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Short communication

# Determination of glibenclamide in human plasma by solid-phase extraction and high-performance liquid chromatography

José R. Valdes Santurio*, Esther González Porto

*Dpto. de Estabilidad, Centro de Investigacion y Desarrollo de los Medicamentos, MINSAP, Ave. 26 No. 1605 Plaza, Ciudad Habana, Cuba*

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**Abstract**

A sensitive high-performance liquid chromatographic method for determination of intact glibenclamide in human plasma has been developed. Sample clean-up prior to chromatographic analysis was accomplished by extraction of the drug using a solid-phase RP-8 or RP-18 cartridge instead of the conventional liquid–liquid extraction methods described. For the separation of the drug from the endogenous components a reversed-phase column (LiChrosorb RP-8) of 5 $\mu$m particle size and 250×4 mm I.D., together with a mobile phase consisting of acetonitrile–12 m*M* perchloric acid (47:53) was selected. The method employs progesterone as an internal standard, and a reversed-phase column combined with UV detection of the drug at 230 nm. The detector response was linear up to the concentration of 400 ng/ml and the average recovery was 100.36%. The sensitivity of the method was 5 ng/ml.

*Keywords:* Glibenclamide

## 1. Introduction

Glibenclamide (Fig. 1), a second generation sulphonylurea, is a drug widely employed in the treatment of non insulin-dependent diabetic patients.

In this respect, some reports can be found in the literature dealing with its bioavailability or bioequivalence [1–5]. Also, different methods were developed in order to quantitate this drug in biological fluids or in dissolution tests [6–17].

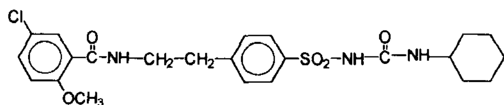


Fig. 1. Structure of glibenclamide.

Among the methods described, there are some spectrophotometric, fluorimetric, GLC [7,8], HPLC [9–15] and RIA methods [16,17].

The spectrophotometric and fluorimetric methods lack, in many cases, specificity and sensitivity, due to the low concentration of glibenclamide found in blood; rarely beyond 300 ng/ml [9,10].

GLC methods require the preparation of volatile and thermally stable derivatives, introducing a time-consuming derivatization step. Also, the derivatives formed are similar, resulting in a limitation on the selectivity, and a probable loss in the final recovery [7,8].

*Corresponding author.

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From analysis of the reported papers, it is concluded that the most favoured method is HPLC, in which ultraviolet detection has been more frequently used. For this purpose either direct measurement or the use of derivatization reactions with compounds such as dinitrofluorobenzene (DNFB) are used [10–15].

There is also a report using fluorescence detection for this determination [9]. In all the cases the extraction method used has been liquid–liquid extraction, with the disadvantage of losses of the drug during the final recovery. This results in an incomplete extraction from the biological fluid, or the formation of emulsions which impair the extraction of the organic layer [10], co-extraction of endogenous components of plasma that interfere later in the determination, less reproducibility in the extraction process and the need of more time spent in sample preparation.

Most of these problems can be avoided in a very efficient way with the use of the solid-phase extraction method.

Considering this possibility, we devised an analytical method for glibenclamide in plasma, with enough sensitivity, specificity, efficiency and speed to allow reliable determination of the drug.

## 2. Experimental

A Model LC-21 high-performance liquid chromatograph (Bruker Franzen Analytik, Karlsruhe, Germany) consisting of a ternary reciprocating pump, UV-Vis variable-wavelength detector, Rheodyne 7125 injection valve, ERMA ERC-3510 degasser and LC-41 CD control and data station was used for the determination of the samples (Bruker).

For the centrifugation of the samples a MLW centrifuge Model T-23 with glass conic tubes of 12-ml capacity was employed (MLW, Germany).

Adsorbex RP-18 and RP-8 solid-extraction phase cartridges of 100 mg perchloric acid, hydrochloric acid and chloroform were obtained from Merck (Darmstadt, Germany). Acetronitrile Hypersolv was purchased from BDH (Poole, UK).

