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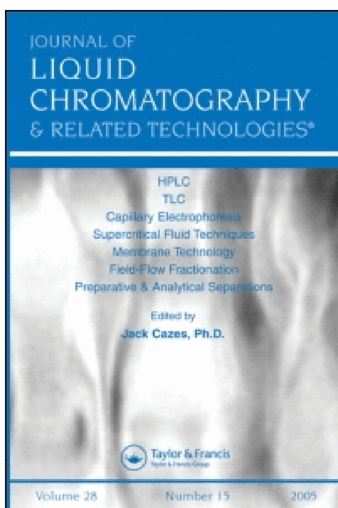

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A SIMPLE, SPECIFIC, AND RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR GLIBENCLAMIDE IN PLASMA

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ABSTRACT

A simple, rapid, sensitive and relatively specific reverse phase HPLC assay procedure for glibenclamide in rat and human plasma is described. The method employs considerably smaller sample volume (0.2 ml) and a single step extraction with dichloromethane. A relatively high degree of sensitivity (detection limit of the assay was 20 $ngml^{-1}$) was obtained by using fluorescence detection at excitation and emission wavelengths of 308 and 360 nm, respectively. The mean percentage recovery of the drug in the concentration range of 30-120 ng was 100.6% while the between-day coefficient of variation for the same concentration range was 7.29%.

The assay procedure offers a much better degree of resolution for the glibenclamide peak than was previously reported. This was achieved through the use of a chromatographic

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column with a finer packing material (Novapak C_{18}, 4 μm) and by selecting the ideal composition, pH and elution rate of the mobile phase - methanol: 0.05 M potassium dihydrogen phosphate (61:39 v/v, pH 4 and pumped at 1.2 $ml.min^{-1}$).

It is hoped that this micro assay technique will find wider application in both clinical and subclinical pharmacokinetic studies involving glibenclamide.

INTRODUCTION

Glibenclamide is a second generation sulfonylurea derivative commonly prescribed for the control of hyperglycemia in Type II (non-insulin dependent, NIDDM) diabetes and thereby to prevent or retard the development of chronic complications of the disease. Glibenclamide produces its hypoglycemic effect by specific interactions with receptors in pancreatic ß-cells to stimulate insulin secretion and to enhance tissue response to endogenous insulin[1]. Glibenclamide is also believed to reduce the hepatic extraction of insulin, thereby increasing its systemic availability to regulate tissue glucose utilization[2].

The hypoglycemic effect of glibenclamide are achieved within a narrow therapeutic range of drug concentration. It has been postulated that there is a threshold plasma glibenclamide concentration which must be exceeded before its hypoglycemic activity can occur[3]. The absolute values for this drug concentration range would essentially depend on the specificity and relative sensitivity of assay method employed.

A number of analytical techniques have been described for glibenclamide in biological samples. These include UV spectrophotometry[4] flurometry[5], radioimmunoassay[6] and high performance liquid chromatographic methods[7-12]. Of these, the

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latter appear to offer simplicity and improved sensitivity. All published HPLC methods do however, require substantial sample volume ($\geq$ 1 ml plasma) and cannot be readily adapted for application in situation involving limited sample volume as in investigational studies with smaller laboratory animals.

In the present study, we report a highly specific, relatively simple and rapid HPLC microassay technique for glibenclamide and discuss its potential for wider application in clinical and subclinical pharmacokinetic investigations.

MATERIALS AND METHODS

Chemicals and Reagents

Glibenclamide was a gift from Saudi Pharmaceutical Industries and Medical Appliances Corporation (SPIMACO, Saudi Arabia) while warfarin was obtained from Sigma Chemical Corpcration (St. Louis, MO, USA). Methanol (HPLC-grade) was purchased from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade and procured from various other sources.

Standard Solutions

For both glibenclamide and warfarin 100 ml of stock solutions of 1 g/L were initially prepared in methanol. Working standards were subsequently obtained by further dilution of the stock solutions to appropriate concentrations.

Instruments

A Waters High-Performance Liquid Chromatographic System (Waters Associates, Milford, MA, USA) was used in the assay. It consisted of a System Controller (M-720), a Data Module (M-730), an Autosampler (WISP-712), a Scanning Fluorescence Detector (M-470), a Novapak C_{18} precolumn and column (4 μm, 150 mm x 3.9 mm ID) and a Solvent Delivery System (M-501).

