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Simultaneous determination of allopurinol and benzbromarone in presence of the active metabolite oxipurinol

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ABSTRACT

Simple, sensitive, selective and precise spectrophotometric, spectrodensitometric and HPLC methods were developed and validated for the simultaneous determination of allopurinol (AL) & benzbromarone (BZ) in presence of oxipurinol (OX) which is the active metabolite of AL. In the spectrophotometric methods, BZ was determined by the application of direct spectrophotometry and measuring its zero-order (D_0) absorption spectra at its λ_{max}=356 nm. AL was determined by measuring the peak amplitude of the second derivative curve (D_2) at 281.4 nm or by measuring the peak amplitude of the second derivative of the ratio spectra curve (DD_2) at 282.4 nm after using a spectrum of 8ug/ml BZ as a divisor. The TLC-densitometric method depends on the difference in R_f values using acetone: chloroform: NH_3 (5: 4: 0.01 v/v/v) as a mobile phase. The spots of the two drugs were scanned at 250 and 356 nm. The HPLC method was based on the separation of the drugs on a reversed phase column using a mobile phase of 0.01 M phosphate buffer pH: 4.0- acetonitrile-methanol (50:30: 20 v/v/v) with UV detection of the effluent at 250 nm. The proposed methods were used to determine both drugs in their pure powder form and in pharmaceutical dosage forms.

KEYWORDS

Allopurinol;
Benzbromarone;
Oxipurinol;
Derivative spectrophotometry;
TLC-densitometry;
RP-HPLC.

INTRODUCTION

Allopurinol (Figure 1) is worldwide the mainstay of modern treatment of gout and prevention of tumor lysis syndrome. Allopurinol, an isomer of hypoxanthine, and its active metabolite oxipurinol (Figure 2) act by inhibiting xanthine oxidase, an enzyme which forms uric acid (urate) from xanthine and hypoxanthine. Allopurinol is

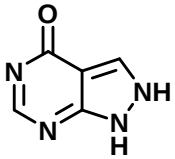

Figure 1 : The structure of allopurinol

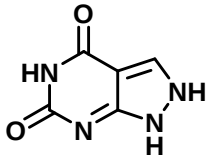

Figure 2 : The structure of oxipurinol

principally metabolized by aldehyde oxidase to the active compound oxipurinol[1]. Benzbromarone (Figure 3) is a uricosuric drug that reduces plasma concentrations of uric acid by blocking renal tubular reabsorption through interaction at the URAT-1 transporter[2,3]. Combination of allopurinol and benzbromarone is frequently used in treatment of hyperuricaemia including that associated with chronic gout[4].

Various analytical techniques were employed for the quantitative analysis of AL; UV spectrophotometry[5], photometry using Folin Ciocalteu reagent[6], fluorimetry based on the quenching effect on the fluorescence of mercurochrome[7], reductive polarographic analysis[8] and flow injection analysis with anodic polarographic detection[9]. Static and flow-through sensors based on xanthine oxidase have been proposed[10,11].

Several methods were also described for the analysis of AL and OX in human serum, using HPLC[12-21] and electrophoresis[22].

Few analytical techniques have been reported in the literature for the quantitative determination of BZ; most of them in biological fluids. These were gas chromatography[23] and HPLC[24,25].

A comprehensive literature survey reveals the lack of any analytical technique for the simultaneous determination of AL and BZ.

The objective of the present study was to develop and validate new simple, rapid and selective analytical methods which enable measurement of AL and BZ in presence of OX. These methods included second-derivative (D_2), second-derivative of the ratio spectra (DD_2), TLC-densitometry and HPLC.

EXPERIMENTAL

Apparatus

Spectrophotometer: SHIMADZU dual beam (Kyoto/ Japan) UV-visible spectrophotometer model UV-1601 PC. TLC plates-Precoated with silica gel F_{254} (20cm × 20 cm, 0.25 mm thickness), E.Merck, (Darmstadt, Germany). Camag Linomat 5 autosampler with Camag microsyringe (100 µl); (CAMAG, Muttenz, Switzerland). Camag TLC scanner- Model 3 S/N 130319 with winCats software (CAMAG, Muttenz, Switzerland).

