Analytical methods

3. ANALYTICAL METHODS

3.1 Estimation method for Docetaxel

3.1.1 High Performance Liquid Chromatography (HPLC) conditions

The drug content was determined using a HPLC system (Shimadzu, Japan). The HPLC system was composed of a UV-visible spectrophotometric detector. The separation was performed on a C 18 150-4.6 HPLC column (Phenomenex Gemini C-18, 250 cm X 4.6mm, 5µm). Mobile phase consisted of acetonitrile: water mixture (70:30 v/v) (Ciccolini et al, 2001; USP33 NF Monograph). The run time was 15 min and the retention time of docetaxel was 4.47 min. UV detection wavelength was 230 nm and mobile phase flow rate 1 ml/min. Data processing was done using Spinchrom CFR (Spinchotech, Japan).

3.1.2 Preparation of standard stock solutions

Stock solution of DTX is to be prepared in acetonitrile by accurately weighing 10mg of DTX in 10mL (1000µg/mL DTX) acetonitrile. Further dilution was performed using acetonitrile:water (70:30).

3.1.3 Preparation of calibration curve

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml of volumetric flask to prepare a working stock solution of DTX (100 µg/mL). Suitable aliquots of working stock solution were accurately measured and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with the solvent acetonitrile:water (70:30) to give final concentrations of 5, 10, 20, 30, 40, 50, and 60 µg/mL. Standards were analysis by RP HPLC at UV detection wavelength 230nm and mobile phase flow rate 1 ml/min. After 15 min elution and results were processed using data processing software Spinchrom CFR. The above procedure was repeated six times and results recorded in the following table 3.1 and figure 3.1. All the estimations were carried out between 20°C - 27°C, and care was taken to prevent solvent evaporation at every stage of estimation. Calibration plot was constructed for the measured area against drug concentration. Accuracy and precision of the method was determined by performing recovery studies after addition of known concentration of DTX.

Table 3.1: Calibration Curve of DTX in DTX in ACN: water (70:30) mobile phase at λ_{max} = 230nm, Data presented as Mean ± SD, n=6.

Concentration (µg/ml)	Retention Time (min)	Area (mV.s)
5	4.520	123.165 ± 1.25
10	4.523	215.048 ± 1.84
20	4.523	467.107 ± 2.17
30	4.527	719.470 ± 3.42
40	4.527	930.587 ± 3.71
50	4.523	1162.711 ± 4.60
60	4.527	1391.341 ± 5.81

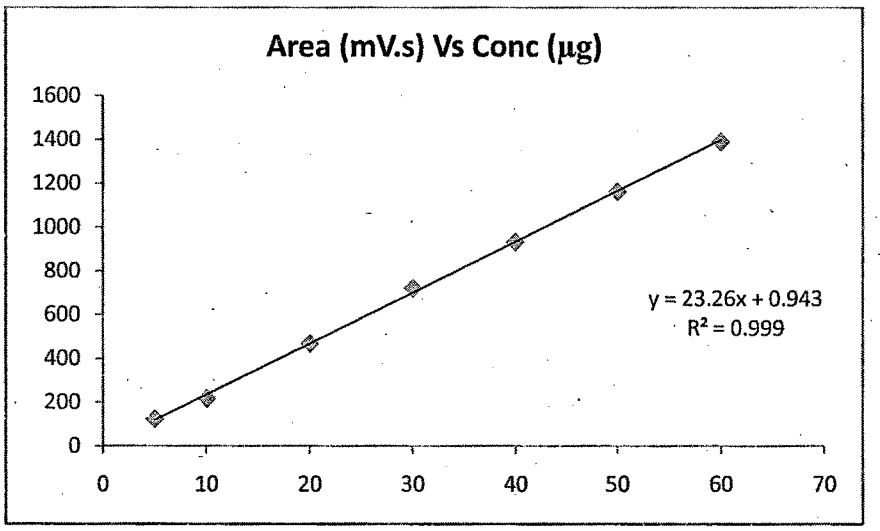

Figure 3.1: Regressed calibration curve of DTX in ACN: water (70:30) mobile phase at λ_{max}=230nm, Data presented as Mean ± SD, n=6.

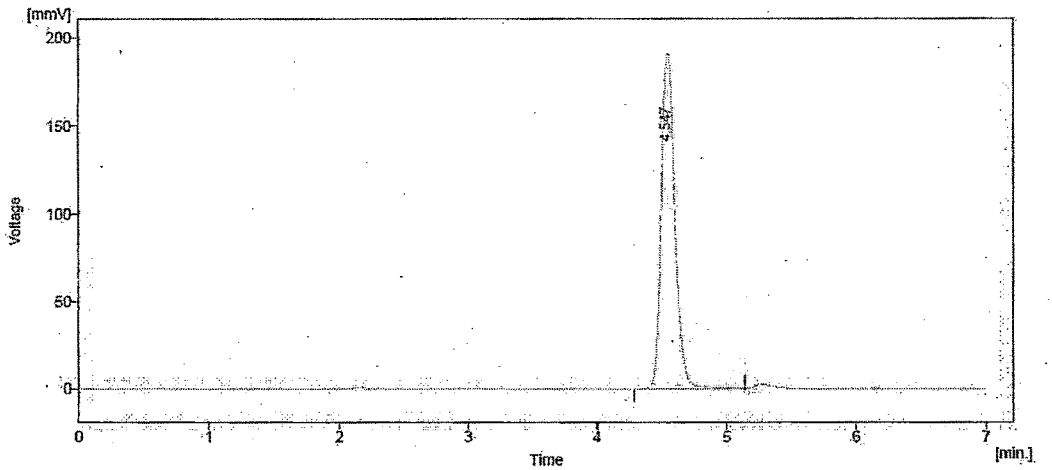

Figure 3.2: Chromagram of Standard DTX solution in ACN: water (70:30) mobile phase at λ_{max}=230nm.

3.1.4 Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of DTX at low medium and high concentration (5, 10, 30 and 50 µg/ml) were subjected to recovery studies as per the procedure described earlier. All standard samples were also performed for intraday and interday variability. The results obtained are tabulated in table 3.2 and 3.3.

Table 3.2: Accuracy of DTX measurement using ACN: water (70:30) mobile phase at λ_{max}=230nm.

Conc. Of DTX (µg)	RT (min)	Area (mV.s)	Conc. Of DTX Obtained (µg)	Accuracy	Precision (RSD %)
5	4.520	123.165 ± 1.25	5.255	105.1	1.01
10	4.523	215.048 ± 1.84	9.205	92.1	0.86
30	4.527	719.470 ± 3.42	30.89	103.0	0.46
50	4.523	1162.711 ± 4.60	49.95	99.9	0.40

Table 3.3: Precision of DTX measurement using ACN: water (70:30) mobile phase at λ_{max}=230nm, Data presented as Mean, n=3.

Conc (μM)	Measured conc (μM)		Acuracy (%)	
	Intra day	Inter day	Intra day	Inter day
5	5.255	5.026	105.1	100.5
10	9.205	9.448	92.1	94.5
30	30.89	29.130	103.0	97.1
50	49.95	49.535	99.9	99.1

3.1.5 Construction of calibration plot in formulation composition

To 0.1 ml blank PLGA and PBCA NP formulation required quantity of drug solution (from standards prepared in acetonitrile) was added to obtain the final concentrations of DTX ranging between 5 to 50 μg/ml. The contents were gently mixed to ensure uniform mixing and kept aside for 30 min at room temperature. The samples were filtered and the filtrate was collected and estimated by RP HPLC at UV detection wavelength 230nm and mobile phase flow rate 1 ml/min. After 15 min elution and results were processed using data processing software Spinchrom CFR.

