Pharmaceutical Sciences
March 2017, 23, 50-58
doi: 10.15171/PS.2017.08
http://journals.tbzmed.ac.ir/PHARM

Research Article

A Very Simple and Sensitive Spectrofluorimetric Method Based on the Oxidation with Cerium (IV) for the Determination of Four Different Drugs in Their Pharmaceutical Formulations

Ahad Bavili Tabrizi[1,2*], Farshad Bahrami[2,3], Hossein Badrouj[3]
[1]*Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*
[2]*Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*
[3]*Department of Medicinal Chemistry, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*

Article Info

Article History:
Received: 26 September 2016
Accepted: 2 November 2016
ePublished: 30 March 2017

Keywords:
-Atenolol
-Metoprolol
-Methyldopa
-Pioglitazone
-Cerium(IV)
-Spectrofluorimetry
-Pharmaceutical formulation

ABSTRACT

Background: Methyldopa is a catecholamine widely used as an antihypertensive agent. Pioglitazone is an oral anti-hyperglycemic agent. It is used for the treatment of diabetes mellitus type 2. A survey of the literature reveals that only one spectrofluorimetric method has been reported for the determination of pioglitazone in pharmaceutical preparations. Atenolol and metoprolol are prescription drugs of the β-blocker class with hypotensive action to treat angina, MI, alcohol syndrome, hypertension, and arrhythmias. A survey of the literature reveals that several spectrofluorimetric methods have been reported for the determination of atenolol and metoprolol in pharmaceutical preparations. In continuing of our studies on the developing of simple and fast spectrofluorimetric methods for determination of drugs and active ingredients, in this work we have developed a spectrofluorimetric method based on the oxidation with cerium (IV) for the determination of studied drugs in their pharmaceutical formulations.

Methods: A simple, rapid and sensitive spectrofluorimetric method was developed for the determination of studied drugs in pharmaceutical formulations. Proposed method is based on the oxidation of these drugs with Ce (IV) to produce Ce (III), and its fluorescence was monitored at 356 ± 3 nm after excitation at 254 ± 3 nm.

Results: The variables affecting oxidation of each drug were studied and optimized. Under the experimental conditions used, the calibration graphs were linear over the range of 25-450, 50-550, 15-800 and 15-800 ng/mL in the case of atenolol, metoprolol, pioglitazone and methyldopa, respectively. The limit of detection was found to be 8.27, 16.5, 1.52 and 5.08 ng/mL in the case of atenolol, metoprolol, pioglitazone and methyldopa, respectively. Intra- and inter-day assay precisions, expressed as the relative standard deviation (RSD), were lower than 3% in all cases.

Conclusion: The proposed method was applied to the determination of studied drugs in their pharmaceutical formulations by good recoveries in the range 92-113%.

Introduction

Methyldopa, a catechol derivative (catecholamine), is chemically named α-methyl-3,4-dihydroxyphenylalanine (Figure 1d) and widely used as an antihypertensive agent. The methyldopa is a centrally acting alpha2-adrenoreceptor agonist, which reduces sympathetic tone and produces a fall in blood pressure.[1] Pioglitazone, chemically known as [(±)-5-[4-[2-(5-ethyl-2-pyridinyl) ethoxy] phenyl]-methyl]-2,4-thiaolidinedionemonohydro-chloride (Figure 1c), is a thiazolidinedione derivative that widely used in patients with type-2 diabetes (non-insulin dependent diabetes).[2] Blockers have been included in the list of forbidden substances by the World Anti-Doping Agency.

