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DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF BENZIL IMPURITY IN PHENYTOIN FORMULATIONS BY REVERSE PHASE HPLC

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ABSTRACT

The HPLC method was developed for the estimation of Benzil impurity present in the Phenytoin. The method was carried out using a stationary phase of Hibar® column RP_{18} (250 X 4.6 mm I.D, 5µm), 25 mM potassium dihydrogen orthophosphate buffer (pH 3.0) and acetonitrile (20: 80 %v/v) used as mobile phase at a flow rate of 1.0 mL min^{-1} with a detection wavelength of 217 nm. The Rt were 5.45 min and 3.22 min for Benzil and Phenytoin respectively. The relative retention time was 1.65 min. The recovery of Benzil obtained was from 97.75 % w/w to 100.41 % w/w. The benzil was produced linear response from 0.1 to 1.5 µg mL^{-1}. The R^2 was 0.995. LOQ and LOD were 7.66 ng mL^{-1} and 23.23 ng mL^{-1} respectively. The proposed method valuable for the quantification of Benzil in Phenytoin for the safety of the drug in bulk and formulation.

KEYWORDS

Reverse Phase HPLC, Phenytoin, Benzil, impurity and Validation.

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INTRODUCTION

Phenytoin (PHY)[1], chemically 5, 5-diphenylimidazolidine-2,4-dione (Figure No.1) is used for treating epilepsy seizures. Impurity (IM)[2] defined as any component of the drug product that is not the drug substance or an excipient in the drug product. The IM resulting from a chemical change in the drug substance brought about during manufacture and / or storage of the drug product by the effect of light, temperature, pH, humidity or by reaction with an excipient and/or the immediate container closure system. Benzil (BZL), chemically 1, 2-diphenyl ethane-1, 2-dione (Figure No.2) IM present in

Phenytoin and it is reported as Impurity B. It is a reactant[3] for the formation of phenytoin.

The literature describe, the PHY and benzophenone (as impurity) were estimated by polarography[4], the PHY and its metabolites were estimated in *equine* plasma by High-Performance Liquid Chromatography (HPLC)[5], simultaneous estimation method were developed to estimate the PHY and carbamazepine in serum by PLS regression and comparison by HPLC[6], A poly(vinyl chloride) membrane electrode for determination of PHY in pharmaceutical formulations[7], the PHY and dextromethorphan simultaneously estimated by HPLC in human[8], The HPLC method was developed to estimate carbamazepine, PHY, phenobarbital, primidone and their metabolites[9], by using Stir bar-sorbtive extraction SBSE/HPLC-UV method for therapeutic drug monitoring of carbamazepine, carbamazepine-10,11-epoxide, PHY and phenol barbital in plasma samples[10], the sodium salt of PHY in plasma was estimated by high-performance capillary zone electrophoresis (CZE)[11], a micro emulsion electrokinetic chromatography separation method was estimated for levetiracetam from other antiepileptic drugs[12], the determination of lamotrigine with primidone, carbamazepine, carbamazepine epoxide, phenobarbital, and PHY human plasma was established by using solid-phase micro extraction and gas chromatography with thermionic specific detection[13], the quantitation of PHY in human breast milk, maternal plasma and cord blood plasma was established by HPLC[14]. Even though the PHY was BZL was analysed separately the benzyl was identified as a very potent genotoxic agent, as well it produces a significant DNA damage[15]. The previous literature emphasizing that the least concentration of benzyl produces significant change changes in DNA hence the BZL estimation has high impact than other impurity estimation in PHY.

EXPERIMENTAL

REAGENTS, CHEMICALS AND SOLVENTS

PHY and BZL were obtained from Harman fine chem Ltd and Loba chemie Ltd respectively, Acetonitrile (Ranchem, HPLC grade), orthophosphoric acid (Fine chemicals, HPLC grade). Potassium di hydrogen orthophosphate (SD fine chemicals). Water HPLC grade (in house Milli-Q RO system) were used.

EQUIPMENTS

Analysis was performed on a liquid chromatographic system of Waters 2487 dual wave length absorbance detector and having 1515 solvent delivery system, with a Rheodyne 7725*i* of 20 µL loop capacity equipped with the data station of Breeze 3.2. The analytical column used for the separation of the components was Hiber® column RP_{18} (250 mm x 4.6 mm i.d, 5 µ).

