Original Article

Simultaneous Determination of Hydrophilic and Lipophilic Drugs in Anti-Cancer Liposomes: Absorptivity Method

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ABSTRACT

Background: Dual loaded liposomes have always been a hot topic of investigation because of their therapeutic advantages over single drug regimens. But simultaneous estimation of both the drugs during *in vitro* and *in vivo* analysis is most of the times a major obstacle for the researchers. This problem is even bigger when the two drugs are of opposite nature (hydrophilic and lipophilic). **Methods:** Here, we formulated anti-cancer liposomes loaded with Dacarbazine (hydrophilic) and Eugenol (lipophilic). Since both the drugs are of different nature, selecting a solvent system that dissolves both the drugs, as well as is a suitable media for determining *in vitro* release, was a challenge. After resolving this issue, we developed a simple and robust UV method based on the principle of solving simultaneous equations generated after Absorptivity measurements of the two drugs. **Results:** UV spectrophotometer is preferred over other analytical techniques because of its availability, broad area of applications, easy and fast handling, robust nature and simplicity. Thus, using the developed method for simultaneous determination, loading of the two drugs and their simultaneous release from the liposomes was determined. **Conclusion:** We could perform the *in-vitro* characterization of formulated anti-cancer liposomes using the developed UV Absorptivity method, which justifies the practical applicability of the developed method and proved that the method is specific and reproducible.

Key words: UV absorptivity method, Simultaneous determination, Dual loaded liposomes, Suitable solvent system, Method Validation.

INTRODUCTION

Cancer still remains the major cause of health concern worldwide as it claims millions of deaths each year.[1] Recent advances in the field of cancer have given us a deeper insight into the pathophysiology of this deadly disease.[2] This enables us to understand the mechanisms of the cancer growth and thus helps us to plan better and more effective strategies against different types of cancers. But these newer strategies come with their own limitations that make the practical use of these strategies very challenging.

Dual loaded nanoparticles are advantageous over single loaded nano-agents as synergistic and combinatorial action can be achieved with only one formulation. Several dual loaded nanoparticles and liposomes have already been synthesized,[3–7] but the choice of drugs for such formulations becomes limited due to the troublesome simultaneous analysis of both the drugs. When the two drugs are of different nature, i.e., one is hydrophilic and the other is lipophilic, simultaneous analysis becomes even more challenging.

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Knowing the potential of eugenol as anti-cancer[8,9] and apoptosis inducing[10] agent, we thought of combining this promising agent with a chemotherapeutic drug with a vision of improving the cytotoxic action of chemotherapy without any additional side effects. Dacarbazine is a chemotherapeutic drug which is commonly used to treat melanoma[11] and hodgkin's lymphoma.[12] Though it is a potent anti-cancer drug, it often fails to produce complete response due to the resistance of cancer cells against apoptosis which is supposed to be induced by chemotherapy.[13] Thus, we combined dacarbazine with eugenol and formulated dual drugs loaded liposomes containing these two agents with a vision that eugenol, being lipophilic, will be entrapped in the lipid bilayers of the liposomes and dacarbazine being hydrophilic will go into the core of the liposomes. Eugenol will be released first, bind to the cancer cells and inhibit their resistant tendency, while dacarbazine will be released and perform better in the absence of resistance and will be able to induce apoptosis more efficiently.

But, the simultaneous estimation of both these drugs during loading and release studies was difficult because of their opposite nature. Also, dacarbazine could not be estimated by HPLC because it produces active metabolites, so LC-MS was a suitable estimation technique for dacarbazine; but then, eugenol could not be estimated by either HPLC or LC-MS, as it is a volatile liquid.

Keeping the easy availability and several other advantages of UV spectroscopy[14,15] in mind, we developed a robust, reproducible UV method based on absorptivity measurement for the simultaneous estimation of these two drugs.[16] UV spectrometric method for quantification of dacarbazine[17] and eugenol.[18] are available separately, but this is the first report on method development for their simultaneous determination.

