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Fluorometric Determination of Bopindolol and Celiprolol in Pharmaceutical Preparations and Biological Fluids

F. Belal · M. Sharaf El-Din · F. Aly · M. Hefnawy · M. El-Awady

Abstract Two sensitive fluorometric methods were developed for the determination of both bopindolol malonate (BOP) and celiprolol HCl (CLP) based on measuring their native fluorescence in methanol and acetonitrile, respectively. For BOP, the fluorescence was measured at 316 nm after excitation at 278 nm. The proposed method was successfully applied to the assay of commercial tablets as well as content uniformity testing. For CLP, the fluorescence was enhanced by the addition of carboxymethylcellulose solution and measured at 455 nm after excitation at 339 nm. The method was successfully applied to the analysis of CLP in tablets and biological fluids. In both methods, interference likely to be introduced from co-formulated, co-administered, or chemically related drugs was studied. The results were statistically compared with those obtained by reference methods and were found to be in good agreement.

Keywords Bopindolol · Celiprolol · Fluorometry · Applications · Pharmaceutical preparations · Content uniformity test · Biological fluids

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Introduction

Bopindolol; (*RS*)-1-(*tert*-butylamino)-3-[(2-methyl-1*H*-indol-4-yl)oxy]propan-2-yl benzoate (Fig. 1); is a non-cardioselective beta blocker used in the management of hypertension and angina pectoris. It is given orally as the malonate salt[1]. It is rapidly hydrolyzed in plasma and no measurable amounts of the parent drug could be detected after oral administration[2]. Celiprolol; (*RS*)-N'-{3-acetyl-4-[3-(*tert*-butylamino)-2-hydroxypropoxy]phenyl}-*N,N*-diethylurea (Fig. 1); is a cardioselective β-blocker used as the hydrochloride salt in the management of hypertension and angina pectoris. The metabolism of CLP is minimal and so it is mainly excreted unchanged in the urine and faeces[1]. Mean peak plasma concentration measured after administration of a 200 mg single oral dose is 687 μg/L[2]. Besides being formulated as a single ingredient preparation, both BOP and CLP are also co-formulated with chlorthalidone in the form of tablet preparations[1].

Few analytical methods have been described for the determination of BOP, in pure form and in pharmaceutical preparations. Different HPLC methods were reported for the determination of BOP[3–8]. Supercritical fluid chromatography was also utilized for the analysis of BOP and its precursor and degradation product[9]. Moreover, BOP was also analysed using different types of capillary electrophoresis[10–12]. Capillary electrochromatography was also investigated for the chiral separation of BOP[6, 13]. In addition, BOP was determined in tablets by the use of flow injection analysis coupled with solid phase extraction[14]. Moreover, BOP was determined by sequential injection analysis with spectrophotometric detection[15].

Different analytical methods were developed for the determination of CLP. A good guide for the work published up to 1991 is presented as a comprehensive monograph in analytical
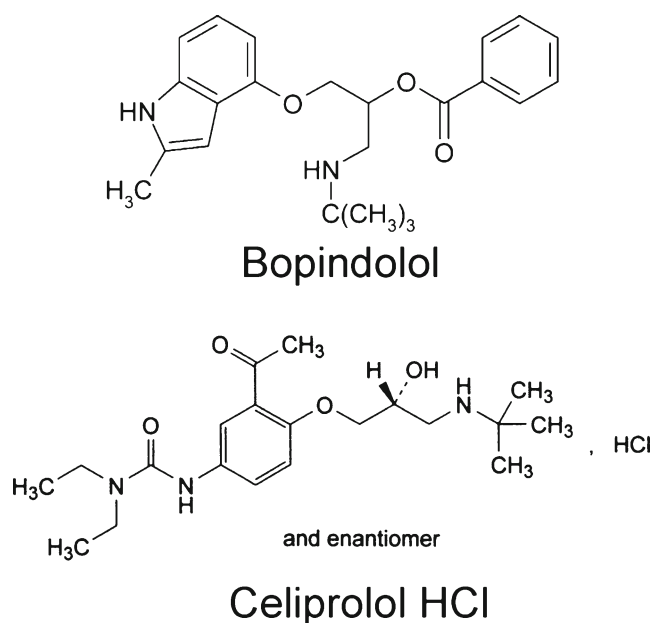
Fig. 1 Chemical structures of BOP and CLP

profiles of drug substances[16]. In addition, the British Pharmacopoeia[17] recommended a titrimetric method for the assay of CLP in pure form based on an acid–base titration using standard NaOH as a titrant and detecting the end point potentiometrically. Different colorimetric methods were developed for the analysis of CLP[18, 19]. The adsorptive voltammetric behavior of CLP was studied by Zeng et al.[20]. Densitometric determination of CLP was reported by Vujic and Radulovic[21]. Plasma analysis for CLP by HPTLC was also conducted[22]. Several HPLC methods were published for the determination of CLP in pure form, pharmaceuticals, and biological fluids [23, 24]. LC/MS was also utilized for the determination of CLP in combination with several drugs with the application to biological fluids[7, 25–27]. Different capillary electrophoretic methods were developed for the assay of CLP[28]. Capillary electrochromatography was also used for the determination of CLP [13, 29, 30].