SELECTION OF DETECTION WAVELENGTH (Λ_{MAX})

An UV spectrum of 10µg mL^{-1} BZL and PHY were separately recorded by scanning in the range of 200-400nm. The UV spectrums were superimposed on the spectrum UV-Probe 3.2 software. The overlaid spectra have shown the isobestic point at the wave length of 217 nm as shown in Figure No.3.

EFFECT OF pH

The standard solution of the mixture of BZL and PHY were chromatographed for 15 min using acetonitrile and buffer solution of different pH ranging from 3.0 to 6.0 (25mM Phosphate buffer of pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) at a flow rate of 1 mL min^{-1} using Hibar® C_{18} column and the retention times obtained for BZL and PHY were 5.24, 5.26, 6.20, 6.22, 6.35, 7.2 and 7.51 min 3.14, 3.15, 4.34, 4.54, 4.97, 5.00 and 5.35 min respectively. It was observed that pH of the mobile phase has altered the elution pattern of the PHY and BZL. Buffer pH of 3.0 was selected based on good retention and peak shape.

EFFECT OF SOLVENT RATIO

In the method development initially the different solvent ratio of 50% of 25 mM phosphate buffer and 50% of acetonitrile were used. The retention times of BZL and PHY were found to be 10.2 min and 12.2 min respectively. The capacity factor, resolution, asymmetry factor and column efficiency were calculated. Results the peak was asymmetric. To decrease the retention time and to obtain the peak

symmetry of the drug, the acetonitrile ratio in the mobile phase was increased to 60 % and the chromatogram was recorded. The retention times of the BZL and PHY were 6.4 and 7.5 min respectively and peak symmetry improved. The phosphate buffer ratio was reduced to 20 % and the peaks were eluted at 5.45 min and 3.22 min for BZL and PHY respectively with well resolved and symmetric peaks and appreciable relative retention time.

SELECTION OF STATIONARY PHASE

The different stationary phases of Princeton SPHERE 100 C_{18} (250 x 4.6 mm i.d., 5µ), and Hibar® C_{18} (250 x 4.6 mm i.d., 5µ) were used to study the chromatogram response changes and the theoretical plates were observed.

PREPARATION OF BUFFER SOLUTION

Buffer solution was prepared by dissolving 3.46 g potassium dihydrogen phosphate in 1000 mL water and the final pH was adjusted to 3.0 ± 0.05 with dilute ortho-phosphoric acid and filtered through 0.45 µ before use.

DEVELOPMENT OF STANDARD CHROMATOGRAM

The 20 µl standard solution of mixture of PHY (50 µg mL^{-1}) and BZL (2.5 µg mL^{-1}) were injected in to the Waters 2487 HPLC. The retention time obtained were 5.45 min and 3.22 min for BZL and PHY respectively, as shown in Figure No.4.

ESTIMATION OF BZL IN TABLET DOSAGE FORM

The twenty tablets of PHY were weighed and tablet powder equivalent to 10 mg of PHY was weighed and dissolved with few mille liters of mobile phase sonicated for 30 min and the volume was made up to 10 mL with mobile phase. The solution was filtered and the resulting filtrate was injected in HPLC to obtain for chromatogram as shown in the typical chromatogram in Figure No.5.

ESTIMATION OF BZL IN BULK DRUG

The PHY sodium bulk drug (10 mg) was weighed and transferred in to the 10 mL standard flask dissolved and made up to the 10mL with mobile phase and the resulting solution was injected into the HPLC to obtain the chromatogram as shown in the typical chromatogram in Figure No. 6. Using Breeze 3.2 data station the system suitability parameters were calculated such as relative retention time, asymmetry factor and theoretical plates.

LINEARITY AND RANGE

Standard stock solutions of BZL and PHY (1mg mL^{1}) were prepared separately by dissolving 10 mg of respective materials in the mixture of acetonitrile and phosphate buffer (80:20 % v/v) and the volume was made up to 10 mL and it was stored under refrigerator condition. Further to study the linearity response of the BZL a serial dilutions from 0.1 to 1.5 µg mL^{-1}were prepared from the standard stock solution of BZL. A graph was plotted between concentration of drugs and peak area response.

RESULTS AND DISCUSSION

OPTIMIZED CHROMATOGRAPHIC CONDITIONS

The analysis was carried out on Hibar® C_{18} (250mm × 4.6mm I.D, 5µ) using a mobile phase consisting of phosphate buffer (pH 3.0) and acetonitrile (20:80, v/v) with UV detection at 217 nm at a flow rate of 1 mL min^{-1}. The column was maintained at room temperature during the analysis.