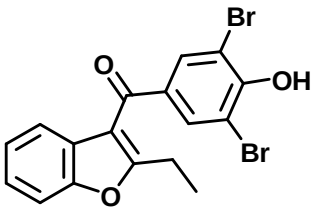

Figure 3 : The structure of benzbromarone

A liquid chromatograph consisted of an isocratic pump (Agilent Model G1310A), an ultraviolet variable wavelength detector (Model G1314A, Agilent 1100 Series), a Rheodyne injector (Model 7725 I, Rohnert Park, CA, USA) equipped with 20µL injector loop, Agilent (USA). Stationary phase; a 250mm × 4.6mm i.d. C18 Zorbax™ 5µm analytical column, Agilent (USA). The mobile phase was filtered through a 0.45µm Millipore membrane filter and was degassed for ~15 min in an ultrasonic bath prior to use. UV-detection was done at 250 nm. The samples were filtered also through a 0.45µm membrane filter, and were injected by the aid of a 25µL Hamilton® analytical syringe.

Chemicals and reagents

Allopurinol and benzbromarone were kindly supplied by (Marcyrl Pharmaceutical Industries, El-Obour City, Egypt), their purity were certified to be 100.26±0.981and 99.89±1.023 respectively. Oxipurinol was obtained from Sigma (St. Louis, U.S.A). Its purity was labelled to be 99.69 %.

All chemicals used throughout this work were of analytical grade, and the solvents were of spectroscopic grade. Methanol, acetone: chloroform, ammonium hydroxide 33%-Prolabo (VWR International, West Chester, PA). Acetonitrile and potassium dihydrogenphosphate; Sigma (U.S.A).

Harpagin® tablets, labeled to contain 100 mg Allopurinol and 20 mg Benzbromarone per tablet, batch No. 93050 , manufactured by Marcyrl Pharmaceutical Industries, El-Obour City, Egypt, under license of Merz pharmaceuticals GmbH-Germany.

Standard solutions

Allopurinol standard solutions (0.1mg/ml) in methanol for the D_2, DD_2 and HPLC methods, and (1mg/ml) in methanol for the TLC-densitometric method.

Benzbromarone standard solutions (0.1mg/ml) in methanol for the D_0 and HPLC methods, and (1mg/ml)

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TABLE 1 : Parameters required for system suitability test of HPLC method

Parameter	Obtained value			Reference value
	OX	AL	BZ	
Resolution (R)	2.74		2.42	R > 0.8
T (tailing factor)	1.0	1.0	1.12	T = 1 for a typical symmetric peak
α (relative retention time)	1.590		1.597	> 1
K (column capacity)	1.552	3.059	5.483	1- 10 acceptable
N (column efficiency)	416.8	732.2	343.1	Increases with efficiency of the separation
HETP	0.06	0.034	0.072	The smaller the value, the higher the column efficiency

in methanol for the TLC-densitometric method.

Oxipurinol standard solutions (0.1 mg/ml) in methanol for the D_2, DD_2 and HPLC methods, and 1mg/ml in methanol for the TLC-densitometric method.

Procedures

(A) Construction of calibration graphs

(a) Spectrophotometric methods

1 **For BZ (D_0 method):** Aliquots equivalent to 20-140 μg of BZ were transferred from its standard solution (0.1 mg/ml) into a series of 10-ml volumetric flasks and completed to mark with methanol. The zero order (D_0) absorption spectra of these concentrations were recorded at 356 nm using methanol as a blank and the calibration curve was constructed.

2 **For AL (D_2 method):** Aliquots equivalent to 20-200 μg of AL were transferred from stock solution (0.1 mg/ml) into a series of 10-ml volumetric flasks and completed to mark with methanol. The zero order (D_0) absorption spectra of these concentrations were recorded using methanol as a blank. The second derivative (D_2) spectra was computed and the peak amplitude at 281.4 nm was recorded for AL, using scaling factor = 100 and $\Delta\lambda$ = 8. Calibration curve was constructed relating the peak amplitudes of the second derivative of AL at 281.4 nm, to the corresponding drug concentrations.