***Corresponding Author:** Ahad Bavili Tabrizi, E-mail: a.bavili@tbzmed.ac.ir

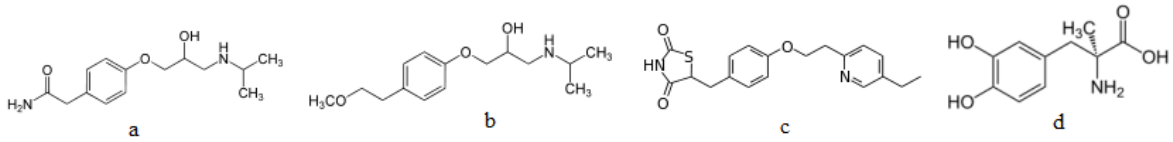

Figure 1. Structure of studied drugs: a) atenolol, b) metoprolol, c) pioglitazone, d) methyldopa.

They have been designed primarily as drugs for the management of cardiac arrhythmias and cardioprotection after myocardial infarction. β-blockers also improve the heart's ability to relax and exhibit calming neurological effects decreasing anxiety, nervousness and stabilizing motor performance.[3]

Different methods have been employed for the analysis of methyldopa in pharmaceutical formulations including electrochemical methods,[4-7] spectrophotometry,[1,8-10] kinetic methods,[11] chemiluminescence,[12] capillary electrophoresis (CE)[13] and thin layer chromatography.[14] The main methods of the determination of pioglitazone in pharmaceutical preparations are chromatography,[2,15-17] spectrophotometry,[18,19] electrochemical,[20,21] and thermal methods.[22] There is only one report on the spectrofluorimetric determination of pioglitazone in pharmaceutical preparations.[23]

Different techniques have been used to determine β-blockers in pharmaceutical preparations including spectrophotometry,[24-27] high performance liquid chromatography (HPLC),[27-30], gas chromatography (GC),[31] electrochemical method,[32] CE[33,34] and chemiluminescence.[35] Several spectrofluorimetric methods[36-40] have also been used for the determination of β-blockers in pharmaceutical preparations.

The chromatographic and electrophoresis methods are very sensitive and reliable, but they are relatively time-consuming and expensive. The majority of other reported methods utilize reagents which are expensive or may in some cases present with certain stability problems. Therefore, developing of simple, sensitive and rapid analytical methodologies as practical alternatives to these methods can be very attractive. Fluorescence spectrometry due to great sensitivity and selectivity as well as relatively low cost for the operation is widely used in quantitative analysis of pharmaceuticals. Thus, a simple, sensitive and inexpensive spectrofluorimetric method has been proposed here for the determination of these drugs in their pharmaceutical formulations.

Several spectrofluorimetric techniques have used Ce(IV) as an oxidizing agent for the determination of certain drugs.[41-48] Its reaction with targeted drug in sulfuric acid medium can easily produce Ce(III) that shows a characteristic fluorescence. The literature survey revealed that this system has not been used for the spectrofluorimetric analysis of studied drugs, so there is only one report on the spectrophotometric analysis of atenolol by the use of Ce(IV).[49] Thus, in continuing of our studies on the developing of simple and fast spectrofluorimetric methods for determination of drugs and active ingredients, in this work we have developed a spectrofluorimetric method based on the oxidation with Ce(IV) for the determination of studied drugs in their pharmaceutical formulations.

Materials and Methods

Apparatus

A Shimadzu RF-5301 PC spectrofluorophotometer, equipped with a 150 W Xenon lamp and 1.00 cm quartz cells, was used for the fluorescence measurements. Both excitation and emission slits were adjusted to 3 nm and the sensitivity adjusted to low.

Reagents

Atenolol, metoprolol, pioglitazone and methyldopa were obtained as gifts from Pars Darou Co. (Tehran, Iran), Sobhan Darou Co. (Tehran, Iran), Daroupaksh (Tehran, Iran) and Zahravi (Tabriz, Iran), respectively. Sulfuric acid and Ce(IV)-sulfate-tetrahydrat were obtained from E. Merck (Darmstadt, Germany).