MATERIALS AND METHODS

Dacarbazine was kindly provided as gift by Intas Pharmaceuticals. Eugenol (99.9%) was purchased from Sigma Aldrich. Lipoid S100 was a generous gift from Lipoid, Germany. All other reagents were of analytical grade and were used without any further purification.

UV-Vis spectrophotometer (Shimadzu, Japan) was used for the measurement of absorbance of the samples. Beckman Coulter (Optima™ L-100K) ultracentrifuge was used for the centrifugation of the liposomes.

The method was validated according to ICH guidelines, Q2 (R1) with respect to linearity and range, precision, accuracy, limit of detection (LoD) and limit of quantification (LoQ).

Selection of an appropriate solvent system

Selecting a suitable solvent system was a challenge since both the drugs are of different nature (one hydrophilic and other lipophilic). For drug loading determination, a solvent which could dissolve both dacarbazine and eugenol as well as lipid (the entire drugs loaded liposome) was needed. For the release study, the solvent had to be appropriate which could dissolve both the drugs but not the lipid. So, various solvent systems like PBS + Triton X, PBS + methanol, PBS + Tween 80 and PBS + propylene glycol were tried to select an appropriate solvent with good suitability and stability.

Stock solution of Dacarbazine

Stock solution of dacarbazine (100 µg/ml) was prepared by dissolving 100 mg dacarbazine in 1000 ml PBS (pH 7.4): propylene glycol (9:1) in 1000 ml volumetric flask with vigorous shaking. This stock solution was diluted further to obtain working solutions of different concentrations (1-10 µg/ml).

Determination of absorbance maximum (λ_{max})

Dacarbazine solution of 5 µg/ml concentration was prepared by appropriately diluting the stock solution. This solution was scanned in UV spectrophotometer in the UV range (200–400 nm) to determine the λ_{max} of the dacarbazine which was found to be 331 nm (denoted as λ_1) for this solvent system.

Stock solution of Eugenol

Stock solution of eugenol (1000 µg/ml) was prepared by dissolving 100 mg eugenol in 100 ml of PBS (pH 7.4): propylene glycol (9:1) in 100 ml volumetric flask with vigorous shaking. This stock solution was diluted further to obtain working solutions of different concentrations (10-100 µg/ml).

Determination of absorbance maximum (λ_{max})

Eugenol solution of 50 µg/ml concentration was prepared by appropriately diluting the stock solution. This solution was scanned in UV spectrophotometer in the UV range (200–400 nm) to determine the λ_{max} of the eugenol which was found to be 281.5 nm (denoted as λ_2) for this solvent system.

Method Development

Drug Release

Following the method developed by Murtaza *et al.*[16] the concept of Absorptivity is employed to develop a method for simultaneous estimation of both these drugs. The absorptivity (a) is extinction coefficient which is calculated using following equation:

$$A = a.C$$

$$\mathbf{a = A/C} \quad (1)$$

Where, a → Absorptivity, A → Absorbance and C → Concentration (mg/100 ml)

Using equation (1), absorptivities of Dacarbazine and Eugenol were calculated at both λ_1 (331nm) and λ_2 (281.5nm), for which, the absorbance of any three dilutions (n=3) of Dacarbazine was determined at λ_1 (331nm). Then the values of absorbance were divided with corresponding concentrations to calculate the absorptivity of Dacarbazine at λ_1. Average of three absorptivity values was taken and denoted as $\mathbf{a_{d1}}$, where 'a' denotes absorptivity, 'd' denotes dacarbazine and '1' denotes λ_1 i.e. 331 nm, meaning, absorptivity of dacarbazine at λ_1. Same way, absorbance of dacarbazine dilutions (n=3) was determined at λ_2 also, i.e. 281.5nm and $\mathbf{a_{d2}}$ was calculated, where 'a' denotes absorptivity 'd' denotes dacarbazine and '2' denotes λ_2 i.e. 281.5 nm, meaning, absorptivity of dacarbazine at λ_2.