3 **For AL (DD_2 method):** Alternatively, the zero order spectra were divided by the spectrum of (8 μg/ml) BZ. Then the second derivative of the obtained ratio spectra were recorded using scaling factor = 100 and $\Delta\lambda$ = 8 nm. Calibration curve was constructed relating the peak amplitudes of the second derivative of the ratio spectra of AL at 283.4 nm, to the corresponding drug concentrations and the regression equation was computed.

(b) TLC-densitometric method

Aliquots equivalent to 2-12 μg of AL and BZ were spotted from their stock solutions (1.0 mg/ml) onto a TLC plate using Camag Linomat autosampler with microsyringe (100 μl). Spots were spaced 24.5 mm apart from each other and 15 mm from the bottom edge of the plate with a band length of 2 mm. The plates were developed in a chromatographic tanks previously saturated with the mobile phase acetone: chloroform: NH_3 (5: 4: 0.01 v/v/v), by ascending chromatography. The plates were dried; spots were scanned at 250 nm for AL, and 356 nm for BZ. Two calibration graphs relating the optical density of each spot to the corresponding concentration of AL and BZ were constructed.

(c) HPLC method

Aliquots equivalent to 10-100 μg of AL and BZ were transferred from their stock solution (0.1 mg/ml) separately into a series of 10mL volumetric flasks. The contents of each flask were completed to volume with the mobile phase. The samples were then chromatographed using a mobile phase of; 0.01 M phosphate buffer pH: 4.0-acetonitrile-methanol (50:30: 20 v/v/v) with a flow rate of; 1mLmin^{-1} and detected at 250 nm. The relative peak area ratios of AL and BZ to that of external standard (5μg/ml of each drug) were then plotted versus the corresponding concentrations of AL and BZ to construct the calibration curves.

(B) Analysis of laboratory prepared mixtures

Laboratory prepared mixtures containing different ratios of BZ, AL and OX were prepared and analyzed by the proposed methods.

(C) Application of the proposed methods to the analysis of AL and BZ in pharmaceutical preparation

(1) Spectropthotometric and HPLC methods

Ten tablets of Harpagin® were weighed, powdered finely and mixed thoroughly. An accurately weighed portions of the powder equivalent to 15 mg AL and 3 mg BZ for the spectrophotometric methods or equivalent to 10 mg AL and 2 mg BZ for the HPLC method

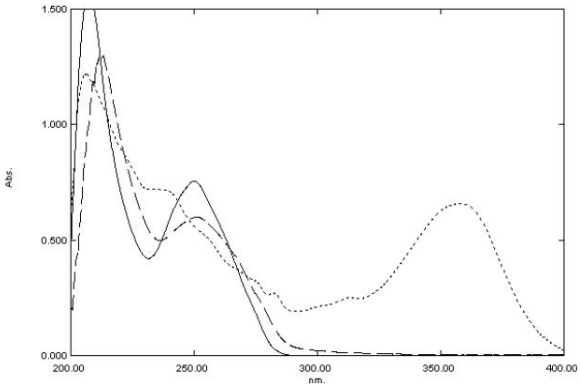

Figure 4 : Zero order absorption spectra of 10 µg/ml allopurinol (—), 10 µg/ml benzbromarone (·····)and 6 µg/ml oxipurinol (———) using methanol as a blank

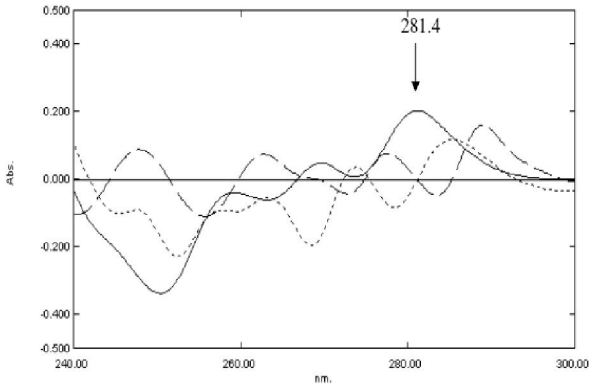

Figure 5 : Second derivative absorption spectra of 8 µg/ml allopurinol (—), 8 µg/ml oxipurinol (·····) and 8 µg/ml benzbromarone (- - - -) using methanol as a blank

