Journal of the Association of Arab Universities for Basic and Applied Sciences (2017) **22**, 70–75

University of Bahrain

Journal of the Association of Arab Universities for Basic and Applied Sciences

www.elsevier.com/locate/jaaubas
www.sciencedirect.com

ORIGINAL ARTICLE

HPLC method development, validation and its application to investigate *in vitro* effect of pioglitazone on the availability of H_1 receptor antagonists

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Received 21 June 2015; revised 17 November 2015; accepted 17 December 2015
Available online 3 February 2016

KEYWORDS

Pioglitazone;
Fexofenadine;
Cetirizine;
Levocetirizine;
Interaction;
HPLC

Abstract The method has been developed and validated for the simultaneous determination of pioglitazone and H_1-receptor antagonists (fexofenadine, cetirizine and levocetirizine) in formulations and human serum. Utilizing HPLC techniques, an assay was designed to determine the *in vitro* effects of pioglitazone on H_1-receptor antagonists. Obtained results were verified using the UV spectrophotometric technique. First-derivative values versus concentrations were used to plot calibration curves of these drugs and were found to similar with the HPLC data. The availability of pioglitazone remained unchanged in absence or presence of fexofenadine, cetirizine and levocetirizine. This *in vitro* analysis confirms the harmless co-administration of pioglitazone and H_1-receptor antagonists, and can serve as the foundation for designing further *in vivo* studies.

1. Introduction

In current days, a close inter-relationship has been identified between inflammation, insulin resistance and lipid disorders in diabetic and non-diabetic patients (Haffner et al., 2002). Thiazolidinediones are peroxisome proliferator-activated receptor gamma (PPARγ) agonists that target insulin resistance directly (Irons et al., 2006; Qayyum and Schulman, 2006). Pioglitazone reduced the risk of the primary endpoint and secondary macro-vascular trial in a patient with type 2 diabetes (Erdmann and Wilcox, 2008).

Histamine H_1-receptor antagonists are used for the treatment against allergic disorders, particularly rhinitis, conjunctivitis, dermatitis, urticaria and asthma (Chen et al., 2003). Long term administration of histamine H_1-receptor antagonists may have a depressing effect on central nervous system in diabetic patients (Kamei et al., 2005). Moreover, second generation histamine H_1-receptor antagonists cause sedation by crossing blood brain barrier in diabetic patients (Stauber et al., 1981).

Controlling blood sugar levels are crucial for diabetics having several allergic disorders and depression is more prevalent in diabetic patients than the general population (Anderson

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Peer review under responsibility of University of Bahrain.

http://dx.doi.org/10.1016/j.jaubas.2015.12.001
1815-3852

et al., 2001). Co-administration of anti-diabetic drugs with H_1 receptor antagonists is common. This co-administration increases the risk for significant drug–drug interactions. *In vitro* drug interaction studies of H_1-receptor antagonists have also been reported in the literature (Kenawi et al., 2005a,b), but these studies contains lacks of physiological conditions and typically the lack of other confounding physiological processes.

The robust and non classical methods have an important application in the pharmaceutical industry associated with drug development and drug interaction processes such that strong, dependable information can be put into a clinical framework. These assays must be able to clinically examine for suitable drug interaction studies. As in combination therapies, co-administration of drugs increases the risk for significant drug–drug interactions.

Currently, it is important to ponder drug–drug interactions using appropriate *in vitro*/*in vivo* studies to guide clinical interaction studies. The goal of designing a method for *in vitro* interaction studies is the prediction of clinical parameters. *In vitro* techniques offer a complete means to create a huge amount of data using minimum resources. Investigation of *in vitro* drug interactions has also been reported by using minimum standards (Arayne et al., 2008, 2010c,d; Mirza et al., 2013b; Shamshad et al., 2014). These suggested that *in vitro* approaches should be properly characterized; validated and proper controls should be incorporated in routine use. Of the available *in vitro* techniques, dissolution test model offers several advantages. In pharmaceutical industry, this is commonly used to conduct formulation design. It is the only test that measures the rate of *in vitro* drug release, which can simulate *in vivo* drug release (Brown et al., 2004).

