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Spectrophotometric and RP-HPLC Methods for Simultaneous Determination of Pharmaceutical Formulation for Anticancer Drugs

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ABSTRACT

In this study, three simple and rapid methods were developed for determination of some anticancer drugs Gemcitabine (GMB) and Etoposide (ETP) simultaneously in their pharmaceutical formulation. The first derivative amplitudes at 264 nm and 302 nm were selected for the assay of GMB and ETP, respectively. Calibration curves were established at 5 µg/mL-20 µg/mL for GMB and 2 µg/mL-15 µg/mL. The LOD of 0.89 µg/mL and 0.75 µg/mL, LOQ of 2.97 µg/mL and 1.42 µg/mL for GMB and ETP, respectively. The ratio derivative at 270 nm and 238 nm, calibration curves were established at 5 µg/mL–20 µg/mL and 2 µg/mL–15 µg/mL, LOD of 0.66 µg/mL and 0.25 µg/mL, LOQ of 2.19 µg/mL and 0.88 µg/mL for GMB and ETP, respectively. In the RP-HPLC method separation, the detection wavelength were 273 nm and 246 nm, the retention time was found to be 1.713 min and 5.021 min, linearity ranges of 0.5 µg/mL–35 µg/mL and 1 µg/mL-20 µg/mL, LOD of 0.09 µg/mL and 0.21 µg/mL and LOQ of 0.31 µg/mL and 0.80 µg/mL for GMB and ETP, respectively. The methods were applied for pharmaceutical formulation and were validated according to the procedure described in ICH guidelines.

Keywords: Gemcitabine; Etoposide; Anti-cancer; Spectrophotometric; Simultaneous; RP-HPLC

INTRODUCTION

Gemcitabine HCI (GMB) is chemically know as 2'-deoxy-2', 2'-difluorocytidine monohydrochloride (Figure 1) is a pyrimidine analogue that is proven to be active against a variety of solid tumours, such as non-small cell lung cancer, cancers of pancreas, lung, breast, bladder, kidney and biliary tract either singly or in combination with other cytotoxic agents [1,2]. On the basis of these results, gemcitabine has generally been accepted as a standard chemotherapeutic agent for advanced pancreatic cancer [3,4] and urothelial carcinoma [5,6]. As a prodrug, gemcitabine undergoes intracellular phosphorylation to form two active metabolites, 2′,2′-difluorodeoxycytidine-5′-diphosphate and triphosphate. 2′,2′-difluorodeoxycytidine-5′-diphosphate inhibits ribonucleotide reductase, and triphosphate can incorporate into DNA [7], as a result, DNA synthesis is inhibited and finally causes cancer cell dead [8,9]. Meanwhile, gemcitabine is inactivated into 2′,2′- ifluorodeoxyuridine by cytidine deaminase in plasma and liver [10].

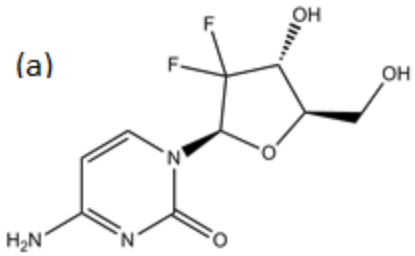

Figure 1: Chemical structure of Gemcitabine (GMB)

Etoposide (ETP) is chemically known as 4-demethylepipodophyllotoxin ethylidene-b-D-glucoside (Figure 2). It is a semisynthetic derivative of podophyllotoxin 1. Etoposide is a superior chemotherapy drug for many types of cancer treatment, including treatment of small cell leukemia, testicular cancer, ovarian cancer, lung cancer, lymphoma, nightmare sarcoma, glioblastoma, and polymorphic glioma [11,12]. Etoposide was administered intravenously by injection or orally [13,14]. It can form a ternary complex with topoisomerase II enzyme and DNA, which induces DNA double-strand breaks and prevents DNA from repairing, leading to an accumulation of DNA breaks and cell death [14].

