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Analytical methodology for the assessment of Novel Cytostatic Drugs in Hospital effluent, WWTPs influent and river water

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A B S T R A C T

Due to the increase in a cancerous patient with time consumption of cytostatic medication increased globally which results in the occurrence of these cytostatic compounds in watercourses globally. This study successfully developed and validates a highly selective and efficient analytical method for the determination of highly prescribed cytostatics Gemcitabine (GEM) and Capecitabine (CAP) compound from Hospital effluent, WWTP influent and in the river at the point of discharge from WWTP. The cytostatic separation had been performed through the SPE method with Oasis HLB 6 cc, 200 mg. The quantification was performed through HPLC coupled with a dual-wavelength UV-Vis spectrophotometer. The correlation coefficient value found above 0.99 and the recovery was more than 80% for all investigated Cytostatic drug. The detection limit (LOD) is 136.8 ppb for GEM, 23.9 ppb for CAP and The quantification limit is 414.6 ppb for GEM and 72.5 ppb for CAP. All the validation parameter matches with the analytical system validation parameter and guidelines. The validated method study applied in the determination of GEM and CAP concentration in Hospital effluent, Pirana WWTPs influences, and WWTPs effluent discharge into the Sabarmati river of Ahmedabad city. This results out that both GEM and CAP analytes not detected in WWTP influent and river water, CAP detected below LOQ in hospital effluent frequently but GEM is not.

K E Y W O R D S

Cytostatic | Gemcitabine | Capecitabine | Analytical method | Ahmedabad

C I T A T I O N

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Introduction

The maturing of the human populace causes a rise in the worldwide utilization of pharmaceuticals. (Arnold *et al*., 2014) There is a developing enthusiasm for the impacts they could have on the environment. (Heijnsbergen and Schmitt, 2008) More than 600 pharmaceuticals observed above the detection limit in soil, seas, lake, river, and groundwater around the world. (Aus der Beek *et al*., 2016) The information on the event of pharmaceuticals in water is significant due to the conceivable negative impacts on the ecosystem in both the short and long term. Shockingly, just a few class of pharmaceuticals were studied, for example, antibiotics and hormones. (Heijnsbergen and Schmitt, 2008) Even though, The International Cancer Research Agency has estimated that the global cancer burden has risen to 18.1 million new cases per year.(WHO, 2018), Because of this, the consumption of Cytostatics drugs is increasing along with the time. (Besse *et al*., 2012) although less attention has been paid towards Cytostatic drug. (Zounkova *et al*., 2010).

Cytostatic drug is a class of pharmaceutical which is composed of synthetic and natural chemical compound and it is used for cancer treatment. (Heijnsbergen and Schmitt, 2008) (Zounkova *et al*., 2010)It acts on cancerous cell by inhibiting or complete blocking of the DNA replication of tumour cell which result in death of cell. (Balcerzak and Rezka, 2014) (Heijnsbergen and Schmitt, 2008)(Zounkova *et al*., 2010) As it affects the DNA replication process, So it is considered to be potentially carcinogenic, phenotoxic, teratogenic and mutagenic.(Rowney *et al*., 2009)

Cytostatic compounds and its metabolites are entering in to the water courses through Hospital Effluents (especially from Cancer Hospital) and as excretion of outpatient of cancer treatment. Significantly, Hospital effluent is considered as a primary source of cytostatic drugs in the water courses.(Balcerzak and Rezka, 2014) (Mudgal *et al*., 2013) (Zhang *et al*., 2013) The occurrence of these medications influence by numerous components, such as- the quantity of patients, the kinds of the medications utilized, Amount of dose, Discharge rates, transport and pretreatment.(Zhang *et al*., 2013). So far, the availability of these medication in influents and effluents of WWTPs are generally in the ppb range,(Booker *et al*., 2014)(Santana-Viera *et al*., 2016) But the implications of their continued addition in water courses are unknown, Particularly regarding their toxic impact on humans and environment.

