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CONTENTS

REVIEW ARTICLES

- Cholesteryl Ester Transfer Protein: A Potential Target for the Treatment of Coronary Artery Disease**
HARSHA PATEL, JIGNA SHAH, SUNITA PATEL AND I. S. ANAND 735-740
- Properties and Formulation of Oral Drug Delivery Systems of Protein and Peptides**
A. SEMALTY, MONA SEMALTY, R. SINGH, S. K. SARAF AND SHUBHINI SARAF 741-747

RESEARCH PAPERS

- Fabrication and Evaluation of Asymmetric Membrane Osmotic Pump**
C. S. CHAUHAN, M. S. RANAWAT AND P. K. CHOUDHURY 748-752
- Studies of Disintegrant Properties of Seed Mucilage of *Ocimum gratissimum***
RAVIKUMAR, A. A. SHIRWAIKAR, ANNIE SHIRWAIKAR, S. LAKHSHMANA PRABU, R. MAHALAXMI, K. RAJENDRAN AND C. DINESH KUMAR 753-758
- Simultaneous Spectroscopic Estimation of Ezetimibe and Simvastatin in Tablet Dosage forms**
S. J. RAJPUT AND H. A. RAJ 759-762
- Formulation and Optimization of Carbamazepine Floating Tablets**
D. M. PATEL, N. M. PATEL, N. N. PANDYA AND P. D. JOGANI 763-767
- Effects of *Medicago sativa* on Nephropathy in Diabetic Rats**
M. S. MEHRANJANI, M. A. SHARIATZADEH, A. R. DESFULIAN, M. NOORI, M. H. ABNOSI AND Z. H. MOGHADAM 768-772
- Development of Hospital Formulary for a Tertiary Care Teaching Hospital in South India**
R. J. D'ALMEIDA, LEELAVATHI D. ACHARYA, PADMA G. M. RAO, J. JOSE AND RESHMA Y. BHAT 773-779
- Simultaneous Spectrophotometric Estimation of Rosiglitazone Maleate and Glimepiride in Tablet Dosage Forms**
ANJU GOYAL AND I. SINGHVI 780-783
- Preparation, Characterization and Antimicrobial Activity of Acrylate Copolymer Bound Amoxicillin**
J. S. PATEL, H. R. PATEL, N. K. PATEL AND D. MADAMWAR 784-790
- Haematitic Evaluation of *Lauha Bhasma* and *Mandura Bhasma* on HgCl₂-Induced Anemia in Rats**
P. K. SARKAR, P. K. PRAJAPATI, A. K. CHOUDHARY, V. J. SHUKLA AND B. RAVISHANKAR 791-795
- RPHPLC Method for the Estimation of Glibenclamide in Human Serum**
S. D. RAJENDRAN, B. K. PHILIP, R. GOPINATH AND B. SURESH 796-799
- 2D QSAR of Arylpiperazines as 5-HT_{1A} Receptor Agonists**
URMILA J. JOSHI, SONALI H. TIKHELE AND F. H. SHAH 800-804
- Antiproliferative and Cancer-chemopreventive Properties of Sulfated Glycosylated Extract Derived from *Leucaena leucocephala***
AMIRA M. GAMAL-ELDEEN, H. AMER, W. A. HELMY, H. M. RAGAB AND ROBA M. TALAAT 805-811

