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Research Article

VALIDATED HPLC METHOD FOR SIMULTANEOUS QUANTITATION OF BENFOTIAMINE AND METFORMIN HYDROCHLORIDE IN BULK DRUG AND FORMULATION

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ABSTRACT

A simple, sensitive and rapid reverse phase high performance liquid chromatographic method was developed for the estimation of Benfotiamine (BEN) and Metformin Hydrochloride (MET) in pure and in pharmaceutical dosage forms. Thermo Hypersil BDS–C_{18} Column (250 mm × 4.6 mm, 5.0 µ Germany) with isocratic conditions was used with a mobile phase containing mixture of Methanol and Aq. Phosphate buffer (10mM of Potassium Dihydrogen Phosphate adjusted to 3.2 with ortho phosphoric acid) in the ratio of 80: 20. The flow rate was 1 ml/min and effluents were monitored at 239nm and eluted at 2.583 min (BEN) and 3.233 min (MET). Calibration curve was plotted with a range of 1-6 µg/ml for BEN and 0.1-5 µg/ml for MET. The assay was validated for the parameters like accuracy, precision, robustness and system suitability parameters. The proposed method can be useful in the routine analysis for the determination of Benfotiamine and Metformin Hydrochloride in pharmaceutical dosage forms.

Keywords: Benfotiamine, Metformin Hydrochloride, HPLC, Validation.

INTRODUCTION

Benfotiamine, *S*-[(2*Z*)-2-{[(4-amino-2-methylpyrimidin-5-yl) methyl] (formyl) amino}-5-(phosphonooxy) pent-2-en-3-yl] benzenecarbothioate, [**Fig. 1]** is used in the treatment of Diabetes. It increases the transketolase activity, an important enzyme in glucose metabolism which results into blockage of three major molecular pathways leading to hyperglycemic damage. It prevents the increase in UDP-N-acetyl glucosamine (UDPGlc-NAc) and increases hexosamine pathway activity that decreases the buildup of detrimental glucose metabolites leading to advanced glycation end products (AGE). It also normalizes protein kinase C (PKC) activity and prevents nuclear factor –kappa (NF-Kb) activation in the retina of diabetics. It can also be used to correct the imbalance in the polyol pathways by decreasing aldose reductase activity, sorbitol concentrations and intracellular glucose thereby protecting endothelial cells from glucose induced damage by normalizing cell replication rates and decreasing apoptosis. Animal models of diabetic limb ischemia demonstrated these mechanisms are responsible for improving post–ischemic healing. Benfotiamine enhancement in transketolase activity in erythrocytes and renal glomeruli protects the kidneys from glucose induced damage and prevents the development of diabetic neuropathy. In alcoholics and patients of chronic renal disease Benfotiamine corrects thiamine deficiency and can decrease the incidence of neuropathies. [1]

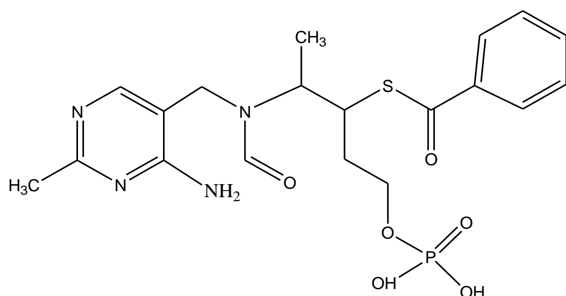

Fig. 1: Structure of Benfotiamine

Metformin Hydrochloride 1, 1-dimethylbiguanide hydrochloride [**Fig. 2]** is taken as first line drug of choice for the treatment of type-2 diabetes and also used in treatment of polycystic ovary disorder. It can also be used for other diseases where insulin resistance is an important factor. Metformin improves hepatic and peripheral tissue sensitivity to insulin without the problem of serious lactic acidosis. [2]

