Densitometric and Spectrometric Determinations of Florfenicol and Buparvaquone

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FFB, SAAR and MMF designed and wrote the protocol of the study. Authors MMF and FAF managed the analyses of the study. Author D managed the literature searches, performed the statistical analysis and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Three new accurate and sensitive methods were developed for the determination of two veterinary drugs. Florfenicol was analyzed by a stability-indicating TLC/densitometric method in presence of its amine degradation product obtained via acid hydrolysis. It was based on the difference in R_f values of the drug and its degradation product at 254 nm on silica gel 60 GF_{254} plates using chloroform–methanol (7:3 v/v) as developing solvent (Method A). However, buparvaquone was analyzed through reduction with $NaBH_4$ in methanolto yielda red colored product measured spectrophotometrically at 489 nm (Method B). This reduction product was also measured spectrofluorometrically at 450 nm after excitation at 334 nm (Method C). Good linearity was obtained in the range of 10-100 µg/spot for florfenicol in method A and 10-120 or 0.3-2.4 µg mL^{-1} for buparvaquone by methods B and C with mean accuracy of 99.94%±0.89, 99.99%±0.99 or 99.66%±1.80, respectively. The densitometric method A proved to be stability-indicating in presence of 10-90% of buparvaquone amine degradation product. The three proposed methods were validated according to ICH guidelines and successfully applied for the assay of the cited drugs in their pharmaceutical formulations.

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1. INTRODUCTION

Florfenicol, a fluorinated analogue of thiamphenicol is a broad spectrum antibiotic used for the treatment of respiratory and in urinary tract infections. It acts by binding to the 50S ribosome, thereby inhibiting bacterial protein synthesis [1] and is approved for use in cattle, sheep and goat for the treatment of [2]. Literature survey revealed that florfenicol was analysed by UV-spectrophotometry [3], cyclic voltametry [4], micellar electrokinetic chromatography [5,6], HPTLC [7], GC [8-11] and HPLC [12-17].

Buparvaquone is a second generation highly lipophilic hydroxynaphthoquinone, used as antiprotozoal for the treatment of bovintheileriosis [18]. Only few methods have been reported in the literature for its assay including HPLC [19,20] and a spectrofluorimetric one depending on quenching effect of buparvaquone on the fluorescence intensity of Tb^{3+}-deferasirox complex as a fluorescence probe [21]. Recently, we have reported a spectrophotometric method for its assay through metal chelation or reaction with KOH [22].

The aim of this work is to introduce a selective and reproducible stability-indicating densitometric method for the selective quantitation of pure florfenicol and to develop new simple and sensitive spectrophotometric and spectrofluorometric methods for determination of buparvaquone in its pure form as well as in pharmaceutical formulations.

2. MATERIALS AND METHODS

2.1 Apparatus

- Camag TLC scanner 3, with WINCATS computer software (Switzerland).
- Precoated TLC plates, silica gel 60 GF$_{254}$ (20 × 20 cm), (Flukachemie, Switzerland).
- 20-μL microsyringe (Hamilton, Germany).
- UV lamp with short wavelength, 254 nm (Desega-Germany).
- Chromatographic tank (25 × 25 × 9 cm).
- Jenco digital pH/temp meter with Jenway glass electrode (UK).
- Shimadzu UV-Vis 1601 PC spectrophotometer (Tokyo, Japan).
- Jasco FP-6200 spectrofluorometer (USA).

2.2. Samples

Pure florfenicol was kindly supplied by Pharma Swede (Cairo, Egypt) and pure buparvaquone was supplied by Waycome Ph. Co. (Cairo, Egypt) with purity of 99.1% and 99.87%, respectively as stated by the suppliers.

Panflor® oral solution; B.N. 437, each 100 mL claimed to contain 10 g florfenicol (the product of Marcyrl Pharmaceutical Industries, El Obour City, Egypt) and Butalex® injections, B.N. 6666105; each 1mL contain 50 mg buparvaquone (product of Schering – Plough, Germany) were purchased from local market.

2.3 Chemicals and Reagents

All chemicals and solvents used through this study were of analytical grade and distilled water was used throughout this study.