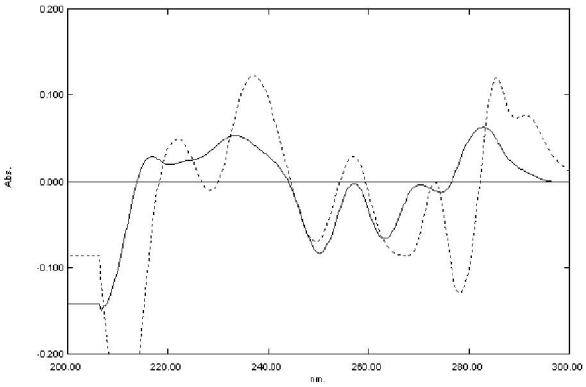

Figure 6 : Second derivative of ratio spectra of allopurinol 10 µg/ml (—), 10µg/ml oxipurinol (———) using the spectrum of 8 µg/ml of benzbromarone as a divisor, methanol was used as a blank

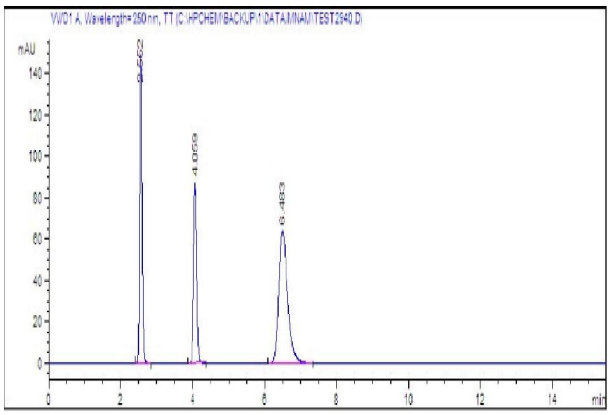

Figure 7 : HPLC chromatogram of 6 µg/ml oxipurinol (1), 6 µg/ml allopurinol (2) and 8 µg/ml benzbromarone (3)

were transferred into two 250 ml beakers and then 50 ml methanol was added to each, Stirred for 10 minutes using a magnetic stirrer then filtered into a 100-ml volumetric flask. The residue was washed three times with 10 ml methanol and completed to mark with methanol. 1 ml of the filtrate was accurately transferred into a 10-ml volumetric flask, completed to mark with methanol. Then the procedure was completed as described in spectrophotometric and HPLC method.

(2) TLC-Densitometric method

Ten tablets of Harpagin® were weighed, finely powdered and mixed. An accurately weighed portion of the powder was transferred equivalent to 25 mg AL and 5 mg BZ into a 50-ml beaker and then 10 ml methanol was added, Stirred for 10 minutes using a magnetic stirrer then filtered into a 25 ml volumetric-flask. Wash the residue three times each with 5 ml methanol then complete to the mark with the same solvent. 11.0 µl of the prepared solution was spotted in triplicate using Linomat applicator onto a TLC plate, and the procedure was completed as described in TLC-Densitometric method.

RESULTS AND DISCUSSION

D_2 and DD_2 methods

The zero order spectra of the three components (Figure 4) shows that BZ can be directly measured at 356 nm without any interference from AL and OX, while AL couldn't be measured directly in the presence of BZ and OX due to severe overlap.

A rapid, simple and low cost spectrophotometric method based on measuring the peak amplitude of D_2 spectrum of AL at 281.4 nm (corresponding to zero-crossing of BZ and OX) was developed with good selectivity without interference of BZ and OX as shown

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TABLE 2 : Determination of benzbromarone and allopurinol in laboratory prepared mixtures by the proposed spectrophotometric and HPLC methods

Concentration (µg/ml)			BZ	AL	AL	BZ	AL
BZ	AL	OX	D_0 method	D_2 method	DD_2 method	HPLC method	HPLC method
2.00	10.00	0.00	99.56	101.23	98.89	99.12	98.56
2.00	8.00	2.00	99.81	98.29	99.81	98.34	101.72
4.00	6.00	4.00	99.23	99.87	99.23	101.58	98.90
4.00	6.00	4.00	99.75	101.57	100.98	101.77	98.52
6.00	4.00	4.00	101.23	112.23*	101.23	100.25	101.23
8.00	2.00	6.00	100.92	117.92*	101.58	101.58	101.72
Mean			100.08	99.91	100.29	100.21	99.79
SD			0.800	1.153	1.126	1.500	1.559
RSD %			0.800	1.154	1.123	1.497	1.562