3.1.6 Accuracy and precision in formulation

In order to determine the accuracy and precision of the developed method, known amounts of DTX at low medium and high concentration (5, 30 and 50 μg/ml) were subjected to recovery studies as per the procedure described earlier. The results obtained are tabulated in table 3.4.

Table 3.4: Accuracy of DTX measurement in PLGA formulation using ACN: water (70:30) mobile phase at λ_{max}=230nm, Data presented as Mean, n=3.

Conc. Of DTX (μg)	RT (min)	Area (mV.s)	Conc. Of DTX Obtained (μg)	Accuracy (%)	Precision (RSD %)

5	4.524	116.138 ± 1.63	4.795	95.9	1.40
30	4.527	689.342 ± 2.38	29.60	98.7	0.35
50	4.528	1205.637 ± 4.61	51.79	103.6	0.38

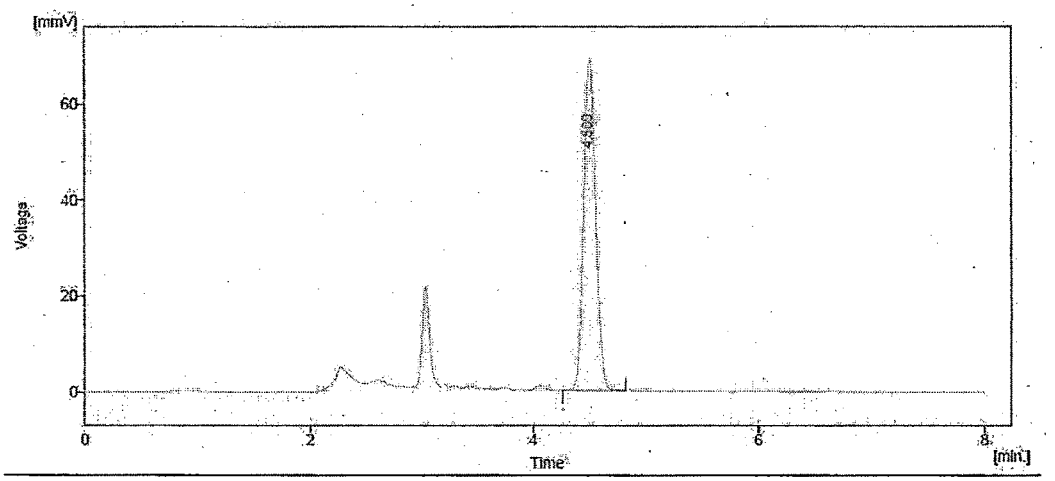

Figure 3.3: Chromatogram of Standard DTX in PLGA NP formulation using ACN: water (70:30) mobile phase at λ_{max}=230nm.

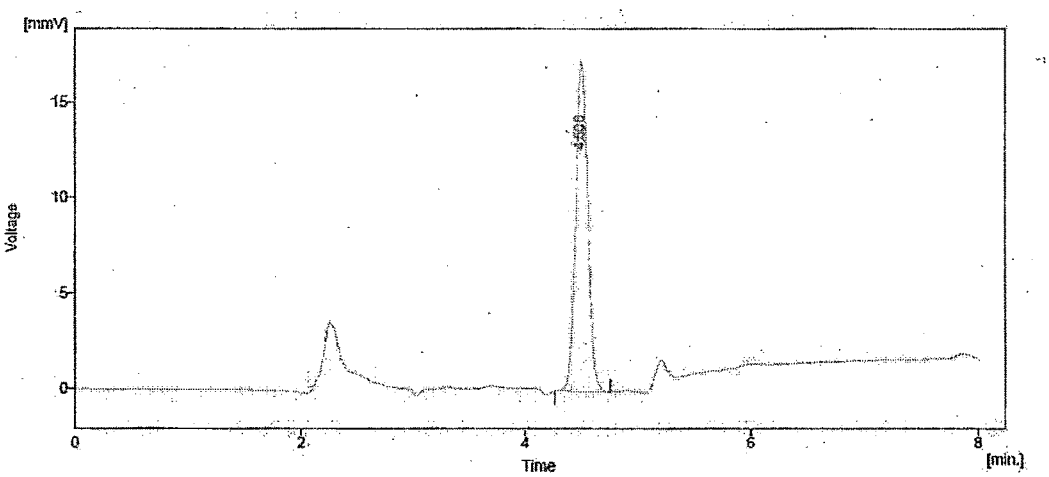

Figure 3.4: Chromatogram of Standard DTX in PBCA NP formulation using ACN: water (70:30) mobile phase at λ_{max}=230nm.

3.2 Estimation of IPP and ApppI

3.2.1 Cells treatment and sample preparation for LC-MS

Sample treatment and preparation was performed as per earlier reported method (Marjo Jauhiainen et al 2009). MCF7 and BO2 cells were seeded in 6 well plates at the density of 4 × 105 cells per well and incubated for 24 h to allow cell attachment. After 24 h, cells were incubated/treated with 1 µg/ml, 2 ml of ZOL solution and PLGA-PEG-ZOL NP along with control group (PBS) for 24 h. To extract anylates from cell line samples, cell culture plates was treated with ice cold acetonitrile (300 µL) followed by ice cold milliQ water (200 µL). The adherent cell line was scrapped and performed pipette mixing followed by centrifugation (13,000×g, 3 min, 4°C). The supernatant was transferred in another tube and evaporated until complete dryness using vacuum centrifugation and stored at -20°C until LC-MS analysis. Before analysis, the content was dissolved in MilliQ water (150 µL) having 0.25 mM NaF and Na3VO4 phosphatase inhibitors and 1.0 µM AppCp as internal standard. The cell lysate precipitate was digested with 1M NaOH at 60 °C for 2 h and performed for total protein content estimation using modified Bradford procedure (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard protein on plate counter (Wallac Victor2) at 595 nm. The final concentration of IPP and ApppI was presented as nM per mg of protein.

3.2.2 Ion-pair HPLC

The highly hydrophilic compounds were separated on a C18 reversed phase column using volatile dimethylhexylamine (DMHA) as an ion-pair agent. The method optimization is presented earlier by our group. The MRM chromatograms of the analytes are shown in Fig. 3.5 to 3.8. The ion-pair modifiers added to the solvents are considered to interfere with the ESI process, however, It was proven that addition of 2mM DMHA does not significantly suppress the signal of the analyte. Hence, in the gradient elution of the analytes, the DMHA concentration was decreased from 20mM to 2mM with simultaneous increasing of the organic eluent to avoid suppression during the elution of the analytes as well as assist the elution. All compounds including the internal standard eluted at retention times within 55 s and therefore the ionization environment can be expected to be similar for each compound.

3.2.3 Identification of IPP and ApppI using LC-MS

All conditions have been previously described in detail by Monkkonen et al. (2006) and Marjo Jauhiainen et al (2009). On-line HPLC-ESI MS measurements were carried out using an Agilent 6410 Triple Quad LC/MS for the analysis of IPP, ApppI and AppCCl2 (standard). Instrument was equipped with an electrospray ionization source (EIS) and operated on negative ion mode. For the Agilent triple quadrupole instrument optimized parameters were following: drying gas temperature was kept at 300°C, gas flow at 8 mL/min, nebulizer gas pressure operated at 40 psi and capillary voltage was kept at - 4500V. Negative full scan mass spectra were performed in the mass range of m/z 60–650 at above mentioned condition. Parent ion abundance with highest intensity was indentified using fragmentor voltages of 120 V for IPP, and 140V for ApppI and internal standard (ISTD) AppCp. Individual product ion (MS2) spectra were recorded using collision induced dissociation (CID) in the collision cell with nitrogen gas. Most intense product ion signal was achieved when offset voltages kept at 15 eV, 27 eV and 30 eV for detection of IPP, ApppI and internal standard (AppCp) respectively. Following transitions were optimized for multiple reactions monitoring (MRM): m/z 245→79 for IPP, m/z 574→227 for ApppI and m/z 504→157 for internal standard (AppCp). Agilent Mass Hunter Workstation software was used for data acquisition.