A stock standard solution of each drug at a concentration of 500 µg/mL was prepared by dissolving appropriate amount of each drug in 5 mL doubly distilled water and diluting to 25 mL with this water. These solutions were stored under dark conditions in refrigerator when not in use for two weeks. Working standard solutions were obtained daily by appropriately diluting this stock solution with double distilled water. Ceric sulfate, 0.01 mol/L was prepared in 2.0 mol/L sulfuric acid and was kept in the refrigerator at 4 °C for two weeks. All other reagents were of analytical-reagent grade (E. Merck) and all solutions were prepared in doubly distilled water.

Recommended procedure for calibration

An aliquot of sample solution containing atenolol, metoprolol, pioglitazone or methyldopa in the range of 25-450, 50-550, 15-800 and 15-800 ng/mL, respectively, was transferred into 15-mL calibrated centrifuge tubes. This was followed by addition of sulfuric acid (*e.g.* 1, 1, 3 and 2 mL of 2.0 mol/L solution) and then Ce(IV) (*e.g.* 50, 50, 240 and 40 µL of 0.01 mol/L solution), respectively. The content of each tube was mixed well and diluted to 10 mL with double distilled water. The resultant solutions of atenolol and

metoprolol were equilibrated at 65 ºC for 40 min, while solutions of pioglitazone and methyldopa equilibrated at room temperature and 50 ºC for 20 min, respectively. Then, the fluorescence intensity of each solution was measured at 356 ± 3 nm while excited at 254 ± 3 nm against reagent's blank.

Preparation of Pharmaceutical Formulations

Tablets and capsules

Ten atenolol (Jalinous, Tehran, Iran), metoprolol (Alborzdarou, Tehran, Iran), pioglitazone (Daroupaksh, Tehran, Iran) or methyldopa (Zahravi, Tabriz, Iran) tablets, each containing 50, 50, 30 and 250 mg atenolol, metoprolol, pioglitazone and methyldopa, respectively, were accurately weighed individually and finely powdered. Powdered sample containing 50 mg each of atenolol, metoprolol or methyldopa and 30 mg pioglitazone, was weighed and placed into a 15-mL glass tube, dissolved in 5-mL water and vigorously shaken on a vortex mixer for 1 min. The solution was then filtered and transferred into a 100-mL volumetric flask. The residue was washed in enough water and the solution was finally made up to the mark with double distilled water. Thus, a 500 µg/mL solution of atenolol, metoprolol or methyldopa and 300 µg/mL solution of pioglitazone was obtained.

These solutions were diluted to 1 µg/mL in the case of atenolol and metoprolol, and 10 µg/mL in the case of pioglitazone and methyldopa. Then, aliquots of 500 µL of these prepared samples were used for determination of each drug as mentioned in the procedure.

Ampoule

The content of metoprolol ampoule (Alborzdarou, Tehran, Iran), containing 5 mg/5mL metoprolol, was completed to 100 mL with double distilled water, thus a 50 µg/mL solution was obtained. This solution was diluted to 1 µg/mL with water, than 500 µL of this diluted sample was subjected to metoprolol determination as mentioned in the procedure.

Results and Discussion

Certain functional groups in the drug substances can be oxidized with Ce(IV), as a well-known oxidation agent. The produced Ce(III) is usually more fluorescent than the other oxidation products and also un-reacted Ce(IV), thus the monitoring of its fluorescence has been used as analytical signal for the establishment of useful analytical techniques for certain drugs.[41-48] In the present work the Ce(IV) has been used for the chemical oxidation of studied drugs in sulfuric acid medium and the fluorescence of produced Ce(III) was monitored in desired wavelengths. The excitation and emission spectra for methyldopa-Ce(IV) system, obtained in the optimum conditions, have been shown in Figure 2. Other drug-Ce(IV) reaction systems produced very similar spectra.