The present paper describes the design and validation of simultaneous methods for the determination of pioglitazone, fexofenadine, cetirizine or levocetirizine using HPLC techniques, which are robust, convenient and specific, have good dynamic range, make interpretable results of significance to clinicians and have reasonable throughput. These are the co-prescribed and co-administered drugs. The present work also seeks to assess the actual suitability and general applicability of *in vitro* methods for interaction of the drugs, pioglitazone with fexofenadine, cetirizine and levocetirizine, through the application of dissolution test, HPLC and UV spectrophotometry techniques. An *in vitro* assay was established to demonstrate and identify potential drug–drug interaction. Existing assays carried out to assess the interactions of cetirizine, lacked uniformity of approach and were designed as such, not fulfilling the information requirements of clinical relevance neither proving of eminence to industries, but for routine application in drug discovery and development process (Kenawi et al., 2005a,b). The present study has not been made previously and therefore it provides a potential explanation for the observed effect.

Two negative *in vitro* interactions of H_1 receptor antagonists (Arayne et al., 2010d; Sultana et al., 2010) and one *in vitro* interaction of pioglitazone with losartan have been reported by UV spectrophotometry and HPLC techniques (Mirza et al., 2013a).

It is also observed that, there are several simultaneous HPLC methods reported for the determination of pioglitazone (Arayne et al., 2010a, 2011; Mirza et al., 2015, 2013a) and H_1 receptor antagonists (Arayne et al., 2010b) and a UV spectrophotometric method of H_1 receptor antagonist (Raghu and Basavaiah, 2012) but there no method for the simultaneous determination of pioglitazone, fexofenadine, cetirizine or levocetirizine has been reported by HPLC. The method was validated for the parameters like linearity, specificity, accuracy and intermediate precision, limit of detection and quantitation.

2. Experimental

2.1. Materials

Pioglitazone hydrochloride reference standard were gifted from PharmEvo (Private) Limited Karachi. Poze® (Pioglitazone 45 mg) tablets were purchased from a local pharmacy. The H_1-receptor antagonists, fexofenadine, cetirizine and levocetirizine were obtained from various pharmaceutical companies. Fexet® (30 mg), Zyrtec® (10 mg) and Xyzal® (45 mg) tablets were purchased from a local pharmacy. HPLC grade methanol was purchased from Merck, Germany.

2.2. Instrumentation

Double beam UV visible spectrophotometer (Shimadzu UV-1601) and HPLC, with LC-10 AT *VP* pump, SPD-10A *VP* UV–*vis* detector utilizing a Purospher® STAR RP-18 end capped (5 μm, 25 × 0.46 cm) column were used. Shimadzu Class-GC 10 software (version 2) was used for data acquisition. JP XIV type 2 apparatus (rotating paddle method, Model NTR-6100A, Toyama-sangyo, Osaka, Japan) with B.P. 2005 specifications was utilized for dissolution profiles (British Pharmacopoeia, 1998).

2.3. Chromatographic technique

2.3.1. Preparation of stock solutions

Stock solutions of pioglitazone and H_1-receptor antagonists of 100 μg mL^{-1} were prepared in methanol. Twenty tablets of each drug were weighed and triturated to obtain a homogeneous mixture. By dissolving the suitable amount of each powder in methanol, sample solutions at 100 μg mL^{-1} concentration of active substance were prepared.

2.3.2. Chromatographic conditions

Isocratic elution with mobile phase of methanol: water (65:35 v/v), pH of 2.55 adjusted with phosphoric acid with a flow rate of 1 mL min^{-1} and isosbestic wavelength at 230 nm was used for the development of the method. Diluents consisted of methanol: water (70:30 v/v) with analysis performed at room temperature (24 ± 2 °C). Representative chromatogram is shown in Fig. 1.

3. Method development

3.1. Assessment of linearity and recovery studies

Linearity was assessed by using two sets of five standard and recovery and matrix effects was observed using solutions at concentrations of 2.5–25 μg mL^{-1} for pioglitazone and 5–50 μg mL^{-1} for each H_1-receptor antagonists in methanol. The linearity of each standard curves were assessed by plotting

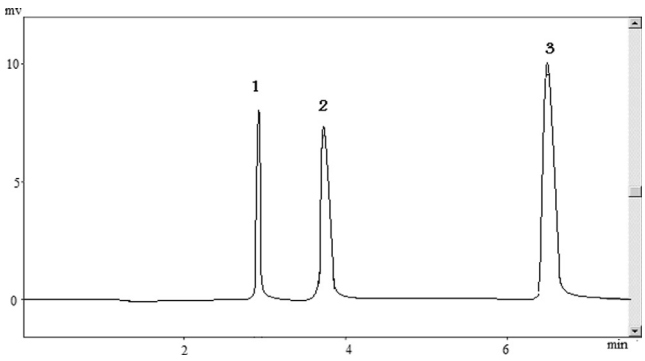

Figure 1 Representative chromatogram of pioglitazone (1), fexofenadine (2) and levocetirizine (3).