Precision was determined by performing inter and intra day studies, six repeated injections for three days of the standard BZL and PHY solutions analysed and %RSD was calculated as given in Table No.1. The accuracy of the method was determined by performed the assay for six times and the percentage relative standard deviation (%RSD) was calculated. The recovery study was performed by spiking method. The 100 µg mL^{-1}standard impurity were spiked, the developed method had a capacity to produced percentage recovery from 99.06 % w/w to 100.41 % w/w for BAZ. The accuracy and recovery was as given in Table No.2 and 3 respectively.

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were calculated by slope equation method. The developed method LOD and LOQ values were 7.66 ng $mL^{-1}$and 23.23 ng mL^{-1}respectively.

SYSTEM SUITABILITY

The system suitability parameters such as relative retention time between the PHY and BZL, asymmetry factor, theoretical plates, tailing factor were calculated. The number of theoretical plates of peak should not be less than 3000 and peak symmetry should be in between 0.7 and 1.2. The obtained results were shown in Table 4. The developed HPLC method was validated for all the validation parameters as per ICH guidelines[16]. The ruggedness and robustness of the method was determined by slightly changing the parameters of the optimized method and performed by different analysts. The percentage ratio of acetonitrile in mobile phase was altered by ± 1%. pH of mobile phase was altered by ± 0.5 , concentration of the buffer was altered by ± 5 mM and the flow rate was altered by ± 0.1 mL.

Table No.1: Inter day and Intra day precision study

S.No	Precision study	Inter-day precision*		Intra-day precision*	
		BZL	PHY	BZL	PHY
1	Average	1997821	1223946	2008414	1251861
2	Standard Deviation	7959.90	10539.43	5864.74	526.79
3	RSD	0.398	0.8611	0.292	0.0420

***n=6**

Table No.2: Assay for BZL in different formulations

S.No	Drug and formulation	BZL present in 100mg drug (µg)	BZL present in for 300 mg drug (µg)
1	Bulk Drug	04.58	13.74
2	100 mg/tablet	29.58	88.74
3	100mg/tablet	130.46	391.38

Table No.3: Recovery Data

S.No	Amount found (µg mL^{-1})	Standard amount added (µg mL^{-1})	Amount present as per assay	Percentage Recovery	Standard deviation	% RSD
1	104.99	100	4.58	100.41	0.066533	1.461198
2	127.33	100	29.58	97.75	0.198132	0.66857
3	229.52	100	130.46	99.06	0.372742	0.28575

Table No.4: System suitability and method validation parameters

S.No	Validation parameters	Observation
1	Linearity and Range*	0.1-1.5µg mL^{-1}
2	Correlation co-efficient (R^2)	0.995
3	Slope equation	y = 0.025x – 0.001
4	Limit of detection (LOD)	7.66 ng mL^{-1}
5	Limit of Quantification (LOQ)	23.23 ng mL^{-1}
6	Number of theoretical plates	9743 m^{-1}
7	Relative retention time	1.65 min
8	Asymmetric factor	1.0
9	Tailing factor	1.0

***n=6**

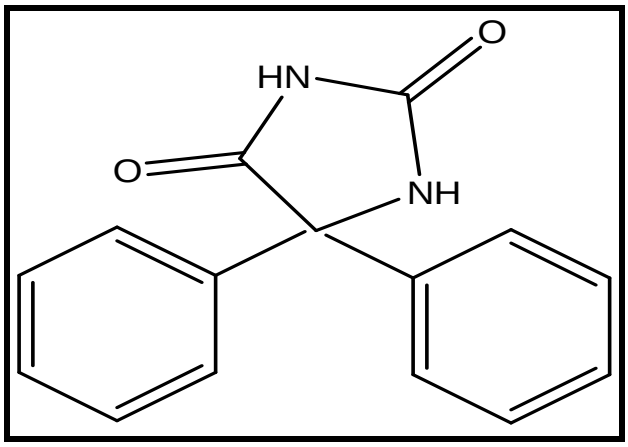

Figure No.1: Structure of Phenytoin (PHY)