***Rejected values**

TABLE 3 : Determination of allopurinol and benzbromarone in laboratory prepared mixtures by the proposed TLC-densitometric method

Concentration (µg/spot)			Recovery % of AL	Recovery % of BZ
AL	OX	BZ	**98.56**	**99.12**
10.00	00.00	2.00	101.72	98.34
8.00	2.00	2.00	98.90	101.58
6.00	2.00	4.00	98.52	101.77
4.00	4.00	4.00	101.23	100.25
2.00	4.00	6.00	98.56	99.12
Mean			99.79	100.21
S.D.			1.559	1.500
RSD%			1.562	1.497

in (Figure 5). In order to optimize D_2 method, different smoothing and scaling factors were tested, where a smoothing factor $\Delta\lambda$= 8 and a scaling factor = 100 showed a suitable signal to noise ratio and the spectra showed good resolution. Linear calibration curve was obtained for BZ in concentration range of 2-14 µg/ml, and for AL in concentration range of 2-20 µg/ml by recording the peak amplitude of D_2 at 281.4. The regression equations were computed and found to be:

A = 0.0623 C + 0.0704 r = 0.9999 at 356 nm for BZ

D_2 = 0.0257C + 0.0035 r = 0.9998 at 281.4 nm for AL

where A and D_2 is the absorbance and peak amplitude for BZ and AL, respectively, C is the concentration of the drug in µg/ml and r is the correlation coefficient.

In order to improve the selectivity of the analysis of AL in presence of BZ and OX, DD_2 was established. The main advantage of the method was that the whole spectrum of interfering substance was cancelled. Accordingly, the choice of the wavelength selected for calibration wasn't critical as in the D_2 method. DD_2 method demonstrated high potentiality and more selectivity for simple and rapid determination of AL in ternary mixture with BZ and OX without interference.

In order to optimize DD_2 method, several divisor concentrations 2, 4, 6 and 8 µg/ml of BZ was tried, the best result was obtained when using 8 µg/ml of BZ as a divisor. Different smoothing and scaling factors were tested, where a smoothing factor $\Delta\lambda$ = 8 and a scaling factor = 100 were suitable to enlarge the signal of AL to facilitate its measurement and to diminish error in reading the signal. Dividing the absorption spectra of AL and OX by the absorption spectrum of 8 µg/ml of BZ (as a divisor); the obtained ratio spectra were differentiated with respect to wavelength. DD_2 values showed good linearity and reproducibility at 283.4 nm for AL, where OX has no contribution (Figure 6).

The linearity of the peak amplitudes of the of the DD_2 curves at 283.4 nm was studied for AL. A linear relationship was obtained in the range from 2-20 µg/ml for the drug, and the regression equation was computed and found to be:

DD_2 = 0.0064C + 0.0001 r = 0.9998 at 283.4 nm for AL

where DD_2 is the peak amplitude, C is the concentration of the drug in µg/ml and r is the correlation coefficient.

TLC-densitometric method

The TLC-densitometric method for the simultaneous determination of AL and BZ in the presence of OX

TABLE 4 : Determination of benzbromarone and allopurinol in Harpagin® tablets by the proposed and the manufacturer methods and application of standard addition technique

Product	D_0 method	D_2 method	DD_2 method	TLC method		HPLC method		Manufacturer method[a]	
	BZ	AL		BZ	AL	BZ	AL	BZ	AL
	%Found ± S.D.[b]								
Harpagin® tablets 20mg BZ and 100mgAL/tablet Batch No. 93050	100.78± 0.858	100.15± 0.703	100.46± 0.881	99.64± 0.779	100.14± 0.791	99.82 ± 0.670	100.08± 0.898	99.98± 0.890	99.85± 0.981
Recovery of standard added %	100.61± 0.977	100.08± 1.507	99.64± 1.919	101.42± 0.382	99.75± 1.521	100.39± 1.512	99.44± 0.918	99.98± 0.890	99.85± 0.981