SHORT COMMUNICATIONS

- Simultaneous Derivative and Multi-Component Spectrophotometric Determination of Drotaverine Hydrochloride and Mefenamic Acid in Tablets**
P. P. DAHIVELKAR, V. K. MAHAJAN, S. B. BARI, A. A. SHIRKHEDKAR, R. A. FURSULE AND S. J. SURANA 812-814
- Design and Synthesis of Substituted 2-Naphthylxyethylamines as Potential 5-HT_{1A} Antagonists**
URMILA J. JOSHI, R. K. DUBE, F. H. SHAH AND S. R. NAIK 814-816
- Diuretic Activity of *Lagenaria siceraria* Fruit Extracts in Rats**
B. V. GHULE, M. H. GHANTE, P. G. YEOLE AND A. N. SAOJI 817-819
- Determination of Racecadotril by HPLC in Capsules**
S. L. PRABU, T. SINGH, A. JOSEPH, C. DINESH KUMAR AND A. SHIRWAIKAR 819-821
- Novel Spectrophotometric Estimation of Frusemide Using Hydrotropic Solubilization Phenomenon**
R. K. MAHESHWARI, S. DESWAL, D. TIWARI, N. ALI, B. POTHEH AND S. JAIN 822-824
- In Vivo* Pharmacokinetic Studies of Ibuprofen**
ABHA DOSHI AND S. G. DESHPANDE 824-827
- Protective Effect of *Tamarindus indica* Linn Against Paracetamol-Induced Hepatotoxicity in Rats**
B. P. PIMPLE, P. V. KADAM, N. S. BADGUJAR, A. R. BAFNA AND M. J. PATIL 827-831
- Simultaneous Estimation of Atorvastatin Calcium and Amlodipine Besylate from Tablets**
P. MISHRA, ALKA GUPTA AND K. SHAH 831-833
- Development and Validation of a Simultaneous HPTLC Method for the Estimation of Olmesartan medoxomil and Hydrochlorothiazide in Tablet Dosage Form**
N. J. SHAH, B. N. SUHAGIA, R. R. SHAH AND N. M. PATEL 834-836
- Orodispersible Tablets of Meloxicam using Disintegrant Blends for Improved Efficacy**
P. V. SWAMY, S. H. AREEFULLA, S. B. SHIRSAND, SMITHA GANDRA AND B. PRASHANTH 836-840
- Spectrophotometric Method for Ondansetron Hydrochloride**
SRADHANJALI PATRA, A. A. CHOUDHURY, R. K. KAR AND B. B. BARIK 840-841
- HPTLC Determination of Artesunate as Bulk Drug and in Pharmaceutical Formulations**
S. P. AGARWAL, A. ALI AND SHIPRA AHUJA 841-844
- Simultaneous Spectrophotometric Estimation of Metformin and Repaglinide in a synthetic mixture**
J. R. PATEL, B. N. SUHAGIA AND B. H. PATEL 844-846
- Synthesis and Antiinflammatory Activity of Substituted (2-oxochromen-3-yl) benzamides**
V. MADDI, S. N. MAMLEDESAI, D. SATYANARAYANA AND S. SWAMY 847-849
- Evaluation of Hepatoprotective Activity of Ethanol Extract of *Prosopium acerifolium* Ster Leaves**
S. KHARPATE, G. VADNERKAR, DEEPTI JAIN AND S. JAIN 850-852
- New Antihistaminic Agents: Synthesis and Evaluation of H1-Antihistaminic actions of 3-[(N,N-Dialkylamino)alkyl]-1,2,3,4-tetrahydro-(1H)-thioquinazolin-4(3H)-ones and Their oxo Analogues**
M. B. RAJU, S. D. SINGH, A. RAGHU RAM RAO AND K. S. RAJAN 853-856

RPHPLC Method for the Estimation of Glibenclamide in Human Serum

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Rajendran, *et al.*: Estimation of Glibenclamide in Human Serum

A high performance liquid chromatographic method is described for estimation of glibenclamide in human serum. After precipitation with methanol, the separation of glibenclamide and internal standard was accomplished using reversed phase chromatography. The mobile phase, a combination of acetonitrile and 25 mM phosphate buffer (pH 3.5) at 3:2 ratio was run isocratically through a C18 analytical column. The UV detection was done at 253 nm for glibenclamide. Analytical run time was less than 12 min. Mean recovery was 92% for 0.5 µg/ml concentrations. The assay exhibited good linear relationship between peak area ratios and serum concentration. Quantification limit was at least 25 ng/ml of glibenclamide and accuracy and precision were over the concentration range of 50-500 ng/ml. Assay was successfully applied to the measurement of glibenclamide in serum for therapeutic drug monitoring.