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Sample Preparation

To 0.2 ml of rat or human plasma, 15 μl of internal standard (5 mg/L - warfarin) and 3 ml of dichloromethane were added. The sample was vortex-mixed for 1 min, shaken on a rotary mixer for 5 min and then centrifuged at 1000 g for 15 min. The organic layer was transferred to another glass centrifuge tube and evaporated to dryness under a stream of purified nitrogen gas. The residue was reconstituted with 0.1 ml of mobile phase and 80 μl was injected onto the HPLC column.

Chromatographic Conditions

The mobile phase consisted of 0.05 M ammonium dihydrogen phosphate and methanol (39:61%, v/v) with the pH adjusted to 4.0. The mobile phase was pumped at 1.2 $ml.min^{-1}$ and the fluorescence detector was set at excitation and emission wavelengths of 308 nm and 360 nm, respectively.

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RESULTS AND DISCUSSION

The need to develop a new HPLC assay procedure for glibenclamide arose from our failure to adapt a number of the published methods for use in pharmacokinetic interaction studies of the drug in the rat model. The volume of blood samples that can serially collected from such a model without inducing serious haemodynamic changes is fairly limited ($\leq$ 0.5 ml). Despite appropriate modification of the published extraction procedures [8,11] to reflect the reduced sample volume glibenclamide was invariably co-eluting with an endogenous plasma component. As a result, quantitation of the drug was greatly compromised. This problem was also encountered by Abu-Nasif and co-workers (personal communication) in their attempts to adapt the methods described by previous authors [9,10], to assay glibenclamide in

rat plasma. It was apparent that most of the published methods had employed columns with similar packings (spherisorb 5 μm) and ultra violet (UV) detection in the region of 200-230 nm - a band where absorption by extracted endogenous plasma components is relatively high. These points were taken into consideration when we started to develop the present method. Extraction with dichloromethane and the use of finely packed column (Novapack C_{18}, 4 μm) together with fluorescence detection gave better resolution for glibenclamide. Furthermore, the sensitivity of the assay estimated on the basis of sample volume used was considerably improved. The detection limit of the assay was 20 ng/ml.

Figures 1 and 2 show typical chromatograms obtained from rat and human plasma. The chromatograms for the rat plasma were obtained from analysis of samples collected following *in vivo* administration of glibenclamide (0.9 mg kg^{-1}, p.o.) while the chromatogram for the human plasma were obtained after spiking plasma samples with the drug. The relative retention times for warfarin (the internal standard) and glibenclamide were 6.7 and 12.3 min., respectively. Blank human plasma gave a much cleaner chromatogram than rat plasma. The reportedly coeluted interfering plasma component is well resolved from glibenclamide, having a retention time of 11.4 min.

Quantitation

Plasma glibenclamide was quantitated by relating peak height ratios (glibenclamide/internal standard) to a calibration curve prepared from triplicate determination of fresh plasma (rat and human) spiked with known drug solutions (concentration range 25 - 200 ng/ml). There was very good correlation between drug concentration and peak height ratio as evidenced by the equation for the regression analysis.

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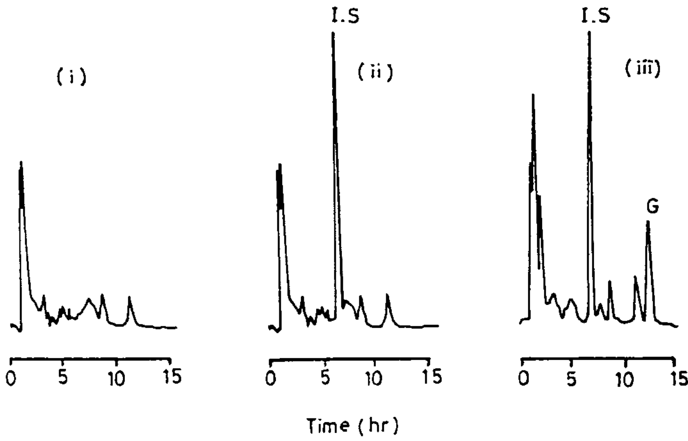

Fig. 1 Typical chromatograms obtained from extracts of (i) blank rat plasma and (ii) blank plasma spiked with internal standard (IS) and (iii) plasma collected 2 hr following an oral dose of (G) glibenclamide (0.9 $mg.kg^{-1}$).

