_{0-\infty}$ (ng h/ml)	878.3 ± 306.5	(483.1–1324.4)
AUC_{0-t} (ng h/ml)	808.6 ± 279.4	(460.6–1279)
T_{max} (h)	3.6 ± 2.3	(0.7–6)
$t_{1/2}$ (h)	3.4 ± 2.9	(1.5–13.4)

plasma concentration–time profile of glibenclamide. Pharmacokinetic parameters were estimated using standard noncompartmental methods [16]. After oral administration of a 5-mg dose of glibenclamide to 18 healthy volunteers the following pharmacokinetic parameters (mean ± SD) were estimated: $AUC_{0-\infty}$ 878.3 ± 306.5 ng h/ml, AUC_{0-t} 808.6 ± 279.4 ng h/ml, C_{max} 167.7 ± 63.4 ng/ml, T_{max} 3.6 ± 2.3 h and $t_{1/2}$ 3.4 ± 2.9 h (Table 2).

4. Conclusions

A specific, sensitive, rapid, precise and accurate HPLC assay for the determination of glibenclamide was developed and validated, suitable for the analysis of large numbers of plasma samples. The assay was used for the analysis of plasma samples obtained from 18 volunteers in the conduct of pharmacokinetic study, following oral administration of 5 mg of glibenclamide.

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