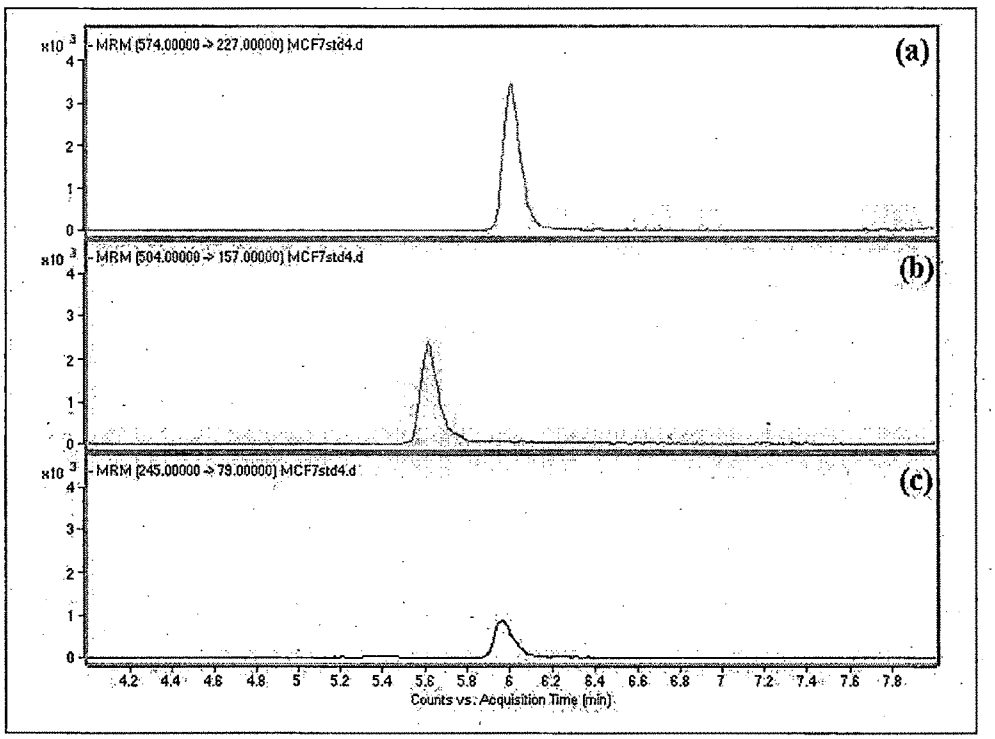

Figure 3.5: MRM chromatograms of ApppI (a), IPP (b) and ISTD (c) in standard preparation in MCF7 cell line.

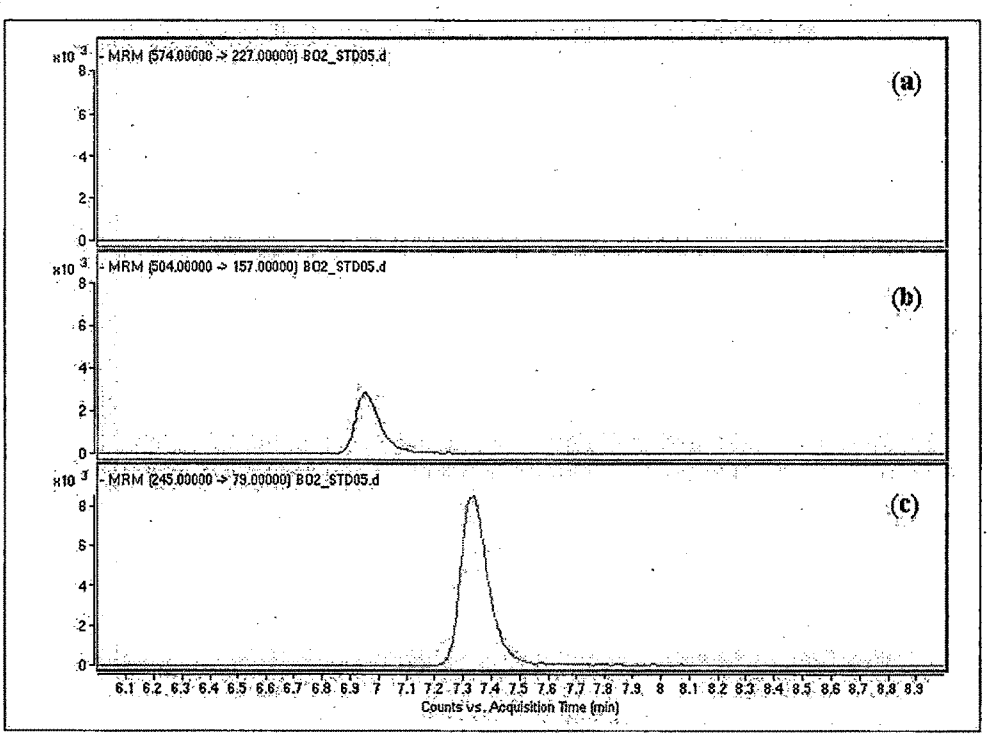

Figure 3.6: MRM chromatograms of ApppI (a), IPP (b) and ISTD (c) in standard preparation in BO2 cell line.

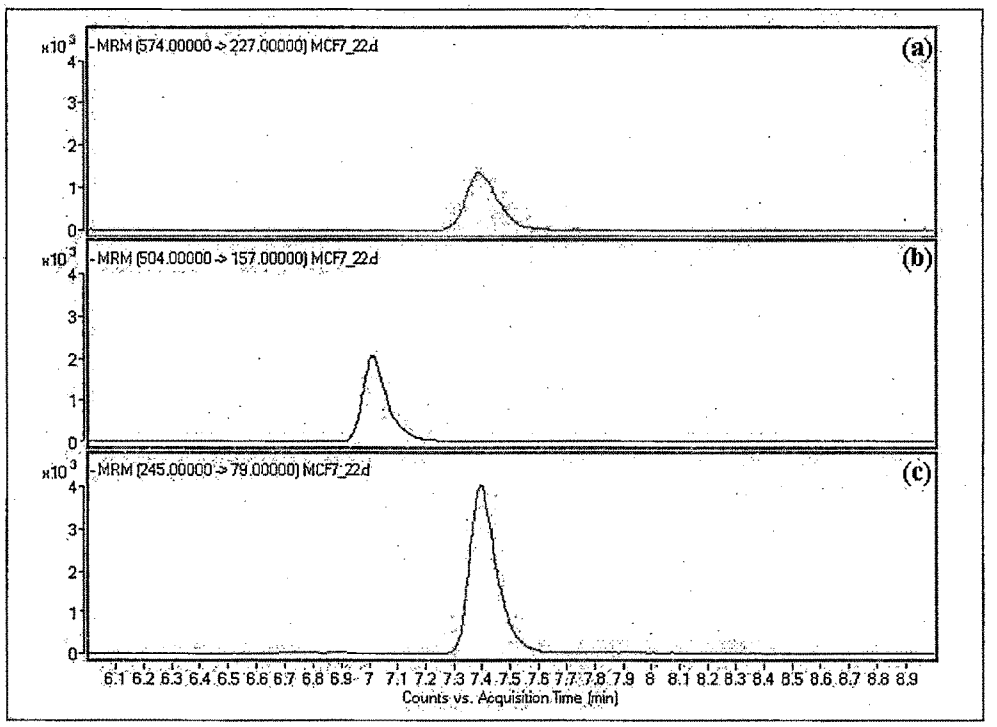

Figure 3.7: MRM chromatograms of ApppI (a), IPP (b) and ISTD (c) in test preparation in MCF7 cell line.