Same process was repeated for eugenol, that is, absorbance of three dilutions of eugenol was determined at λ_1 (331nm) and λ_2 (281.5nm) separately and $\mathbf{a_{e1}}$ and $\mathbf{a_{e2}}$ were calculated, where 'a' denotes absorptivity, 'e' denotes eugenol, '1' denotes λ_1 (331nm) and '2' denotes λ_2 (281.5nm).

Next, the method involves the solving of following two simultaneous equations derived from equation (1) in order to determine the concentrations of both drugs in unknown samples.

$$\mathbf{A_1 = a_{d1}C_d + a_{e1}C_e} \quad (2)$$

$$\mathbf{A_2 = a_{d2}C_d + a_{e2}C_e} \quad (3)$$

$\mathbf{A_1}$ → Absorbance of test sample at λ_1
$\mathbf{A_2}$ → Absorbance of test sample at λ_2
$\mathbf{a_{d1}}$ → Absorptivity of Dacarbazine at λ_1
$\mathbf{a_{d2}}$ → Absorptivity of Dacarbazine at λ_2
$\mathbf{a_{e1}}$ → Absorptivity of Eugenol at λ_1
$\mathbf{a_{e2}}$ → Absorptivity of Eugenol at λ_2
$\mathbf{C_d}$ → Concentration of Dacarbazine (to be determined)
$\mathbf{C_e}$ → Concentration of Eugenol (to be determined)

Drug Loading

For the determination of loading of drugs in the liposomes, the entire process was developed with Ethanol as solvent. Absorbance maximum of dacarbazine in ethanol (λ_1^{et}) was found to be 333 nm; absorbance maximum of eugenol in ethanol (λ_2^{et}) was found to be 282.5 nm. So, absorptivities of dacarbazine and eugenol (in ethanol) were measured at λ_1^{et} and λ_2^{et} separately as described above.

Method Validation

For the validation of the developed method, the standard stock mixture solution of both the drugs was prepared where 100 mg of dacarbazine and 1000 mg of eugenol was dissolved in 1000 ml of the selected solvent system to obtain dacarbazine concentration of 100 µg/ml and eugenol concentration of 1000 µg/ml. This stock solution was then suitably diluted to obtain different concentrations of the drugs in the mixture.

Linearity and range

To determine the linearity and range of both the drugs, the stock solutions of individual drugs were diluted and used. Dilutions of Dacarbazine stock solution were prepared in the range 1-10 µg/ml and calibration curve was plotted between concentration and absorbance at 331 nm and 281.5 nm separately. Similarly, dilutions of Eugenol stock solution were prepared in the range 10-100 µg/ml and calibration curve was plotted at 331 nm and 281.5 nm separately.

Accuracy

To determine the accuracy of the developed method, three dilutions of the stock mixture solution were prepared. These solutions of known concentrations were then analyzed by the developed method as unknown samples. To further check the accuracy of the developed method, the pre-analyzed sample was separately spiked with extra 50%, 100% and 150% of the drugs concentrations and the mixtures were again analyzed by the developed method.

Precision

To check the precision of the developed method, three different dilutions of stock mixture solution were made and the precision (intra-day and inter-day precision) of the method was assessed by determining the concentrations of both the drugs in the mixture.

(i). Intra-day Precision (Repeatability):
Repeatability of the method was assessed by determining the concentrations of dacarbazine and eugenol in three different dilutions of stock mixture solution at three different times a day.

(ii). Inter-day (Intermediate) Precision:
Intermediate precision of the method was assessed by determining the concentrations of dacarbazine and eugenol in three different dilutions of stock mixture solution for three consecutive days.