- Chloroform (Sigma – Aldrich, USA).
- Ethanol absolute (Riedell - detlean, Germany).
- Hydrogen peroxide (El Nasr Co., Egypt); 5% aqueous solution.
- Sodium hydroxide (El Nasr Co., Egypt); 5 M aqueous solution.
- $NaBH_4$ (Merck, Germany), 0.03%, 2% and 5×10^{-3} M solution in methanol. The latter was prepared by dissolving 0.019 g in 100 mL methanol.
- Methanol (Sigma – Aldrich, USA).
- Gelatin (Adwic, Egypt); 1% aqueous solution.
- β-cyclodextrin (Fluka, Switzerland); 1% aqueous solution.
- Sodium dodecyl sulphate, SDS (Oxford Lab., Mumbai); 1% aqueous solution.
- HCl (Prolabo, France); 0.1M and 5M aqueous solutions and 2% ethanolic solutions.
- H_2SO_4 (Merck, Germany); 0.1M solution prepared in water.
- Acetonitrile (Sigma – Aldrich, USA).
- Absolute ethanol for HPLC (Fisher-Chemical, UK).
- Methanol for HPLC (POCH, Poland).

2.4 Standard Solutions

Ethanolic solution of florfenicol (10 mg mL^{-1}) was prepared for method A.

Standard methanolic solution of buparvaquone, 0.4 mg mL^{-1} and 5×10^{-3} M solutions, the later was prepared by dissolving 0.163 g of the drug in methanol and completing to 100 mL to be used for method B.

Fresh stock standard solution of reduced buparvaquone (0.4 mg mL^{-1}) was prepared by dissolving 10 mg of pure drug in 4 mL methanol, adding 1 mL of 2% $NaBH_4$ and completing to 25 ml with 2% ethanolic HCl in a volumetric flask. One mL of this solution was transferred into a 100-mL volumetric flask and diluted to mark with methanol to obtain a working solution labeled to contain 4 µg mL^{-1} to be used in method C.

2.5 Preparation of Degraded Florfenicol

Florfenicol (250 mg) were accurately weighed and transferred to a 100-mL round bottomed flask to which 50 mL of 5 M HCL were added and refluxed for 4 h. Then cooled, neutralized with 5 M NaOH and evaporated to dryness under vacuum. The obtained residue was extracted with ethanol (3×10 mL), filtered into a 25-mL volumetric flask and diluted to volume with ethanol to obtain a solution labeled to contain amine degradation product derived from 10 mg mL^{-1} florfenicol.

2.6 Procedures

2.6.1 Linearity

Method A- Into a series of 10-mL volumetric flasks, aliquots of standard florfenicol solution (10 mg mL^{-1}) equivalent to 5-50 mg drug were transferred and diluted to volume with

ethanol. Twenty μL of each solution were applied to a 20×20 cm TLC silica gel 60 GF254 plate using Hamilton microsyringe. The plate was developed in a presaturated tank with a mobile phase of $CHCl_3$- methanol (7:3). The plate was then air dried, detected under UV-lamp and scanned at 254nm. Calibration curve was constructed by plotting area under the peak versus the corresponding drug concentrations in μg/spot and the regression equation was computed.

Method B- Into a series of 10-mL volumetric flasks, aliquot volumes of standard solution of buparvaquone (0.4 mg mL^{-1}) containing (0.1-1.2 mg) of the drug were introduced. 1.5 mL of 0.03% $NaBH_4$ was added and the volumes were completed with methanol. The absorbance of the formed red color was measured at 489 nm against a reagent blank. The absorbance was plotted against concentration to construct the calibration curve from which the regression equation was computed.

Method C- Aliquots of standard reduced working buparvaquone solution in methanol (4 μg mL^{-1}) prepared under (2.4. Standard Solutions) equivalent to (3-24 μg) were transferred into a series of 10-mL volumetric flasks and diluted to volume with methanol. The fluorescence intensity was measured at 450 nm after excitation at 334 nm against methanol as blank. Relative fluorescence intensity was plotted versus final drug concentrations and the regression equation was derived.