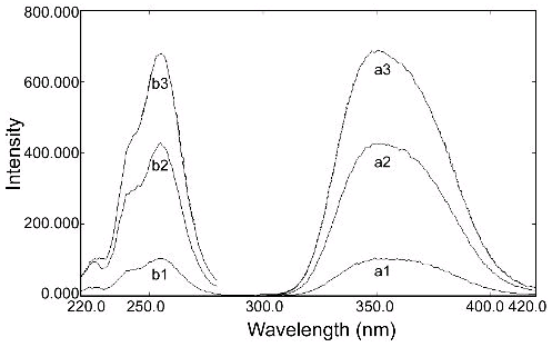

Figure 2. Emission and excitation spectra: (a_1 & b_1) reagent's blank, (a_2 & b_2) sample prepared from tablet (350 ng/mL), (a_3 & b_3) standard solution of methyldopa (600 ng/mL); other conditions: Ce(IV) (0.4×10^{-4} mol/L), sulfuric acid (0.4 mol/L), equilibration at 50 ºC for 20 min.

Effect of Ce(IV) concentration

The effect of Ce(IV) concentration on the fluorescence intensities was studied in the range of 0.1-5.0 ($\times10^{-4}$) mol/L and the results were presented in Figure 3.

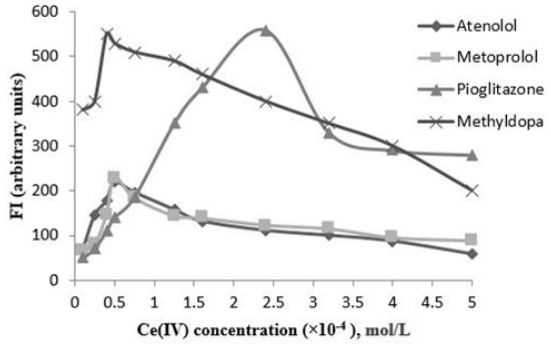

Figure 3. The effect of Ce(IV) concentration on the analytical signals, 500 ng/mL of each metoprolol, pioglitazone and methyldopa and 300 ng/mL of atenolol was used for optimization; other conditions: sulfuric acid: 0.2 mol/L in the case of atenolol and metoprolol and 0.6 and 0.4 mol/L in the case of pioglitazone and methyldopa; temperature: equilibration at 65 ºC for 40 min in the case of atenolol and metoprolol and equilibration at room temperature and 50 ºC for 20 min in the case of pioglitazone and methyldopa.

This figure revealed that the fluorescence intensity was reached to its maximum amount at concentration of 0.5, 0.5, 2.4 and 0.4×10^{-4} mol/L of Ce(IV), in the case of atenolol, metoprolol, pioglitazone and methyldopa, respectively, and then decreased gradually. A dramatic decrease in the fluorescence intensity was observed at concentrations lower than this range due to insufficient Ce(IV) for the chemical oxidation of each drug. On the other hand, at concentrations higher than this range the fluorescence intensity decreased probably due to quenching effect of

Ce(IV).[41-43] Hence, the above mentioned concentrations were taken as optimum amount for other experiments.

Effect of sulfuric acid concentration

The influence of sulfuric acid concentration on the fluorescence intensity of the reaction product was studied when increasing volumes of 2.0 mol/L sulfuric acid solution, equivalent to final concentrations of 0.02-0.9 mol/L, were added to the reaction system. From Figure 4 it was found that maximum and constant fluorescence intensity was attained when sulfuric acid concentration was 0.2, 0.2, 0.6 and 0.4 mol/L, in the reaction medium of atenolol, metoprolol, pioglitazone and methyldopa, respectively. Thus, these concentrations were used for the oxidation of studied drugs in the rest of work.