[a]HPLC method using C_{18} column, aqueous 0.01 M potassium dihydrogenphosphate , pH: 3.0-acetonitrile (30:70, v/v) as a mobile phase and UV detection at 237 nm. [b]Average of three determination

TABLE 5 : Statistical analysis of the results obtained by applying theproposed methods and the manufacturer methods for the determination of benzbromarone and allopurinol

Item	BZ				AL				
	D_0 method	TLC method	HPLC method	Manufacturer method[a]	D_2 method	DD_2 method	TLC method	HPLC method	Manufacturer method[a]
Mean	100.09	100.53	100.53	99.98	100.53	100.49	100.39	100.39	99.85
S.D	0.552	0.482	0.482	0.890	0.602	0.718	0.559	0.552	0.981
n	7	6	10	5	10	10	6	10	5
Variance	0.305	0.232	0.232	0.792	0.352	0.516	0.312	0.305	0.952
Student's t-test[b]	0.266 (2.228)	1.31 (2.262)	1.622 (2.228)		1.78 (2.160)	1.51 (2.160)	1.16 (2.31)	0.938 (2.228)	
F value[b]	2.60 (4.53)	3.41 (5.19)	3.38 (5.05)		2.23 (3.63)	1.52 (3.63)	3.05 (5.19)	2.60 (5.05)	

[a]HPLC method using C_{18} column, aqueous 0.01 M potassium dihydrogenphosphate, pH: 3.0-acetonitrile (30:70, v/v) as a mobile phase and UV detection at 237 nm. [b]The values in the parenthesis are the corresponding theoretical values of t and F at (P= 0.05)

depended on the difference in R_f values is also described. Several trials were done to choose a developing system. Complete separation was obtained using acetone: chloroform: NH_3 (5: 4: 0.01 by volume) as a mobile phase. The R_f values were 0.27, 0.70 and 0.11 for AL, BZ and OX, respectively. The spots were scanned at 250 nm for AL and 356 nm for BZ without any interference from OX.

Linear relationship was found to exist between the integrated area under the peak of the separated spots at the selected wavelength (250 and 356 nm) and the corresponding concentrations of AL and BZ in the range of (2-12 μg/spot) for both drugs. The regression equations were computed and found to be:

A= 0.14340 C + 0.0331 r = 0.9997 for AL

A= 0.2233 C + 0.0093 r = 0.9998 for BZ

where A is the integrated area under the peak x 10^{-4}, C is the concentration in μg/spot and r is the correlation coefficient.

The proposed TLC method was very simple, rapid and consumed minimal volume of solvents compared with other separation techniques. Furthermore, an extremely large numbers of samples could be analyzed at the same time without compromising accuracy. The proposed method is suitable for quality control laboratories where economy and time is essential.

HPLC method

A simple isocratic RP-HPLC method with UV detection was developed for the simultaneous determination of AL and BZ in the presence of OX, using C_{18} analytical column with a mobile phase was of 0.01 M phosphate buffer pH: 4.0-acetonitrile-methanol (50:30: 20 v/v).

System suitability parameters were tested by calculating the capacity factor, tailing factor, the sensitivity factor and resolution (TABLE 1).

The average retention times under the conditions described were 2.56 ± 0.02 min for OX, 4.05 ± 0.02 min for AL and 6.48 ± 0.02 min for BZ (Figure 7).

Calibration curves were obtained by plotting the relationship between the relative peak area at the se-

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TABLE 6 : Assay validation sheet of the proposed methods for the determination of benzbromarone and allopurinol