2.6.2 Laboratory prepared mixtures of intact and degraded florfenicol

Into a set of 10-mL volumetric flasks, different volumes (4.5-0.5 mL) of intact florfenicol solution (10 mg mL^{-1}) were transferred and mixed with (0.5-4.5 mL) of its acidic degradate solution prepared under (2.5. Preparation of Degraded Florfenicol). Volumes were completed to the mark with ethanol then proceeded as under "2.6.1. Linearity, Method A". Peak areas of the obtained chromatograms were measured and the concentration of intact drug was calculated from corresponding regression equation.

2.6.3 Application to pharmaceutical formulations

Method A- The Contents of 3 Panflor® oral solution was mixed. A volume equivalent to 250 mg florfenicol was transferred into a 25-mL volumetric flask then completed to volume with ethanol to obtain a solution labeled to contain 10 mg mL^{-1} florfenicol to be analyzed by the proposed densitometric method (A) as detailed under "2.5.1.Linearity, Method A" and the drug concentrations were calculated from the corresponding regression equation.

Method B- Three Butalex injections were mixed well, an aliquot equivalent to 40 mg buparvaquone was transferred into 100-mL volumetric flask and the volume was completed with methanol to obtain a solution claimed to contain 0.4 mg mL^{-1} drug to be analyzed spectrophotometrically at 489 nm as detailed under "2.5.1.Linearity, Method B".

Method C- An aliquot of the above mixed Butalex® injections solutions equivalent to 10 mg drug was transferred accurately into a 25-mL volumetric flask and mixed with 4 mL methanol. The details under "2.4.Standard Solutions " were followed starting from(adding 1 mL of 2% methanolic $NaBH_4$) to give a solution labeled to contain a final concentrationof 4 μg mL^{-1} reducedbuparvaquone, to be analyzed spectrofluorometrically as detailed under "2.5.1.Linearity, Method C".