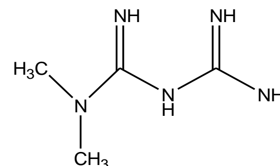

Fig. 2: Structure of Metformin Hydrochloride

Literature review reveals that methods have been reported for analysis of Benfotiamine and Metformin Hydrochloride. HPLC method for determination of Benfotiamine alone or in combination with other drugs in pharmaceutical dosage form has been reported[3,4,5], determination of Metformin Hydrochloride alone or in combination with other drugs by HPTLC [6,7], simultaneous estimation of Metformin Hydrochloride by RP-HPLC method from solid dosage forms [8,9,10,11,12,13,14], simultaneous analysis of Metformin and other drugs by RP-HPLC and its applications in pharmaceuticals and human serum [15], ssimultaneous determination of Metformin, Cimetidine, Famotidine and Ranitidine in Human serum and dosage formulations using HPLC with UV detection[16], stability-indicating HPTLC method for simultaneous determination of Nateglinide and Metformin Hydrochloride in pharmaceutical dosage form[17], simultaneous determination of Metformin and Rosiglitazone in human plasma by LC-MS with Electro spray Ionization[18], LC-MS method for simultaneous determination of Antidiabetic drugs Metformin and Glyburide in human plasma[19], simultaneous spectrophotometric estimation and validation of Metformin alone or in combination with other drugs[20,21,22], spectrophotometric quantitation of Metformin in bulk drug and pharmaceutical formulations using multivariate technique[23].

To date, there have been no published reports about the simultaneous quantitation of Benfotiamine and Metformin Hydrochloride by HPLC in bulk drug and in tablet dosage form. This present study reports simultaneous quantitation of Benfotiamine and Metformin Hydrochloride by HPLC in bulk drug and in tablet dosage form. The proposed method is validated as per ICH guidelines.

MATERIALS AND METHODS

Materials

Zeel Pharmaceuticals Pvt. Ltd Mumbai supplied pure drug sample of BEN of Batch No.: 20090304 and Sohan Healthcare Pvt. Ltd. Kurkumbh (Pune). India, kindly supplied pure drug sample of MET as a gift sample of Batch No.: MH/49707/11. They were used without further purification and certified to contain 99.80 % (w/w) for BEN and 99.6 % (w/w) for MET on dried basis. All chemicals and

reagents used were of HPLC grade and were purchased from Merck Chemicals, India.

Instrumentation

The HPLC system consisted of a Pump (model Jasco PU 2080); Intelligent LC pump with sampler programmed at 20 µl capacity per injection was used. The detector consisted of UV/ VIS (Jasco UV 2075) model operated at a wavelength of 239 nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system. The column used was Thermo Hypersil BDS–C_{18} (250 mm × 4.6 mm, 5.0 µ) from Germany.

Preparation of Standard Stock Solutions

Standard stock solution of concentration 1000 µg/ml of BEN and 1000 µg/ml of MET was prepared using methanol. From the standard stock solution, the mixed standard solutions were prepared to contain 3.75 µg/ml of BEN and 25 µg/ml of MET. The stock solution was stored at 2-8 °C protected from light.

Optimization of HPLC Method

The HPLC procedure was optimized with a view to develop a simultaneous assay method for BEN and MET respectively. The mixed standard stock solution (3.75 µg/ml of BEN and 25 µg/ml of MET) was injected in HPLC. For HPLC method optimization different ratios of methanol and aqueous buffer (10mM of Potassium Dihydrogen Phosphate pH-3.2 adjusted with OPA) were tried but it was found that methanol: aqueous buffer in the ratio 80: 20 v/v, at a flow rate 1 ml/min gives acceptable retention time (tR), plates and good resolution for BEN and MET **[Fig. 3]**.