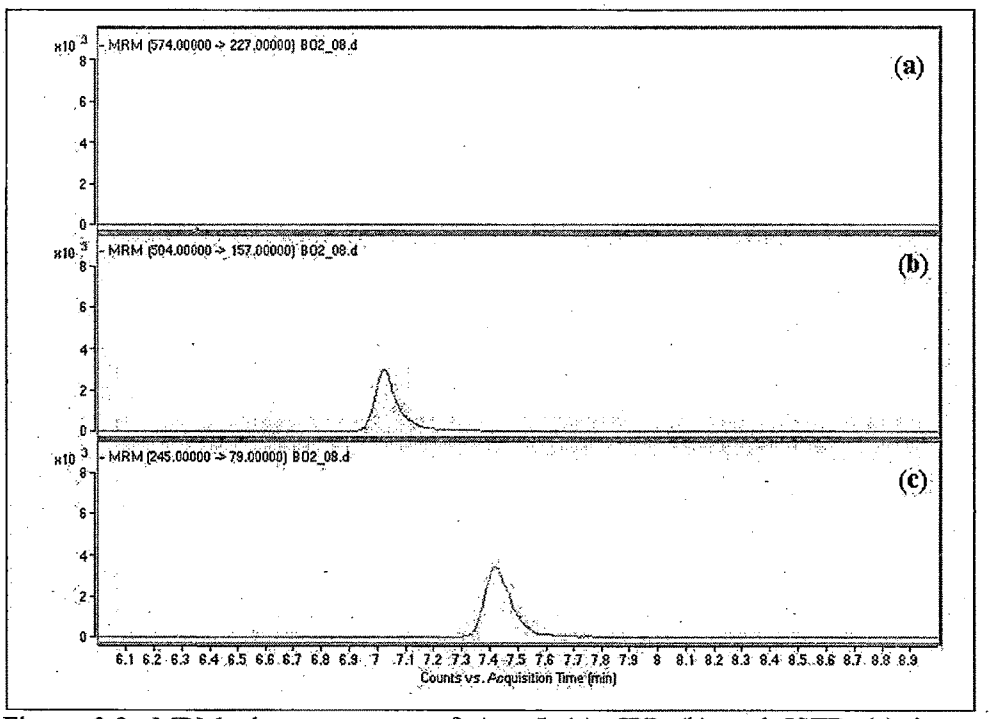

Figure 3.8: MRM chromatograms of ApppI (a), IPP (b) and ISTD (c) in test preparation in BO2 cell line.

3.2.4 Standardization and calibration curve preparation of IPP and ApppI

The analysis method was validated using IPP and ApppI standards. The limit of quantification (LOQ) was estimated as the lowest estimation with the percent relative standard deviation (RSD %) value lower than 20% and signal-to-noise ratio remained greater than 10:1. All calibration samples were prepared freshly on each day of analysis. The calibration area was selected in range from 0.010 µM to 90.0 µM for IPP and from 0.030 µM to 30.0 µM for ApppI. The calibration curve was obtained in three replicate measurements. Ratio of peak area of analytes and internal standard were plotted against the concentration of the standards.

3.2.5 Quantification of the total amounts of IPP and ApppI

The assay for the analysis of total concentration of IPP and ApppI mixtures was validated using IPP and ApppI standards. The limit of quantitation (LOQ) was determined as the lowest concentration with the percent relative standard deviation (RSD %) value lower than 20% and signal-to-noise ratio greater than 10:1. Calibration and quality control (QC) samples were prepared fresh daily from stock

solutions. The calibration area ranged from 0.030 µM to 15.0 µM for IPP and from 0.020 µM to 15.0 µM for ApppI. The calibration curve was generated by three replicate measurements and nine standard points. The peak area ratios of the MRM chromatograms of the analytes and internal standard were plotted against the concentration of the standards. A linear $1/x$ fit, where x is concentration, was employed. QC samples were prepared at low, middle and high concentration range of the standard curve, e.g. at 0.045 µM, 0.30 µM, 0.60 µM and 12.0 µM for IPP and at 0.030 µM, 0.20 µM, 0.60 µM and 12.0 µM for ApppI.

Accuracy was reported as the percentage of the expected concentration and precision as a percent relative standard deviation (RSD %). Intra-day accuracy and precision were evaluated by six (n = 6) replicate measurements of the QC samples on the same day. Inter-day accuracy and precisionwere obtained by analysing the QC samples in three or six replicate injections on three different days (n = 9 - 12) over period of one week. Matrix effect was determined by spiking six (n = 6) untreated MCF-7 cell extracts with known amount (1.0 µM) of IPP, ApppI and AppCp and comparing the obtained peak areas to the ones of matrix free standard of same concentration. Spiked sample also contained the phosphatase inhibitors (0.25 mM NaF and Na_3VO_4). The recovery and precision of the extraction method were determined by analyzing the total content of IPP and ApppI in six (n = 6) MCF-7 cell samples, treated with 25 µM ZOL for 24 h. Extraction recovery was obtained by repeating the extraction procedure twice for the cell precipitates.

Table 3.5: Calibration of ApppI using standard known concentration in MCF7 cell line

Tested Conc. (nM)	ApppI standard				Internal Standard	
	RT	Response	Final Conc	Accuracy	RT	Response
10	6.00	855	11	110.6	5.61	14232
	5.99	899	12	106.4	5.62	14253
30	6.00	2242	31	102.9	5.61	13766
	6.00	2157	30	101.0	5.61	13491
100	6.00	6392	91	90.5	5.62	13535

	6.00	6552	92	91.8	5.60	13682
300	6.01	19716	261	86.9	5.62	14555
	6.00	20115	277	92.5	5.61	13949
1000	6.01	74907	1026	102.6	5.62	14064
	6.00	75652	1050	105.0	5.61	13880

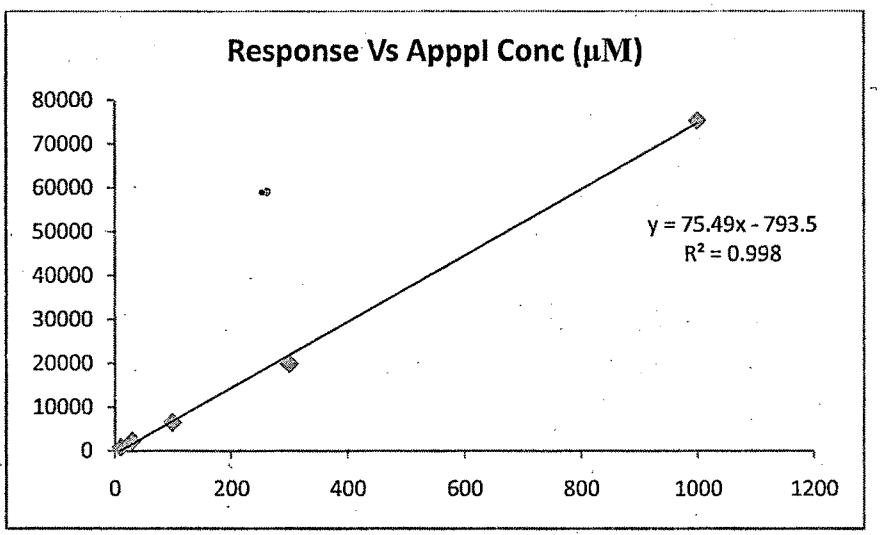

Figure 3.9: Calibration curve and regression analysis for ApppI in MCF7 cell line

Table 3.6: Calibration of IPP using standard known concentration in MCF7 cell line

Tested Conc. (nM)	IPP standard				Internal Standard	
	RT	Response	Final Conc	Accuracy	RT	Response
10	5.97	459	10	100.1	5.61	14232
	5.39	367	5	94.8	5.62	14253
30	5.96	770	28	94.0	5.61	13766
	5.97	851	34	112.3	5.61	13491
100	5.96	2048	101	101.4	5.62	13535
	5.96	2035	99	99.4	5.60	13682
300	5.97	5549	278	92.6	5.62	14555