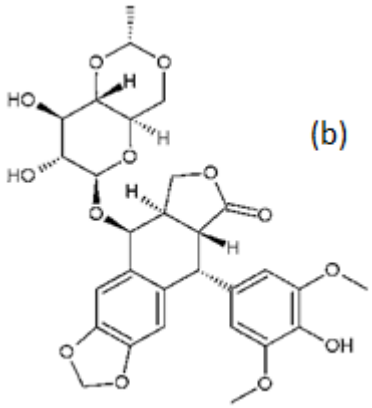

Figure 2: Chemical structure of Etoposide (ETP)

A GMB drug for pharmaceutical significance, there are several methods for determination GMB in both pharmaceutical preparations and biological fluids in the literature. GMB is ordinarily determined by high-performance liquid chromatography coupled with UV detection [15-17], capillary gas chromatography [18], ultra-performance liquid chromatography (UPLC) with tandem mass spectrometry (UPLC-MS/MS) [19,20], liquid chromatography-tandem mass spectrometric (LC-MS/MS) [21,22], The fluorescence, electroanalytical [23,24], electroanalytical investigations on the interaction with DNA [25,26], capillary electrophoresis [27], UV-spectrophotometric [28,29]and spectrophotometric[28-30].

There are many different literary studies describing several analytical methods for determination ETP in several matrices including forms of injectable pharmaceutical preparations, biological fluids, and cancer cells. The high-

performance liquid chromatography (HPLC) [31], high-performance liquid chromatography with electrochemical detection [32], URP-HPLC–MS/MS [10], RP-HPLC with fluorescence detections [33], capillary electrophoresis [27], selective electrochemical using a polypyrrole coated glassy carbon electrode [34] UV-Visible spectrophotometric [35-37].

There are no published spectrophotometric methods for estimating both drugs simultaneously. This article discusses three simple, rapid, accurate, precise, reproducible, and cost-effective spectrophotometric and chromatographic methods for estimating gemcitabine and etoposide simultaneously in their pharmaceutical formation.

MATERIALS AND METHODS

A GENESYS 10S UV-Vis double beam spectrophotometer (Thermo Spectronic, USA) with a fixed slit width (1.8 nm) connected to an IBM computer loaded with Thermo Spectronic VISION Lite version 4 software and quartz cuvettes (1.00 cm) were matched and used for all absorbance measurements.

A powerful liquid chromatographic system (Varian Prostar 310 UV/Vis Detector and Varian 230 SDM). At 30°C, an octadecylsilane (ODS), Phenomenex Luna, C18 (2) 100 A, 250 4.6 mm, 5 analytical column was used for separation.

Etoposide and Gemcitabine HCl (99%) chemicals reference substances 99% from Sigma-Aldrich Chemicals (USA). Acetonitrile is RP-HPLC grade and other solvents, and reagents used were of analytical grade.

The pharmaceuticals were purchased as an injection from the market, Gemcitabine GEMZAR 200 mg per vial from Lilly-France and Etoposide 100 mg/5 mL from EBEWE Pharma Ges-Austria.

Preparation of Standard Solutions and Calibration

Stock standard solutions of ETP and GMB were prepared for the spectroscopic and RP-HPLC method methods weighed 10 mg of and transferred to a 100 ml volumetric flask, dissolved in methanol, and diluted up to mark to obtain stock solution of 100 µg/mL. All solutions were prepared on the day of analysis.

The spectrophotometry a method, volume was completed with methanol to prepare solutions in concentration ranges of 2.0 µg/ml-15.0 µg/mL for ETP and 4.0-20.0 µg/mL for GMB. The absorption spectra have been recoded and show absorption maxima for GMB at 264 and 288 nm, and ETP at 230 nm and 253 nm.

RP-HPLC method, series of working solutions of ETP and GMB were prepared by the appropriate dilution of the stock solutions with same solvent to reach the concentration ranges of 1 µg/ml-20 µg/mL for ETP and 0.5 µg/ml-35 µg/mL for GMB. Triplicate 50 µL injections were made for each concentration using the following chromatographic conditions: Mobile phase consisting of acetonitrile: 0.05 M KH_2PO_4 (80:20 v/v) adjusted by phosphoric acid to pH 3.6 [38]. Detector wavelengths are 270 nm for GMB and 264 nm for ETP, with a flow rate of 0.8 mL/min at room temperature. To create a calibration curve, the peak area for each concentration was plotted versus the same concentration.