There is an urgent need to develop a sufficiently sensitive and specific method for the determination of BOP in pharmaceutical preparations as it is formulated in a very minute amount in tablets (1 mg/tablet). Assay of CLP by conventional spectrophotometric measurements suffers from poor sensitivity and this problem is more aggravated if the compound needs to be estimated in biological fluids. Fluorometric analysis, by virtue of its high inherent sensitivity, could successfully overcome these problems.

To the best of our knowledge, no fluorometric methods have been yet described for the determination of either BOP or CLP.

Experimental

Apparatus

All fluorescence measurements were made with a Perkin-Elmer UK model LS45 luminescence spectrometer, equipped with a 150 Watt Xenon arc lamp. The slit widths were 10 nm for both excitation and emission, and the photomultiplier voltage was set to auto. Quartz 1-cm cuvette was used.

Reagents and Materials

All the chemicals used were of Analytical Reagents grade, and the solvents were of HPLC grade.

- Bopindolol malonate, 99.0% was kindly provided by (Novartis Pharma Schweiz AG, Bern, Switzerland) and was used as received without further purification.
- Sandonorm® tablets (Novartis Pharma Schweiz AG, Bern, Switzerland) were purchased from commercial sources.
- Celiprolol HCl was kindly provided by (Aventis Pharma Ltd, Auckland, New Zealand). The purity of the sample was found to be 99.69±0.51 according to the BP[17] method.
- Cordiax® tablets (CRINOS S.p.A., Via Pavia 6, Milano, Italy) were purchased from commercial sources.
- Methanol (Prolabo, France) and acetonitrile (Sigma-Aldrich Chemie GmbH, Germany).
- Carboxymethylcellulose (El-Nasr Pharmaceutical Chemicals Company (ADWIC), Egypt), used as 0.1% w/v aqueous solution.
- Plasma samples were obtained from Mansoura University Hospital (Mansoura, Egypt) and kept frozen until use after gentle thawing.
- Urine samples were obtained from a healthy volunteer (male, around 18 years old).

Standard Solutions

Stock solutions (0.20 mg/mL) of BOP and CLP were prepared by transferring 0.020 g of each drug, accurately weighed, to a 100-mL volumetric flask, adding about 50 mL of methanol and acetonitrile for BOP and CLP, respectively, and dissolving by swirling and with the aid of sonication. The solutions were then diluted with the same solvents to volume, and mixed. These two solutions were stable for at least 7 days when kept in the refrigerator.

Construction of Calibration Curves

Working standard solutions of BOP were prepared from the stock solution in 10-mL volumetric flasks by serial dilutions

with methanol to 0.02–1.00 μg/mL (final concentration). The fluorescence intensities of the solutions were measured at 316 nm after excitation at 278 nm. The fluorescence intensity was then plotted against the final concentration to get the calibration graph. Alternatively, the corresponding regression equation was derived.

Working standard solutions (5.0 μg/mL) and (1.0 μg/mL) of CLP were prepared from the stock solution by serial dilution with acetonitrile. Aliquot volumes of the suitable working standard solution were transferred into a series of 10-mL volumetric flasks so that the final concentration was in the range of 0.05–1.20 μg/mL. A 0.5 mL of 0.1 % aqueous carboxymethylcellulose (CMC) solution was added to each flask. The solutions were mixed and then diluted to volume with acetonitrile.

The fluorescence intensities of the solutions were measured at 455 nm after excitation at 339 nm and then plotted against the final concentration to get the calibration graph. Alternatively, the corresponding regression equation was derived.

Assay Procedure for Tablets

For each drug, 20 tablets were weighed and then powdered. For BOP: An accurately weighed amount of the powder equivalent to 1.0 mg of bopindolol (equivalent to 1.27 mg bopindolol malonate) was transferred into a 100-mL volumetric flask, and diluted to the mark with methanol. For CLP: An accurately weighed amount of the powder equivalent to 20.0 mg of CLP was transferred into a 100-mL volumetric flask, and diluted to the mark with acetonitrile. The two flasks were sonicated for 30 min, filtered and then analyzed as described under *Construction of calibration curves*. The concentrations of the drugs were determined using either the calibration curve or the corresponding regression equation. The results obtained were compared to those given with the comparison colorimetric methods[19, 31] for BOP and CLP, respectively.