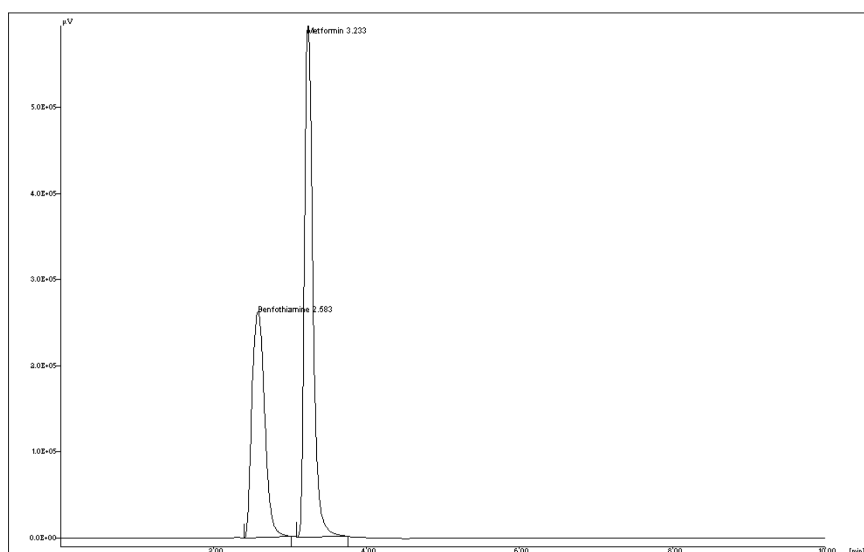

Fig. 3: HPLC chromatogram of standard BEN and MET (3.75µg/ml and 25µg/ml)

Validation of the Method

Validation of the optimized HPLC method was carried out with respect to the following parameters.

Linearity and Range

The mixed standard stock solution (3.75µg/ml of BEN and 25 µg/ml of MET) was further diluted to get BEN and MET concentration in the range of 1-6 µg/ml and 0.1-5 µg/ml respectively. Linearity of the method was studied by injecting six concentrations of the drug prepared in the mobile phase in triplicate into the LC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations 1, 3, 5 µg/ml for BEN and 0.1, 2, 4 µg/ml for MET six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days.

Limit of detection and limit of quantitation

Limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ respectively. To determine the LOD and LOQ serial dilutions of mixed standard solution of BEN and MET was made from the standard stock solution. The samples were injected in LC system and measured signal from the samples was compared with those of blank samples.

Robustness of the method

To evaluate robustness of a HPLC method, few parameters were deliberately varied. The parameters included variation of flow rate, percentage of methanol in the mobile phase and solvents from different lot were taken. Robustness of the method was done at three different concentration levels 1, 3, 5 µg/ml and 0.1, 2, 4 µg/ml for BEN and MET respectively.

Specificity

The specificity of the method towards the drug was established through study of resolution factor of the drug peak from the nearest resolving peak.The peak purity of BEN and MET was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E). Effect of excipients of formulation was studied for whether it interfered with the assay.

Accuracy

Accuracy of the method was carried out by applying the method to drug sample (BEN and MET combination tablet) to which known amount of BEN and MET standard powder corresponding to 80, 100 and 120 % of label claim had been added (Standard Addition Method), mixed and the powder was extracted and analyzed by running chromatogram in optimized mobile phase.

Analysis of a marketed formulation

To determine the content of BEN and MET in conventional tablet (Brand name: Benforce-M, Label claim: 75 mg BEN and 500 mg MET per tablet), twenty tablets were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate

equivalent to75 mg of BEN and 500 mg of MET was transferred into a 100 ml volumetric flask containing 60 ml methanol, sonicated for 30 min and diluted up to 100 ml with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and the drug content of the supernatant was determined (7.5 µg/ml and 50 µg/ml for BEN and MET respectively). Supernatant was taken and after suitable dilution the sample solution was then filtered using 0.45 µ filter (Millipore, Milford, MA). The above stock solution was further diluted to get sample solution of 3.75µg/ml and 25µg/ml for BEN and MET respectively. A 20 µl volume of sample solution was injected into HPLC, six times, under the conditions described above. The peak areas were measured at 239nm and concentrations in the samples were determined using multilevel calibration developed on the same HPLC system under the same conditions using linear regression equation.