Limit of Detection

The limit of detection (LoD) is the lowest amount of analyte in a sample which can be detected but

not necessarily quantified.[16] LoD is calculated using equation:

$$LoD = 3.3 \times N/B$$

where 'N' is standard deviation of the peak areas of the drugs ($n = 3$), taken as a measure of noise and 'B' is the slope of the corresponding calibration curve.[19]

Limit of Quantification

The limit of quantitation (LoQ) is the lowest amount of analyte in a sample which can be quantified with appropriate precision and accuracy.[16] LoQ is calculated using equation:

$$LoQ = 10 \times N/B$$

where 'N' and 'B' mean same as above.

Analysis of Liposomes

The synthesized liposomes[20] were analyzed using the developed UV absorptivity method for drug loading and drug release profile.

Drug Loading

To determine the loading of drugs in the synthesized liposomes, the liposomal suspension was centrifuged at 36,000 rpm to remove the unentrapped drugs. The supernatant which contained unentrapped drugs was separated and the pellets of liposomes were dissolved in ethanol. Ethanol, which dissolved the liposomes as well as both drugs, was a suitable solvent for determination of drug loading, which is why, the above explained UV absorptivity method was redeveloped with ethanol also. This ethanolic solution of drugs loaded liposomes was suitably diluted and absorbance was measured at λ_1^{et} and λ_2^{et} against ethanolic solution of unloaded liposomes as blank.

Drug Release

For the determination of rate of release of drugs from liposomes, a fresh batch of liposomes was prepared with same formula and centrifuged at 36,000 rpm to remove unentrapped drugs. After discarding the supernatants, the pellets of liposomes were redispersed in 10 ml of PBS (pH 7.4): Propylene glycol (9:1). 5 ml of this liposomal suspension was used for release study.

The *in-vitro* drug release study was performed by dialysis method with PBS (pH 7.4): Propylene glycol (9:1) as release medium.[21] The dialysis membrane (MW cut off 8-10 kDa; Spectra/Por® Spectrum Laboratories, Inc, USA) was activated before using as per the instructions given on the packaging. 5 ml liposomal suspension was put in dialysis bag and the bag was suspended in 200 ml of receiving phase i.e. PBS (pH 7.4): Propylene glycol (9:1) and placed into an incubator shaker maintained at 37°C and 100 rpm. Aliquots each of 3 ml were withdrawn at various time points (up to 72 h). Sink condition was maintained throughout the experiment. Absorbance of samples withdrawn at different time points (and suitably diluted when needed) was measured at λ_1 and λ_2 against pure release media as blank.

Statistical Analysis

All the experiments are performed in triplicate. MS Excel 2010 is used to calculate mean ± standard deviation of the experimental data. All statistical analyses is carried out using one-way analysis of variance (ANOVA). The data are considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

UV spectrophotometric method based on absorptivity measurements for simultaneous estimation was successfully developed and employed for the determination of drug loading and drug release form the dual drugs loaded liposomes.

Selection of suitable solvent media

For determination of loading and release of drugs from the liposomes, suitable and appropriate solvents were to be selected. Since dacarbazine is hydrophilic and eugenol is lipophilic in nature, solvents which could dissolve both hydrophilic and lipophilic agents were needed. For this, various solvent systems were screened.

For drugs release study, various solvent systems were tried. PBS (pH 7.4) was combined with co-solvents, such as methanol, to increase the solubility of eugenol, but these systems were unstable because of the volatile nature of these co-solvents. Then PBS + Triton X was tried but it interfered with the absorbance of both drugs. Tween 80, when added to PBS, interfered with the absorbance of dacarbazine. Finally, PBS (7.4) + Propylene glycol (9:1) was selected as it was stable, could dissolve both drugs and did not interfere with the absorbance of any of the drugs.

For the determination of drugs loading, Ethanol was found to be a suitable solvent because it could dissolve both the drugs as well as the lipid (i.e. the entire liposome).

Method Development

Absorptivity values of both the drugs at both the λ_{max} (for PBS: Propylene glycol and Ethanol) were calculated and were put in equations (2) and (3).

