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Original research paper

An analytical "quality by design" approach in RP-HPLC method development and validation for reliable and rapid estimation of irinotecan in an injectable formulation

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The objective of the present study was to develop a robust, simple, economical and sensitive HPLC-UV method using the "quality-by-design" approach for the estimation of irinotecan (IRI) in marketed formulations. RP-HPLC method was developed by applying Box-Behnken design with Hyper-Clone (Phenomenex®) C_{18} column (250 × 4.6 mm id, particle size 5 μm, ODS 130 Å) as a stationary phase. Acetonitrile and 20 mmol L^{-1} potassium phosphate buffer (pH 2.5) containing 0.1 % triethylamine in a ratio of 45:55 % (*V*/*V*) was used as a mobile phase. The sample was injected in a volume of 20 μL into the HPLC system. UV detector at 254 nm was used to estimate and quantify IRI. Isocratic elution was opted while the flow rate was maintained at 0.75 mL min^{-1}. The retention time of IRI was found to be 4.09 min. The responses were found to be linear for concentration range of 0.5 to 18.0 μg mL^{-1} and the coefficient of determination value was found to be 0.9993. Percent relative standard deviation for intra- and inter-day precisions was found in the range of 0.1 to 0.4 %. *LOD* and *LOQ* values were found to be 4.87 and 14.75 ng mL^{-1}, resp. Robustness studies confirmed that the developed method is robust with RSD of a maximum 0.1 %. The method is simple, precise, sensitive, robust and economical making it applicable to the estimation of IRI in an injectable formulation.

Keywords: irinotecan, HPLC-UV, "quality by design", full factorial design, Box-Behnken design

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With the increasing number of deaths worldwide due to cancer, the number of chemotherapeutic drugs entering the market has been progressive. Irinotecan (IRI, CPT-11), a United States Food and Drug Administration (FDA) approved drug is presently used to treat colon cancer, colorectal cancer and lung cancer (1, 2). IRI is a semi-synthetic analogue of camptothecin. The bi-piperidinocarbonyloxy side-chain is attached to camptothecin to

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enhance the water solubility of SN-38 (7-ethyl-10-hydroxycamptothecin) giving rise to the development of IRI (3, 4). CPT-11 is an amphiphilic weak base with pK_a value 8.1 (5).

Many methods are available in the literature for the estimation of IRI in analytical and bioanalytical samples. Most of the literature suggests the use of HPLC with a fluorescent detector or using liquid chromatography coupled with mass spectroscopy (6–10). However, the use of a fluorescent detector or mass spectroscopy is better for sensitive methods for analysis, but these detectors are more expensive compared to a UV-detector (11). Apart from the economical setback of using fluorescent detectors, IRI exhibits a difference in fluorescence depending on the acidity or basicity of the medium (11). Prior literature confirmed the availability of few methods for quantification of IRI using HPLC with a UV detector (12–14). Although few methods are available for the estimation of IRI in the analytical and/or bioanalytical samples, still there is a need to work on the method sensitivity, robustness and analysis cost. To achieve these targets with a one-factor-at-a-time (OFAT) approach is difficult and also tedious and expensive. On the other hand, the "design of experiments" (DoE) is a well-known and well-established approach for cost control, saving also method's development time, still used for developing a sensitive and robust method for estimation of a drug in formulations or marketed products (15–17).

Nowadays, regulatory agencies are also suggesting the use of "quality by design" (QbD) in pharmaceutical development. When the QbD is applied for the development and optimization of the analytical method then it is also known as "analytical quality by design" (AQbD) (18). International Conference for Harmonization (ICH) has suggested few guidelines (such as Q8, Q9 and Q10) for QbD. These guidelines are not meant for method development only, but the concepts can be implemented to develop a sensitive and robust analytical method (19). Hence, this study was focused on the development of a simple, precise, sensitive, robust and economical HPLC method with a UV detector, by applying "quality by design" (QbD) approach, and its application for the estimation of IRI in pharmaceutical products.

EXPERIMENTAL

Chemicals and reagents

IRI (purity 99.7 %) was obtained from Laurus Laboratories Ltd. (India). Irnocam (20 mg mL^{-1}) injection manufactured by Dr. Reddy's Laboratories (India) was obtained from a local hospital pharmacy.