RESULTS AND DISCUSSION

The results of validation studies on simultaneous estimation method developed for BEN and MET in the current study involving methanol: Aqueous phosphate buffer (80: 20 v/v) (10 Mm Potassium Dihydrogen Phosphate pH-3.2 adjusted with ortho phosphoric acid) is given below.

Linearity

BEN and MET showed good correlation coefficient (r^2 = 0.9991 for BEN and 0.9993 for MET) in given concentration range (1-6 µg/ml for BEN and 0.1-5 µg/ml for MET). The mean values of the slope and intercept were 132464 ± 1.18 and 2180.2 ± 1.82 for BEN and 179670 ± 1.21 and 73459 ± 1.09 for MET respectively.

Precision

The results of the repeatability and intermediate precision experiments are shown in **Table 1.** The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2 %, respectively as recommended by ICH guidelines.

LOD and LOQ

Signal-to-noise ratios of 3:1 and 10:1 were obtained for the LOD and LOQ respectively. The LOD and LOQ were found to be 0.01µg/ml and 0.1µg/ml for BEN and 0.005 µg/ml and 0.01µg/ml MET respectively.

Robustness of the method

Each factor selected (except columns from different manufacturers) was changed at three levels (−1, 0 and 1). One factor at the time was changed to estimate the effect. Thus, replicate injections (n = 6) of mixed standard solution at three concentration levels were performed under small changes of three chromatographic parameters (factors). Insignificant differences in peak areas and less variability in retention time were observed **Table 2.**

Table 1: Precision studies

Concentration (µg/ml)	Repeatability (n=6)			Intermediate precision (n=6)		
	Measured conc. ± SD	(%) RSD	Recovery (%)	Measured conc. ± SD	(%) RSD	Recovery (%)
Benfotiamine						
1	1.01 ± 1.10	1.22	101.00	0.99 ± 0.76	1.18	99.00
3	2.98 ± 5.81	1.18	99.33	2.97 ± 2.03	0.96	99.00
5	4.90 ± 7.98	1.20	98.00	4.98 ± 5.84	0.99	99.06
Metformin Hydrochloride						
0.1	0.09 ± 0.59	0.78	99.00	0.098 ± 0.72	0.99	98.00
2	1.99 ± 1.85	1.07	99.5	1.98 ± 1.77	1.53	99.00
4	4.025 ± 2.65	1.43	100.5	3.99 ± 2.34	1.76	99.75

Table 2: Robustness testing [a] (n = 3)

Drug	Parameter	Factor [a]	Level	Retention time	Retention factor	Asymmetry
BEN	**A:**	0.9	-1	2.65	0.20	1.25
	Flow rate (ml/min)	1.0	0	2.58	0.24	1.21
		1.1	+1	2.51	0.29	1.18
		Mean ± SD		2.58 ± 0.07	0.24 ± 0.05	1.21 ± 0.04
	B: % of methanol in the mobile phase (v/v)	79	-1	2.63	0.19	1.24
BEN		80	0	2.58	0.22	1.21
		81	+1	2.53	0.26	1.19
		Mean ± SD		2.58 ± 0.05	0.22 ± 0.08	1.21 ± 0.03
	C: Solvents of different lots	First lot		2.58	0.29	1.21
BEN		Second lot		2.59	0.28	1.22
		Mean ± SD		2.58 ± 0.01	0.29 ± 0.01	1.21 ± 0.01
MET	**A: Flow rate (ml/min)**	0.9	-1	3.26	0.43	1.18
		1.0	0	3.21	0.39	1.14
		1.1	+1	3.17	0.45	1.10
		Mean ± SD		3.21 ± 0.05	0.39 ± 0.04	1.14 ± 0.04
MET	**B: % of methanol in the mobile phase (v/v)**	79	-1	3.27	0.44	1.19
		80	0	3.21	0.38	1.14
		81	+1	3.15	0.40	1.09
		Mean ± SD		3.21 ± 0.06	0.38 ± 0.02	1.11 ± 0.05
MET	**C: Solvents of different lots**	First lot		3.21	0.42	1.14
		Second lot		3.22	0.40	1.15
		Mean ± SD		3.21 ± 0.01	0.40 ± 0.01	1.14 ± 0.01