Water was bidistilled twice in glass. No further purification technique was applied to the other reagents.

### 2.1. Chromatographic conditions

The mobile phase was acetonitrile–12 m*M* perchloric acid (47:53), but sometimes it was slightly adjusted in order to have a retention time (RT) for glibenclamide of about 8 min.

A LiChrosorb RP-8 column of 5 $\mu$m particle size and 250×4 mm I.D. was used. (H. Knauer, Berlin, Germany).

The flow-rate was set at 1.2 ml/min and the wavelength at 230 nm.

### 2.2. Internal standard solution

A working internal standard solution with a final concentration of 5 $\mu$g/ml was prepared in mobile phase from a stock solution containing 1 mg/ml progesterone in acetonitrile. This solution was used to redissolve the residue after evaporation to dryness.

### 2.3. Model preparations

From a glibenclamide stock solution having a final concentration of 1 $\mu$g/ml a series of solutions were prepared in a range of concentrations between 10 and 400 ng/ml in acetonitrile.

From the different concentrations 1 ml was taken, evaporated to dryness in a thermostatic bath at 50°C with nitrogen, diluted with 1 ml plasma and vortexed for 30 s.

The spiked plasma was used for the calibration curve and for the calculation of the recovery method.

### 2.4. Sample extraction

The extraction column (RP-8 or RP-18) was conditioned with one column volume of acetonitrile, one column volume of distilled water and 500 $\mu$l of 0.1 *M* hydrochloric acid. After this, 1 ml of distilled water, 1 ml of sample or model preparation, previously centrifuged at 6000 rpm (approx. 3900 *g*), and 200 $\mu$l of 0.1 *M* hydrochloric acid were added.

The column was inverted three or four times in order to mix its contents thoroughly, and slowly eluted, applying air pressure with a 5-ml syringe fitted onto the column.

The extraction column was later washed twice

with one column volume of distilled water and one column volume of acetonitrile–water (30:70).

Finally, glibenclamide was eluted from the column to a 12-ml centrifuge tube with 500 μl of acetonitrile, and evaporated to dryness with nitrogen in a thermostatic bath at 50°C. The evaporated sample was redissolved with 100 μl of the internal standard solution, vortexed for 15 s and 50 μl was injected onto the chromatographic system.

## 3. Results and discussion

Some of the previously reported techniques for the determination of glibenclamide in biological fluids were tested without success, obtaining a low reproducibility and recoveries [9,10,13–15].

The method reported by Uhlein and Sistovaris [10] did not offer the expected sensitivity, giving a limit of only 20 ng/ml, in agreement with the results found by other authors [7,8].

Due to the low wavelength used in order to increase the sensitivity of the method, and the extraction method employed, a lot of interfering peaks were seen in the chromatogram on which glibenclamide was not totally resolved, causing problems with quantification. Also, they used an internal standard that is not commercially available.

Although the mobile phase in our system is similar to the mobile phase of Pötter and Hulm [15], which was selected taking into account that there is no buffer in its composition, the extraction method presented difficulties, extracting a great number of endogenous components that interfere with glibenclamide and with the tolbutamide used as internal standard. This produced erratic results and a low recovery.

The method reported by Adams [9] employs an unusual internal standard, and working with this procedure it was not possible for us to detect the glibenclamide peak, even at a concentration of 400 ng/ml.

The method described by Zecca [11] introduces a time-consuming derivatization step with DNFB, and uses chloroform as an extracting solvent. This produces an emulsion with plasma, making manipulation very difficult.

Emilson also found a similar problem with different extracting solvents [10].

Also tested were extractions with toluene, benzene or ethyl acetate, buffered in acid medium. In all cases the recovery did not exceed 90%.

Other extraction-solvent systems were employed, mixing different proportions of ethyl ether and chloroform but, in all cases, the recovery was low.