the peak area ratio of analytes versus its concentrations (Table 1). The mean relative standard deviation (%R.S.D.) was $<2.0\%$ and % recovery was above 99.74% for pioglitazone, 99.29% for fexofenadine, 99.01% for cetirizine and 99.60% for levocetirizine (Table 2). In the second set, five standard lines were constructed using plasma. The plasma samples were subjected to protein precipitation with methanol prior to a HPLC analysis. The reported method was used for serum analysis (Mirza et al., 2015, 2013a). 10 mL blood samples from ten healthy volunteers were collected and centrifuged at 3000 rpm for 10 min followed by the addition of 1 mL of methanol for protein precipitation. After centrifugation at 15,000 rcf for 5 min, clear extracts were collected, fortified with pioglitazone and H_1 receptor antagonists solutions and were injected into the HPLC column (Fig. 2). The recovery was expressed as the ratio of mean peak area of all drugs spiked to the mean peak area of the same analyte standards multiplied by 100 (Table 3).

3.2. Specificity

According to ICH "specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present in the sample matrix" (ICH, 2005). In order to determine the specificity of the method in the presence of pharmacopeial excipients (i.e. microcrystalline cellulose, croscarmellose sodium, magnesium stearate, lactose and hydro xypropylmethylcellulose), no peak of excipients was found in chromatogram, which proved that the method can be applied successfully to dosage formulation.

3.3. Accuracy and intermediate precision

Three consecutive measurements at three different concentrations of pioglitazone (8, 10 and 12 $\mu g\,mL^{-1}$) and H_1 receptor antagonists (16, 20 and 24 $\mu g\,mL^{-1}$) were performed for each concentration within the same day and on 3 different days (Table 4).

3.4. Limit of detection and quantitation

Limit of detection (LOD) and limit for quantitation (LOQ) of pioglitazone and H_1 receptor antagonists were determined by the following equations according to ICH guidelines (ICH, 2005).

$$\mathrm{LOD} = \frac{3.3\sigma}{S} \quad (1)$$

$$\mathrm{LOQ} = \frac{10\sigma}{S} \quad (2)$$

3.5. Interaction studies

Powder equivalent to 20 mg of crushed tablets of each drug, was transferred to separate 100 mL volumetric flasks using buffer solution as diluent. 100 $\mu g\,mL^{-1}$ of each drug was mixed in reaction flasks by taking equal volume of pioglitazone and each H_1 receptor antagonists. These flasks were maintained in water bath at 37 °C with constant stirring and 2 mL was withdrawn at zero minute and periodically after every 15 min interval for continuous two hours. Withdrawn aliquots after dilution with methanol to 10 mL were chromatographed.

Table 1 Regression statistics and LOD and LOQ.

Drug	Regression equation	Slope %RSD	r^2	LOD $\mu g\,mL^{-1}$	LOQ $\mu g\,mL^{-1}$
Pioglitazone	$y = 7741x + 44036$	0.02	0.9963	0.19	0.66
Fexofenadine	$y = 4404x - 12523$	0.09	0.9961	0.71	2.37
Cetirizine	$y = 8563x + 42072$	0.09	0.9958	0.30	1.00
Levocetirizine	$y = 8554x + 42470$	0.10	0.9961	0.29	0.95

Table 2 Results from recovery studies of pioglitazone, fexofenadine, cetirizine and levocetirizine.

Levels in spiked samples	Recovery %	% RSD	Levels in spiked samples	Recovery %	% RSD	Recovery %	% RSD	Recovery %	% RSD
μgmL^{-1}	Pioglitazone		$\mu g\,mL^{-1}$	Fexofenadine		Cetirizine		Levocetirizine	
5	102.4	1.11	10	97.9	1.01	98.2	1.21	99.5	0.79
10	102.1	0.92	20	99.3	0.98	98.6	0.88	99.4	1.21
15	101.4	0.78	30	99.3	0.91	98.9	0.87	99.2	1.09

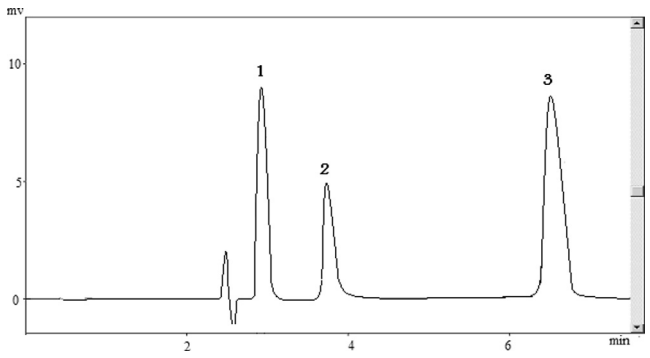

Figure 2 Representative chromatogram of pioglitazone (1), fexofenadine (2) and levocetirizine (3) in human serum.