Sample Preparation

An amount of Etoposide (ETP) injectable solution, claimed to contain 100 mg ETP per 5.0 mL, was transfer to 1000 mL volumetric flask and dissolve in methanol [34]. GMB injectable solution GEMZAR 200 mg per vial was transferred to a 2000 mL volumetric flask and dissolved in methanol. The final concentration 100 µg/mL of ETP and GMB 100 µg/mL was used for the estimation.

RESULTS AND DISCUSSION

Spectroscopic Methods

GMB and ETP absorption spectra were measured from 200 nm to 400 nm against methanol as a blank. The absorption spectra of ETP and GMB, as well as their mixture, have been recorded. The absorption spectrum of GMB solution 12 g/mL is shown in Figure 3, with three absorptions at wavelengths 208, 278, and 319 nm. Spectrum (b) portrays the absorption spectrum of ETP solution 12 g/mL, with three absorption maxima at 222, 264, and 295 nm. The total spectrum of a GMB/ETP mixture is shown in curve (c), with absorption maxima of 225 and 278 nm between the two components.

Individual zero order absorption spectra of GMB and ETP were converted into their first order derivative spectra using the simultaneous first derivative method. Figure 3 depicts the zero crossing points in the first order derivative spectra of GMB at 238.5, 275.6, and 308 nm, and those of ETP at 215, 246.3, 268.5, and 287.1 nm. GMB can be measured from the peak to the best line at 264 and 288 nm, whereas ETP can be measured from the peak to the best line at 230 and 302 nm.

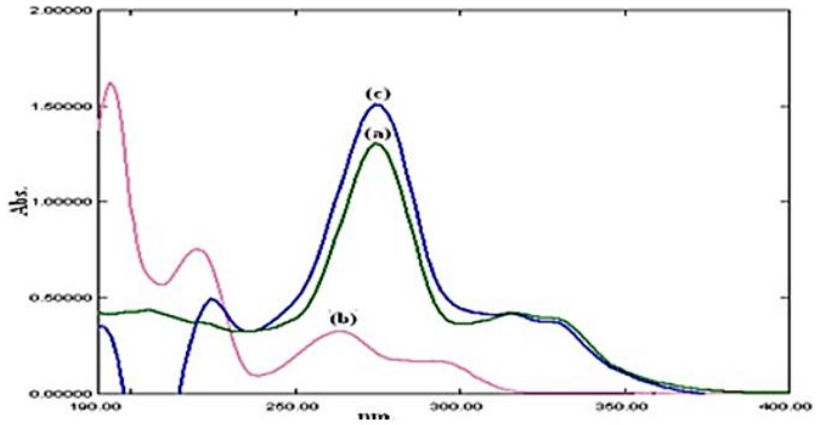

Figure 3: Absorption spectra of (a) 12 µg/mL GMB, (b) 8 µg/mL GMB (c) Mixture GMB and ETP

Aliquots of GMB equivalent to 5 g/mL-20 g/mL were accurately transferred from stock solution to a 10 ml volumetric flask using the ratio derivative method. The prepared solution's absorption spectra were scanned and recorded in the 200 nm-400 nm range. ETP at a concentration of 8 g/mL was added to these solutions. When the obtained spectrum is converted to first derivative, the maxima and minima of the spectrum are found to be 270 nm and 287.5 nm, respectively, in Figure 4. Table 1 shows the calibration curve with the amplitude on the y-axis against the concentrations. Similarly, different concentrations of ETP in the range of 2 g/mL-15 g/mL were scanned and recorded in the 200 nm-400 nm range. Using inbuilt software, these solutions were supplemented with 12 g/mL of GMB. When the obtained spectrum is converted into first derivative, the maxima and minima of the spectrum are found to be 238 nm and 224 nm, respectively, as shown in Figure 5. Table 1 shows the calibration curve with the amplitude on the y-axis and the concentrations on the x-axis (Figure 6).