Procedure for Content Uniformity Testing for BOP

The same procedure applied for the analysis of the studied compound in tablets was followed using one tablet as a sample. Ten different tablets were analyzed and the uniformity of their contents was tested by applying the official USP[32] guidelines (*Chapter 905: Uniformity of Dosage Units*).

Application of the Proposed Method to the Analysis of CLP in Spiked Human Plasma and Urine

A standard calibration curve was constructed using spiked human plasma or urine with varying amounts of CLP as follows:

1.0 mL aliquots of plasma or urine were transferred into a series of centrifugation tubes. Aliquots of standard aqueous solution of CLP were added so that the final concentration is in the range of 0.05–1.20 μg/mL. The tubes were mixed well and then 1.0 mL of 1 mol/L NaOH was added to liberate CLP base. The tubes were shaken and then CLP base was extracted using 2.0 mL ethyl acetate and centrifugation at 3000 rpm for 30 min. 1.0 mL aliquots of the ethyl acetate layers were evaporated at room temperature and the residues were dissolved in few milliliters of acetonitrile and then quantitatively transferred into 10-mL volumetric flasks. The previously mentioned general procedure was then followed. A blank experiment was carried out simultaneously. The fluorescence intensity was then plotted against the final concentration to obtain the calibration graph. Alternatively, the corresponding regression equation was derived.

Samples of spiked plasma or urine with different amounts of CLP in the working concentration range were treated in the same manner and the nominal contents of the drug were determined using the corresponding regression equation.

Results and Discussion

Depending on the intense native fluorescence exhibited by BOP and CLP solutions, highly sensitive fluorometric methods for their determination have been developed.

Effect of Diluting Solvent

The effect of different solvents on the fluorescence intensity for both drugs was studied. The highest fluorescence intensity was obtained in methanol and acetonitrile for BOP and CLP, respectively (Table 1). This may be attributed to the change in the medium polarity that may result in some sort of physical interaction between methanol or acetonitrile and the excited singlet state of the drug molecules. The effect of different organic solvents on the fluorophore differs depending on the relative strengths of the solvent-solvent and solute-solvent interactions. Moreover, the solvent molecules are usually non symmetric dipoles and display different relaxation rates along their different axes. Most importantly, intermolecular rearrangements of the fluorophore, formation of specific complexes in the excited state (exciplexes), and reorganization of fluorophore-solvent hydrogen bonds may result in temporal shifts of fluorescence spectra[33].

Moreover, the effects of volumes of these solvents were also investigated and it was found that maximum fluorescence intensity was obtained when the flask is completely

Table 1 Effect of solvents on the fluorescence intensity of BOP and CLP (1 µg/mL of each)

Solvent	BOP			CLP		
	$\lambda_{ex.}$ (nm)	$\lambda_{em.}$ (nm)	Fluorescence intensity	$\lambda_{ex.}$ (nm)	$\lambda_{em.}$ (nm)	Fluorescence intensity
Water	286	316	7	341	442	9
Acetonitrile	284	308	8	339	460	296
Ethanol	284	311	52	—	—	No fluorescence
Methanol	278	316	320	335	472	46
0.1 mol/L NaOH	287	317	4	350	436	21
0.05 mol/L H_2SO_4	—	—	No fluorescence	335	441	5
Acetate buffer (pH 4.5)	—	—	No fluorescence	331	451	7
Borate buffer (pH 7)	282	312	1	341	438	13
Borate buffer (pH 9.4)	287	318	4	341	438	9

diluted with methanol and acetonitrile for BOP and CLP, respectively.

Effect of Organized Media

The effect of different organized media (amphiphile aggregates or polymers which form anisotropic microstructures in solution) on the fluorescence intensity of both drugs was studied by adding 1 mL of an aqueous solution of each one of them to the drug solution. Different surfactants, like sodium dodecyl sulfate (SDS) [anionic surfactant], cetrimide [cationic surfactant], triton X 100 and tween 80 [non-ionic surfactants] as well as carboxymethylcellulose (CMC), gelatin and β-cyclodextrin were tried. For BOP: as shown in Table 2, SDS, cetrimide, CMC, and tween 80 caused an inhibitory effect on the fluorescence intensity. Triton X 100 was not suitable as it showed a very high fluorescence intensity of the blank. The use of Gelatin or β-cyclodextrin resulted in the formation of white turbidity. Therefore, no organized media were used with BOP. The typical fluorescence spectra of BOP in methanol are illustrated in Fig. 2 showing $\lambda_{ex.}$ at 278 nm and $\lambda_{em.}$ at 316 nm.