The absorptivity of the Dacarbazine at λ_1 (a_{d1}) was found to be 949.64 ± 11.43

The absorptivity of the Dacarbazine at λ_2 (a_{d2}) was found to be 319.93 ± 6.67

The absorptivity of the Eugenol at λ_1 (a_{e1}) was found to be 30.59 ± 0.98

The absorptivity of the Eugenol at λ_2 (a_{e2}) was found to be 138.36 ± 1.32

Putting all the four absorptivity values in equations (2) and (3), we get following equations:

$$A_1 = 949.64\ C_d + 30.59\ C_e \quad (4)$$

$$A_2 = 319.93\ C_d + 138.36\ C_e \quad (5)$$

The concentrations of dacarbazine and eugenol in the test samples could then be determined by simply putting absorbance of the test samples at both λ_1 and λ_2 (A1 and A2) in equations (4) and (5) and solving them for C_d (concentration of dacarbazine) and C_e (concentration of eugenol).

When ethanol was taken as the solvent, the absorptivity values were found to be:

$a_{d1}^{et} = 1005 \pm 15$

$a_{d2}^{et} = 616 \pm 02$

$a_{e1}^{et} = 72.24 \pm 1.62$

$a_{e2}^{et} = 144.21 \pm 6.29$

Putting these values in equations (3) and (4), we obtain following equations:

$$A_1^{et} = 1005\ C_d^{et} + 72.24\ C_e^{et} \quad (6)$$

$$A_2^{et} = 616\ C_d^{et} + 144.21\ C_e^{et} \quad (7)$$

Now, same as earlier, putting values of absorbance of the test samples in equations (6) and (7), concentrations of the two drugs in the ethanol could be determined. Multiplying by the dilution factor, entrapped (loaded) amounts of both the drugs could be calculated.

Method Validation

Linearity and range

The absorbance vs concentration curve of both the drugs at both the λ_{max} obeyed Beer-Lambert's law in tested concentration range (1-10 µg/ml for Dacarbazine and 10-100 µg/ml for Eugenol). The values of regression coefficient (R^2) for Dacarbazine and Eugenol at both λ_{max} indicate a good correlation between the concentration and absorbance within the concentration range tested. Figure 1 shows all the four curves while the data is summarized in Table 1 with PBS: Propylene glycol as solvent. Figure 2 and Table 2 represent the data when ethanol was taken as solvent.

Accuracy

As the standard addition technique was followed by adding 50, 100 and 150% of the drugs concentration in the pre-analyzed samples, the % recoveries of the three concentrations were found to be 99.23 ± 0.85 for dacarbazine and for 101.04 ± 0.98 eugenol, which indicates high accuracy of the developed analytical method. Data is summarized in Table 3.

Precision

(i) Intra-day Precision (Repeatability)

The absorbance of three different dilutions of stock mixture solution was measured three times a day and % RSD values (Table 3) were calculated to obtain the intraday variations. %RSD was found to be 0.54 – 1.16 for dacarbazine and 0.94 – 1.58 for

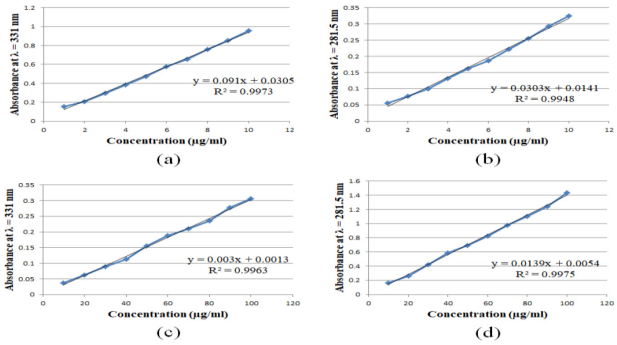

Figure 1: (a) Standard curve of Dacarbazine in PBS: Propylene glycol at λ_1 (331 nm); (b) Standard curve of Dacarbazine in PBS: Propylene glycol at λ_2 (281.5 nm); (c) Standard curve of Eugenol in PBS: Propylene glycol at λ_1 (331 nm); (d) Standard curve of Eugenol in PBS: Propylene glycol at λ_2 (281.5 nm).