4. Spectrophotometric studies

Standard stock solution of 35.6 μg mL^{-1} was prepared by taking 0.0356 g pioglitazone hydrochloride in buffer of pH 1. Calibration curves for linearity studies were performed between the concentration of 3.5–35.6 μg mL^{-1} for pioglitazone, 5–25 μg mL^{-1} for cetirizine, 5–25 μg mL^{-1} for levocetirizine and 10–50 μg mL^{-1} concentrations for fexofenadine, in simulated gastric juice (buffers of pH 1) (Fig. 3). *In vitro* availabilities of pioglitazone and H_1-receptor antagonists were performed in individual dosage forms, using 500 mL of simulated gastric juice, which was used as dissolution medium (Table 5). This dissolution medium was selected on the basis of availability of pioglitazone (Mirza et al., 2013a).

4.1. Interaction studies

Reported methods were utilized for interaction (Arayne et al., 2008, 2010c,d; Mirza et al., 2013a; Shamshad et al., 2014). Briefly, in each set of experiment, pioglitazone tablet was added into the dissolution medium along with fexofenadine, cetirizine or levocetirizine tablets at zero minute. Aliquots were withdrawn periodically from 0 to 120 min, at 15 min intervals, and assayed and calculations were performed (Table 6).

5. Results and discussion

Simple and reliable simultaneous HPLC method for the determination of these drugs in active and serum have been developed for the first time. The method has an advantage of being simple, rapid and free of any extensive sample preparation, purification and extraction processes.

In order to comprehensively determine the potential for clinical drug–drug interactions, number of *in vitro* drug interaction models will be required. Reported assays have deficiency in approach, robust quality standards and were not designed to provide information of clinical relevance (Kenawi et al., 2005a,b). The present study provides a consistent and relevant *in vitro* parameter, which could be used to identify a potential drug–drug interaction. No significant change in the availability of pioglitazone was found in the presence fexofenadine, cetirizine and levocetirizine (Table 7). No changes in the availability of pioglitazone in the absence or presence of fexofenadine, cetirizine and levocetirizine were observed. Similar results were obtained using both techniques such as HPLC and spectrophotometer.

Beer's equation was used to quantitate the *in vitro* availability of pioglitazone, fexofenadine, cetirizine and levocetirizine as shown in Table 6. The absorption maxima of pioglitazone, fexofenadine, cetirizine and levocetirizine were at 269, 220, 231 and 231 nm, respectively (Fig. 3) and were interfered with each other. Derivative spectroscopic technique was used to resolve this problem. This technique presents the solution for the elimination of analytical interference and allows the determination of one or more wave-lengths, where the compound of interest can be analyzed with zero absorption from the formulation matrix (Wang and Asgharnejad, 2000). First derivative values versus concentration was used to draw calibration curves of these drugs which were found to be linear over the range. The absence of any interaction was evident from the similar availability values as shown in Table 6. The results acquired were in accordance with the HPLC data. The results of the HPLC method demonstrate that simultaneous determination of pioglitazone, fexofenadine, cetirizine or levocetirizine is very beneficial for pharmaceutical manufacturers and clinicians. The proposed method is simple and suitable for the analysis

Table 4 Accuracy and precision.

Analyte	Spiked concentration (μg mL^{-1})	Precision %RSD	Accuracy %
Pioglitazone	8	1.23	98.75
	10	1.58	101.07
	12	0.66	100.56
Fexofenadine	16	1.61	99.86
	20	1.05	99.30
	24	0.65	100.76
Cetirizine	16	1.60	99.61
	20	1.80	99.66
	24	2.59	99.02
Levocetirizine	16	1.65	99.71
	20	1.75	99.61
	24	2.07	100.09

Table 3 Results of recovery studies of pioglitazone, fexofenadine, cetirizine and levocetirizine in human serum.