These results led us to think of the solid-phase extraction method as the most suitable technique due to its advantages over the liquid–liquid extraction methods in relation to the recovery of the drug, the elimination of emulsions and the speed of the extraction process [18].

For this purpose, the RP-18 and RP-8 solid-phase extraction columns were selected, taking into consideration their similarity to the column used in the chromatographic system.

It could be seen in the initial experiments that one important point in the retention of glibenclamide in the sorbent was the pH. It was observed that the retention of the drug was a pH-dependent factor, increasing with the decrease of the pH.

This result is consistent with the data reported [13] in the partition study of glibenclamide in an octanol–water system, where a higher distribution of the drug was found in the organic layer with the decrease of the pH in the aqueous phase. It was also found that plasma dilution contributed to a higher retention of glibenclamide and made elution easier, so 1 ml of water was added to it.

The retention of the drug in the extraction cartridge was controlled through HPLC, checking the different eluted fractions. The best retention was obtained when the plasma pH reached a value of 2.5 obtained by the addition of 200 μl of 0.1 *M* hydrochloric acid.

Different proportions of acetonitrile–water were tried in order to wash the column and discard the endogenous component retained in the sorbent. It was found that a 30:70 mixture cleaned the extract to a greater extent without affecting the recovery of the drug.

In the different experiments we conducted during this study, we compared the efficiency of the RP-8 and RP-18 extraction cartridges in the sample clean-up.

We found that there were no differences between

Table 1
Results corresponding to the experiments done with spiked plasma at different concentration levels

| Glibenclamide added (ng/ml) | Glibenclamide found (ng/ml) | Recovery (%) | Standard deviation | Standard error | Coefficient of variation (%) | $n$ |
|---|---|---|---|---|---|---|
| 11.20 | 10.86 | 97 | 1.0 | 0.4 | 9.5 | 8 |
| 55.16 | 54.91 | 100 | 1.8 | 0.6 | 3.2 | 8 |
| 109.00 | 109.88 | 101 | 1.2 | 0.4 | 1.0 | 8 |
| 207.20 | 216.07 | 104 | 6.7 | 3.0 | 3.1 | 5 |
| 251.80 | 251.93 | 100 | 2.6 | 1.1 | 1.0 | 5 |
| 414.24 | 416.48 | 100 | 2.8 | 1.3 | 0.7 | 5 |

Average total recovery = 100.36±2.36%. Coefficient of variation = 2.35%.