For CLP: SDS, cetrimide, and tween 80 caused an inhibitory effect on the fluorescence intensity. Triton X 100 did not show any marked effect on the fluorescence intensity. The use of gelatin or β-cyclodextrin resulted in the formation of white turbidity. On the other hand, the addition of CMC showed a marked enhancement of the fluorescence intensity so it was used throughout the present study (Table 2). The effect of volume of CMC solution was also investigated and it was found that maximum fluorescence intensity was obtained when 0.5 mL of 0.1% aqueous CMC solution was added. The typical fluorescence spectra of CLP in acetonitrile after the addition of CMC solution are illustrated in Fig. 2 showing $\lambda_{ex.}$ at 339 nm and $\lambda_{em.}$ at 455 nm.

Validation of the Method

Linearity and Range

The calibration graphs for the determination of BOP and CLP by the proposed methods were constructed by plotting the fluorescence intensity versus the concentration (Figs. 3 and 4). The graphs were found to be rectilinear over the concentration range cited in Table 3.

Table 2 Effect of organized media on the fluorescence intensity of BOP and CLP (1 µg/mL of each)

Organized Media	Fluorescence Intensity Change	
	BOP	CLP
None	100%	100%
0.1% Sodium Dodecyl Sulfate (SDS)	85%	94%
0.1% Carboxymethylcellulose (CMC)	95%	119%
0.1% Gelatin	Appearance of turbidity	Appearance of turbidity
0.1% Triton X 100	Blank reading over the range	99%
0.1% Cetrimide	90%	89%
0.2% Tween 80	82%	75%
0.1% β-Cyclodextrin	Appearance of turbidity	Appearance of turbidity

Fig. 2 Fluorescence spectra of 0.6 μg/ml BOP in methanol and 0.6 μg/ml CLP in acetonitrile after the addition of 0.5 ml 0.1% aqueous CMC solution. **a** Excitation spectrum; **b** Emission spectrum
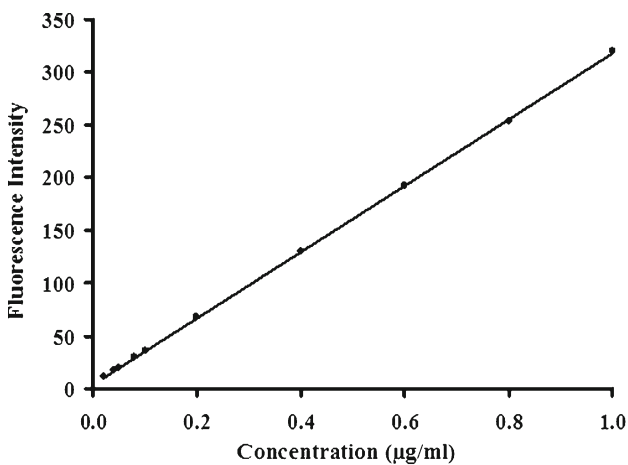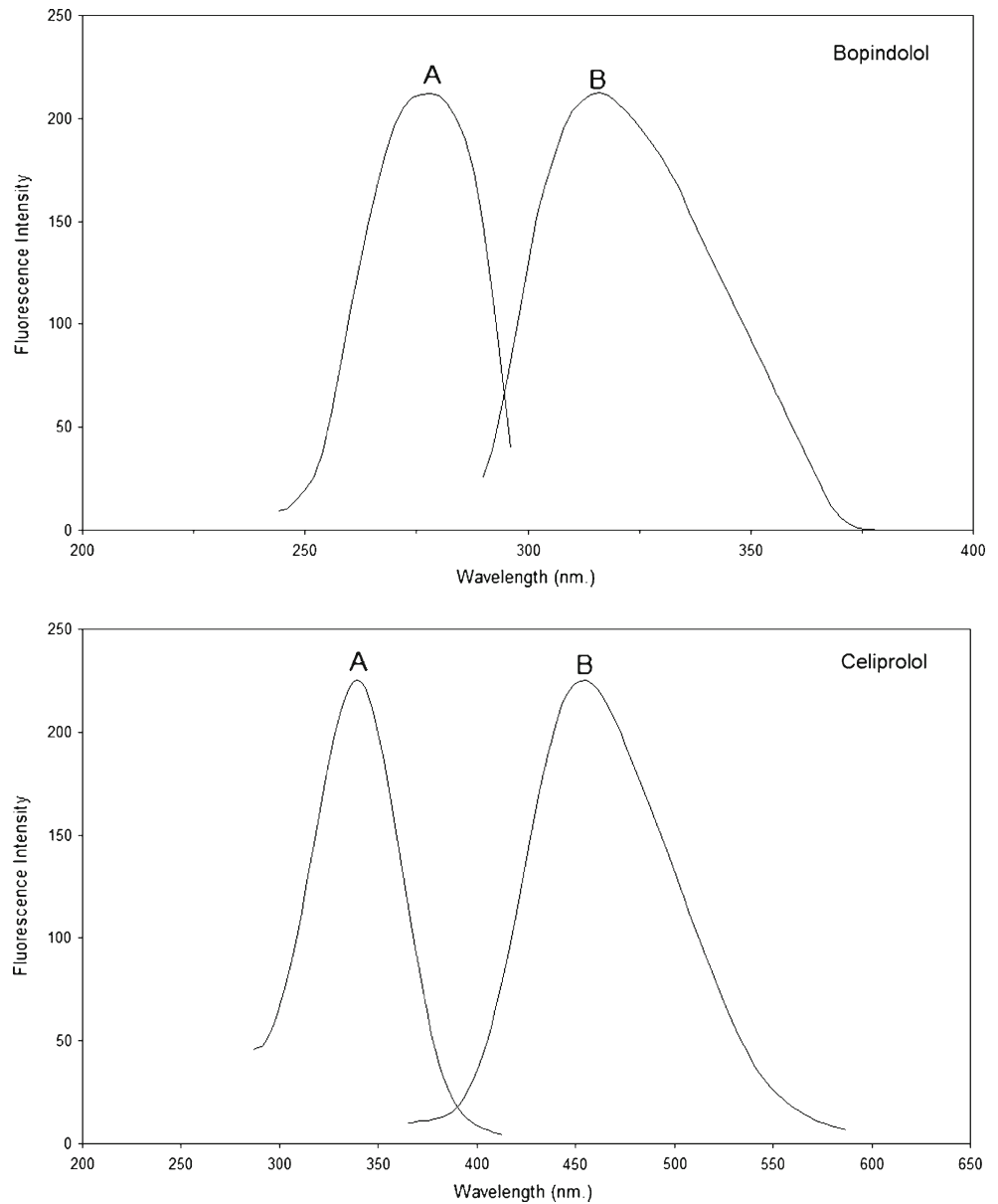

