Glibenclamide in Serum: HPLC Determination with Pre-Column Derivatization

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Key Words:

HPLC fluorescence detection
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Serum drug levels

1 Introduction

The problem of the determination of glibenclamide, one of the sulfonylurea drugs most widely used in the treatment of diabetes mellitus [1], has been overcome by employing chemical methods [2, 3], radioimmunoassay [4], and gas chromatography [5, 6]. Due to the extremely low therapeutic dose of the drug (2.5 to 15 mg/day) blood levels are also extremely low and create a growing need for specific analytical methods with sufficient sensitivity. This has led us to the development of a method using high performance liquid chromatography following pre-column derivatization. HPLC methods published so far [7-9] make use of UV detection and have difficulty in meeting the required limit of detection. Our method is more sensitive, since it is based on reaction with NBD chloride prior to chromatography on a silica column and fluorometric detection [10].

2 Experimental

2.1 Drug Standards and Chemicals

Glibenclamide and the internal standard, tolbutamide, have been synthesized in our laboratories and were of analytical grade. Other sulfonylurea drug standards, like chlorpropamide, glipizide, glibornuride, and tolazamide were of analytical grade and obtained from the various manufacturers. Analytical grade NBD chloride (7-chloro-4-nitrobenzofurazan) was purchased from E. Merck, Darmstadt. All other reagents employed were of analytical grade and were used without further purification.

2.2 Apparatus

Chromatographic separation was performed using an Altex 110 HPLC pump (Kontron Technik GmbH, Eching, GFR), a silica-gel column (250 × 4.1 mm, packed with Lichrosorb Si-60, 10 µm, from E. Merck A.G., Darmstadt, GFR), and a fluorescence spectrophotometer (Hitachi-Perkin Elmer MPF-3, Perkin Elmer & Co. GmbH, Überlingen, GFR) equipped with an HPLC cell (Hellma GmbH & Co., Müllheim, GFR). The chromatograms were recorded on a chart recorder (Siemens Kompensograph III, Siemens A.G., München, GFR). The samples were injected by an autosampler (WISP, Waters GmbH, Königstein, GFR).

2.3 Mobile Phase

The elution system was prepared by adding 30 ml of ethanol (99%) to 600 ml of n-heptane and 400 ml of diisopropyl ether. The mixture was flushed with nitrogen and degassed with a sonicator for 10 minutes.

2.4 Standard Solutions

Aqueous solutions of glibenclamide between 0 and 3000 ng/ml were prepared in deionized water. 100 µl of each were added to 1 ml of pooled human serum to yield standard solutions from 0 to 300 ng/ml. The internal standard was prepared at a concentration of 1500 ng tolbutamide/ml in deionized water.

2.5 Assay Procedure

100 µl of 1 N HCl, 100 µl of internal standard, and 1 ml of isoamyl acetate was added to 1 ml of serum. The mixture was shaken for 10 minutes on a reciprocating shaker. After centrifugation for 10 minutes at 3000 × g, the organic layer was transferred and washed with 2 ml of 0.04 M Britton-Robinson buffer, pH 7.0. After centrifugation for 5 min, the buffer phase was discarded and the ether phase dried with solid Na_2SO_4 for 20 minutes. The residue of about 0.5 ml dried organic phase was transferred into a glass tube. After addition of 25 µl of freshly prepared NBD chloride solution (30 mg/ml in isoamyl acetate), the tube was sealed tightly and after mixing incubated at 120°C for 60 minutes. After cooling the solution was transferred into WISP vials and 25 µl injected onto the column.

2.6 Isoamyl Acetate

Isoamyl acetate was kept over solid Na_2SO_4 for approximately 60 minutes before use with occasional stirring. This solution was used both for the purpose of extraction and for the preparation of the derivatizing solution.

2.7 Chromatographic Conditions

Solvent flow rate was 2 ml/min at 800 psi, and the fluorometer set at 468/522 nm with slit widths of 12 and 16 nm, respectively. The chromatograms were recorded with a chart speed of 0.2 cm/min. The elution volume was 16 ml for glibenclamide and 21 ml for tolbutamide. Standard curves were constructed by plotting the ratio of the peak height of glibenclamide to that of tolbutamide versus the concentration of glibenclamide.

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3 Results

A chromatogram obtained from blank human serum is shown in **Figure 1 A. Figure 1 B** shows a chromatogram from standard serum (5 ng/ml glibenclamide and 100 ng/ml tolbutamide). There was no peak interfering with glibenclamide and tolbutamide in any case. The peaks are sharp, symmetrical, and well defined with respect to the baseline.

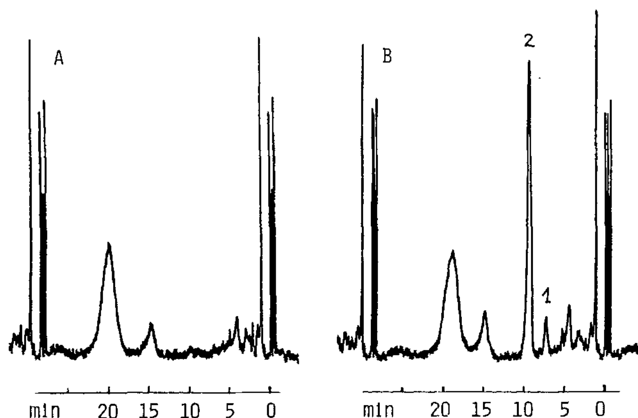

Figure 1

Chromatograms of (A) blank human serum, (B) standard serum containing glibenclamide and tolbutamide derivatives, 5 ng/ml and 100 ng/ml respectively.
1 = Glibenclamide;
2 = tolbutamide.