Key words: Glibenclamide, human serum, TDM, RPHPLC

Glibenclamide is an effective, long-acting, second generation sulfonylurea used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM)¹. It occurs as a white or almost white, odourless crystalline powder. Solubility of the drug in water is approximately 4 µg/ml at pH 4 and 600 µg/ml at pH 9 and 3 mg/ml in alcohol. The drug has a pK_a of 6.8. It decreases blood glucose levels by stimulating insulin release from functioning beta cells in the pancreas. After prolonged administration, the hypoglycemic effects of the drug appear to be related to extra-pancreatic effects possibly including reduction of basal hepatic glucose production and enhanced peripheral sensitivity to insulin, the later may result either from an increase in the number of insulin receptors or from changes in events subsequent to insulin binding².

Studies conducted in western population reveal that the terminal elimination half-life of the drug is 1.4-1.8 h (0.7-3 h)³. The usual initial adult dosage is 2.5-5 mg daily. The adult maintenance dose for type II diabetes ranges from 1.25-20 mg daily⁴. The maximum recommended dosage is 20 mg daily. Following oral administration of a single 5 mg dose, the drug appears

in the plasma or serum within 15-60 min and average peak plasma or serum concentration of approximately 140-350 ng/ml usually are attained within 2-4 h^{5,6}. The glibenclamide at a concentration of 0.05-10 µg/ml *in vitro*, is more than 99% bound to serum proteins and its metabolite 4-trans-hydroxyglibenclamide, is more than 97% bound to serum proteins⁷. A number of assay methods for use in pharmacokinetic studies are available for determination of glibenclamide in biological specimen⁸⁻¹⁴ (Table 1). Here we describe a high performance liquid chromatographic method for the estimation of glibenclamide in human serum. The method can be used in pharmacokinetic studies of glibenclamide where multiple, serial blood sample collection is desired in individuals.

MATERIALS AND METHODS

Glibenclamide (Zydus Cadila, India) and glimepride (Micro Laboratories, India) were obtained as gift samples. Methanol, acetonitrile, potassium dihydrogen ortho-phosphate and ortho-phosphoric acid were purchased from Qualigens Fine chemicals (Mumbai, India). Water for analytical purpose was obtained from Milli-Q R-O system.

Chromatographic conditions:

The HPLC system consisted of a Shimadzu LC-

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TABLE 1: PREVIOUSLY PUBLISHED ASSAY METHODS FOR GLIBENCLAMIDE IN BLOOD FLUIDS

Species	Matrix	Volume (ml)	Validated LLQ (ng/ml)	Reference
Human	Serum	1	5	[8]
Human	Plasma	0.2	5-10	[9]
Human	Plasma	0.5	10-400	[10]
Human	Serum	0.5	NS	[11]
Human	Serum	0.5	NS	[12]
Human	Serum	0.5	20-400	[13]
Dog	Serum	1	20	[14]

The above table provides the references of the previously published studies on the development of high-performance liquid chromatographic method for the analysis of glibenclamide from blood of animals and human. NS: Not Stated

10AT liquid chromatographic pump, SIL-10a manual injector and SPD-10A UV/Vis UV absorbance detector (Shimadzu, Kyoto, Japan). Data collection, integration and calibration were accomplished using Class VP Chromatography Data System Version 4.2 computer software (Shimadzu, Kyoto, Japan). The chromatographic separations of glibenclamide and internal standard (glimepride) were accomplished using a 250 mm×4.6 mm ID Luna phenomenex 5u C18 analytical column (Phenomenex, USA). A Guard-Pak precolumn module (Phenomenex, USA) containing an ODS cartridge insert was placed serially just before the analytical column. The mobile phase consisted of acetonitrile:25 mM phosphate buffer (pH: 3.5) in a combination of 60:40 v/v. Before use the mobile phase was degassed by passing it through a 0.22 µm filter.

The mobile phase was pumped at an isocratic flow rate of 1 ml/min at room temperature. The UV detection wave length was set at 253 nm. The wave-length of 253 nm represented the UV maximum of glibenclamide in acetonitrile: water in 1:1 ratio.