Fig. 3 Calibration curve for the fluorometric determination of BOP at 316 nm after excitation at 278 nm

Statistical analysis[34] of the data gave high values of the correlation coefficients (r) of the regression equations, small values of the standard deviation of residuals ($S_{y/x}$), of intercept (S_a), and of slope (S_b), and small value of the percentage relative standard deviation and the percentage relative error (Table 3). These data proved the linearity of the calibration graphs for both BOP and CLP, respectively.

Accuracy and Precision

To prove the accuracy of the proposed method, the results of the assay of both drugs were compared with those obtained by reference comparison methods both in pure form and in pharmaceutical preparations. For BOP:
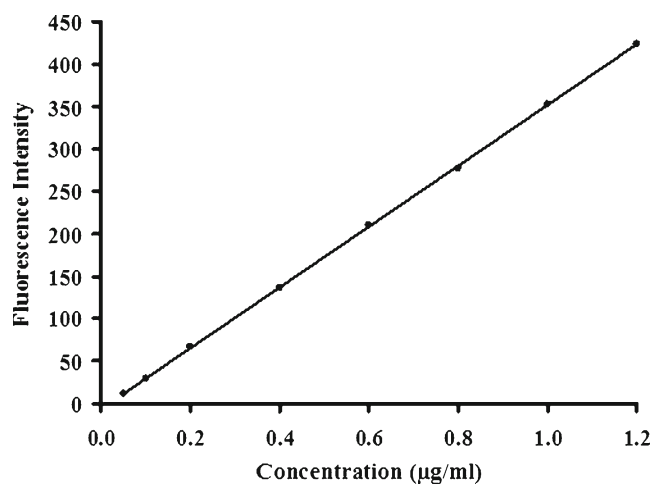
Fig. 4 Calibration curve for the fluorometric determination of CLP at 455 nm after excitation at 339 nm

The procedure[31] adopted for the assay of pindolol was applied on BOP based on a derivatization reaction with 4-dimethylaminobenzaldehyde (Ehrlich's reagent), which is used for the derivatization of secondary aromatic amines. This reaction was used by Satinsky et al.[15] for determination of BOP by sequential injection analysis (SIA) but using less drastic conditions.

The results of the assay of CLP in pure form were compared with those of the official method[17] and the results of the assay of the pharmaceutical preparation were compared with those of a reported comparison method[19]. Statistical analysis[34] of the results obtained by the proposed and comparison methods for both drugs using Student's *t*-test

and variance ratio *F*-test showed no significant difference between them regarding accuracy and precision, respectively.

Specificity

The specificity of the methods was investigated by observing any interference encountered from common tablet excipients. It was shown that these compounds did not interfere with the results of the proposed methods as indicated by the assay results shown in Table 4. The mean percents found were (99.37±0.47) and (101.24±0.77) for BOP and CLP, respectively.