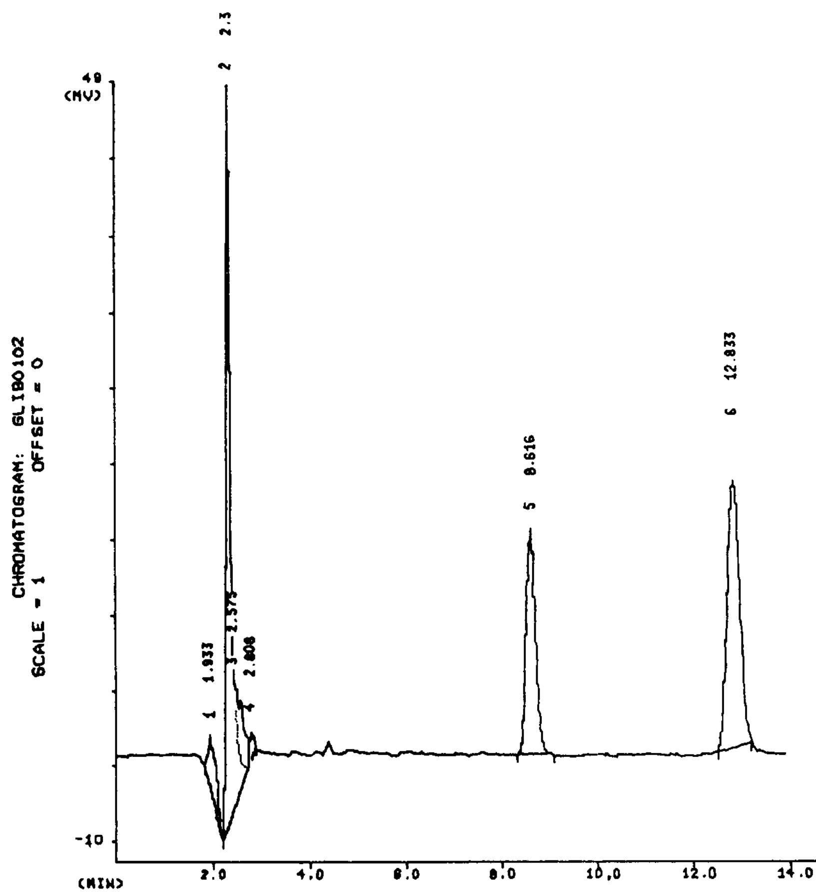


Fig. 2. Chromatograms from a calibration standard (100 ng/ml) containing glibenclamide (peak No. 5, $t_R$ = 8.61 min) and progesterone as internal standard (peak No. 6, $t_R$ = 12.03 min).

the two classes of cartridge with regard to the final chromatographic profile and quantitative results. Therefore, it did not matter which class of cartridge was used for this purpose.

The acetonitrile used in the final elution step of glibenclamide from the cartridge should be of the best quality to avoid additional peaks produced by the concentration of the impurities in the evaporation of the eluted fractions to dryness.

It could be proved, in the liquid–liquid extractions tested in the first experiments, that when the organic phase was evaporated to dryness, especially in those systems using ethyl ether, a lot of interfering peaks were obtained, mainly in the first part of the chromatogram, which could be assigned erroneously to endogenous plasma components. This problem was avoided by employing acetonitrile of good quality. Plasma centrifugation avoids blockage of the column by mechanical contamination and makes the elution easier.

It was found during the development of the method that the injections were not reproducible.

The internal standard, with a shorter retention time than glibenclamide, appears in a zone where it generally co-elutes with some of the endogenous plasma components, changing the values of the area or peak height ratios, giving incorrect results.

Synthetic derivatives of glibenclamide eluted after glibenclamide, but they are not commercially available and have to be synthesised.

Progesterone, although it cannot be considered a true internal standard, presented the desired conditions, because it elutes after the glibenclamide and is commercially available.

Due to the fact that progesterone did not elute properly from the extraction column in the selected