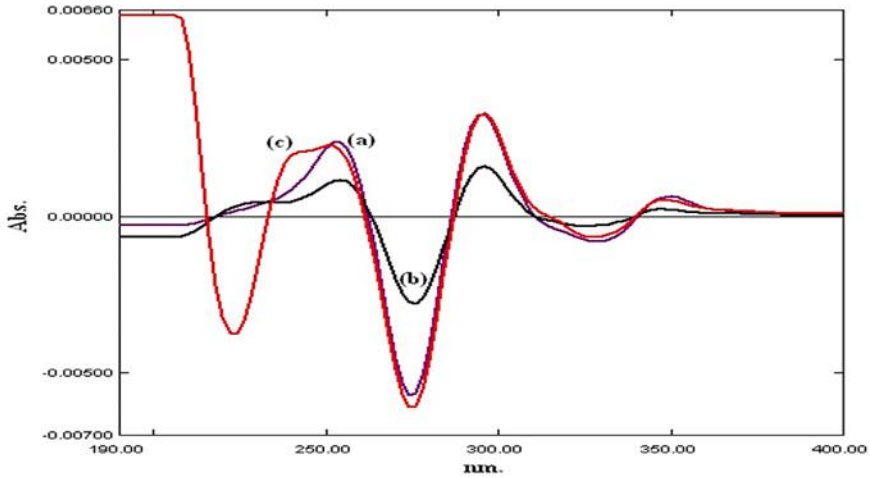

Figure 4: First derivatives spectra (1D): (a) 12 µg/mL GMB (b)8 µg/mL ETP (c) Mixture GMB and ETP

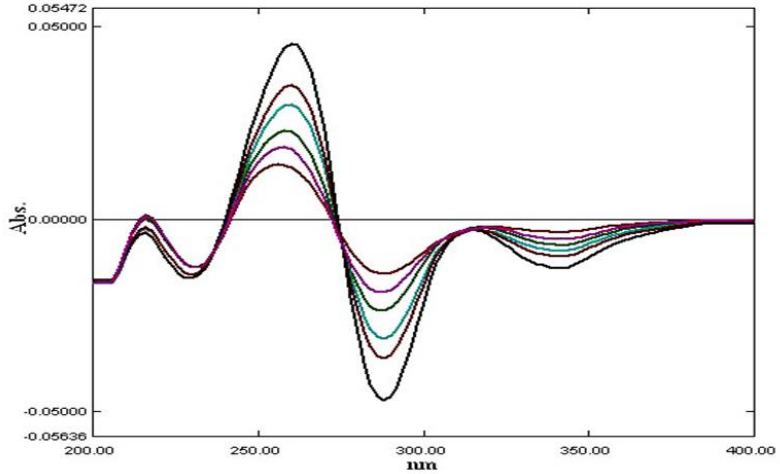

Figure 5: First derivative spectra (1D) of mixtures containing 5 µg/mL-20 µg/mL GMB and 8 µg/mL of ETP

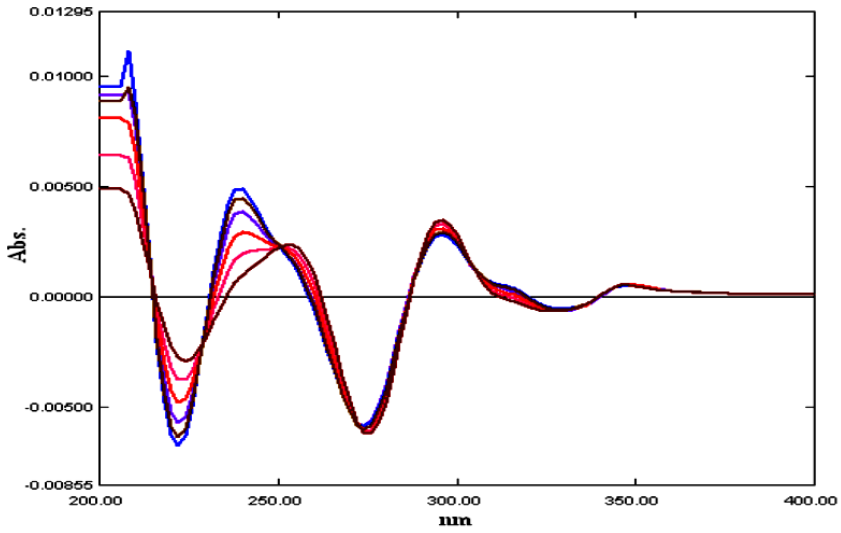

Figure 6: First derivative spectra (1D) of mixtures containing 2 µg/mL-15 µg/mL ETB and 12 µg/mL of GMB