Interferences likely to be introduced from co-formulated drugs, such as chlorthalidone, co-administered drugs, such as hydrochlorothiazide, or other related drugs, such as timolol, labetalol, alprenolol, propranolol, atenolol, and pindolol were studied under the same experimental conditions using a methanolic solution (in case of BOP) or acetonitrile solution (in case of CLP) of each of the above mentioned drugs. The tolerance limit (concentration of interfering drug

Table 3 Analytical performance data for the fluorometric determination of BOP and CLP

Parameter	BOP	CLP
Wavelength [$\lambda_{ex.}$ / $\lambda_{em.}$] (nm)	278 / 316	339 / 455
Linearity range (µg/mL)	0.02–1.00	0.05–1.20
Intercept (*a*)	4.602	−6.015
Slope (*b*)	313.4	357.5
Correlation coefficient (*r*)	0.9999	0.9999
S.D. of residuals ($S_{y/x}$)	1.147	1.518
S.D. of intercept (S_a)	0.507	0.904
S.D. of slope (S_b)	1.075	1.338
% RSD[a]	0.93	0.68
% Error[b]	0.29	0.24
LOD (µg/mL)[c]	0.005	0.02
LOQ (µg/mL)[d]	0.02	0.05

[a] Percentage relative standard deviation for ten replicate samples [= 100 (S.D. / mean)]

[b] Percentage relative error for ten replicate samples [= % $RSD/\sqrt{n}$]

[c] Limit of detection

[d] Limit of quantitation

Table 4 Assay results for the determination of BOP and CLP in commercial tablets by the fluorometric and comparison methods

Dosage form	% Found[a] of the drug	
	Proposed method	Comparison methods [31] for BOP, [19] for CLP
Sandonorm® tablets[b] (BOP)	98.82	98.37
	99.24	99.48
	99.87	100.26
	99.07	99.87
	99.18	
	100.02	
Mean±S.D.	99.37±0.47	99.50±0.81
t	0.32 (2.31)[d]	
F	2.969 (5.409)	
Cordiax® tablets[c] (CLP)	101.84	100.86
	101.96	98.83
	100.63	99.11
	100.51	99.27
Mean±S.D.	101.24±0.77	99.52±0.91
t	2.87 (2.45)[d]	
F	1.403 (9.277)	

[a] The average of three separate determinations

[b] Labeled to contain 1 mg bopindolol (equivalent to 1.27 mg BOP) per tablet; manufactured by Novartis Pharma Schweiz AG, Bern, Switzerland; batch number W0009X

[c] Labeled to contain 200 mg CLP per tablet; manufactured by CRINOS S.p.A., Via Pavia 6, Milano, Italy; batch number C024

[d] The figures between parentheses are the tabulated values of *t* and *F* at *P*=0.05

Table 5 Effect of some related drugs on the fluorometric determination of 0.5 µg/mL of BOP and 0.6 µg/mL of CLP

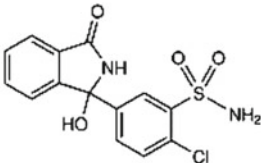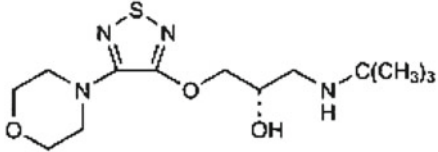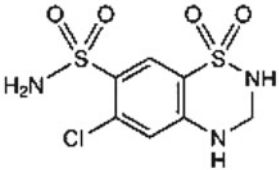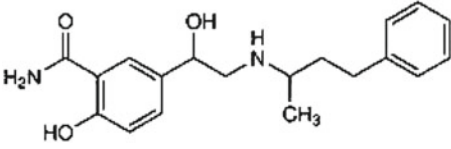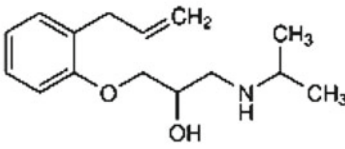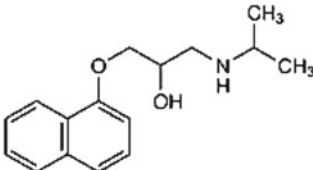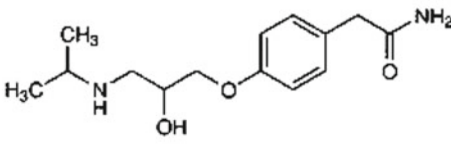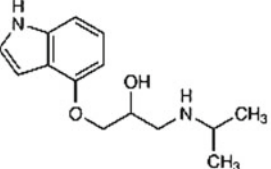| Drug	Structure	Tolerance limit (µg/mL)	
		BOP	CLP
Chlorthalidone		24.2	21.0
Timolol		25.8	14.0
Hydrochlorothiazide		3.8	160.2
Labetalol		1.5	0.02
Alprenolol		0.007	86.3
Propranolol		0.005	10.1
Atenolol		0.001	84.4
Pindolol		0.001	5.6