3. RESULTS AND DISCUSSION

3.1 Optimization of Experimental Conditions

Method A: Stressed degradation of florfenicol was performed by refluxing the drug using different media; NaOH, HCl and 5% H_2O_2 for different time intervals. Complete degradation took place after heating the drug under reflux with 5 M HCl for 4 h. To confirm and isolate the corresponding degradate of the studied drug, solution was adjusted to about pH 7 using 5 M NaOH, evaporated to dryness under vacuum and residue was extracted with ethanol. TLC separation of the obtained solution was performed on silica gel 60 GF_{254} plates using a mobile phase of chloroform–methanol (7:3 v/v) where a spot for intact florfenicol at R_f 0.87 and another one at R_f 0.68 corresponding to its degradation product were obtained. The prepared ethanolic solution was then evaporated to dryness and confirmed by IR using KBr discs. Where, the pure drug exhibited an intense band at 1684 cm^{-1}, characteristic to the amidic carbonyl group (–CO); Fig. (1a). Disappearance of this band in the spectrum of the degradation product; Fig. (1b) confirmed the breakage of this amide bond which in turn suggested the following degradation pathway:
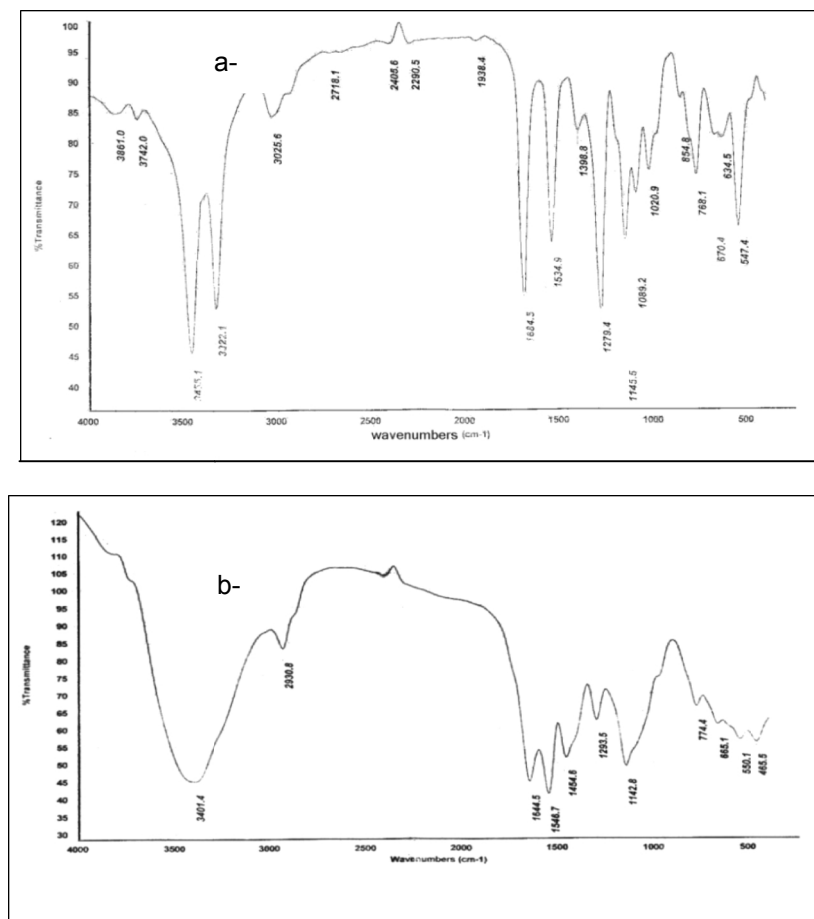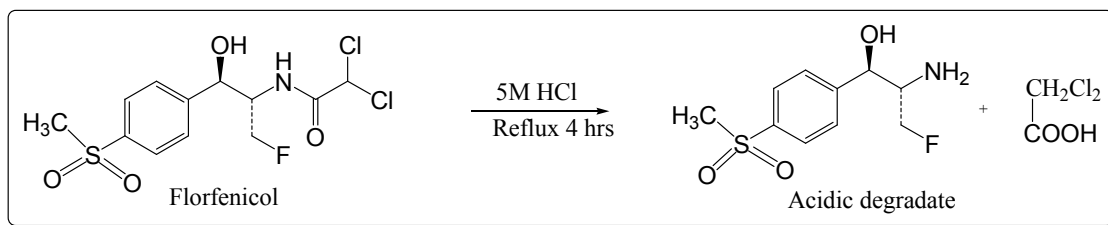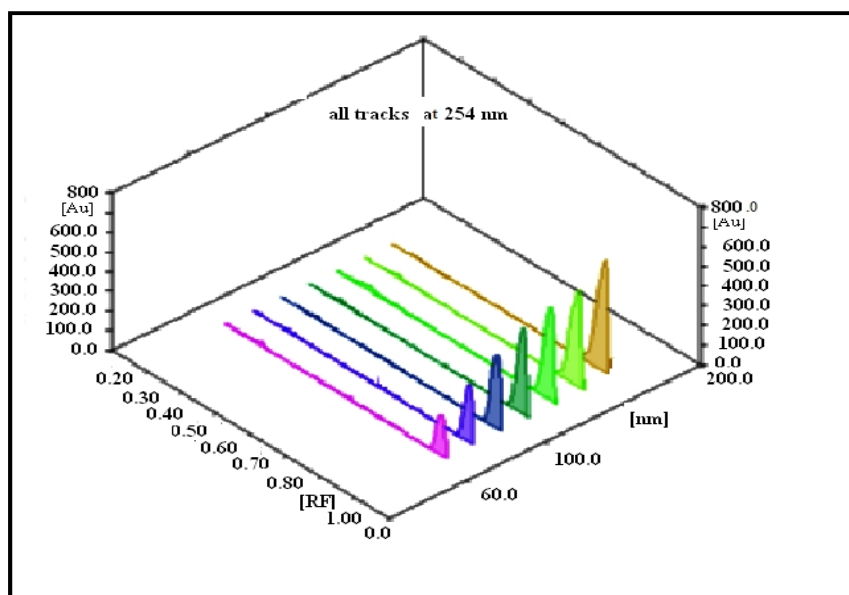

Fig. 1. IR Spectra of: a) Intactflorfenicol   b) Florfenicol degradation product on KBr disc.

**Scheme 1. Proposed acidic degradation pathway of florfenicol**

This encouraged, the densitometric evaluation of thin layer chromatograms of florfenicol using chloroform-methanol (7:3 v/v) as developing solvent and 254 nm for scanning, Fig. 2.