	5.96	5599	293	97.8	5.61	13949
1000	5.97	18929	1018	101.8	5.62	14064
	5.95	18499	1008	100.8	5.61	13880

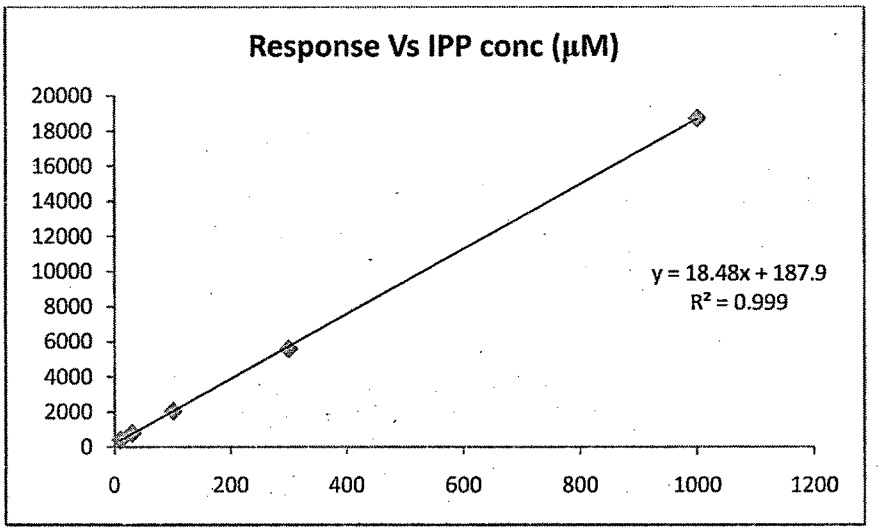

Figure 3.10: Calibration curve and regression analysis for IPP in MCF7 cell line

Table 3.7: Calibration of IPP using standard known concentration in BO2 cell line

Tested Conc. (nM)	IPP standard				Internal Standard	
	RT	Response	Final Conc	Accuracy	RT	Response
10	5.99	325	0	0	5.61	14331
	5.96	266	0	0	5.61	13665
30	5.97	699	16	53.6	5.61	12755
	5.95	651	13	43.8	5.61	12868
100	5.96	2509	113	112.7	5.61	12960
	5.97	2460	110	110.4	5.61	12923
300	5.96	5688	280	93.4	5.60	13093
	5.96	5587	281	93.8	5.61	12821
1000	5.97	18185	922	92.2	5.61	13418
	5.95	17938	916	91.6	5.60	13315
3000	5.96	58215	3134	104.5	5.60	12840
	5.95	56717	3043	101.4	5.60	12881

6000	5.94	91264	5784	96.4	5.61	12658
	5.97	95391	6438	107.3	5.60	13193

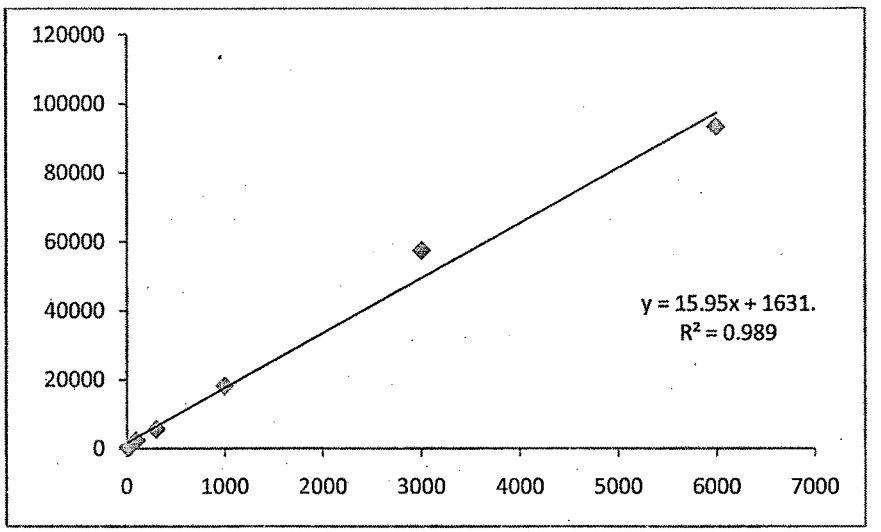

Figure 3.11: Calibration curve and regression analysis for IPP in BO2 cell line

3.2.6 Stability of IPP and ApppI in sample

The stability of the analytes during the analysis process was tested by evaluating the short-term and freeze-thaw stability of the working solutions. IPP and ApppI were diluted with water to final concentration of 1.0 µM and the samples were kept in room temperature for 24 h. Freeze–thaw stability was tested by repeating the freeze and thaw cycle three times before analyzing the content. Post-preparative stability of the samples was tested by resuspending untreated MCF-7 cell samples by vortex mixing to 150 µL of MilliQ water containing 1.0 µM IPP, ApppI and AppCp, either with or without 0.25mM NaF and Na3VO4 as phosphatase inhibitors. The samples were kept either at room temperature or in a cooled autosampler (4 °C) for maximum of 24 h. The contents of ApppI and IPP were measured at 0 h, 8 h and 24 h after the sample preparation and the degradation percentages were calculated as comparison of the peak areas of the analytes in the sample at 8 h and 24 h to the ones at 0 h.

3.2.7 Linearity, accuracy and precision of IPP and ApppI

Limit of quantitation was 0.030 µM for IPP and 0.020 µM for ApppI. The range of the method of analyzing the total amount of IPP and ApppI was relatively high (2000-

fold). the calibration curve was linear over their calibration range. Intra-day accuracy for each QC sample was within 100±21.7% for IPP and 100±14.8% for ApppI. Intra-day precisions were better than 13.4% and 10.3% for IPP and ApppI, respectively. The inter-day results did not differ significantly from the intra-day results. The measured concentrations, accuracies and precisions for each QC sample are displayed in Table 3.8 and 3.9.

Table 3.8: Accuracy and precision for inter and intraday measurement of IPP. Data presented as Mean ± SD, n=3.

Sample No.	Conc (μM)	Measured conc (μM)		Acuracy (%)		Precision (RSD %)	
		Intra day	Inter day	Intra day	Inter day	Intra day	Inter day
QC1	0.045	0.039 ± 0.005	0.042 ± 0.003	88.8	94.0	13.4	6.0
QC2	0.30	0.37 ± 0.02	0.34 ± 0.03	121.7	114.7	6.4	8.4
QC3	0.60	0.68 ± 0.01	0.66 ± 0.08	115.0	101.5	4.6	14.9
QC4	12.0	13.5 ± 0.2	13.0 ± 0.7	113.2	104.5	1.6	9.2

Table 3.9: Accuracy and precision for inter and intraday measurement of ApppI. Data presented as Mean ± SD, n=3.

Sample No.	Conc (μM)	Measured conc (μM)		Acuracy (%)		Precision (RSD %)	
		Intra day	Inter day	Intra day	Inter day	Intra day	Inter day
QC1	0.030	0.034 ± 0.002	0.032 ± 0.004	114.8	107.7	10.3	13.6
QC2	0.20	0.22 ± 0.01	0.21 ± 0.02	110.3	105.0	7.3	7.5
QC3	0.60	0.65 ± 0.04	0.67 ± 0.06	108.0	111.0	6.2	9.4
QC4	12.0	13.2 ± 0.2	12.7 ± 1.0	110.3	106.0	1.6	7.9

3.2.8 Extraction recovery, repeatability and matrix effect

The recovery percentage of the extraction method was examined by repeating the acetonitrile: water extraction twice for the same ZOL treated cell sample and

comparing the contents. ZOL treated samples were used for the experiments since they contain a significant amount of IPP and ApppI, whereas in the untreated control samples these metabolites were not detected. It appeared that relative to the first extraction, only approximately 5% of the total amount was covered during the second extraction. Thus, it was concluded that the extraction using acetonitrile: water was sufficient to extract the majority of the analytes from the cell culture samples with relatively low amount of interferences. Also, it simultaneously precipitates the proteins that can be easily separated for the protein concentration measurement. The repeatability of the extraction method, expressed as percent relative standard deviation (RSD %), calculated from the peak area ratios of the MRM chromatograms of the analytes and internal standard were 5.9% and 6.2% for IPP and ApppI, respectively. Matrix induced ion suppression was not significant for IPP and ApppI standards.