Table 6 Results of content uniformity testing of BOP tablets using the fluorometric method

Parameter	Tablet[a] no.	Percentage of the label claim
Data	1	101.38
	2	99.56
	3	100.44
	4	101.02
	5	99.82
	6	100.44
	7	98.12
	8	98.36
	9	99.42
	10	102.35
Mean		100.09
S.D.		1.31
% RSD[b]		1.31
% Error[c]		0.42
Acceptance value (AV)[32]	3.1	
Max. allowed AV (L1)[32]	15.0	

[a] Sandonorm® tablets: Labeled to contain 1 mg bopindolol (equivalent to 1.27 mg bopindolol malonate) per tablet; manufactured by Novartis Pharma Schweiz AG, Bern, Switzerland

[b] % RSD=100 (S.D. / mean)

[c] %Error $= \% RSD / \sqrt{n}$

causing less than 3% relative error) was calculated for each drug. In case of BOP, chlorthalidone and timolol showed very high tolerance limits indicating negligible interference with the determination. Hydrochlorothiazide and labetalol showed smaller tolerance limits indicating moderate interference with the assay. On the other hand, alprenolol, propranolol, atenolol, and pindolol showed strong interference with the fluorometric determination of BOP as indicated by the very small tolerance limit values. While in the case of CLP, hydrochlorothiazide, alprenolol, and atenolol showed very high tolerance limits indicating negligible interference with the assay. Chlorthalidone, timolol, propranolol and pindolol showed smaller tolerance limits but the interference with the assay was still neglected especially with chlorthalidone as it is always present as the minor component when co-formulated with CLP. On the contrary, labetalol showed strong interference with the fluorometric determination of CLP as indicated by the very small tolerance limit values (Table 5).

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were determined according to the USP[32] guidelines. LOD was determined by establishing the minimum level at which the analyte can reliably be detected (signal-to-noise ratio is 3:1) while LOQ was determined by establishing the lowest concentration of analyte that can be determined with acceptable precision and accuracy (signal-to-noise ratio is 10:1). The results are shown in Table 3.

Ruggedness

To examine the ruggedness of the procedures, the intraday and interday precisions were evaluated. The precision of the proposed method was fairly high, as indicated by the low values of the percentage relative standard deviation (% RSD).

Applications

Dosage form analysis For both drugs, the proposed methods were successfully applied to their assay in tablets. The average

Fig. 5 Fluorescence emission spectra of plasma spiked with CLP after enhancement with 0.5 ml 0.1% aqueous CMC solution **a** Blank, **b** 0.2 μg/ml, **c** 0.6 μg/ml, **d** 0.7 μg/ml, and **e** 0.9 μg/ml
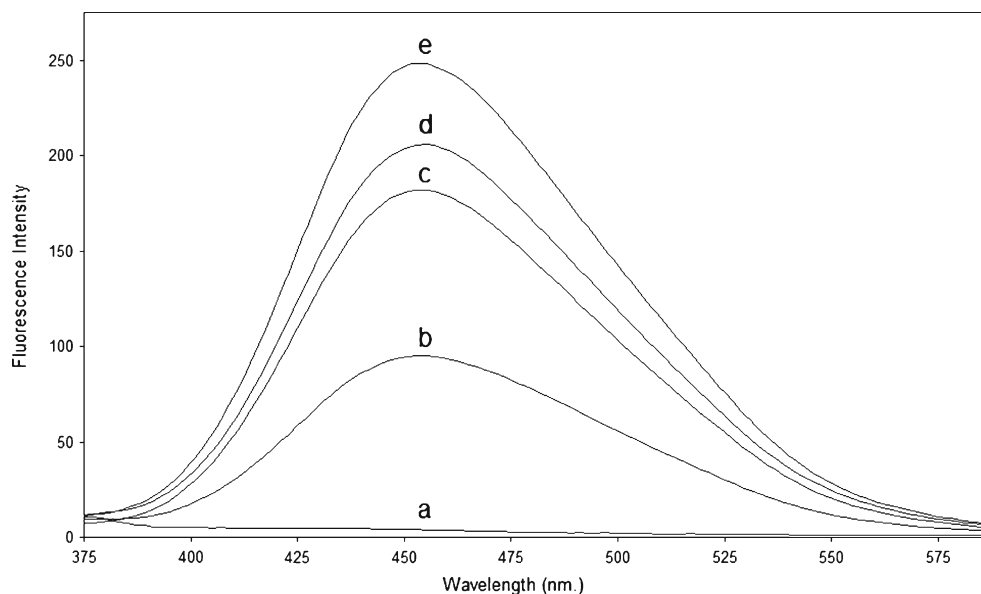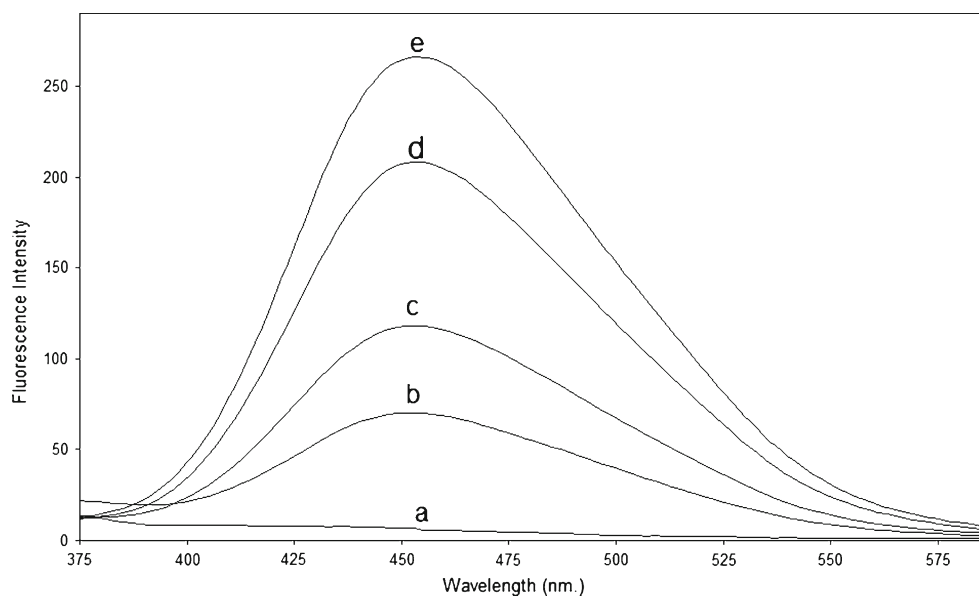
Fig. 6 Fluorescence emission spectra of urine spiked with CLP after enhancement with 0.5 ml 0.1% aqueous CMC solution. **a** Blank, **b** 0.2 μg/ml, **c** 0.4 μg/ml, **d** 0.8 μg/ml, and **e** 1.0 μg/ml