Fig. 2. Densitometric chromatogram of florfenicol (10-100 μg/spot) at 254 nm.

Method B: Buparvaquone in methanol was found to be reduced with $NaBH_4$ which in alkaline medium giving a red colored product with λ_{max} 489 nm; Fig. **3**. Different experimental conditions were studied. Concerning the effect of volume of reagent, 1-3 mL of 0.03% methanolic $NaBH_4$ gave the same color intensity, hence 1.5 mL of it was used. The reaction was optimized at room temperature, where maximum color intensity was obtained after 20 min and remained stable for further 40 min; no appreciable effect on the color intensity upon heating reaction mixture. Different solvents were tried as diluent for reaction mixture; acetonitrile, ethanol, methanol, isopropanol and butanol. Methanol and ethanol gave almost the same absorbance, thus methanol was used.

It was note worthy to mention that buparvaquone is insoluble in H_2O, hence the reduction reaction was carried out in methanol. The molar ratio [23] was applied using 0.5×10^{-4} M $NaBH_4$ methanolic solution and the drug was found to react with the reagent in 1:1 ratio. Scheme (2) represented the suggested mechanism of the reduction reaction.
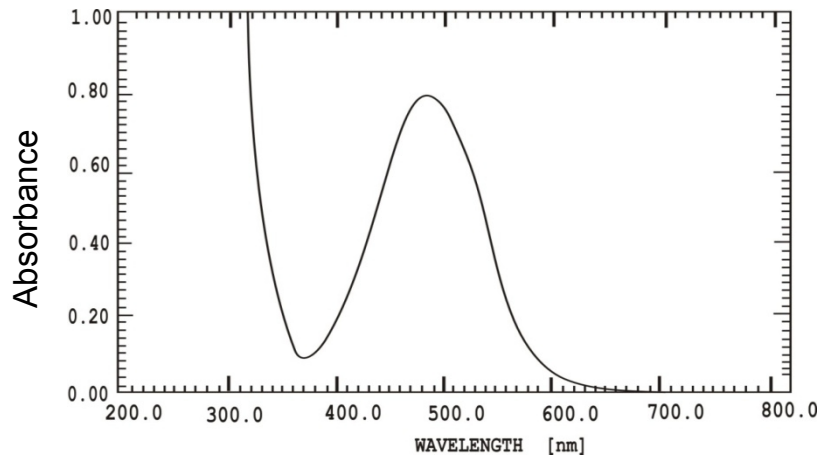
Fig. 3. Absorption spectrum of buparvaquone (100 μg mL^{-1}) reaction product with 0.03% NaBH$_4$ in methanol.
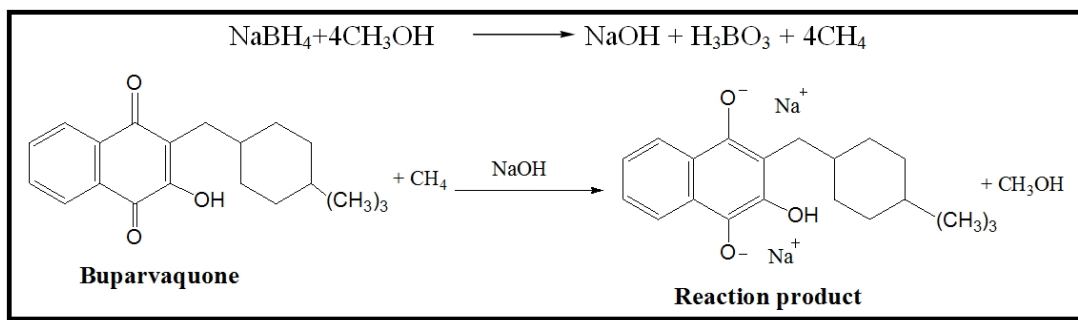
Scheme 2. Suggested reaction mechanism of buparvaquone with NaBH$_4$.

Method C: Regarding the structure of buparvaquone, it was expected to have native fluorescence, but unfortunately preliminary tests showed that it exhibited weak fluorescence intensity. However, the phenate anions produced by reduction of two quinonoid groups of the drug with NaBH$_4$ in alkaline medium together with the already existing phenolic OH resulted in a highly resonating structure [24]; scheme (2). The later when excited at 334 nm produced high fluorescence intensity picked at 450 nm; Fig. 4.