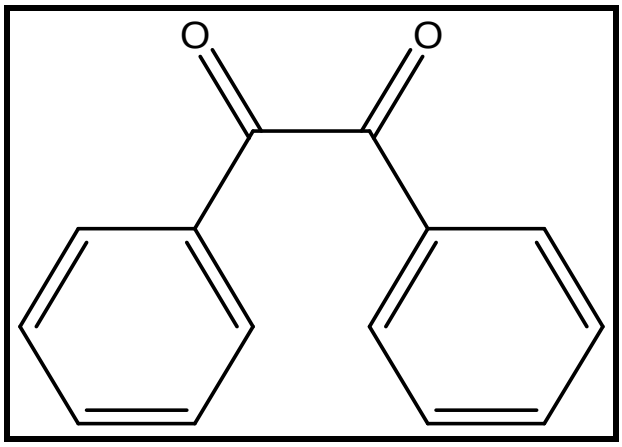

Figure No.2: Structure of Benzil (BZL)

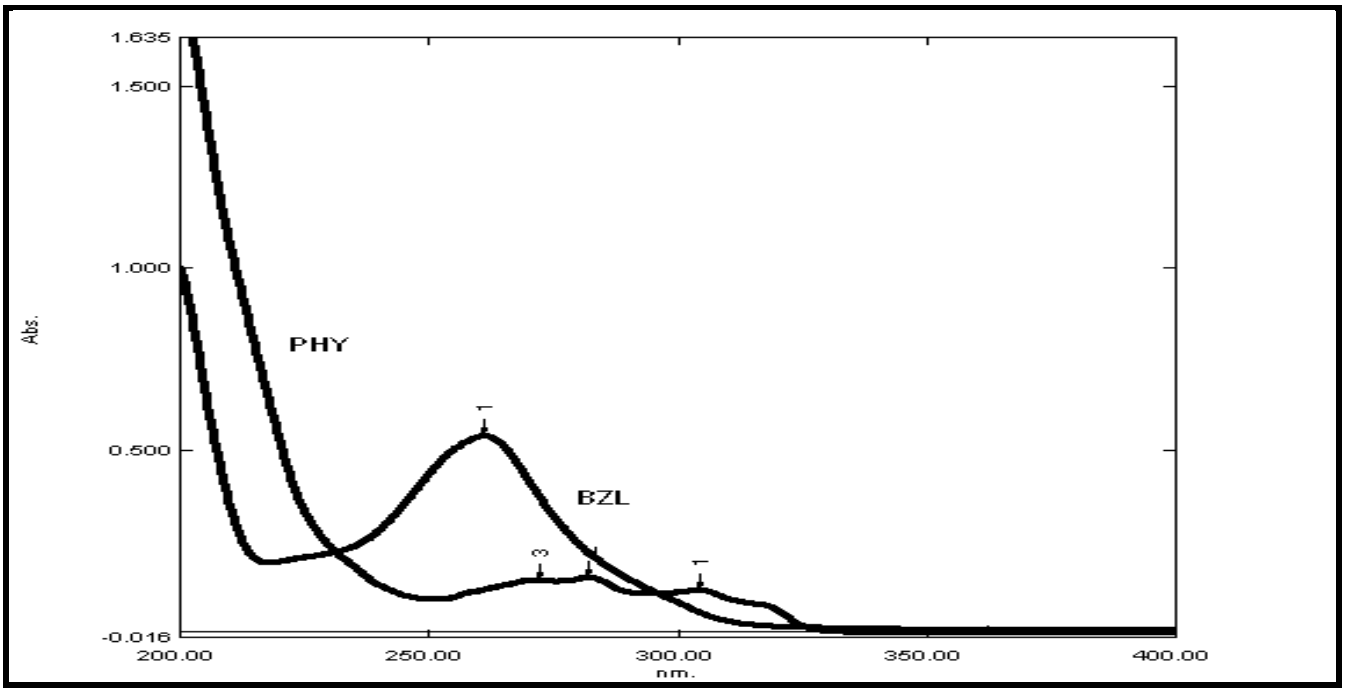

Figure No.3: UV-visible overlaid spectrum of BZL and PHY