3.2.9 Stability

Working solutions of IPP and ApppI were stabile at room temperature for at least 24 h (accuracies 105.7% and 107.9%, respectively) and after three freeze-thaw cycles (accuracies 102.2% and 104.1%, respectively). Post-preparative stability of the samples was tested by storing the samples at four different conditions: (1) at room temperature without phosphatase inhibitors, (2) at room temperature with 0.25mM phosphatase inhibitors (NaF and Na3VO4), (3) in a cooled autosampler at 4°C without phosphatase inhibitors, and (4) in a cooled auto-sampler at 4°C with 0.25 mM phosphatase inhibitors. Significant degradation of the analytes occurred without cooling and without presence of the phosphatase inhibitors after 8 h, and after 24 h only less than 20% of IPP and 3% of ApppI compared to the initial amount was detected. It was concluded that ApppI is stable for at least 24 h at 4°C with 0.25 mM phosphatase inhibitors (98.2% recovery) and that in similar conditions the recovery for IPP was 81.5%. No significant degradation of AppCp was found after 24 h storage in any conditions.

3.3 Estimation of coumarin-6

3.3.1 Method of estimation

All fluorimetric estimations were performed on a fluorescence microplate reader (Perkin Elmer EnVision 2014 multilabel) equipped with a xenon lamp. The various experimental conditions like excitation wavelength ($\lambda_{excitation}$) and emission wavelength ($\lambda_{emission}$) were optimized ($\lambda_{excitation}$ = 430nm; $\lambda_{emission}$ = 485).

3.3.2 Preparation of Calibration Plot in ACN

Stock solution of coumarin-6 in acetonitrile was prepared by accurately weighing 10mg of coumarin-6 in 100mL of solvent. After ensuring that the coumarin-6 has totally dissolved, suitable aliquots of the 100µg/mL stock solution of coumarin-6 was pipetted into 10mL volumetric flasks. The volume was made upto 10mL using the same solvent. The contents were shaken well and the relative fluorescence intensity was measured setting the $\lambda_{excitation}$ at 430nm and the corresponding $\lambda_{emission}$ peak intensity was measured at 485nm using a fluorescence microplate reader (Perkin Elmer EnVision 2014 multilabel) against suitable blank. The above procedure was repeated 3 times and the mean relative fluorescence intensity values were determined. The mean relative fluorescence intensity obtained tabulated in Table 3.10 and figure 3.12.

Table 3.10: Calibration Curve of coumarin-6 ($\lambda_{excitation}$ = 430nm; $\lambda_{emission}$ = 485 nm), Data presented as Mean ± SD, n=3.

Concentration (µg/ml)	Mean Fluorescence
0.02	12.62 ± 0.46
0.05	27.26 ± 0.64
0.08	38.16 ± 0.79
0.10	51.62 ± 0.86
0.15	78.36 ± 0.96
0.20	106.62 ± 1.13

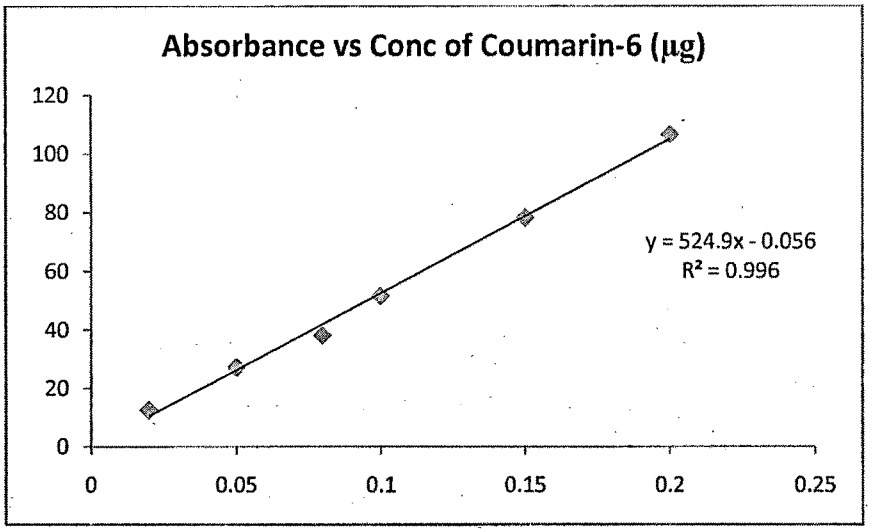

Figure 3.12: Calibration curve and regression analysis of coumarin-6 in ACN (λ_{ex} = 430nm; λ_{em} = 485 nm)

3.3.3 Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of coumarin-6 (50, 100 and 200 ng/mL) are subjected to recovery studies as per the procedure described earlier in presence of formulation. The measured quantity of coumarin-6 was dissolved in ACN, suitable quantity of blank formulation (PLGA NP and PBCA NP) was added and make up volume using aliquots to 100µg/mL stock. Required amount of solution of coumarin-6 was pipetted into 10mL volumetric flasks and made upto 10mL using the same solvent. The contents were shaken well and the relative fluorescence intensity was measured setting the $\lambda_{excitation}$ at 430nm and the corresponding $\lambda_{emission}$ peak intensity was measured at 485nm using a Fluorescence microplate reader (Perkin Elmer EnVision 2014 multilabel) against suitable blank. Obtained results are tabulated in Table 3.11 and 3.12.

Table 3.11: Accuracy coumarin-6 measurement in PLGA formulation using ACN (λ_{ex} = 430nm; λ_{em} = 485 nm) Data presented as Mean ± SD, n=3.

Conc. Of	Absorbance	Conc. Of Coum-6	Accuracy	Precision

Coum-6 (µg)		Obtained (µg)		(RSD %)
0.05	29.27 ± 0.78	0.0557	111.4	2.66
0.10	53.36 ± 1.16	0.1016	101.6	2.17
0.20	108.83 ± 1.84	0.2072	103.6	1.7

Table 3.12: Accuracy of coumarin-6 measurement in PBCA formulation using ACN mobile phase at λ_{max}=230nm, Data presented as Mean ± SD, n=3.

Conc. Of Coum-6 (µg)	Absorbance	Conc. Of Coum-6 Obtained (µg)	Accuracy	Precision (RSD %)
0.05	27.13 ± 0.64	0.0516	103.2	2.36
0.10	52.68 ± 0.87	0.1003	100.3	1.65
0.20	101.45 ± 1.32	0.1932	96.6	1.30

3.4 Estimation of Poloxamer P188

3.4.1 Experimental method

The was carried out in eppendorf tunes containing 0.5 ml of an dichloromethane, 0.5 ml ammonium ferroisothiocynate solution, and 0.5 ml phosphate buffer (pH 7.4) containing the required quantity of poloxamer P188 (Al-Hanbali et al, 2007). The system was shaken vigorously for 20 min, followed by centrifugation (3 min) using at 16,000 g at room temperature. The lower solvent layer was extracted and placed into a quartz cuvette with a 1 cm pathlength and the covered with 100 µl of the ammonium ferroisothiocyanate reagent. The absorbance was measured at 510 nm in a spectrophotometer Shimadzu RF-540 spectrofluorometer (Shimadzu Corporation, Japan). The experiment was performed in triplicates.