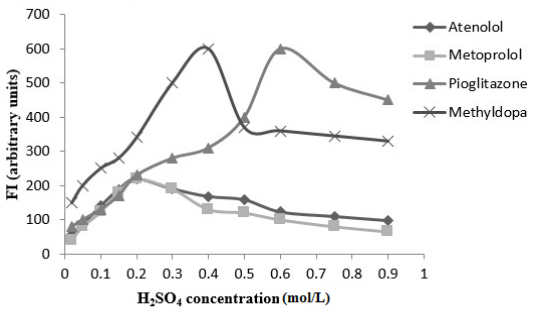

Figure 4. The effect of sulfuric acid concentration on the analytical signals, 500 ng/mL of each metoprolol, pioglitazone and methyldopa and 300 ng/mL of atenolol was used for optimization; other conditions: Ce(IV): 0.5×10^{-4} mol/L in the case of atenolol and metoprolol and 2.4 and 0.4 ($\times10^{-4}$) mol/L in the case of pioglitazone and methyldopa; equilibration at 65 ºC for 40 min in the case of atenolol and metoprolol and equilibration at room temperature and 50 ºC for 20 min in the case of pioglitazone and methyldopa.

Effect of temperature and time

Heating the reaction solution was necessary for proceeding the reaction and increasing the fluorescence intensity, thus the oxidation reaction of studied drugs was carried out at different temperatures ranging from 25-90 °C. As shown in Figure 5, the maximum signals were obtained at 65, 65 and 50 °C, in the case of atenolol, metoprolol and methyldopa, respectively. It was found that temperature has not any significant effect on the chemical oxidation of pioglitazone, thus its reaction was performed at ambient temperature. Also, the oxidation reactions were carried out at these temperatures for periods ranging from 5 to 60 min. The results revealed that equilibration time of 40 min was sufficient for atenolol and metoprolol systems, while 20 min was sufficient for pioglitazone and methyldopa systems.

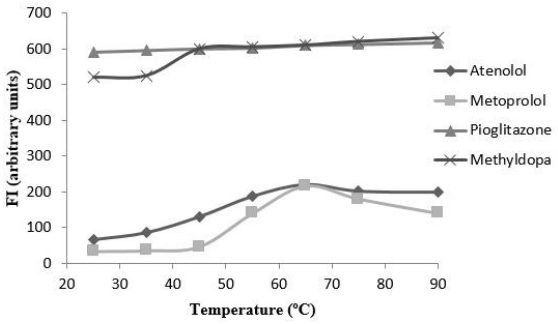

Figure 5. The effect of temperature on the analytical signals, 500 ng/mL of each metoprolol, pioglitazone and methyldopa and 300 ng/mL of atenolol was used for optimization; other conditions: Ce(IV): 0.5×10^{-4} mol/L in the case of atenolol and metoprolol and 2.4 and 0.4 ($\times10^{-4}$) mol/L in the case of pioglitazone and methyldopa; sulfuric acid: 0.2 mol/L in the case of atenolol and metoprolol and 0.6 and 0.4 mol/L in the case of pioglitazone and methyldopa.

Analytical characteristics

The method was validated according to ICH Q2(R1) guidelines[50] and considering the parameters such as linearity, sensitivity, precision, accuracy and recovery. The calibration curves were established by measuring the fluorescence intensity of standard solutions of studied drugs. In all cases linear relations between fluorescence intensity and concentration of each drug was found in the range of 25-450, 50-550, 15-800 and 15-800 ng/mL of atenolol, metoprolol, pioglitazone and methyldopa, respectively. The equations for these calibration curves are summarized in Table 1. The limit of detection (LOD) and limit of quantification (LOQ) were calculated by considering the three and ten times the standard deviation of the blank signals (S_b) and based on $3S_b/m$ and $10S_b/m$ equations, respectively, where m is the slope of the calibration line. The analytical figures of merit of the proposed method are summarized in Table 1.

Table 1. Analytical characteristics of the proposed method for studied drugs.