Table 1: Optical Parameters and Regression Values of Dacarbazine and Eugenol in PBS: Propylene glycol.

PARAMETERS	DACARBAZINE		EUGENOL	
Wavelength	331 nm (λ_1)	281.5 nm (λ_2)	331 nm (λ_1)	281.5 nm (λ_2)
Beer's Law limit	1-10 (µg /ml)	1-10 (µg /ml)	10-100 (µg /ml)	10-100 (µg /ml)
Regression equation(Y)	Y = 0.091x + 0.0305	Y = 0.0303x + 0.0141	Y = 0.003x + 0.0013	Y = 0.0139 + 0.0054
Slope (m) **Intercept (c)**	0.091 0.0305	0.0303 0.0141	0.003 0.0013	0.0139 0.0054
Correlation coefficient (R^2)	0.9973	0.9948	0.9963	0.9975

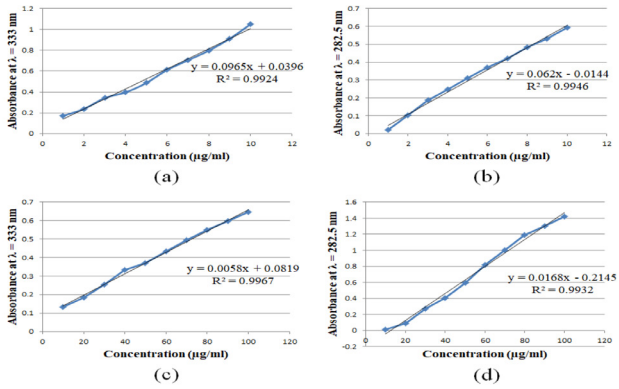

Figure 2: (a) Standard curve of Dacarbazine in Ethanol at λ_1^{et} (333 nm); (b) Standard curve of Dacarbazine in Ethanol at λ_2^{et} (282.5 nm); (c) Standard curve of Eugenol in Ethanol at λ_1^{et} (333 nm); (d) Standard curve of Eugenol in Ethanol glycol at λ_2^{et} (282.5 nm).

Table 2: Optical Parameters and Regression Values of Dacarbazine and Eugenol in Ethanol.

PARAMETERS	DACARBAZINE		EUGENOL	
Wavelength	333 nm (λ_1)	282.5 nm (λ_2)	333 nm (λ_1)	282.5 nm (λ_2)
Beer's Law limit	1-10 (µg /ml)	1-10 (µg /ml)	10-100 (µg /ml)	10-100 (µg /ml)
Regression equation (y)	y = 0.0965x + 0.0396	y = 0.062x - 0.0144	y = 0.0058x + 0.0819	y = 0.0168x - 0.2145
Slope (m) Intercept (c)	0.0965 0.0396	0.062 -0.0144	0.0058 0.0819	0.0168 -0.2145
Correlation coefficient (R^2)	0.9924	0.9946	0.9967	0.9932

Table 3: Method validation data for quantification of dacarbazine and eugenol in PBS (7.4): Propylene glycol (9:1).

PARAMETERS		DACARBAZINE	EUGENOL
Linearity Range		1-10 µg/ml	10-100 µg/ml
Accuracy (Mean Recovery)		99.23 ± 0.85	101.04 ± 0.98
Precision	Intra-day	0.54 – 1.16	0.94 – 1.58
	Inter-day	0.82 – 1.60	1.15 – 1.86
Limit of Detection (LoD)		0.32 µg/ml	4.60 µg/ml
Limit of Quantification (LoQ)		0.62 µg/ml	7.56 µg/ml

eugenol, which indicate good repeatability of the method.

(ii) Inter-day (Intermediate) Precision

The absorbance of three different dilutions of stock mixture solution was measured daily for three consecutive days to calculate % RSD values (Table 3) and calculate inter-day variations. Results of precision studies are summarized in Table 3. Low values of % RSD for inter-day precision (0.82 – 1.60 for dacarbazine and 1.15 – 1.86 for eugenol) suggest good intermediate precision of the developed UV absorptivity analytical method.