Different sensitizing agents were tried to increase fluorescence intensity (1% aqueous gelatin, β-cyclodextrin and SDS), but no effect was observed, hence non of them was used in this work. The effect of diluting solvent was also studied by diluting methanolic aliquots of the reduced drug solution with 0.1 M H$_2$SO$_4$, 0.1 M HC1, ethanol, acetonitrile and methanol; the later gave maximum fluorescence intensity and thus used through out the procedure.
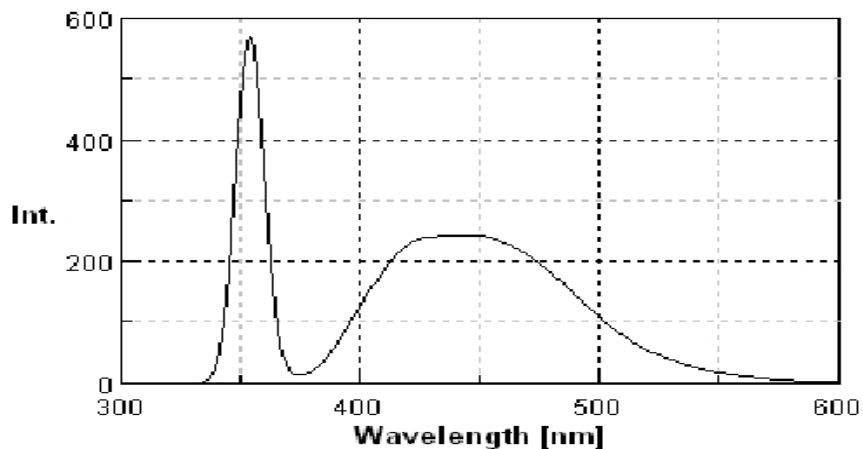
Fig. 4. Excitation and emission spectra of buparvaquone (1.2 µg mL^{-1}) in methanol

3.2 Method Validation

The proposed methods were validated for linearity, accuracy, precision in according with ICH guidelines on analytical validation [25].

3.2.1 Linearity

Beer' s Law was found to be obeyed in the range of 10-100 µg/spot for florfenicol by densitometry (method A) and in the range of 10-120 or 0.3-2.4 µg mL^{-1} of buparvaquone using spectrophotometry (method B) or spectrofluorometry (method C), respectively. The regression parameters were computed and presented in Table 1.

3.2.2 Accuracy and precision

They were tested by analyzing four different florfenicol or buparvaquone concentrations on the same day on three successive days. Calculated accuracies were 99.91- 102.39% by using method A for florfenicol and 98.78-101.19% or 97.71-101.44% for method B or method C for buparvaquone. While precision calculated as intra and interday RSD% were found to be 0.305-1.76, 0.13-1.74 or 0.34-1.70 % by the above three methods.

3.2.3 Selectivity

Laboratory prepared mixtures of intact and degraded florfenicol were analyzed by the proposed densitometric method A. The results presented in Table (**2**) proved that this method could be used for the selective determination of pure florfenicol in presence of 10-90% of its amine degradation product with mean recovery of 102.08±0.98.

3.2.4 Robustness

For densitometric method (A), it was checked by small variations in the mobile phase ratio, where RSD did not exceed 1.1%. Whereas for methods B and C, changing reagent volume

($NaBH_4$) from one to 3 mL gave RSD of 1.02%. This proved that the three proposed method were robust.