Assay procedure:

A stock solution representing 100 µg/ml of glibenclamide was prepared in acetonitrile: water in 1:1 ratio. These solutions were stored at -20° until use. Glibenclamide was found to remain stable for at least 3 mo. under these conditions, based on our preliminary findings. The working standard solutions were prepared prior to use from the stock solution by sequential dilution with a combination of acetonitrile: water in 90:10 ratio to yield final concentrations of 50, 100, 200, 400, and 500 ng/ml of glibenclamide. The internal standard stock solution was prepared by dissolving 1 mg of glimepride in 100 ml of acetonitrile: water in 1:1 ratio. This solution was stored at -20° until use.

In a 2 ml microcentrifuge tube, 500 µl of serum was added along with 500 µl of internal standard solution. The serum was precipitated by the addition of 500 µl of methanol, and then the tubes were vortexed for 30 s and centrifuged at 5000 g for 8 min. The supernatant was transferred to a clean, similarly labeled tube and was subsequently re-centrifuged for 2 min. The resulted solution was injected in to the HPLC.

Assay parameters:

The extraction efficiency was determined by comparing the peak area of known amounts glibenclamide (unextracted) in mobile phase directly injected to peak area of samples containing the same amounts of glibenclamide in plasma after extraction.

Quantification was based on calibration curves constructed using peak area ratios of drug to internal standard vs. nominal concentration. Intra-day reproducibility was tested by using four different concentrations (50, 100, 200, 400, 500 ng/ml). The procedure was repeated on three separate days to allow determination of inter-day precision and accuracy. Intra-day accuracy was estimated based on the mean percentage error, and the inter-day accuracy was calculated as the mean of the intra-day accuracy determinations. The precision, expressed as a percentage, was evaluated by calculating the intra- and inter-day relative standard deviations. The standard drug solutions in varying concentrations ranging from 50 ng/ml to 500 ng/ml were examined by the assay procedure. The peak area was calculated. The calibration curve was plotted using peak area vs concentration of the standard solutions.

Patient samples:

Patient serum samples were obtained from patients who were on long term and short term oral glibenclamide therapy. The protocol was approved by the institutional ethics committee. Five blood samples were collected i.e 0.5-2 h, 2-5 h, 5-10 h and 24 h post dose of glibenclamide administration. The samples were placed on ice immediately after collection. Subsequently, serum samples were kept frozen at -70° until analysis. Blank serum samples were collected from volunteers for method validation and were stored at -70°.

RESULTS AND DISCUSSION

Peaks corresponding to internal standard, and

glibenclamide eluted free of interfering substances, at 9.6 and 8.5 min, respectively (fig.1). The analytical run time was 12 min for each serum sample. The mean extraction efficiencies of glibenclamide from 500 μ l of serum at a concentration of 50-500 ng/ml were 90-93%. Good linear relationships were obtained between peak area ratios and the corresponding plasma concentration over a range of 50-500 ng/ml of glibenclamide. The calibration curve shows a linear response over the range of concentrations used in the assay procedure. The calibration curve passes through the origin, which justifies the use of single point calibration.

The inter-day and intra-day coefficients of variation were less than 2% (Table 2). Over the range of

concentrations from 50-500 ng/ml, the intraday accuracies ranged from 95.1% to 98.9%, and average interday accuracy ranged from 94.9% to 98.8%. Based on this data, the validated lower limit of quantification of the method was 25 ng/ml based on 500 μ l of human plasma. In patients taking a dose of 5-15 mg/day of glibenclamide, serum samples were analyzed using the described HPLC method (fig. 2).

The HPLC method described here was accurate and precise, and capable of determining concentrations of glibenclamide in small volumes of human serum. The extraction procedure was simple and the procedure used a commercially available internal standard (glimepride). The method performed well with respect to reproducibility and accuracy over the range of