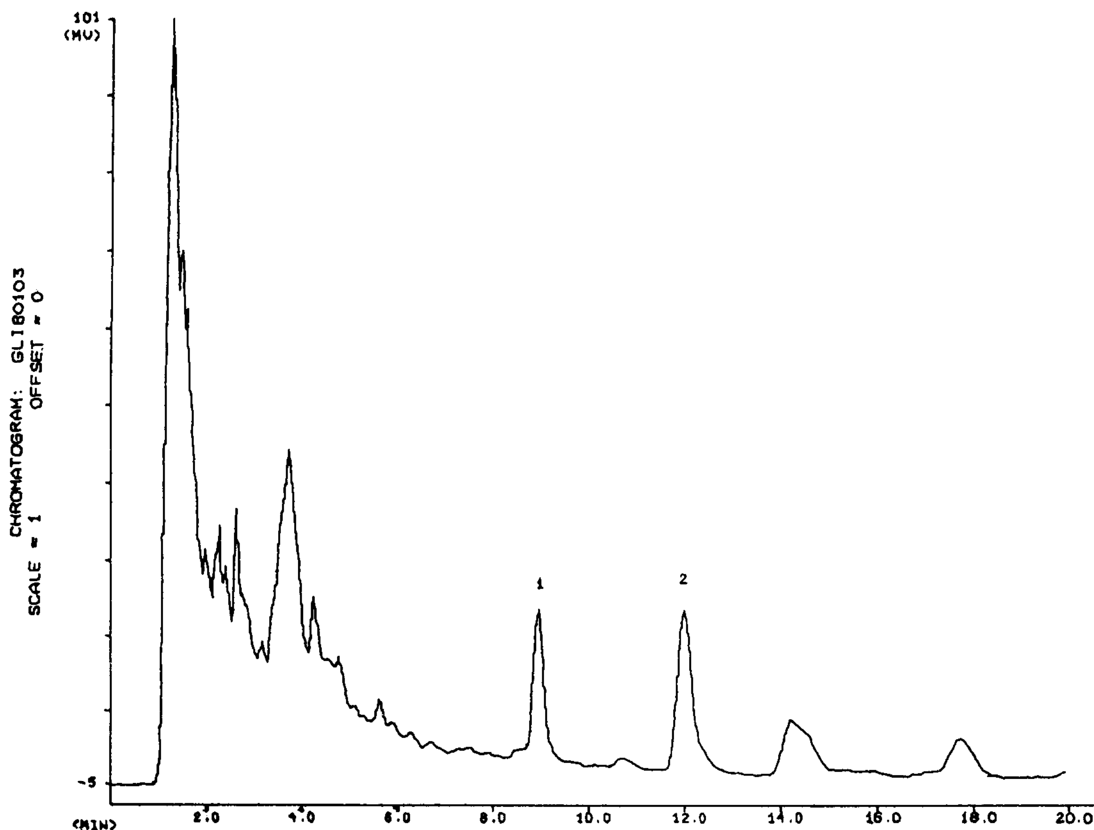


Fig. 3. Chromatogram from the plasma of a human volunteer, showing the peaks corresponding to glibenclamide (peak No. 1) and the internal standard (peak No. 2).

conditions for glibenclamide, it was decided to add the internal standard at the end of the reconstitution step. This procedure made it possible to obtain reproducible injection.

We tried to keep the retention time of glibenclamide in the range 7–10 min because in this zone there were practically no interfering components.

The run time was about 20 min per sample, to ensure that all endogenous components were eluted from the column.

The sensitivity of the method was 5 ng/ml with a signal-to-noise ($S/N$) ratio of 2, comparable to other reported techniques. The lowest concentration determined experimentally was 10 ng/ml (SIN=3).

Under the conditions described for this method a good linearity could be obtained up to 400 ng/ml.

It was experimentally found that the quantification was more accurate employing the peak-height ratios rather than the peak-area ratios.

The total average recovery obtained was 100.36±2.36%, higher than the previously reported ones, which ranged from 85 to 96%. The coefficient of variation determined for this result was 2.35% ($n$=39).

In Table 1 the precision, repeatability and accuracy of the method are shown. The replicated samples were processed as separate batches and in every case a new calibration curve was constructed for these determinations.

In Fig. 2 a chromatogram from the calibration standard is shown, and in Fig. 3 and Fig. 4 the chromatograms corresponding to a volunteer's plasma and a blank are shown. The peak concentration level was found between 2–3 h. In Fig. 5 the absorption profiles of glibenclamide corresponding to different formulations are shown.

This results are in good agreement with those previously reported by other authors [1–5]. The