percent recoveries of different concentrations were based on the average of three replicate determinations. The results obtained were in good agreement with those obtained with the comparison method[19, 31].

Content uniformity test for BOP Due to the high sensitivity of the proposed method and its ability to rapidly measure the fluorescence intensity of a single tablet extract with sufficient

accuracy, the method is ideally suited for content uniformity testing which is a time-consuming process when using conventional assay techniques. The steps of the test were adopted according to the USP[32] procedure. The acceptance value (AV) was calculated and it was found to be smaller than the maximum allowed acceptance value (L1). The results demonstrated excellent drug uniformity as shown in Table 6.

Analysis of spiked human plasma and urine for CLP The high sensitivity of the proposed method allowed the determination of CLP in biological fluids. The method was successfully applied for the analysis of spiked human plasma (Fig. 5) and spiked human urine (Fig. 6). The nominal content of the drug in spiked plasma or urine was determined using the corresponding regression equation. The assay results using the proposed method are summarized in Table 7.

Table 7 Assay results for the fluorometric determination of CLP in spiked human plasma and urine

Parameter	Amount taken (μg/mL)	Amount found (μg/mL)	% Found
Spiked human plasma	0.30	0.308	102.67
	0.40	0.391	97.75
	0.50	0.504	100.80
	0.80	0.776	97.00
Mean			99.56
S.D.			2.65
% RSD[a]			2.66
% Error[b]			1.33
Spiked human urine	0.30	0.295	98.33
	0.50	0.510	102.00
	0.60	0.587	97.83
	0.70	0.692	98.86
Mean			99.26
S.D.			1.88
% RSD[a]			1.89
% Error[b]			0.95

[a] % RSD=100 (S.D. / mean)

[b] %Error $= \% RSD / \sqrt{n}$

Conclusion

The proposed fluorometric method for BOP could be successfully used for the determination of the drug in pharmaceutical formulations without interference of co-formulated drugs. The detection limit of the proposed method was found to be 0.005 μg/mL while the quantitation limit was 0.02 μg/mL. The proposed method is very suitable to be applied in content uniformity testing.

The proposed fluorometric method for CLP could be applied for its determination of in pharmaceutical formulations and biological fluids without interference of co-formulated or co-administered drugs. The detection limit of the proposed method was found to be 0.02 μg/mL while the quantitation limit was 0.05 μg/mL.

The proposed procedures are simple, sensitive, reliable, accurate, and rapid. The good validation criteria of the proposed methods allow their use in the routine quality control of pharmaceutical preparations as well as in pharmacokinetic and toxicological studies with a relatively inexpensive instrumentation.

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