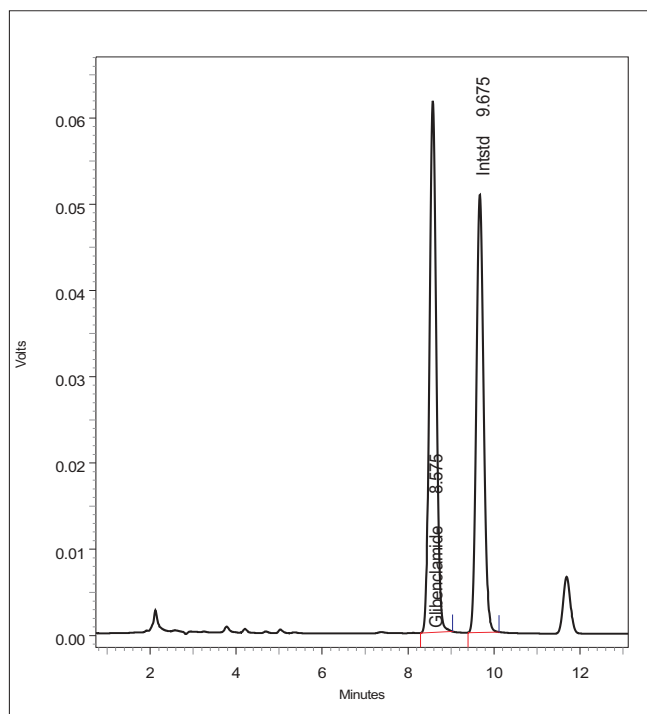


Fig. 1: Chromatogram of standard solution and internal standard. The above figure shows the chromatogram obtained from the HPLC analysis of standard solutions of glibenclamide along with the internal standard, glimepride

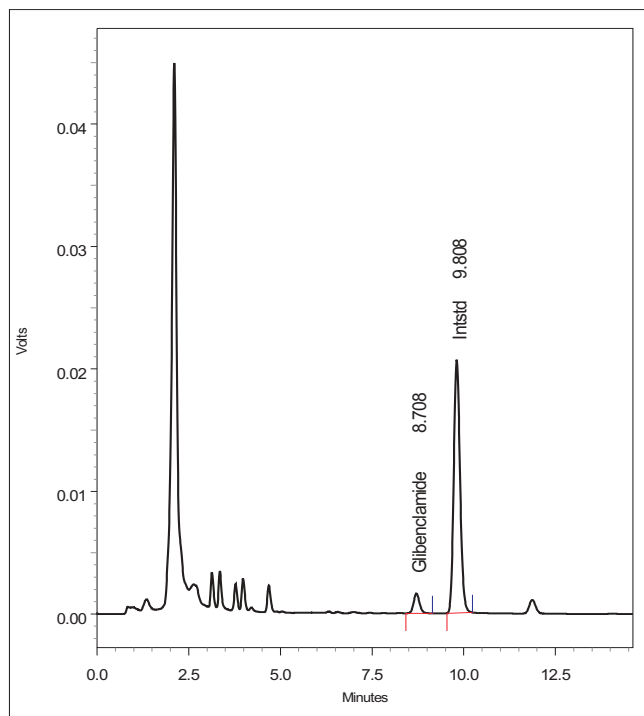


Fig. 2: Chromatogram of standard solution and internal standard. The above figure shows the chromatogram obtained from the HPLC analysis of standard solutions of glibenclamide along with the internal standard, glimepride, in serum.

TABLE 2: INTRADAY AND INTERDAY PRECISION AND ACCURACY DATA

Drug	Added Concentration (ng/ml)	Intraday (n=5)			Interday (n=5)		
		Observed concentration (ng/ml)	CV %	Accuracy %	Observed concentration (ng/ml)	CV %	Accuracy %
Glibenclamide	50	47.56	1.9728	95.13	47.46	1.1634	94.92
	100	97.65	0.9963	97.64	97.48	0.8689	97.48
	200	196.92	0.4442	98.45	196.55	0.3853	98.27
	400	395.99	0.1673	98.99	395.63	0.2476	98.90
	500	494.55	0.1054	98.90	494.47	0.1697	98.89

The above table provides information regarding the intra-day and inter-day precision and accuracy data for the analysis of glibenclamide from human serum samples

concentrations studied (Table 2). This assay method was rapid; preparation of 110 samples took 8 h from initial protein precipitation to final placements of samples in the HPLC. The chromatographic run time was less than 12 min. The lower limits of quantification and the small plasma necessary for this assay make the assay suitable for studying glibenclamide pharmacokinetics.

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