Limit of Detection

Limit of detection of dacarbazine was found to be 0.32 µg/ml. Limit of detection of eugenol was found to be 4.60 µg/ml.

Limit of Quantification

Limit of quantification of dacarbazine was found to be 0.62 µg/ml. Limit of quantification of eugenol was found to be 7.56 µg/ml.

Table 3 summarizes the validation data of the developed UV analytical method for the quantification of dacarbazine and eugenol in PBS (7.4): Propylene glycol (9:1).

As the same complete process was repeated to also develop the estimation method with ethanol as solvent, the method was validated too. Table 4 summarizes the validation data for both drugs when ethanol was taken as solvent.

Analysis of Liposomes

We used the above developed method of simultaneous estimation to determine the drug loading and rate of drug release from liposomes co-loaded with Dacarbazine and

Table 4: Method validation data for quantification of dacarbazine and eugenol in Ethanol.

PARAMETERS		DACARBAZINE	EUGENOL
Linearity Range		1-10 µg/ml	10-100 µg/ml
Accuracy (Mean Recovery)		98.82 ± 1.15	101.46 ± 0.89
Precision	Intra-day	0.45 – 1.62	0.84 – 1.68
	Inter-day	1.02 – 1.94	1.23 – 1.75
Limit of Detection (LoD)		0.24 µg/ml	3.46 µg/ml
Limit of Quantification (LoQ)		0.49 µg/ml	5.80 µg/ml

Table 5: Cumulative release of Dacarbazine and Eugenol from Liposomes.

Time (Hours)	Cumulative % Release of Dacarbazine	Cumulative % Release of Eugenol
0	0	0
0.5	10.3	18.2
1	17.56	24.44
2	22.67	33.8
4	28.5	41.45
8	36.21	54.83
12	44.24	68.62
24	61.78	81.73
36	72.45	86.49
48	79.5	91.74
72	84.67	97.1

Eugenol. Liposomes were formulated by ethanol injection method where initial amount of dacarbazine added was 12.5 mg and amount of eugenol added was 30 mg.

Drug Loading

The absorbance of the ethanolic solution of drugs loaded liposomes was measured at λ_1 and λ_2 and substituted in the equations (6) and (7) which were then solved to find out the values of C_d^{et} and C_e^{et}. After multiplying these values with the dilution factor, the concentration of dacarbazine was found to be 4.036 mg and concentration of eugenol was found to be 13.47 mg in the liposomes. Percentage of drug loading was calculated as follows:

$$\frac{\text{Amount of drug in Liposomes}}{\text{Total weight of the Liposomes (lyophilized)}} \times 100$$

Total weight of lyophilized liposomal formulation was 29.35 mg.

$$\% \text{ loading of Dacarbazine: } 4.036 \text{ mg} / 29.35 \text{ mg} \times 100 = 13.75\ \%$$

$$\% \text{ loading of Eugenol: } 13.47 \text{ mg} / 29.35 \text{ mg} \times 100 = 45.89\ \%$$

So, the Dacarbazine loading was 13.75 % and Eugenol loading was 45.89 %.

Drug Release

Absorbance of aliquots of release media withdrawn at different time points were measured at λ_1 and λ_2 (A_1 and A_2) against pure release media as blank. The amount of drugs present (cumulative amount of drugs released) in release media at different time intervals was calculated by putting these absorbance values in equation (4) and (5). A plot was made between the time interval and cumulative percent of drug released. Table 5 and Figure 3 depict the results of *in-vitro* drugs release study. Prepared liposomes exhibited sustained release of the two drugs as determined by the developed UV absorptivity method. In 24 h, around 45% of dacarbazine and 68% of eugenol was released. 85% dacarbazine and 97% eugenol was released in a span of 72 h.