[a] Three factors were slightly changed at three levels (-1, 0, 1)

Table 3: Recovery study of BEN and MET (n = 6)

	Label claim (mg/tablet)	Amount added (mg)	Total amount (mg)	Amount Recovered (mg) ± % RSD	% Recovery
BEN	75	60 (80%)	135	98.87 ± 0.96	100.62
	75	75 (100%)	150	99.86 ± 1.01	99.86
	75	90 (120%)	165	111.26 ± 0.78	101.30
MET	500	400 (80%)	900	98.11 ± 1.16	99.33
	500	500 (100%)	1000	100.6 ± 1.40	100.85
	500	600 (120%)	1100	110.01 ± 0.98	102.25

Table 4: Analysis of commercial formulation

Lot	Amount added	Mean ± SD (n= 6)	Recovery (%)
BEN (75 mg)			
1st Lot	(500mg)	74.85 ± 0.98	99.80
2nd Lot	(500 mg)	75.08 ± 1.45	100.10
MET(500 mg)			
1st Lot	(75 mg)	498.32 ± 1.23	99.66
2nd Lot	(75 mg)	501.23 ± 1.01	100.24

Specificity

The peak purity of BEN and MET was assessed by comparing their respective spectra at the peak start, apex and peak end positions i.e., r (S, M) = 0.9989 and r (M, E) = 0.9996. A good correlation (r = 0.9994) was also obtained between the standard and sample spectra of BEN and MET respectively. Also, excipients from formulation were not interfering with the assay.

Recovery Studies

As shown from the data in **Table 3** good recoveries of the Benfotiamine and Metformin Hydrochloride in the range from 99.33 to 102.25 % were obtained at various added concentrations.

Analysis of formulation

Experimental results of the amount of BEN and MET in tablets, expressed as a percentage of label claims were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients which are normally present. The drug content was found to be 99.2 % for BEN and 100.6 % for MET. Two different lots of BEN and MET combination tablets were analyzed using the proposed procedures as shown in **Table 4.**

CONCLUSION

HPLC method was developed and validated as per ICH guidelines. UV detection allowed an accurate quantitation of chromophoric compounds.

The drug was analysed by HPLC method using Thermo Hypersil BDS–C_{18} (250 mm × 4.6 mm, 5.0 µ) from Germany with isocratic conditions and simple mobile phase containing methanol: Aqueous phosphate buffer (10mM of Potassium Dihydrogen Phosphate adjusted to pH-3.2 with OPA) (80: 20 v/v) at flow rate of 1 ml/min using UV detection at 239nm. The procedure has been evaluated for the linearity, accuracy, precision and robustness in order to ascertain the suitability of the analytical method. The method was also applied to marketed samples. It has been proved that the method is selective and linear between concentration range 1-6 µg/ml for BEN and 0.1-5 µg/ml for MET. LOD was found to be 0.0 1 µg/ml and LOQ was found to be 0.1µg/ml for BEN and LOD was found to be 0.005 µg/ml and LOQ was found to be 0.01µg/ml for MET.

Statistical analysis proves that the method is suitable for the analysis of BEN and MET as bulk drug and in pharmaceutical formulation without any interference from the excipients. It may be extended to study the degradation kinetics of BEN and MET and also for its estimation in plasma and other biological fluids.

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