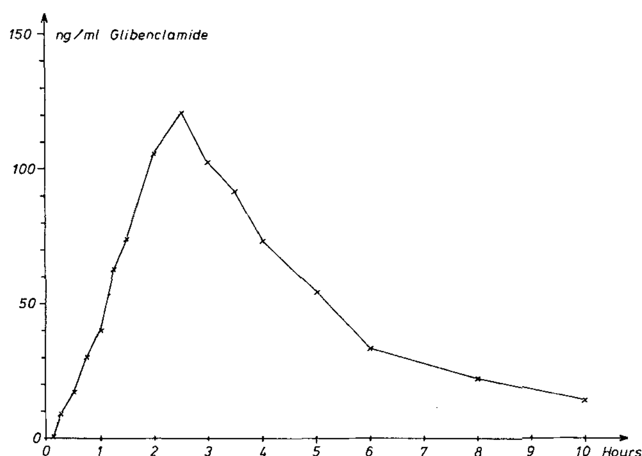

Figure 2

Serum glibenclamide concentration vs. time curve obtained for a volunteer subject following the ingestion of 2.5 mg of the drug.

The relationship between the glibenclamide concentration and the peak height ratio (glibenclamide/tolbutamide) was linear in the range from 5 to 300 ng/ml (correlation coefficient r = 0.9967). A much higher linear range is very likely but has not yet been established experimentally since therapeutic levels are well within the above range.

The detection limit was 5 ng/ml assuming a signal to noise ratio of 3 to 1. The recovery of glibenclamide, determined by derivatizing and chromatographing identical amounts of drug: a) extracted from serum, and b) dissolved directly in the reaction mixture, was 102±2% (N = 10). The retention times of tolbutamide and glibenclamide were 10.5 and 8 minutes under the conditions of the assay. The total analysis time was 25 minutes. The assay procedure can also be used for the selective determination of other hypoglycemic sulfonylureas such as carbutamide (t_R = 10.5 min), glipizide (8 min), and glibornuride (7 min). The method, however, is not applicable for the determination of tolazamide, nor does it detect the 4-trans-hydroxycyclohexyl derivative, the main metabolite in man and rabbit [11].

4 Discussion

The assay method described is specific and sensitive. A representative plot of serum glibenclamide concentration versus time for a volunteer subject is shown in **Figure 2**.

The procedure can also be used for the determination of glibornuride and is applicable for glipizide and carbutamide. Both these substances show retention times identical to those of glibenclamide and tolbutamide respectively.

As is well known [12], these sulfonylurea compounds decompose to the corresponding alkylamines upon heating. Sulfonylureas that yield identical amino compounds will give identical reaction products with identical chromatographic behavior.

For instance, carbutamide and tolbutamide will decompose to n-butylamine and glipizide, while glibenclamide affords cyclohexylamine. In the case of glibenclamide 7-cyclohexylamino-4-nitrobenzofurazan is expected as a reaction product. This structure has been confirmed by making use of mass spectrometry. In addition, the derivative of reagent grade cyclohexylamine has been synthesized and characterized by HPLC and mass spectrometry. The data obtained proved structural identity to exist between this compound and the derivative obtained from glibenclamide [13].

The method described does not determine the 4-hydroxy metabolite because it is not extracted under the conditions of the assay. Moreover, its decomposition product–which is more polar in nature–shows a retention time much longer than 25 minutes.

The method described is a modification of the derivatization procedure for the fluorometric determination of glibornuride [3]. However, it combines the high sensitivity achieved by derivatization with the specificity provided by chromatography and has been developed to improve sensitivity necessary for extensive pharmacokinetic studies. It is not recommended for those sulfonylurea drugs administered in higher doses and yielding higher blood levels. Those drugs do not have to be determined by sophisticated methods, since using the method described here, improved sensitivity has to be bought at the expense of higher variation (a 9% coefficient of variation, day-to-day, interassay).

5 Conclusion

The method presented for the determination of serum glibenclamide appears to be selective, rapid, and sensitive enough to allow accurate measurements for pharmacokinetic purposes.

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Separation of Digitalis Cardenolides by HPLC

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Summary

The separation of *Digitalis* cardenolides has been carried out by HPLC on an adsorbent column. By choice of suitable mobile phase, isocratic elution permitted resolution of mixtures of a) aglycones, b) secondary glycosides, and c) primary glycosides, while gradient elution provided a means of resolving more complex mixtures of these cardiac steroids. HPLC could therefore be used in the quality control of cardiotonic drugs replacing the TLC tests for related compounds currently used, and by suitable calibration could replace the colorimetric assay procedure normally used for such drugs.

1 Introduction

Separation techniques for the study of *Digitalis* cardenolides have relied extensively on paper and thin-layer chromatography [1,2] although in some instances other methods have been used including electrophoresis [3] and gel filtration [4,5]. Gas-liquid chromatography has met with limited success for quantitative work and in each instance derivatization [6-8] was required. However, GLC remains unusable for the high molecular weight primary glycosides which normally constitute the greater proportion of cardiac glycosides in the plant. Modern liquid chromatography appears to offer the most suitable technique for the separation of primary and secondary glycosides and is particularly indicated where quantitative evaluation is required. This note reports a preliminary study on the use of high-pressure liquid chromatography for the separation of some glycosides and aglycones present in *Digitalis purpurea* and *D. lanata*.

2 Experimental

2.1 Apparatus

The chromatograph used was a Chromatronix Model 3100 fitted with a UV detector monitoring at a fixed wavelength of 254 nm.

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