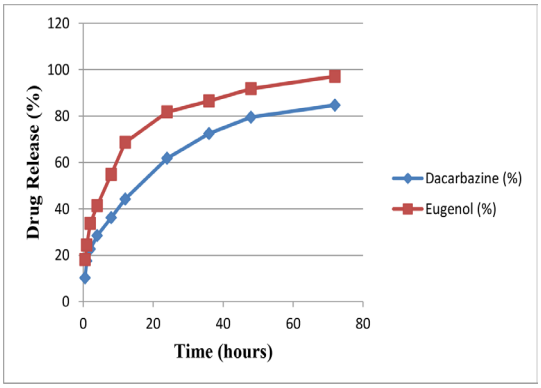

Figure 3: Cumulative release of Dacarbazine and Eugenol from liposomes.

CONCLUSION

We synthesized dual drugs (dacarbazine and eugenol) loaded liposomes by solvent injection method to be used against resistant cancers. Since the combination of dacarbazine and eugenol could not be analyzed by HPLC or LCMS, we developed a UV spectroscopic method based on absorptivity calculation. This method was robust and reproducible. PBS (pH 7.4): Propylene glycol (9:1) was found to be a suitable solvent media and hence was selected for drugs release study. Ethanol was found to be suitable for drugs loading determination.

Thus, UV method was developed for these solvents. The developed method was appropriately validated and was found to be accurate and precise. Finally, the method was applied for the determination of loading and release rate of the drugs from the liposomes. The loading of the two drugs was found to be 13.75 % for dacarbazine and 45.89 % for eugenol and the release of the drugs from the liposomes was sustained. After 24 h, around 61 % dacarbazine and 81 % of eugenol was released from the liposomes. So, the developed UV analytical method based on absorptivity measurement could be practically used for the *in-vitro* analysis of dual drugs loaded anti-cancer liposomes.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

HPLC: High Performance Liquid Chromatography; **LCMS:** Liquid Chromatography: Mass Spectrometry; **UV:** Ultra Violet; **ICH**: International Council for Harmonisation; **LoD:** Limit of Detection; **LoQ:** Limit of Quantification; **PBS:** Phoshate Buffer Saline; **MW:** Molecular Weight; **ANOVA:** Analysis of Variance; **R^2:** Regression Coefficient.

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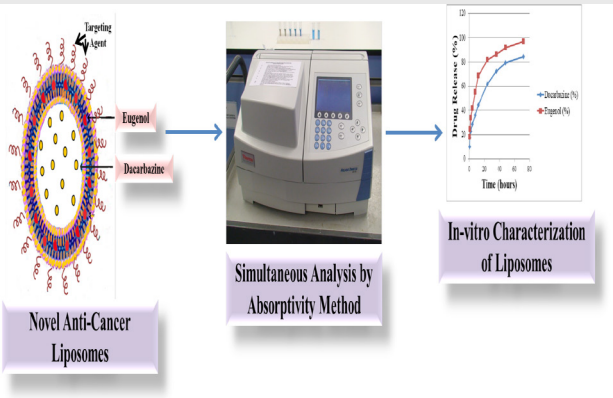
PICTORIAL ABSTRACT



SUMMARY

- Dacarbazine and Eugenol loaded liposomes were formulated by solvent injection method to be used against resistant cancers. For the simultaneous estimation of these two drugs during drug loading and release studies, a UV method based on absorptivity measurement was developed. Absorptivities of both the drugs were measured at both the λ_{max} and the values were put in simultaneous equations. This method was validated for linearity, range, precision and accuracy. Limit of detection and limit of quantification were also determined. For determination of the loading of drugs, the absorbance values were put in the simultaneous equations and the equations were solved to obtain the concentration of the drugs present in the formulation. Similarly, release of drugs was also tracked by measuring the absorbance values of the release media at different time points and solving the equations to determine the amount of drugs released. Thus, the method was developed, validated and successfully used for the *in-vitro* characterization of the dual drugs loaded anti-cancer liposomes.

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