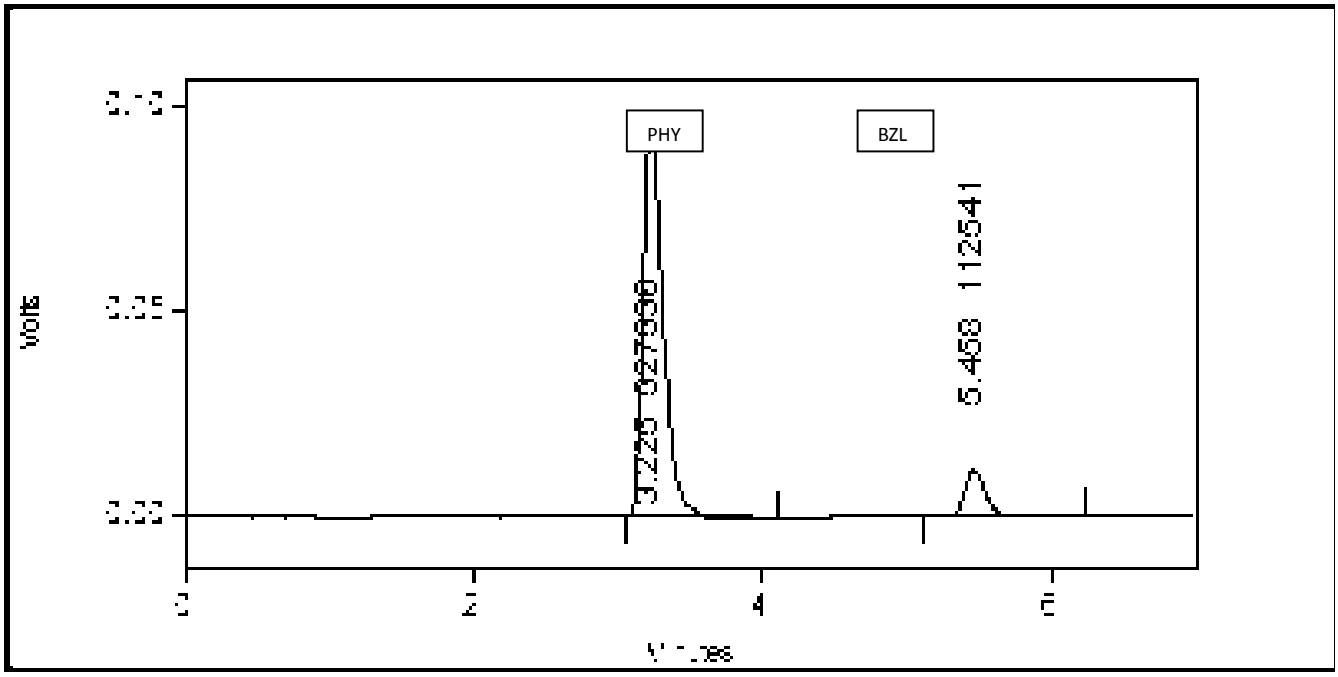

Figure No.4: Typical chromatogram of the mixture of PHY (50µg mL^{-1}) and BZL (2.5µg mL^{-1})