RP-HPLC Method

A simple isocratic RP-HPLC method was developed for the simultaneous determination of GMB and ETP in pure form and pharmaceutical formulation. Different chromatographic conditions were optimized to achieve simultaneous elution of GMB and ETP peaks. The composition of the mobile phase was investigated by using gradient elution with acetonitrile and 0.05 M of KH_2PO_4 in various ratios, with the pH adjusted to 3.6 by phosphoric acid. Retention times (Rf) for GMB were found to be 1.713 min and 5.021 min for ETP, as shown in Figure 7.

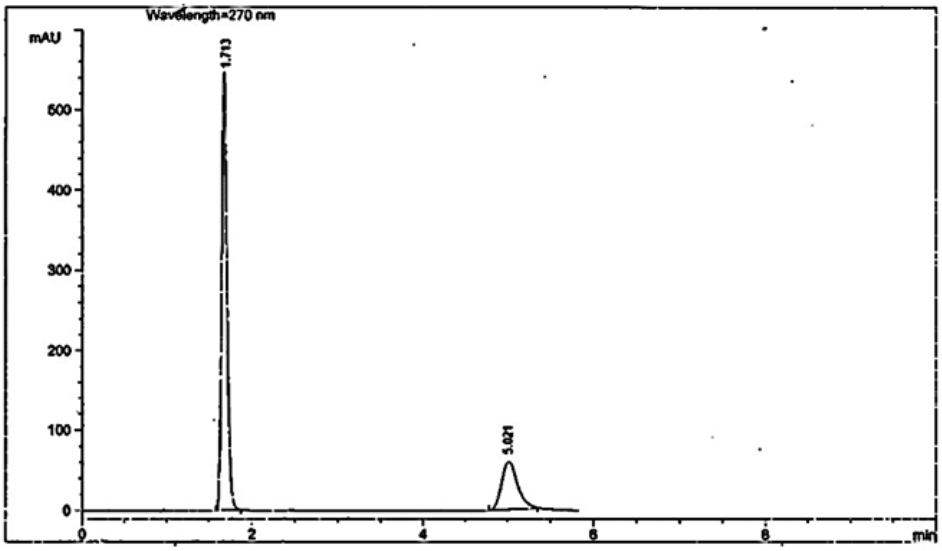

Figure 7: RP-HPLC Chromatogram of 25 µg/mL GMT (Rf=1.713 min) and 10 µg/mL ETP (Rf=5.021 min) in pharmaceutical formulations

Method Validation

The first and ratio derivative spectrophotometric methods, as well as the RP-HPLC method, were fully validated in accordance with ICH guidelines. The methods' performance parameters were linearity, accuracy, precision, specificity, quantitation limit, and reproducibility of sample applications [39,40].

Linearity

The calibration curve was tested with five standard GMB and ETP stock solutions, it was linear in in the range 5 µg/ml-20 µg/ml for the first derivative spectroscopy method, 2 µg/ml-15 µg/ml for the ratio derivative method and 0.5 µg/ml-35 µg/ml and 1 µg/ml-20 µg/mL for RP-HPLC method, respectively. The solutions are prepared in triplicate. The data for the linear regression equations are represent in Table 1. The correlation coefficient ranged from 0.9989 to 0.9996 for MET and GLB, respectively, which attested to the linearity of methods.

