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Original Article

Development of validated HPLC-UV method for simultaneous determination of Metformin, Amlodipine, Glibenclamide and Atorvastatin in human plasma and application to protein binding studies ☆

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ABSTRACT

A simple, sensitive, fast, and economical HPLC method was developed and validated for simultaneous estimation of two fixed dose combinations frequently prescribed in diabetes (Metformin plus Glibenclamide) and hypertension with dyslipidemia (Amlodipine plus Atorvastatin) in Human plasma for the first time. The validated HPLC method was used to quantify the concentration of selected actives in ultrafiltrate. Optimum separation conditions were obtained with Water's Novapack Phenyl (150 mm × 4.6 mm, i.d., 5.0 μm) column with mobile phase consisting of 0.1% Phosphoric acid (pH 3.0) and acetonitrile (ACN) in gradient mode with column oven temperature maintained at 30 °C and elution monitored by a UV detector at 227 nm. Protein precipitation was employed to extract the selected analyte form human plasma. The recoveries were more than 90% for all analytes in cold aqueous 10% trichloroacetic acid (TCA) and acetonitrile. The optimized HPLC-UV was validated in the calibration range of 10–10,000 ng mL^{-1} for Metformin, 25–5000 ng mL^{-1} for amlodipine, 50–10,000 ng mL^{-1} for glibenclamide and 10–5000 ng mL^{-1} for atorvastatin. The mean relative error was least when weighing of $1/\times^2$ was applied for calibration curve. The accuracy of samples for six replicate measurements at LLOQ level was within limit. The precision and accuracy of samples for six replicate measurements at LLOQ level was within limit. The validated method was applied for quantitation of selected analytes in ultrafiltrate from protein binding experiments. A four to five fold increase in unbound fraction was observed when spiked to human serum albumin. Further the unbound fraction of highly albumin bound drugs was increased nearly to double when incubated with Gly-HSA as compare to HSA.

1. Introduction

Type 2 diabetes (T2DM) involves alterations in a number of metabolic pathways. In addition to alteration in glucose metabolism, patients with T2DM are more likely to have concomitant/secondary complications such as adverse cardiac events [1], hypertension [2] and dyslipidemia [3] that may lead to microvascular and macrovascular complications.

Patients with T2DM have a two to four time higher death toll due to cardiovascular diseases than people without the diabetes [4]. Precise pharmacotherapies for hypertension and dyslipidemia to modify multiple microvascular and macrovascular events reduce the risk of such events [5] and mortality [6] in patients with T2DM. Rigid control over blood pressure and setting goals, below those for the non-diabetic subject, have been shown to be effective in lowering cardiovascular events and mortality in the diabetic patient [7]. To achieve these goals in most cases, three to six actives from different pharmacotherapies need to be prescribed [8–11]. The primary therapy for suppression of the renin–angiotensin system (RAS) should be angiotensin 2 receptor blockers and/or angiotensin-converting enzyme inhibitors/calcium channel blocker with a renin inhibitor [12].

The primary oral antidiabetic (OAD) pharmacotherapies for T2DM are metformin alone or in combination with the second generation sulfonylureas *viz.* gliclazide, glimperide, glipizide and

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Glibenclamide [13]. Combinations of metformin and glipizide or gliclazide or glibenclamide are available commercially as single tablets. Recently, several fixed dose combinations of Metformin with sulfonylurea has been banned by Indian government [14] except for Metformin with glyburide (glibenclamide) [15]. Nevertheless Metformin and Glimepiride combination provides greater reductions in HbA1C and fasting plasma glucose compared with metformin plus glibenclamide in T2DM15 but glibenclamide provides effective, convenient and better tolerance as compare to other sulfonylureas when combined with Metformin [16]. Various analytical procedures have been reported for simultaneous estimation of Metformin and Glibenclamide *viz*. HPTLC, and liquid chromatographic methods coupled with UV-Spectroscopy [17], mass spectrometry [18] and tandem mass spectrometry [19].

Many patients with diabetes and cardiovascular disease require treatment for several different pharmacotherapies, the concept of combining agents and/or fixed dose combinations with distinctly different indications has become a veracity of treatment [20]. A fixed-dose combination of atorvastatin and amlodipine is commonly prescribed as first line concomitant treatment to diabetic hypertensive subjects [21]. Single pill therapy or fixed dose combination of Amlodipine and atorvastatin creates positive effect on fibrinolytic balance and better BP control in hypertensive patients with T2DM [22].

Though Metformin does not bound to serum albumin (albumin binding <5%) but it induce conformational change in serum albumin structure [23]. Metformin inhibits the production of glycated albumin in a concentration dependent fashion [24] and reduce production of toxic dicarbonyls and Advanced Glycation End products (AGEs) [25]. Sulfonylurea drugs are extensively bounded to serum albumin and displacement of sulfonylurea drugs from their respective binding sites, predominantly by non-ionic actives may create short term to severe hypoglycemic state in diabetic patients [26]. Clarithromycin have been reported to displace Glipizide and Glibenclamide from their protein binding sites, thereby increasing the unbound or free fraction of the drug [27].