At present, there are about fifty anticancer medications being used regularly in the treatment of cancerous patient in India. In general, most of the medicinal compounds are polar in nature, hence posses water-soluble characteristic and readily soluble in water. (Verlicchi *et al*., 2012) (Luo *et al*., 2014)(Schaider *et al*., 2014). Principle source

of addition in wastewater is hospital effluent and in passive way cancerous patient (outpatient). Therefore, Sewage Treatment plant (STP) discharges are measured as the source of cytostatic to the aquatic environment .(Gomez-Canela *et al*., 2014)

Most of the drug not fully metabolized in the body and also possess poor biodegradability. So it resists biological and physical removal through STP removal process. (Kosjek *et al*., 2013)A portion of these chemical could be viewed as semi-diligent with continuous discharge into environment.(Johnson *et al*., 2013)(Besse *et al*., 2012) Most of cytostatic compound possess similar pharmacology hence it could be believable that they may produce cocktail effect to the environment, probably enhancing their overall cytotoxicity and increasing the threat to aquatic ecosystem.(Isidori *et al*., 2016).

Here we have studied two anticancer drug Gemcitabine (GEM) and Capecitabine CAP), that is on the basis of pharmacy prescription and daily consumption. Gemcitabine is clinically prescribe top most drug which is used to treat certain types of cancer (including breast, lung, ovarian, pancreatic). It is a chemotherapy drug that works by slowing or stopping the growth of cancer cells. It is given by slow injection into a vein. (Adamska *et al*., 2017) Another drug is Capecitabine that is top most outpatient prescribe drug It is an orally-administered chemotherapeutic agent used in the treatment of metastatic breast and colorectal cancers. Capecitabine is a pro-drug, which is changed to fluorouracil (anti-metabolite) in the cancerous cell, where it blocks DNA replication and intervene the growth of tissue.(Walko and Lindley, 2005)

Before this lots of research have been performed for determination of Pharmaceuticals in waste water by using LC-MS, LC-MS/MS (tandem arrangement) (Besse *et al*., 2012; Knobloch *et al*., 2010; Kosjek and Heath, 2011; Martin *et al*., 2011), which is too costly and availability of these instrument is less, Therefore here we have developed and validated an analytical methodology for the separation of GEM and CAP from waste water through solid phase extraction (SPE) and its quantification using HPLC with dual wavelength UV-visible spectrophotometer.

Experiment

Chemical and Material

GEM and CAP API of purity 98-99% was gifted by Alkem laboratories Ltd., Navi-Mumbai (India) for research purpose only, HPLC-grade of Acetonitrile, methanol, Formic Acid and Sodium Hydroxide of AR-Grade purchased from Rankem, New Delhi (India), Milli-Q-Water is from Laboratory Millipore Purifier. SPE cartridge HLB Oasis is purchased from waters India (Mumbai).

Standard stock solution was prepared by dissolving 5mg of each Active ingredient into 100 ml volumetric flask, then further diluted 2 ml of stock to 50ml with water (2000 ppb).

Solubility and solution stability

The solubility and stability of both compounds studied in milli-Q water. For that, a mix solution containing both GEM & CAP was prepared in purified water (2000 ppb) and the analytical response of both compounds taken immediately after the preparation, at 24 Hour and 48 Hour periods. The effect of pH was also confirmed in terms of solubility and solution stability in purified water at different pHs (pH 2, 4, 7 and 10). The solution were analyzed and the results were compared.

Solid-phase extraction

Extraction methodology has been adopted from Santos et.al work. (Santos Mónica S.F. A4 - Franquet-Griell, Helena A4 - Alves, Arminda A4 - Lacorte, Silvia, 2018) Efficacy of the extraction method has been verified by the recovery study of extraction in Hospital Effluent and River water.