Item	BZ			AL			
	D_0 method	TLC method	HPLC method	D_2 method	DD_2 method	TLC method	HPLC method
Accuracy (mean ± SD)	100.09±0.552	100.53±0.482	100.04±1.061	100.53±0.602	100.49±0.718	100.39±0.482	99.84±0.675
Specificity	100.08±0.800	100.21±1.500	99.78±0.897	99.91±1.153	100.29±1.123	99.79±1.559	99.79±1.559
Precision repeatability [a]	100.53±0.446	99.78 ± 0.965	100.61±0.371	99.82 ± 0.552	100.82 ± 0.959	100.61 ± 0.371	100.25±0.465
Intermediate precision[b]	100.29±1.162	100.89 ± 0.717	100.10±0.464	100.70±0.622	98.98 ±0.574	100.10 ± 0.464	100.65±0.617
Linearity slope	0.0623	0.14340	0.2233	0.0257	0.0064	0.2233	0.2004
Intercept	0.0704	0.0331	0.0108	0.0035	0.0001	0.0093	0.1992
Correlation coefficient(r)	0.9999	0.9997	0.9998	0.9998	0.9996	0.9998	0.9999
Range	2-14 µg/ml	2-12 µg/spot	1-10 µg/ml	2-20 µg/ml	2-20 µg/ml	2-12 µg/spot	1-10 µg/ml

[a]The intraday (n = 3), average of (4,6,8 µg/ml) of BZ and AL by the proposed spectrophotometric and HPLC methods and (4,6,8 µg/spot) for the TLC-densitometric methods. [b]The interday (n = 3), average of (4,6,8 µg/ml) of BZ and AL by the proposed spectrophotometric and HPLC methods and (4,6,8 µg/spot) for the TLC-densitometric methods

lected wavelength (250 nm) and the corresponding concentrations of AL and BZ in the range of (1-10 µg/ml) for both drugs by adopting the external standard method for calibration.

The linear regression equations were computed and found to be:

A= 0.2004 C + 0.0008 r = 0.9999 for AL

A= 0.1992 C + 0.0108 r = 0.9998 for BZ

where A is the relative peak area, C is the concentration in µg/ml and r is the correlation coefficient.

The robustness of the HPLC method was investigated by analysis of samples under a variety of experimental conditions such as small changes in the pH (4.0–4.5), small changes in acetonitrile/methanol ratio (from 30/20 to 20/30) in the mobile phase and changing the column using a250mm × 4.6mm, i.d. C_{18} Lichrosorb™ 10µm analytical column, Alltech (USA). The effect on retention time and peak parameters was studied. It was found that the method was robust when the column and the mobile phase ratio were varied. During these investigations, the retention times were modified, however the areas and peaks symmetry were conserved.

The specificity of the proposed methods was checked by analysis of laboratory prepared mixtures containing different ratios of AL and BZ with OX (TABLE 2 & 3).

All the proposed methods were successfully applied for the determination of AL and BZ in Harpagin® tablets with no interference of the excipients. The validity of the methods was assessed by applying the standard addition technique, (TABLE 4).

Results obtained by the proposed procedures for the determination of pure samples of AL and BZ were statistically compared to those obtained by the manufacturer method[26] and no significant difference was observed, (TABLE 5). Method validation was performed according to USP guidelines[27] for all the proposed methods. (TABLE 6) showed results of accuracy, repeatability and intermediate precision of the methods.

Results obtained by the proposed procedures for the determination of pure samples of AL and BZ were statistically compared to those obtained by the manufacturer method[26] and no significant difference was observed, (TABLE 5). Method validation was performed according to USP guidelines[27] for all the proposed methods. (TABLE 6) showed results of accuracy, repeatability and intermediate precision of the methods.

CONCLUSION

The present work is concerned with the simultaneous determination of AL and BZ in the presence of OX. In this paper simple, sensitive and rapid methods are described for simultaneous determination of AL and BZ in pure form or in pharmaceutical formulations. Reviewing literature in hand no other analytical methods concerned with the simultaneous determination of AL and BZ in presence OX which is pharmacologically active and no synthetic mixtures were prepared to check the specificity of the method. D_2 and DD_2 spectrophotometric methods are well-established techniques that are able to enhance the resolution of overlapping bands.

These methods are simple, more convenient, less time consuming and economic compared to other published methods. The advantages of TLC-densitometric method is that several samples can be determined simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis and provides high sensitivity and selectivity. High values of correlation coefficients and small values of intercepts validated the linearity of the calibration graphs and the obedience to Beer's law. The R.S.D. values, the slopes and the intercepts of the calibration graphs indicated the high reproducibility of the proposed methods. From the results obtained, we concluded that the suggested methods showed high sensitivity, accuracy, reproducibility and specificity. Moreover, these methods are simple and inexpensive, permitting their application in quality control laboratories.

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