Analyte	LR (ng/mL)	r	Calibration equation	LOD (ng/mL)	LOQ (ng/mL)
Atenolol	25-450	0.9940	0.7649C-11.01	8.27	27.6
Metoprolol	50-550	0.9981	0.3832C+25.08	16.5	55.1
Pioglitazone	15-800	0.9995	1.0788C+27.32	1.52	5.08
Methyldopa	15-800	0.9996	1.1105C-0.3396	1.21	4.03

LR = linear range

The precision of the method was measured through repeatability and intermediate precision and expressed as the relative standard deviation (RSD%). Repeatability was evaluated by analyzing samples in three concentration range (*e.g.* low, medium and high) in the same day 6 replicates. The intermediate precision was assessed by duplicate analyzing, during five consecutive days, at the same concentration levels. As shown in Table 2, the RSD values for intra- and inter-day precisions were lower than 1.6% and 3.0%, respectively, thus good precision was achieved.

The analytical characteristics of the proposed method were compared with the performances of other methods in Table 3 to highlight the distinct features of the proposed method. The proposed method does not require high investment and maintenance costs of the instruments compared with sophisticated methods which use HPLC or CE for the determination of targeted analytes. More importantly, our figures of merit were comparable to or even better than those reported in other methods (see Table 3). Especially, our found LOD was better than that reported in all spectrophotometric and two spectrofluorimetric[39,40] methods.

Recovery experiments and interference study

Aliquot volumes of each prepared pharmaceutical preparation, as section of "*Preparation of Pharmaceutical Formulations*", were transferred to clean centrifuge tubes and spiked with drug at three concentration levels, then analyzed following the proposed analytical method. As shown in Table 4, the obtained recoveries ranged from 92 to 113% and seem to be satisfactory. On the other hand, typical spectra for blank sample, methyldopa standard solution and its pharmaceutical preparation are shown in Figure 1. No additional peaks due to interfering compounds were observed at the emission wavelength that was used in this work. Thus, the similarities in the excitation and emission spectra and the reasonable recoveries that were found, revealed that there were no significant matrix effects on this work.

For investigating the effects of interfering species on the proposed method, the influence of frequently encountered excipients and additives was studied by adding different amounts of these substances to aqueous sample containing fixed amount of each drug.

Table 2. Repeatability and intermediate precisions for determination of studied drugs.

C (ng/mL)	Atenolol Repeatability	Intermediate precision	Metoprolol Repeatability	Intermediate precision	C (ng/mL)	Pioglitazone Repeatability	Intermediate precision	Methyldopa Repeatability	Intermediate precision
75	0.85	1.35	0.99	1.15	30	1.04	2.63	1.18	2.97
250	1.01	0.73	0.36	0.39	400	0.94	1.59	1.10	1.76
400	0.68	0.54	0.75	0.88	700	1.07	1.12	0.96	1.43

Repeatability and intermediate precisions expressed as RSD%, and for 6 and 5 replicate determinations, respectively.

Table 3. Analytical characteristics of different methods used for the determination of studied drugs.

Method	Analyte	Concentration range (ng/mL)	r	RSD%	LOD (ng/mL)	Mean R (%)	Ref.
S	Methyldopa	50-200($\times10^3$)	0.9999	0.50-1.20	1.9($\times10^3$)	100.1-101.8	1
Ex.-S	Methyldopa	50-500	0.9990	5.18	16.5	99.4-100.0	8
CL	Methyldopa	69-3520	0.9999	5.20	40.0	-	12
Ex.-S	Pioglitazone	1-65($\times10^3$)	0.9999	4.96	0.16($\times10^3$)	99.1-102.2	18
S	Pioglitazone	5-30($\times10^3$)	0.9995	<2.0	0.16($\times10^3$)	99.5-100.3	19
F	Pioglitazone	5-1300	0.9999	<2.0	1.61	99.3($\bar{R}$)	23
S	Metoprolol	8.5-70($\times10^3$)	0.9980	1.54	5.56($\times10^3$)	100.6	24
S	Atenolol	1.5-18($\times10^3$)	0.9999	1.55-1.72	0.23($\times10^3$)	103.0-109.9	26
F	Atenolol	0.05-4($\times10^3$)	0.9998	<2.97	15.2	99.3($\bar{R}$)	36
F	Atenolol	10-400	0.9999	2.50	-	96.8-110.0	37
F*	Metoprolol	0-500	0.9921	1.0-3.0	1.5	87.0-90.0	38
F	Metoprolol	0.5-10($\times10^3$)	0.9999	1.49	0.11($\times10^3$)	100.4($\bar{R}$)	39
F	Atenolol	1-11($\times10^3$)	0.9999	-	0.2($\times10^3$)	100.7	40
F	Atenolol	25-450	0.9940	0.52-0.71	8.27	98.6-113.2	This work
F	Metoprolol	50-550	0.9981	0.54-1.57	16.5	92.2-105.5	This work
F	Pioglitazone	15-800	0.9995	0.94-1.07	1.52	101.3-104.0	This work
F	Methyldopa	15-800	0.9996	0.96-1.18	5.08	98.1-102.3	This work