3.4.2 Preparation of standards and calibration plot

Stock solution of poloxamer P188 in phosphate buffer (pH 7.4) was prepared by accurately weighing 10mg of poloxamer P188 in 10 mL of solvent. After ensuring that the poloxamer P188 has totally dissolved, suitable aliquots of the 100 µg/mL stock solution of poloxamer P188 was pipetted into 10mL volumetric flasks. The

volume was made upto 10mL using phosphate buffer (pH 7.4). The contents were shaken well and the absorbance intensity was measured at510 nm using a Shimadzu RF-540 spectrofluorometer (Shimadzu Corporation, Japan) against suitable blank. The above procedure was repeated 3 times and the mean absorbance intensity values were determined (Table 3.13 and Figure 3.13).

Table 3.13: Calibration of poloxamer P188 in DCM : phosphate buffer (pH 7.4) at 510 nm, Data presented as Mean ± SD, n=3.

Concentration (µg/ml)	Absorbance
10	0.058 ± 0.009
20	0.093 ± 0.013
40	0.168 ± 0.004
60	0.251 ± 0.011
80	0.306 ± 0.011
100	0.375 ± 0.010

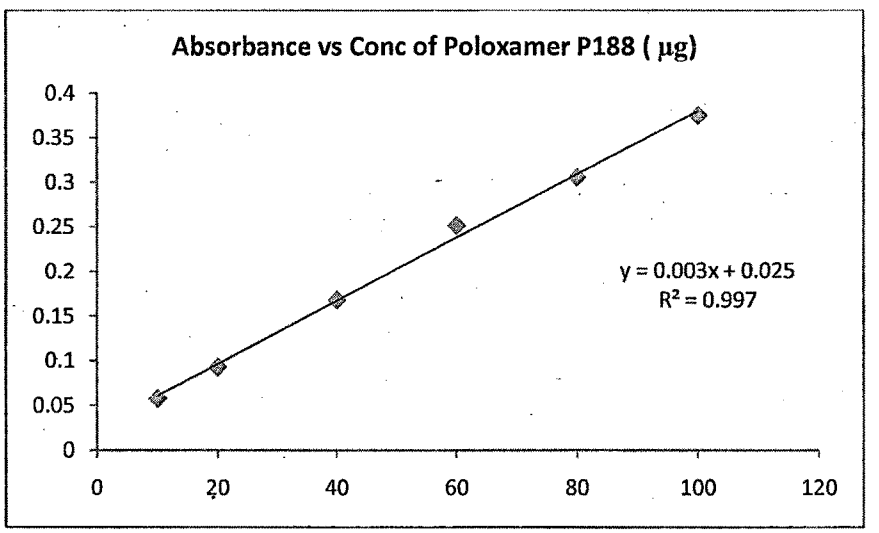

Figure 3.13: Calibration curve and regression analysis of poloxamer P188 in DCM : phosphate buffer (pH 7.4) at 510 nm, Data presented as Mean ± SD, n=3.

3.4.3 Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of poloxamer P188 (20, 60 and 100 µg/mL) are subjected to recovery studies as per the procedure described earlier in presence of formulation. The measured quantity of poloxamer P188 was dissolved in phosphate buffer (pH 7.4), suitable quantity of blank formulation (PLGA NP and PBCA NP) was added and make up volume using aliquots to 1000 µg/mL stock. Required amount of solution of poloxamer P188 was pipetted into 10mL volumetric flasks and made upto 10mL using the solution. The contents were shaken well and absorbance was taken at 520 nm using a Shimadzu RF-540 spectrofluorometer (Shimadzu Corporation, Japan) against suitable blank. Obtained results are tabulated in Table 3.14 and 3.15.

Table 3.14: Accuracy of poloxamer in presence of PLGA NP at 510 nm, Data presented as Mean ± SD, n=3.

Conc. Of P188 (µg)	Absorbance	Conc. Of P188 Obtained (µg)	Accuracy	Precision (RSD %)
20	0.091 ± 0.008	22.0	110.0	8.79
60	0.226 ± 0.017	67.0	111.7	7.52
100	0.357 ± 0.023	110.6	110.6	6.44

Table 3.15: Accuracy of poloxamer in presence of PBCA NP at 510 nm, Data presented as Mean ± SD, n=3.

Conc. Of P188 (µg)	Absorbance	Conc. Of P188 Obtained (µg)	Accuracy	Precision (RSD %)
20	0.088 ± 0.007	21.0	105.0	7.95
60	0.212 ± 0.021	62.33	103.9	9.91
100	0.341 ± 0.019	105.33	105.3	5.57

3.5 Estimation of PEG

3.5.1 Method for PEG estimation

PEG total content in formulation (% of the particles weight) was determined by a calorimetric method, taking advantage of the formation of a complex between PEG

and iodine (Peracchia M.T., 1997). Practically, lyophilized nanoparticles (10 mg) were allowed to completely degrade in NaOH 2N, at 50°C for 4 days. After neutralization with HCl 1 N, 125 µL, I_2/KI solution was added to 5 ml of a diluted solution (1:100) of the degraded nanoparticles. Before lyophilization, nanoparticles were washed four times, in order to remove all the PEG free or weakly bound to the particles. The PEG content was calculated after spectrophotometric measurements at 500 nm.

3.5.2 Preparation of standards and calibration plot

Stock solution of PEG in WFI was prepared by accurately weighing 10mg of PEG in 10 mL of WFI. After ensuring that the PEG has totally dissolved, suitable aliquots of the 100 µg/mL stock solution of PEG was pipetted into 10mL volumetric flasks. The volume was made upto 10 mL using WFI. Now add required quantity Of 2N NaOH and kept at 50°C for 4 days. After neutralization with HCl 1 N, 125 µL, I_2/KI solution was added to 5 ml of the degraded PEG. The contents were shaken well and the absorbance intensity was measured at 510 nm using a Shimadzu RF-540 spectrofluorometer (Shimadzu Corporation, Japan) against suitable blank. The above procedure was repeated 3 times and the mean absorbance intensity values were determined (Table 3.16 and figure 3.14).

Table 3.16: Calibration of PEG in of I_2/KI solution at 500 nm, Data presented as Mean ± SD, n=3.

Conc of PEG (µg)	Absorbance
20	0.081 ± 0.0027
30	0.123 ± 0.0086
40	0.161 ± 0.0091
50	0.192 ± 0.0137
100	0.398 ± 0.0142
200	0.810 ± 0.025

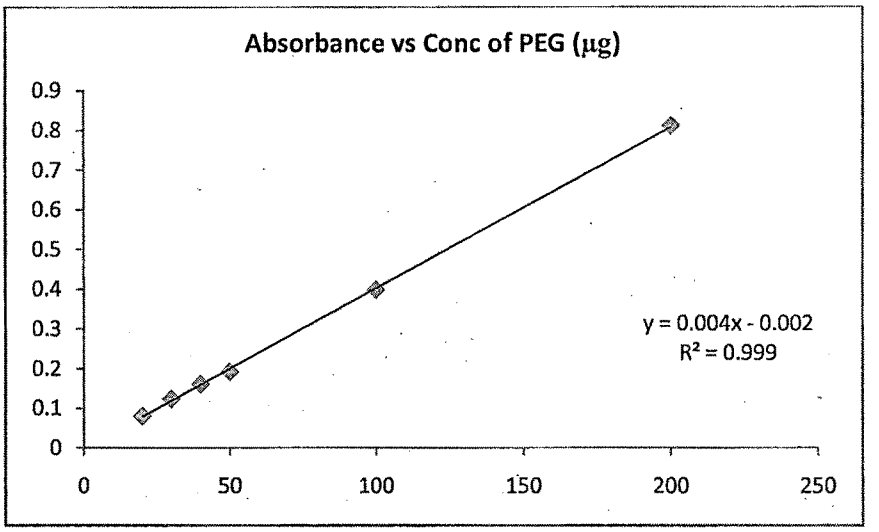

Figure 3.14: Calibration curve and regression analysis of PEG at 500 nm, Data presented as Mean ± SD, n=3.