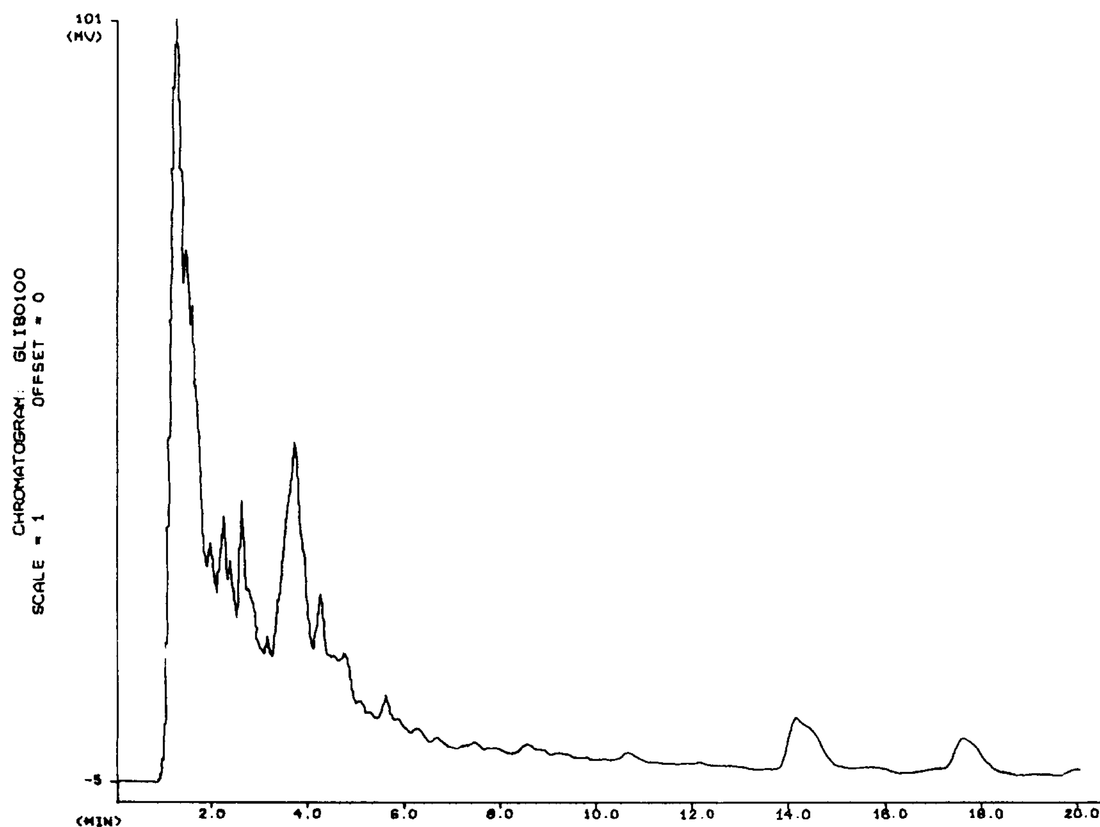


Fig. 4. Chromatogram from a blank plasma sample treated in the same manner as the human volunteer samples.

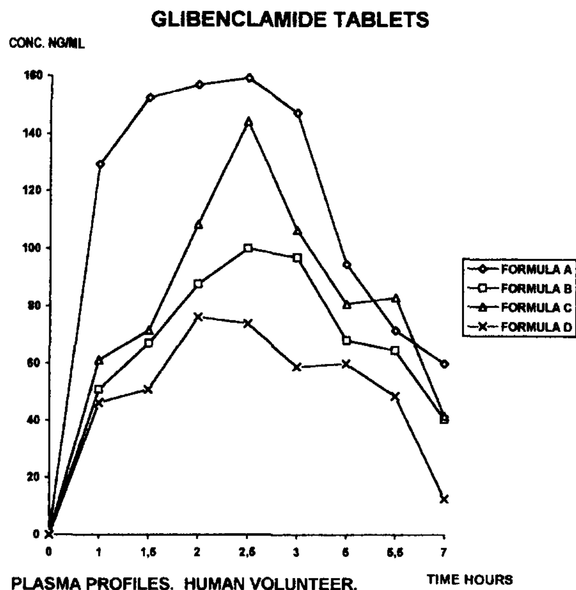


Fig. 5. Plasma profiles of a human volunteer representing the absorption levels of a single oral dose of glibenclamide with the different brands of products (A,B,C,D).

processing time of the sample was about 5 min per sample with a minimal use of reagents.

## 4. Conclusions

The method developed using the solid-phase extraction proved to be accurate, sensitive, precise, faster and with higher recovery than the previously reported methods using the liquid–liquid extraction technique, with the advantage of a minimal use of reagents.

This method may be applied as a routine method in the bioavailability or bioequivalence studies of this drug.

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