S= spectrophotometry; F= spectrofluorimetry; Ex= extractive; R= recovery; *in plasma sample

Table 4. Results of recoveries of spiked samples.

Sample	added (ng/mL)	found ± SD (n = 3), ng/mL	R %
Atenolol tablet	50	49.30 ± 0.5200	98.6
	200	226.4 ± 2.180	113.2
	350	385.5 ± 4.560	110.1
Metoprolol tablet	50	50.5 ± 0.5400	101.0
	200	186.4 ± 2.230	93.2
	350	346.5 ± 4.120	99.0
Metoprolol ampoule	50	52.50 ± 0.5100	105.0
	200	184.4 ± 2.530	92.2
	350	339.5 ± 4.090	97.0
Pioglitazone tablet	30	30.60 ± 0.4600	102.0
	400	416.0 ± 6.660	104.0
	700	709.0 ± 8.510	101.3
Methyldopa tablet	30	30.70 ± 0.3500	102.3
	400	408.0 ± 5.710	102.0
	700	686.7 ± 7.550	98.1

Table 5. Determination of the studied drugs in their pharmaceutical formulations using proposed method.

Sample	Labeled amount (mg)	Found amount ± SD (mg)*	Experimental *t*-values	R%
Atenolol (Tablet)	50	55.1 ± 2.80	3.15	110.2
Metoprolol (Tablet)	50	56.2 ± 3.20	3.36	112.4
Metoprolol ampoule	Each 5 mL containing 5 mg	4.67 ± 0.190	3.01	93.4
Pioglitazone (Tablet)	30	30.5 ± 0.480	2.33	101.7
Methyldopa (Tablet)	250	255 ± 4.23	2.54	101.9

Tabulate *t*-test at P=0.05, t = 4.3 (n = 3) and t = 2.78 (n = 5)

*Three successive determinations in the case of atenolol and metoprolol and five successive determinations in the case of pioglitazone and methyldopa have been done.

The tolerance limit was taken as the concentration causing an error of not more than 7% in the determination of each drug. Lactose, glucose, starch, talk and magnesium stearate showed no interference in the ratios commonly used in pharmaceutical preparations and even when present in 5.00-fold excess over analyte. Thus, a high degree of tolerance was observed for these species.

Applications to the analysis of pharmaceutical formulations

The proposed method was applied successfully for the determination of studied drugs in their pharmaceutical formulations and the results are presented in Table 5.

Conclusion

A validated spectrofluorimetric method has been reported for the determination of atenolol, metoprolol, pioglitazone and methyldopa in their pharmaceutical formulations. The method was validated by considering accuracy and precision for the determination of studied drugs. The obtained LODs and LOQs are comparable to or even better than those reported in other methods (see Table 3). Although, HPLC or CE methods are precise and sensitive (in the case of HPLC), but they use high sophisticated and expensive instruments. Therefore, from the economical point of view, the proposed method is simple, rapid, sensitive and inexpensive method and can be used as an alternative method for quality control or pharmaceutical analysis.

Conflict of interests

The authors claim that there is no conflict of interest.

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