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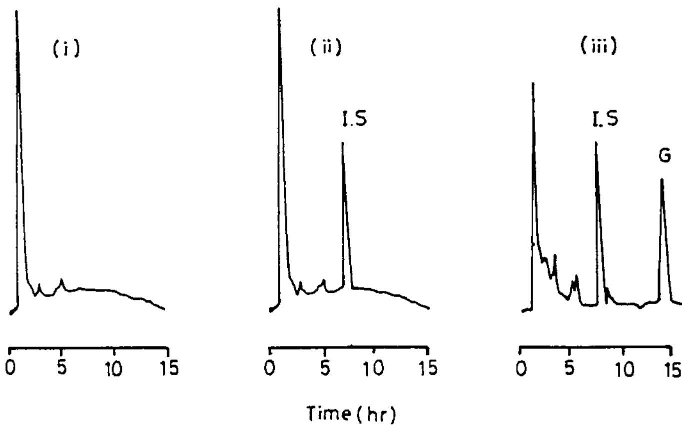

Fig. 2 Typical chromatograms of extracts of (i) blank human plasma (ii) blank plasma with internal standard (IS) and (iii) plasma containing internal standard and glibenclamide (G).

TABLE 1. Between-day precision for the determination of glibenclamide in rat plasma.

Drug Concentration ($ng.\ ml^{-1}$)		
Added		Measured
30	Mean	42.30
	SD	3.80
	CV,%	8.98
60	Mean	72.20
	SD	4.94
	CV,%	6.84
120	Mean	134.00
	SD	8.11
	CV,%	6.05

$Y = 0.0595 + 0.0027\ x$

$r = 0.989$

Precision

The reproducibility of the assay was assessed by assaying known standards (three concentration points in the calibration curve) over a 3-week period. The corresponding values for the

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TABLE 2. Absolute recovery of glibenclamide from rat plasma

Concentration (ng/ml)	Recovery % (mean ± SD, n = 6)
30	101.7 ± 4.86
60	99.18 ± 4.70
120	100.88 ± 6.23

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coefficient of variation were 8.98, 6.8 and 6.05% for the 30, 60 and 120 μgml^{-1}, respectively. These relatively lower values for this paramter indicates a good reproducibility of the assay.

Percentage Recovery

This was assessed by adding known amounts of glibenclamide to drug-free plasma to make concentration of 30, 60 and 120 μgml^{-1}. The samples were subjected to the various steps described in the section on sample preparation before being injected into the chromatograph and subsequently peak heights obtained from the resulting chromatograms. Also obtained were the peak height ratios of absolute drug standards corresponding to the three concentrations (prepared in mobile phase instead of plasma). Absolute recoveries which are summarized in Table 2 were calculated by comparing the two sets of peak height ratios.

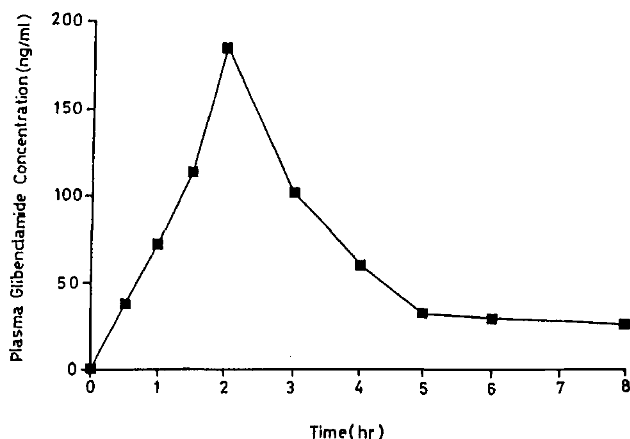

Fig. 3 Plasma glibenclamide concentration versus time profile in normal rats (n = 5).

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Application

A representative pharmacokinetic profile for glibenclamide in rats treated with an oral dose of 0.9 $mgkg^{-1}$ is shown in figure 3. The concentration-time curve was constructed from serially collected plasma samples in femoral artery cannulated rats.

CONCLUSION

The HPLC method described in this report is simple, specific, sensitive and highly reproducible. It requires very small volume of plasma sample. This factor alone may be crucial in investigations involving small laboratory animals or in situations where there is a need for the simultaneous estimation of other related pharmacodynamic parameters such as plasma insulin and glucose levels. Overall, we believe that this method has the potential for a much wider application in both clinical and experimental studies.

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