Limit of Detection and Limit of Quantification

In accordance with the recommendations of ICH [41], the limit of detection, LOD=3 SD/s, where SD=Standard deviation of the response based on either the standard deviation of the blank, the residual standard deviation of regression line of y=intercepts of regression lines and s=slope of calibration curve. The limit of quantitation, LOQ=10 SD/s [41]. In the first derivative spectrophotometric method, the LOD and LOQ were found to be 0.89 µg/ml, 2.97 µg/mL for GMB, 0.75 µg/mL and 1.42 µg/mL for ETP. Whereas, the ratio derivative method were 0.66 µg/mL, 2.19 µg/mL for GMB, 0.25 µg/mL and 0.83 µg/mL for ETP, respectively. As for the RP-HPLC method, LOD and LQD were found 0.09 µg/mL and 0.31 µg/mL for GMB, 0.21 µg/mL and 0.80 µg/mL for ETP, respectively. The LOD and LOQ of the proposed methods are present in Table 1.

Table 1: Analytical parameters for the determination of GMB and ETP by proposed methods

Parameter	**GMB**			**ETP**		
	Derivative spectrophotometric methods		**RP-HPLC method**	**Derivative Spectrophotometric methods**		**RP-HPLC method**
	1D	**Ratio**		**1D**	**Ratio**	
Wavelength (nm)	264	270	273	302	238	246
Linearity	5-20	5-20	0.5-35	2-15	2-15	1-20

range(µg/ml)						
Correlation coefficient (r)	0.9996	0.9989	0.9991	0.999	0.9992	0.9994
Intercept (a)	0.0011	0.0021	0.0018	0.0004	0.0013	0.0047
Slope (s)	0.0138	0.0082	0.1134	0.0063	0.0012	0.0736
Standard deviation of the intercept (SD)	0.0041	0.0018	0.0036	0.0009	0.0001	0.0051
Limit of detection LOD(µg/ml)	0.89	0.66	0.09	0.75	0.25	0.21
Limit of quantification LQD (µg/ml)	2.97	2.19	0.31	1.42	0.83	0.8

Precision

The precision of the methods was determined by repeatability (intra-day) and intermediate precision (inter-day) for by assaying samples, at same concentration and during the same day. The precision the concentration of 5, 10 and 20 µg/mL for GMB and ETP, it was studied by comparing the assays on five days for Five samples solutions. The relative standard deviation values from intra-day and inter-day analysis were lower than 2%, assure the precision of the method, the results are shown in Table 2.

Table 2: The precision studies of GMB and ETP

Compound	**Conc (µg/mL)**	**Intra-day (n=5)**			**Inter-day (n=5)**		
		Recovery% (% ± SD)			**Recovery% (% ± SD)**		
		RSD%			**RSD%**		
		^{1}D method	**Ratio method**	**RP-HPLC method**	**^{1}D method**	**Ratio method**	**RP-HPLC method**

GMB	5	99.2 ± 0.02	100.4 ± 0.04	99.6 ± 0.06	99.0 ± 0.08	99.6 ± 0.08	100.2 ± 0.09
		0.40%	0.79%	1.21%	1.62%	1.61%	2.39%
	10	99.6 ± 0.02	99.2 ± 0.05	99.6 ± 0.13	99.7 ± 0.01	99.1 ± 0.02	99.4 ± 0.02
		0.20%	0.50%	1.31%	0.10%	0.20%	0.20%
	20	99.2 ± 0.21	99.6 ± 0.13	99.5 ± 0.05	996 ± 0.19	99.7 ± 0.31	99.8 ± 0.20
		1.06%	0.65%	0.25%	0.85%	1.55%	1.00%
ETP	5	99.2 ± 0.01	99.0 ± 0.08	99.4 ± 0.06	98.8 ± 0.08	99.2 ± 0.05	99.6 ± 0.04
		0.20%	1.61%	1.21%	1.62%	1.01%	0.80%
	10	99.1 ± 0.11	99.3 ± 0.09	99.2 ± 0.04	99.4 ± 0.02	99.3 ± 0.08	99.8 ± 0.16
		1.11%	0.91%	0.40%	0.20%	0.81%	1.10%
	20	99.6 ± 0.21	99.5 ± 0.14	99.8 ± 0.12	99.0 ± 0.27	99.5 ± 0.23	99.7 ± 027
		1.05%	0.70%	0.60%	1.36%	1.16%	0.85%