Analytical method development

The analysis was carried out by using HPLC (Waters 2695) connected to the dual wavelength UV-detector (Waters 2489). Data were acquired and processed through Empower 2.0 software. In the first step, the wavelength optimization is performed on UV-Visible spectrophotometer (Perkin elmer lambda 35). In this, sample of 2000 ppb concentration scanned in UV spectrophotometer. (Detection Sensitivity and Selectivity, 2012) Then chromatographic parameter was optimized by using LC column Hypersil BDS-C18, (100x4.6)mm, 3.5µ (Make-Thermo), Column compartment temperature kept 25°C, Sample tray temperature kept 10°C, Flow rate of Mobile phase flow kept1.0 ml min^{-1}, For optimizing mobile phase composition, we kept Mobile phase A is 0.1% HC00H and B is ACN. Initial Mobile phase A & B kept 50:50 ratio but after taking so many trial, the final composition is 80% 0.1% HCOOH and 20% Acetonitrile. The runtime of chromatogram and injection volume is set to 15 minutes and 100µl respectively.(“Basics of Separation,” 2012, “The Column,” 2012)

Sampling Procedure and sample preparation

The applicability of Validated method have been assessed in Hospital waste, WWTP effluent and River sample. Water sampling was performed from three locations of Ahmedabad city First Gujarat cancer research institute (GCRI), Second Pirana sewage treatment plant Effluent and third location Sabarmati River (See Fig.1). Hospital wastewater samples were collected in the morning at 9 am. Sampling were repeated on five consecutive days to evaluate the intra-day variability. During the same days, the 24 h composite effluent of WWTP receiving hospital waters and river water were sampled. Samples were kept below 10ºC and processed within 24–48 h. The samples were centrifuged at 5,000 rpm for 10 min then filtered through 1µ nylon membrane filters (Millipore, Merck. India), and further filtered with 0.45 Nylon syringe filter. (Samples were maintain at pH 2.0 with 0.1 N HCl and then extracted using an automated SPE apparatus (Waters 20 position Manifold). (Santos Mónica S.F.

A4 - Franquet-Griell, Helena A4 - Alves, Arminda A4 - Lacorte, Silvia, 2018)

Method Validation

Verification of the intended method had been performed on the basis of the regulatory guideline. Calibration range kept between 50 ppb and 2000 ppb with 7 calibration points.

System precision was performed by injecting 1000 ppb concentration spiked std in six replicate. To check the specificity of method blank, individual compound std spiked in milli-Q water sample was injected. Limit of detection (LOD) and limit of quantification (LOQ) is determined using the slope method of calibration curve,

$$\text{LOD} = \frac{3.3\,\sigma}{\text{S}} \text{ and } \text{LOQ} = \frac{10\sigma}{\text{S}}$$

……Eq.1&2

Where,

σ = Standard deviation error for y to x axis linear regression of least square,

S = Slope of calibration curve

Accuracy of the method studied by spiking both compounds in hospital effluent at LOQ and 1000ppb concentration level (In triplicate). (ICH, 2005)

Result and discussion

Optimization of wavelength

On scanning both the sample of concentration 2000 ppb from a range of 400 nm to 200 nm we found maxima for GEM at 209.8nm and 269.2 nm and minima at 242.2nm and 318.6, similarly For CAP, maxima at 214.6nm, 239.9 nm, 305.4 nm and minima at 226.2 nm, 267.8 nm (See fig.2).On comparing both spectra optimize the λ-max 270 nm and 240 nm for GEM and CAP respectively. Every molecule gives its high response at their own maxima.

Chromatographic separation

In order to achieve optimum resolution and compound detection, we have selected reverse phase chromatography for that stationary phase is relatively non-polar and mobile phase is polar. So in this study C18 Column Brand- Hypersil BDS, 100mmx4.6mm, 3.5µ used for the study, Small particle size of 3.5µ gives sharp peak hence peak broadening not observed. The short column has reduced the time of analysis. C18 column in relatively non polar hence it retains non-polar for more time (as per theory like dissolve like). Here GEM is polar than CAP hence GEM elute before CAP.