Levels in spiked samples (μg mL^{-1})	Pioglitazone		Levels in spiked samples (μg mL^{-1})	Fexofenadine		Cetirizine		Levocetirizine	
	Recov.	%RSD		Recov.	%RSD	Recov.	%RSD	Recov.	%RSD
5	4.97	1.11	10	10.04	0.84	10.06	0.74	10.06	0.74
10	9.89	1.63	20	20.09	0.62	20.19	0.65	20.12	1.51
15	15.21	0.28	30	30.14	0.98	30.14	0.82	30.25	1.05

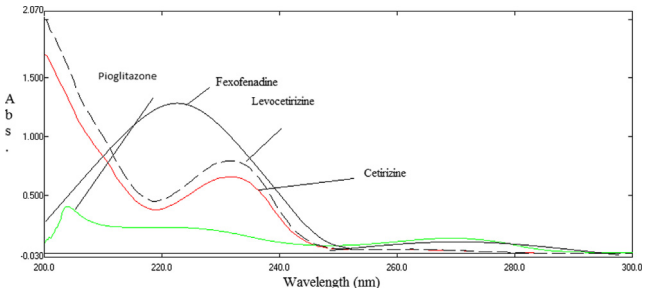

Figure 3 Representative spectra of pioglitazone, fexofenadine, cetirizine and levocetirizine.

of active ingredient in tablet dosage form and human serum. This study thus supports the safe co-administration of pioglitazone and H_1 receptor blockers as an effective diabetic health management regimen.

6. Conclusion

The present HPLC method allowed quantitative determination of pioglitazone, fexofenadine, cetirizine or levocetirizine in formulations and human serum. The proposed method can be used for routine analysis in quality control laboratories owing to its application over other methods as it is fast, precise, accurate, sensitive and efficient. The present study also offers a possibility to a suitable and relevant clinical study to investigate the interaction. Optimized conditions were used to understand interaction of pioglitazone on three separate H_1 receptor antagonists with significant reproducibility and robustness. As a result these methodologies, treatment with fexofenadine, cetirizine or levocetirizine extensively unchanged the availability of pioglitazone compared to its availability alone. It is suggested that receptor or enzymatic level studies should be carried out to further study the possible interaction by the combination of these drugs.

Table 5 % Availability of pioglitazone and H_1 receptor antagonists in individual dosage forms.

Time (Mins)	Pioglitazone %	Fexofenadine %	Cetirizine %	Levocetirizine %
0	2	9	0	0.89
15	99	13.4	27.3	100.42
30	102	13	46.69	102.10
45	103	12.75	65.72	117.58
60	103	13.5	78.76	115.37
75	104	13.05	87.76	100.87
90	104	12.75	94.63	103.59
105	104	12.5	97.33	113.90
120	104	12.95	97.51	108.42

Table 6 % Availability of pioglitazone, fexofenadine, cetirizine and levocetirizine after interaction (UV).

Time (Mins)	Pioglitazone %	Fexofenadine %	Pioglitazone %	Cetirizine %	Pioglitazone %	Levocetirizine %
0	0	0	0	0	0	0
15	54.32	0.91	39.32	35.32	15.32	85.32
30	65.38	5.74	45.65	40.25	31.25	90.21
45	96.32	10.58	89.32	72.36	47.92	98.32
60	97.35	10.28	95.32	78.25	55.32	99.32
75	95.32	9.65	96.78	85.32	81.29	100.58
90	98.21	12.36	98.36	90.21	95.32	105.32
105	100.23	13.01	99.98	95.32	102.32	103.25
120	99.23	12.89	99.32	95.98	102.10	102.32

Table 7 % Availability of pioglitazone, fexofenadine, cetirizine and levocetirizine after interaction (HPLC).

Time (Mins)	Pioglitazone %	Fexofenadine %	Pioglitazone %	Cetirizine %	Pioglitazone %	Levocetirizine %
0	100.00	100.00	100.00	100.00	100.00	100.00
15	103.13	100.13	98.62	97.29	105.72	105.21
30	102.28	104.04	104.79	104.29	107.96	107.74
45	102.93	102.98	105.02	102.50	102.32	101.15
60	106.35	105.69	105.62	105.59	102.06	100.89
75	107.54	102.87	101.48	105.59	104.32	102.91
90	104.27	104.76	102.39	100.57	103.69	100.37
105	103.22	103.25	102.32	100.12	100.25	100.26
120	102.22	103.22	103.02	100.22	102.32	102.00

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgement

The authors acknowledge the Higher Education Commission of Pakistan for providing financial support to Mr. Agha Zeeshan Mirza under Indigenous Ph.D. 5000 Scholarship Program Batch II.

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