- Mean value of five determinations
- Relative standard deviation (%)
- Relative error (%)

Accuracy

To verify the degree of methods accuracy, recovery studies for GMB and ETP were performed samples in triplicates by the standard addition method at 80%, 100% and 120% of the nominal analytical concentration 10 µg/mL for the first derivative spectroscopy, derivative ratios and the concentration is 12 µg/mL for the RP-HPLC method. The relative standard deviation values were 0.34% to 1.61% and recovery 99.0% to 100.4%, the results are show in Table 3.

Table 3: Accuracies studies GMB and ETP

Compound	Method of analysis	Taken (µg/ml)	Mean ± SD (µg/ml)	%RSD	%Recovery (n=3)
GMB	1D	8	17.82 ± 0.064	0.36	99.0
		10	19.78 ± 0.151	0.76	98.9
		12	21.81 ± 0.242	1.11	99.1
	Ratio	8	17.90 ± 0.093	0.52	99.4
		10	19.84 ± 0.172	0.87	99.2
		12	22.03 ± 0.289	1.31	100.1
	RP-HPLC	10	16.06 ± 0.054	0.34	100.4
		12	17.89 ± 0.231	1.29	99.4
		15	20.91 ± 0.337	1.61	99.6
GLB	ID	8	17.91 ± 0.071	0.39	99.5
		10	20.08 ± 0.104	0.52	100.4
		12	21.89 ± 0.201	0.91	99.5
	Ratio	8	17.89 ± 0.084	0.47	99.4
		10	19.94 ± 0.152	0.79	99.7
		12	21.85 ± 0.247	1.13	99.3
	RP-HPLC	10	16.04 ± 0.062	0.34	100.3
		12	17.91 ± 0.207	1.16	99.5
		15	20.91 ± 0.294	1.44	99.6

Assay of GMB and ETP (Injectable solutions)

The proposed methods were applied to samples of pharmaceutical preparations from the market, and to compare them with a reference method. The results in Table 4 show were good accuracy of the proposed methods, the recovery rate ranged between 98.58% to 101.19% and RSD% values lower than 1% for both GMB and ETP. The results obtained with the proposed methods were statistically compared using single factor Analysis of Variance Test (one-way ANOVA) with f-test and t-test, indicating that there is no significant difference between the proposed methods and the reference methods, the results are present in Table 4.

Table 4: Results for analysis of GMB and ETP in pharmaceutical formulation sample

Formulation Brand and Drug	Method of Analysis	Labeled amount	Recovery ± SD[a]	RSD%	T-test[b]	F-test[c]

GMB GEMZAR 200 mg per vial	ID	200 mg	100.79 ± 1.01	0.5	1.43	2.15
	Ratio		98.87 ± 1.83	0.92	1.57	1.52
	RP-HPLC		99.46 ± 1.28	0.64	0.53	1.34
	Reference method [28]		98.56 ± 1.48	0.79	-	-
ETP Etoposid 100 mg/5 ml injectable solution	ID	100 mg	101.19 ± 0.78	0.78	2.12	5.59
	Ratio		99.71 ± 0.37	0.37	0.33	1.26
	RP-HPLC		98.98 ± 0.62	0.62	0.99	3.53
	Reference method [36]		99.77 ± 0.33	0.33	-	-

- Mean value of five determinations
- Tabulated t-value at the 95% confidence level is 2.78
- Tabulated F-value at the 95% confidence level is 6.39

CONCLUSION

GMB and ETP were determined simultaneously in their binary mixture using three different methods: first derived spectrophotometric, ratio derivate spectra and RP-HPLC in pharmaceutical formulation form. The proposed RP-HPLC method for determining GMB and ETP is more sensitive than existing spectroscopic methods but the spectroscopic methods are simpler and less expensive. All of the methods proposed are selective, highly sensitive and fast. The proposed methods can be used to accurately quantify GMB and ETP at the same time and can be easily applied in a quality control laboratory for their analysis.

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