The mobile Phase used 0.1% formic acid with Acetonitrile in the ratio 80% and 20% respectively provide repeatability of the method without shifting in retention time. The dual λ detector had been used at 240 nm and 270 nm for CAP and GEM respectively. The retention time (RT) of GEM was obtained at 1.5±0.2 minutes and for CAP 9.6±0.3 minutes (See Fig. 3) No interference of any other peak is observed at the RT of GEM and CAP.

Stability in analytical solution

The solution stability of GEM and CAP in Milli-Q water revealed that no losses or degradation exist within a period of 48 Hours, Error range for both compound found below 5%, A similar result obtained for the stability

of both compound in Milli-Q water from pH range between 2-10. (See table-1).

Extraction capability

The tests were started with reversed-phase activated polymeric sorbents (Oasis HLB). Oasis HLB is consists of hydrophilic N-vinyl pyrrolidone group with a water-wettable reversed-phase sorbent, a lipophilic divinylbenzene group and a neutral polar "hook" for better retention of polar analytes. The applicability of Oasis HLB was tested on the analysis of wastewater spiked at 1000 ppb at three different pHs. Recovery of GEM and CAP performed in triplicate at pH 2, 4 and 7 (See Table-2).

Analyte	Milli-Q water	pH adjusted Milli-Q Water			
		pH 2.0	pH 4.0	pH 7.0	pH 10.0
GEM	±3.0	±3.1	±3.5	±4.1	±4.1
CAP	±2.6	±2.6	±2.1	±2.6	±1.8

Table-1: Error found in Stability of GEM and CAP in Milli-Q water at different pH for 48 H

Analyte	%Rec. from Spiked Hos. Effluent			%Rec. From Spiked River Water		
	pH 2.0	pH 4.0	pH 7.0	pH 2.0	pH 4.0	pH 7.0
GEM	87±4.1	70±5.5	71±6.2	85±3.1	73±7.1	67±6.2
CAP	96±2.6	95±4.0	95±3.7	95±2.6	94±4.0	94±3.7
		Table-2. %Recovery of GEM and CAP from spiked Hospital effluent with ± SD				

Analyte	Linearity	LOD	LOQ	Recovery ±RSD% (n =3)	
	Range (ppb)	(ppb)	(ppb)	LOQ level	1000 ppb
GEM	50-2000	123.23	373.43	85±9.5%	87±6.2%
CAP	50-2000	21.83	66.17	92±8.4%	94±4.2%

Table 3: Linearity ranges, LOQ, LOD and % Accuracy of the selected compounds

Analyte	Intra-day RSD % (n = 5)		Intra-day RSD % (n = 15)	
	500 ppb	1000 ppb	500 ppb	1000 ppb
GEM	8.4	7.0	8.2	6.9
CAP	5.4	5.9	5.4	5.2

Table 4. Repeatability of the optimized analytical method

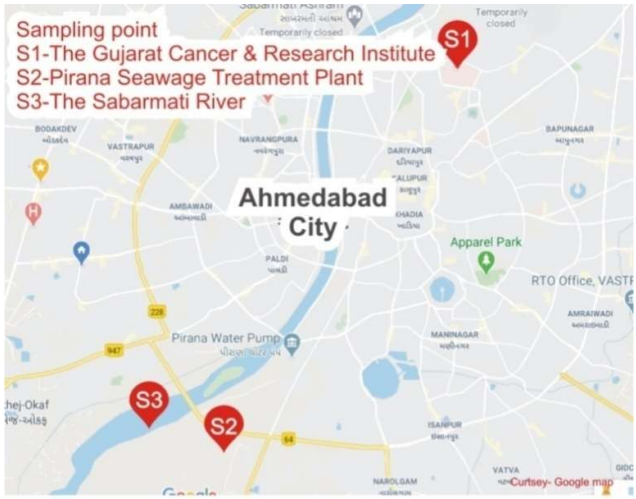

Fig.1: Sampling point in Ahmedabad for monitoring of CAP and GEM in Hospital Effluent, WWTPs Influent and point of discharge in the Sabarmati river.