3.5.3 Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of PEG (20, 50 and 200 µg/mL) are subjected to recovery studies as per the procedure described earlier in presence of formulation. The measured quantity of PEG was dissolved in WFI, suitable quantity of blank formulation (PLGA NP and PBCA NP) was added and make up volume using aliquots to 1000 µg/mL stock. Required amount of solution were allowed to completely degrade in NaOH 2N, at 50°C for 4 days. After neutralization with HCl 1 N, 125 µL, I_2/KI solution was added to 5 ml of a diluted solution into 10mL volumetric flasks and made upto 10mL using the solution. The contents were shaken well and absorbance was taken at 500 nm using a Shimadzu RF-540 spectrofluorometer (Shimadzu Corporation, Japan) against suitable blank. Obtained results are tabulated in Table 3.17 and 3.18.

Table 3.17: Accuracy of PEG determination in presence of PLGA NP at 500 nm, Data presented as Mean ± SD, n=3.

Conc. Of PEG (µg)	Absorbance	Conc. Of PEG Obtained (µg)	Accuracy	Precision (RSD %)
20	0.084 ± 0.003	20.50	102.5	3.57

50	0.204 ± 0.012	50.50	101.0	5.88
200	0.782 ± 0.035	195.00	97.5	4.48

Table 3.18: Accuracy of PEG determination in presence of PBCA NP at 500 nm, Data presented as Mean ± SD, n=3.

Conc. Of PEG (µg)	Absorbance	Conc. Of PEG Obtained (µg)	Accuracy	Precision (RSD %)
20	0.079 ± 0.005	19.25	96.3	6.33
50	0.198 ± 0.017	49.00	98.0	8.59
200	0.816 ± 0.041	203.5	101.8	5.02

3.6 Estimation of Zoledronic acid

3.6.1 High Performance Liquid Chromatography (HPLC) conditions

The drug content was determined using a HPLC system with UV-visible spectrophotometric detector (Shimadzu, Japan). The separation was performed on a C 18 150-4.6 HPLC column (Phenomenex Gemini C-18, 250 cm X 4.6mm, 5µm). Mobile phase consisted of 2.5 L water, 4.7 mL formic acid and pH adjusted to 3.5 using sodium hydroxide (USP33 NF Monograph). The run time was 15 min and the retention time of zoledronic acid was 1.00 min. UV detection wavelength was 210 nm and mobile phase flow rate 1 ml/min. Data processing was done using Spinchrom CFR (Spinchotech, Japan).

3.1.2 Preparation of standard stock solutions

Stock solution of ZOL is to be prepared in mobile phase by accurately weighing 10 mg of ZOL in 10 mL (1000 µg/mL) mobile phase. Further dilution was also performed using mobile phase as required.

3.1.3 Preparation of calibration curve

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml of volumetric flask to prepare a working stock solution of ZOL (100 µg/mL). Suitable aliquots of working stock solution were accurately measured and

transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with mobile phase (2.5 L water, 4.7 mL formic acid, pH 3.5) to give final concentrations of 10, 20, 30, 40, 50, and 60 µg/mL. Standards were analysis by RP HPLC at UV detection wavelength 210 nm and mobile phase flow rate 1 ml/min. After 15 min elution and results were processed using data processing software Spinchrom CFR. The above procedure was repeated six times and results recorded in the following tables 3.19 and figure 16. Calibration plot was constructed for the measured area against drug concentration. Accuracy and precision of the method was determined by performing recovery studies after addition of known concentration of ZOL.

Table 3.19: Calibration Curve of ZOL in mobile phase (2.5 L water, 4.7 mL formic acid, pH 3.5) at λ_{max} = 210 nm, Data presented as Mean ± SD, n = 6.

Concentration (µg/ml)	**Retention Time (min)**	**Area (mV.s)**
10	1.045	22.178 ± 0.93
20	1.028	46.138 ± 1.06
30	1.027	74.821 ± 1.47
40	1.034	96.385 ± 1.83
50	1.019	115.716 ± 2.04
60	1.038	142.924 ± 2.37

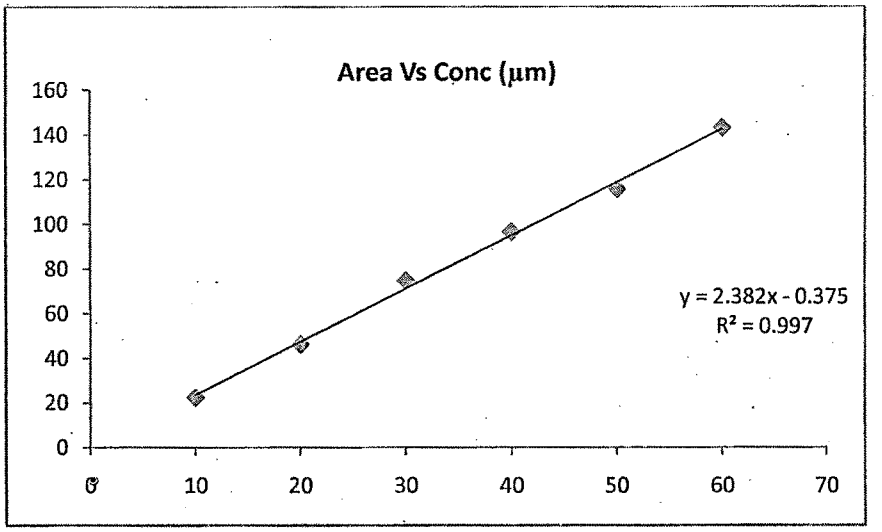

Figure 3.15: Regressed calibration curve of ZOL in mobile phase (2.5 L water, 4.7 mL formic acid, pH 3.5) at λ_{max} = 210 nm, Data presented as Mean ± SD, n = 6.

3.6.3 Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of ZOL (10, 30 and 50 µg/mL) are subjected to recovery studies as per the procedure described earlier in presence of formulation. The measured quantity of ZOL was dissolved in WFI, suitable quantity of blank formulation (PLGA NP and PBCA NP) was added and make up volume using aliquots to 1000 µg/mL stock. Required amount of solution were added to 10 mL volumetric flasks and made upto 10 mL using the mobile phase. The contents were shaken well and analysis by RP HPLC at UV detection wavelength 210 nm and mobile phase flow rate 1 ml/min. Obtained results are tabulated in Table 3.20 and 3.21.

Table 3.20: Accuracy of Zoledronic acid determination in presence of PLGA NP at 510 nm, Data presented as Mean ± SD, n=3.

Conc. Of ZOL (µg)	Area (mV.s)	Conc. of ZOL Obtained (µg)	Accuracy	Precision (RSD %)
10	21.725 ± 0.83	9.278	92.78	3.82
30	75.316 ± 1.17	31.776	105.92	1.55

50	113.918 ± 1.42	47.98	95.96	1.25

Table 3.21: Accuracy of Zoledronic acid determination in presence of PBCA NP at 510 nm, Data presented as Mean ± SD, n=3.

Conc. Of ZOL (µg)	Area (mV.s)	Conc. of ZOL Obtained (µg)	Accuracy	Precision (RSD %)
10	22.216 ± 0.71	9.484	94.84	3.20
30	73.192 ± 1.30	30.884	102.95	1.78
50	116.258 ± 1.57	48.96	97.93	1.35

3.7 Reference

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