An elevated level of glycated albumin has been observed in the diabetic patients [28]. About 30% of serum albumin has been reported to be glycated *via* non-enzymatic addition of reducing sugars or their reactive degradation products to primary or secondary amine groups on albumin [29,30]. The glycosylation of serum albumin is time dependent non-enzymatic process [24] and it impairs the binding capacity of high protein bound drugs [31].

Given this background, the present study was envisaged to evaluate the effect of amlodipine and atorvastatin combination on serum albumin and glycated albumin binding capacity of metformin and Glibenclamide *vice versa*. The selected actives were quantified in filtrate of ultrafiltration protein binding experiments using validated liquid chromatographic method coupled to UV detection. The HPLC-UV method was validated as per USFDA guideline on bioanalytical method validation [32].

2. Experimental

2.1. Chemical and reagents

Qualified standards of Amlodipine besylate, Glibenclamide, Atorvastatin calcium, Ranitidine Hydrochloride and Rosiglitazone were a gift from Torrent Research Centre (Ahmadabad, India). The qualified standards of Metformin Hydrochloride, recombinant Human Serum Albumin (rHSA) and glycated Human Serum Albumin (Gly-HSA) were purchased from Sigma Aldrich (Bangalore, India). Analytical/HPLC grade chemicals and solvents were obtained from Ranbaxy Fine Chemicals Limited (Delhi, India). Amicon® Ultra centrifugal filtration devices (MWCO: 10 kDa) were purchased from Millipore (Bangalore, India).

2.2. Chromatography apparatus and conditions

A high-performance liquid chromatographic system (*JASCO*, Kyoto, Japan) composed of a PU-2089 plus Quaternary pump solvent delivery module, a manual rheodyne injector with a 20 μL fixed loop and a UV-2075 intelligent UV–Visible detector. For statistical calculations in bioanalytical method validation Graphpad PRISM® version 5.1 for Windows, (Graphpad software Inc., California, USA) software was used.

AMLO, GLBN and ATOR were less soluble in water and freely soluble in selected organic solvents like methanol (MeOH), acetonitrile (ACN), whereas, the solubility conditions were *inverse* for MET. The chromatographic conditions were optimized by different means (using different columns, different mobile phase combination and different organic phases). Early chromatographic work was performed with different brands of C_8 and C_{18} columns as stationary phase and various combinations of mobile phase with pH in the range of 2.5–4.0, organic phases (ACN and/or Methanol), stepwise. The flow rate of mobile phase was varied within 1.0–1.5 mL min^{-1}. Wavelength for monitoring the eluent was selected by scanning standard solution of drugs within 400 to 200 nm using double beam UV–Visible spectrophotometer (Shimadzu 1800, Japan).

Different HPLC methods were developed and optimized for retention of MET & GLBN and AMLO & ATOR and combination of all actives.

Optimum separation conditions were obtained with a Water's Nova pack Phenyl (150 mm × 4.6 mm, i.d. 5.0 μm) column with mobile phase consisting of 0.1% Phosphoric acid (pH 3.0) and acetonitrile (ACN) in gradient mode with column oven temperature maintained at 30 °C and elution monitored by a UV detector at 227 nm.

All noted measurements were performed with an injection volume of 20 μL of samples dissolved in a diluent consist of aqueous phase and organic phase in the ratio of 2:3, respectively. During development of bioanalytical procedure, diluent was changed accordingly.

2.3. Preparation of standard and resolution solution

Diluted standard solutions of each analyte representing 10 μg mL^{-1} concentrations were prepared with diluent. Ranitidine (IS-1) was used as internal standards for hydrophilic analyte *i.e.* MET. Rosiglitazone (IS-2) was used as internal standards for lipophilic analytes such as AMLO, GLBN and ATOR. All-in-one resolution solution containing 10 μg mL^{-1} each of MET, AMLO, GLBN and ATOR and 5.0 μg mL^{-1} each of IS-1 and IS-2 was prepared from respective stock solutions.

For optimization purposes, a 20 μL of resolution solution was injected into chromatograph and system suitability parameters *viz*. % RSD of peak area for six injections of all analytes, % RSD of retention time for six injections of all analytes, peak asymmetry factor at 10% peak height and resolution were studied.

2.4. Sample preparation and extraction

The protein precipitation was the preferred choice of separation because of the minimized steps in extraction of drug from matrix. The method was attempted using 10% trichloroacetic acid (TCA) and ACN and combination thereof. It was carried out by and 1 part of plasma, 0.1 part drug and 0.05 parts of I.S. Diluted standard solution containing all analytes (15 μL) was added to 150 μL of plasma previously spiked with internal standards in a 1.5 mL capacity micro-centrifuge tube. The blend was subjected to vortex for about 5 min. The mixture was allowed to stabilize for two minutes then 100 μL of cold aqueous 10% (w/v) TCA was added and subjected to

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