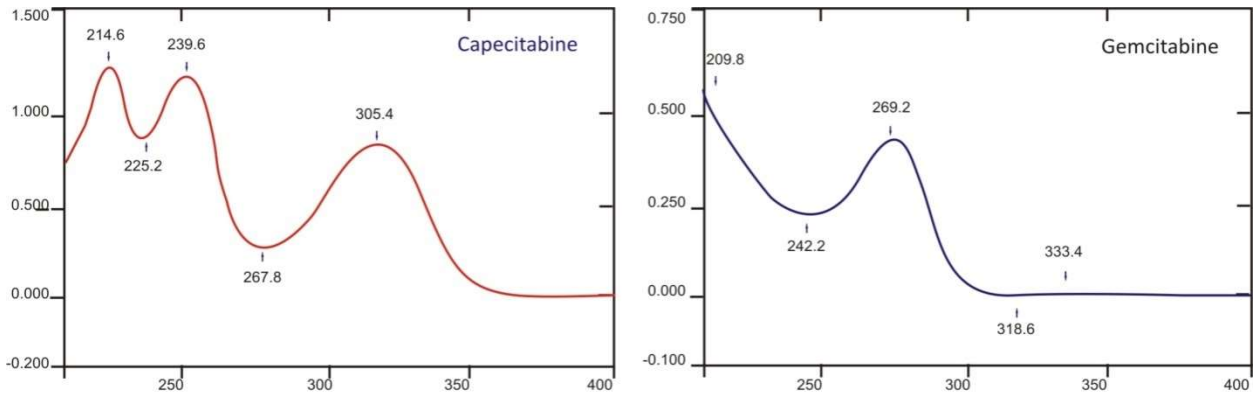

Fig.2: Spectrum of both compounds

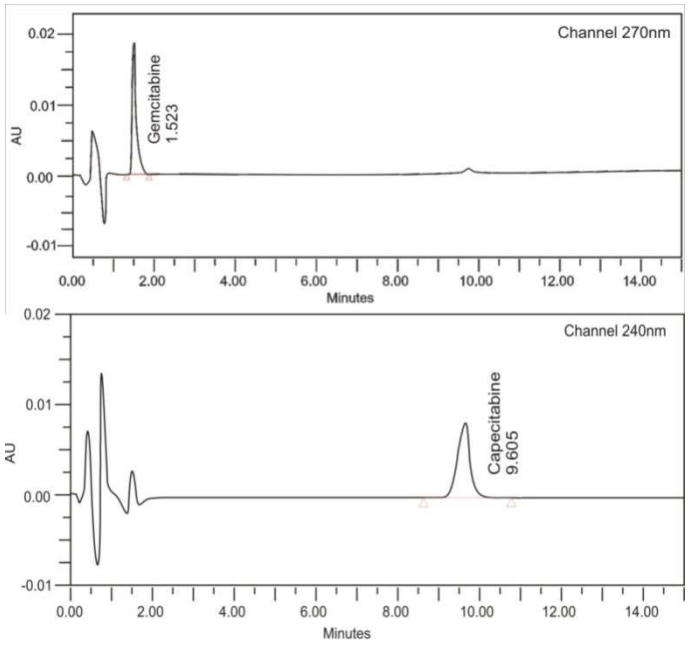

Fig.3: Chromatogram of GEM and CAP

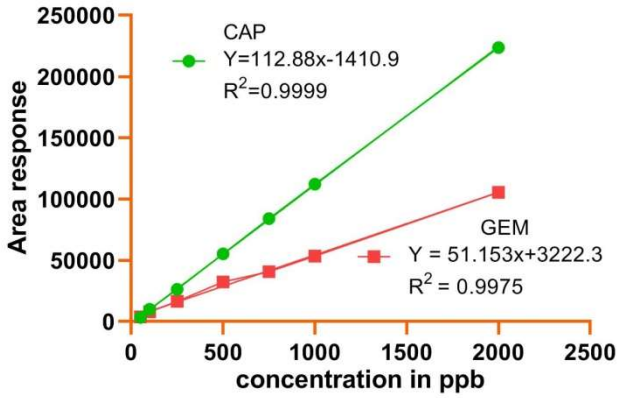

Fig.4 Linearity plot for GEM and CAP

Recovery of GEM found more suitable at lower pH 2.0 because of its polar nature and pKa of GEM is polar in nature and having pKa 3.6, So it gets fully ionized at pH 2.0 produce better retention in the cartridge which result in good recovery of compounds from waste water. While is having pKa value of 8.77 and basic in nature and at lower pH it

found unionized form so it showing full recovery at all pH media.

The recoveries obtained for GEM and CAP are comparable with previous work and found below 5%variance hence found suitable for the intended use. (Santos Mónica S.F. A4 - Franquet-Griell, Helena A4 - Alves, Arminda A4 - Lacorte, Silvia, 2018)

Method Validation

The standard curve for triplicate injection of spiked samples found linear up to 2000ppb for both compounds in waste water. The coefficient for correlation was 0.9975 for GEM and 0.9999 for CAP (See fig.4).

Considering the slope method of calibration for LOD and LOD determination, Limit of detection was calculated 123.23 ppb for GEM and 21.83 ppb for CAP. Limit of Quantification was calculated as 373.43 ppb for GEM and 66.17 ppb for CAP.

Method precision or repeatability of the optimized method was estimated by evaluation of the calibration standards within a sequence (Inter-day, n=5) and on consecutive five days (Intra-day) % RSD observed below 10% for GEM and below 6% for CAP.

Determination of Target Pharmaceuticals in Wastewater

The developed method was applied to the monitoring of these two cytostatic compounds in GCRI Hospital effluent, influent of Pirana WWTPs and Sabarmati river water. Sampling was performed in the first week of August, December and April Month. To assess the variability with season. August month is the Rainy, December is the winter and April is the summer season in India.