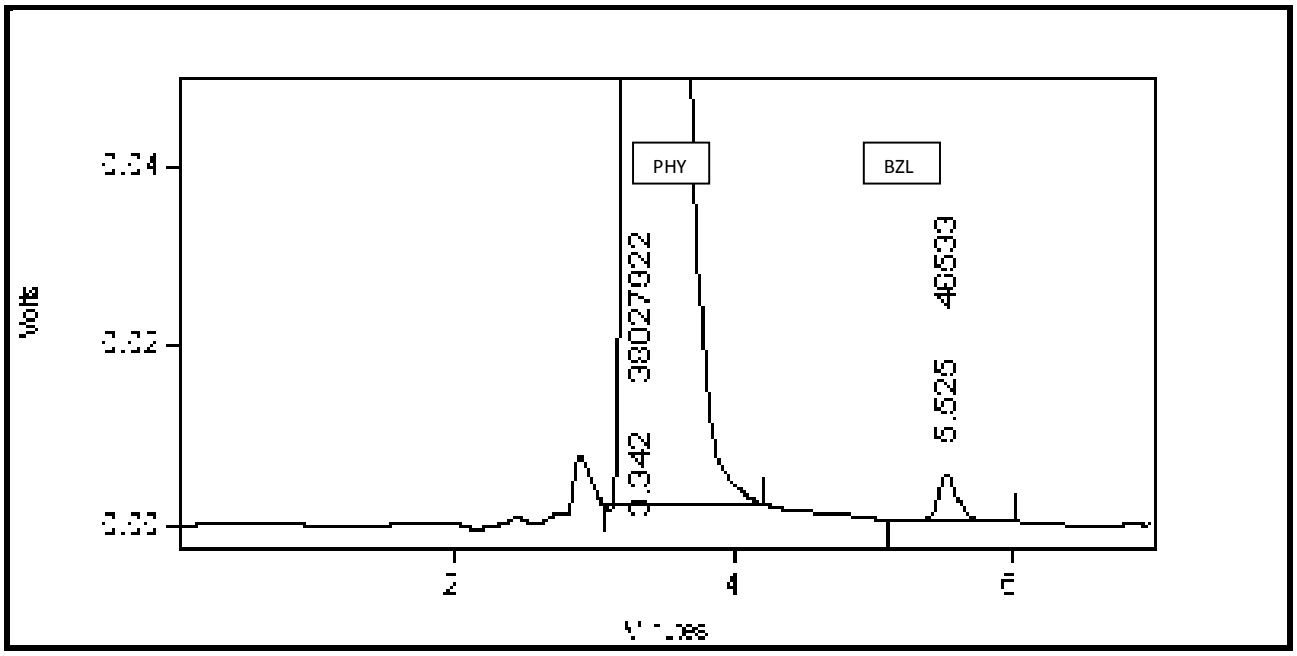

Figure No.5: Typical chromatogram of the formulation

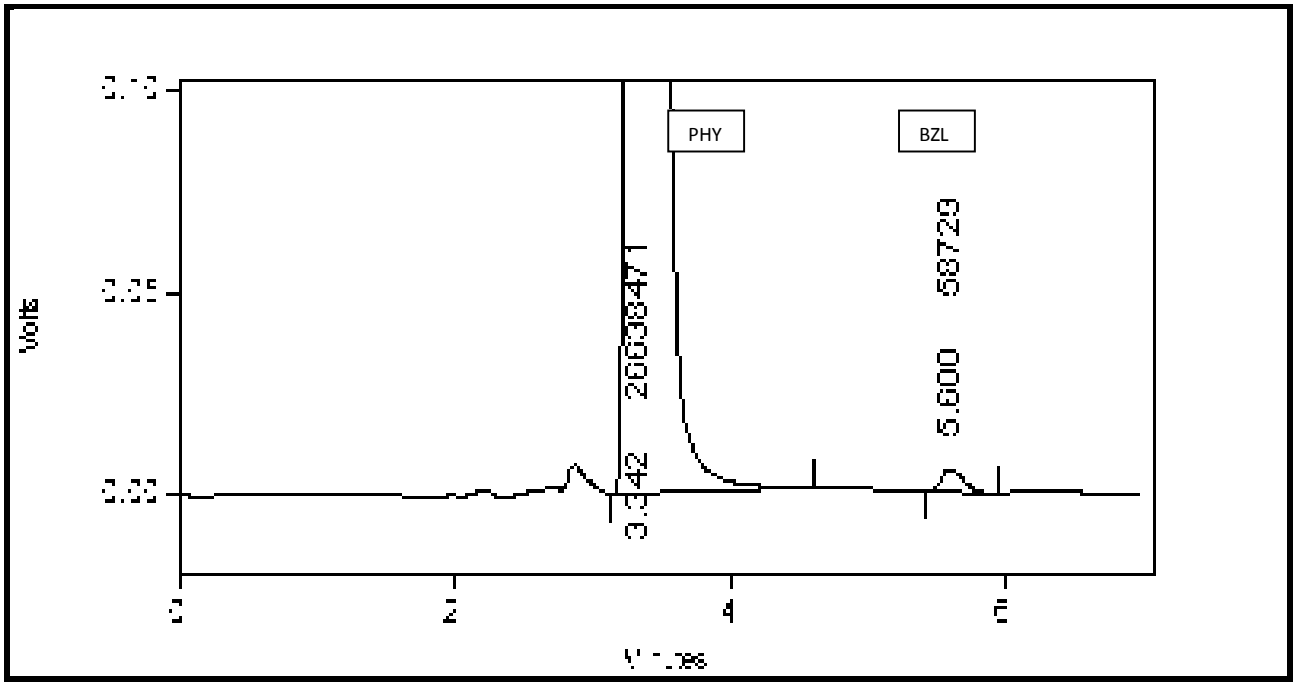

Figure No.6: Typical chromatogram of the bulk drug

CONCLUSION

The IM estimation and control is a major analytical area where still we need more specific to improve the product quality. The developed HPLC method was simple, sensitive, precise and rapid method to analyse the BZL impurity in PHY bulk drug and formulations. The BZL impurity present in the bulk drug and marketed dosage form were analyzed using the developed method. The developed method was validated as per ICH guidelines. It can be used for the analytical research and developmental labs, general quality control labs.

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