Table 1. Assay parameters of the proposed methods for the determination of florfenicol or buparvaquone

Parameters	Method A Florfenicol	Method B Buparvaquone	Method C Buparvaquone
λ_{max} (nm)	254	489	λ_{ex} 334, λ_{em}450
Linearity range	10-100	10-120	0.3-2.4
	(µg/spot)	(ug ml^{-1})	(ug ml^{-1})
<u>Regression Parameters</u>			
Slope±SD (S_b)	195.09 ±1×10^{-3}	0.0078±1.73×10^{-4}	202.71±4.73×10^{-5}
Intercept± SD (S_a)	-449.46± 6.11×10^{-3}	0.0018±1.16×10^{-4}	6.47±3.60×10^{-3}
SD of residuals ($S_{y/x}$)	57.66	3.76×10^{-3}	2.07
Correlation Coefficient (R^2)	0.9998	0.9998	0.9998
Accuracy (R% $\pm$ SD)	99.94 ±0.89	99.99 ± 0.99	99.66±1.80
*Precision (RSD %) Intraday	0.31-1.03	0.13-0.33	0.47-1.63
Intermediate	0.56-1.76	0.83-1.74	0.34-1.70

* average of nine separate determinations

Table 2. Determination of florfenicol in mixtures with its degradation product by the proposed densitometric method

Intact µg/spot	Degradate µg/spot	% degradate	Recovery % of intact
90	10	10	103.62
80	20	20	102.50
60	30	30	102.51
50	50	50	101.38
30	70	70	100.97
10	90	90	101.46
Mean% ±SD	102.08± 0.98		

3.3 Application to pharmaceutical formulations

The proposed densitometric method A was successfully applied for the analysis of florfenicol in panflor oral solution with mean recovery of 100.17%±1.13. Moreover, methods B and C were applied for the analysis of buparvaquone in Butalex injections with good mean recoveries of 99.14%±1.06 and 99.08%±1.07, respectively.

These results were statistically compared to the those obtained by reported methods [3,19], revealing no significant difference; Table (3). The validity of the proposed methods was further assessed by applying the standard addition technique; Table (4).

Table 3. Statistical analysis of the results obtained by the proposed methods compared to reported methods [3,19]

Parameters	Method A Florfenicol	Reported method [3]	Method B Buparvaquone	Method C Buparvaquone	Reported method [19]
Linearity range	10-100 µg/spot	20-160 µg mL^{-1}	10-120 µg mL^{-1}	0.3-2.4 µg mL^{-1}	2-10 µg mL^{-1}
N	5	5	5	6	4
Mean %	100.17	101.08	99.14	99.08	98.20
SD	1.13	1.18	1.06	1.07	0.39
Variance	1.29	1.40	1.13	1.14	0.15
t-	1.24 (2.31)	-	1.37 (2.37)	1.53 (2.31)	--
F-	1.09 (6.39)	-	7.39 (9.12)	7.47 (9.01)	--

- Figures in brackets are the tabulated t- and F- values at P=0.05.
-Ref [3] involved measuring UV absorbance of the drug at 267 nm in ethanol.
-Ref. [19] involved an HPLC method using a Hypersil C$_{18}$ column, mobile phase of ammonium acetate buffer (0.02 M, pH 3.0)- acetonitrile (18:82 v/v), flow rate of 1ml/min and UV detection at 251nm

Table 4. Application of standard addition technique for the determination of florfenicol and buparvaquone by the proposed methods

Preparation	Mean% ± SD	Densitometric method Standard addition			Mean% ± SD	Spectrophotometric method Standard addition			Mean% ± SD	Spectrofluorometric method Standard addition		
		Taken µg mL^{-1}	Added µg mL^{-1}	Recovery% of added		Taken µg mL^{-1}	Added µg mL^{-1}	Recovery% of added		Taken µg mL^{-1}	Added µg mL^{-1}	Recovery% of added
	100.17 ± 1.13				99.14 ± 1.06				99.08 ± 1.07			
1.Panflor® oral solution B.N. 437		10	10	101.22		40	20	96.28		0.4	0.4	101.51
2,3. Butalex® injections B.N. 6666105		10	20	99.95		40	40	98.46		0.4	0.8	100.99
		10	70	99.63		40	60	98.33		0.4	1.2	101.96
		10	80	101.88		40	80	100.67		0.4	1.8	100.08
Mean% ± SD	100.67 ± 1.062				98.44 ± 1.79				101.13 ±0.81			

4. CONCLUSION

The proposed methods can be recommended for the analysis of florfenicol or buparvaquone where sophisticated equipments are unavailable. The suggested methods have the advantage of being simple, rapid, accurate and sensitive. These advantages encourage the application of the developed methods in routine quality control analysis of the two drugs**.**

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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