The sample shows the presence of CAP in effluent of the hospital in concentration range of 123 ppb to 228 ppb, In WWTPs influent 25ppb-58 ppb and in The river water nd to 30 ppb. While GEM show the presence in Hospital Effluent nd to 168 ppb and not detected in influent of WWTPs and river water.

The presence of CAP and GEM is comparable with previous study while CAP observed slightly higher than previously reported value that may be due to increase in consumption of CAP as the patient increase every year worldwide, and GEM is similar to previous study.(Gomez-Canela *et al*., 2014)(Franquet-Griell *et al*., 2017)(Besse *et al*., 2012) (Negreira *et al*., 2013)

As per prescription study conclude that CAP is highly prescribed medicine it consummated orally and as a chemotherapy medication while GEM is consummated only as a chemotherapy medication that why CAP was observed in all sample and GEM was observed only in the Hospital effluent. Seasonal variability observed in WWTPs and River sample because of dilution in the rainy season.

Indeed, if the predictable concentrations in hospital effluents, WWTPs, and river water is low but further research on the occurrence and fate of Cytostatic may be of the relevance of

cancer-causing a potential for human and the environment.

Conclusion

Cytostatics have emerged as a major pollutan t of the aquatic environment because of their increased consumption in cancer therapy, their low removal /degradation at WWTPs and their potential of high cytotoxicity. This work provides an empirical framework for the assessment of Novel Cytostatic drug (GEM and CAP) in Hospital waste water, WWTPs Effluents and River water through HPLC and UV spectrometer. Before that, so many method had been developed and used but that was through LC-MS, which is not easily available at all laboratories so this method is easy and convenient to all scientist, this method was validated as per ICH guideline which shows its reliability to a extend and produce result within their criteria,

On the basis of this research we conclude out that A large and A comprehensive surveillance plan for determining the level of cytostatic compound in the environment should be drawn up.

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