Potassium dihydrogen *ortho*-phosphate (purity ≥ 98 %) and hydrochloric acid (HCl) extra pure, were procured from Finar Ltd. (India). Sodium hydroxide pellets (purity ≥ 98 %) were obtained from Nice Chemicals Ltd. (India). Ammonium formate, anhydrous sodium acetate (purity ≥ 99 %) and glacial acetic acid (purity ≥ 99.5 %) were obtained from Sisco Research Laboratories Pvt. Ltd. (India). Ammonium acetate (purity ≥ 96 %) and formic acid were obtained from Merck Ltd. (India). *Ortho*-phosphoric acid (OPA) (88 %) was obtained from Rankem Chemicals (India). Triethylamine (TEA) (purity ≥ 99 %) and acetonitrile (ACN) of HPLC grade (purity ≥ 99.8 %) were obtained from Spectrochem Ltd. (India). Sodium dihydrogen phosphate (purity ≥ 98 %) was obtained from Loba Chemie Pvt. Ltd. (India). Milli-Q water was produced in our research laboratory.

Chromatographic conditions and equipment

HPLC Shimadzu LC-2010CHT model connected to PDA (photodiode array, model no. SPD-M20A PDA with 220–230 V, Shimadzu, Japan) with dual-wavelength UV detector, column oven and autosampler (Shimadzu) was used in this study. Data acquisition of obtained chromatograms was carried out with LC Solution software (v.5.57). HyperClone (Phenomenex®, USA) C_{18} column (250 × 4.6 mm id, particle size 5 μm, ODS 130 Å) attached with Phenomenex®, 4 × 3.0 mm id, security guard column (Phenomenex®) was used. The mobile phase was filtered through a Millipore® glass filter (using a cellulose nitrate filter paper with pore size 0.22 μm) connected to a glass vacuum filtration unit. The filtered mobile phase was bath sonicated using GT Sonic Professional Ultrasonic Cleaner (Servewell Instruments, India) for 10 min to deaerate the mobile phase system. The pH of the buffer was measured with a pH meter (μ Controller based pH System 361) using a glass electrode (Systronics, India).

Software

Design-Expert software (v.9.0.5.1 software) (Stat-Ease Inc., USA) was used for screening and optimization of analytical method conditions and statistical analysis of obtained results.

Defining QTMP, CAAs and ATP

AQbD approach is applied for the development of a new analytical method for the estimation of IRI in marketed products. To achieve the defined quality target method profile (QTMP), several critical analytical attributes (CAAs) such as peak area, retention time, number of theoretical plates and tailing factor were identified. Analytical target profile (ATP) was fixed based on acceptable criteria of ICH guidelines to achieve the goal of this study (20).

Risk identification

As shown in Table I, few preliminary trials were taken for the selection of appropriate buffer for the mobile phase. Based on preliminary trials and previous knowledge and literature (15, 16, 21) an Ishikawa diagram (as shown in Fig. 1) was constructed to review the effect of all the possible factors on CAAs.

Furthermore, to fulfill the ICH Q9 guidelines, failure mode effects analysis (FMEA) followed by risk ranking and filtering approach were used for risk assessment. As reported in Table II, the risk associated with each factor [critical method parameters (CMPs) and critical process parameters (CPPs)] on responses were critically swotted and then the risks were labeled under the FMEA approach which works with respect to the severity of risk (22). Risk factors were further categorized into three categories such as severity (impact on final method output), the regularity of occurrence and detectability (23). The risk associated with these factors on responses were ranked between 1 and 3. The value 3 represents the highest severity of the risk. The ranking is defined in Table III. The product of severity, regularity and detectability was considered as risk priority number (RPN). The RPN values were used to rank failure modes, CMPs and CPPs as shown in Table IV.

Table I. Preliminary trials for selection of appropriate mobile phase

Buffer	pH	Concentration of buffer (mmol L^{-1})	Mobile phase (ACN/buffer)	Run time (min)	Flow rate (mL min^{-1})	Retention time (t_R, min)	Tailing factor 5 % ($T_{f5\%}$)	Tailing factor 10 % ($T_{f10\%}$)	Area (mV-min)	N
Ammonium formate	3	25	50:50	10	1	3.691	2.756	2.295	267056	2737
Ammonium acetate	3	25	50:50	10	1	3.627	2.629	2.241	262040	2697
Sodium acetate	3	25	50:50	10	1	4.305	3.572	3.043	258168	1455
Potassium dihydrogen phosphate	3	25	50:50	10	1	3.509	2.477	2.096	276628	3102
Ammonium formate	3	35	50:50	8	1	3.395	2.452	2.083	295431	3095
Ammonium acetate	3	35	50:50	8	1	3.33	2.325	2.001	302861	3060
Potassium dihydrogen phosphate	3	35	50:50	8	1	3.221	1.889	1.687	327892	3989
Ammonium formate with 0.1 % TEA	3	35	50:50	8	1	3.029	1.417	1.339	320200	5193
Ammonium acetate with 0.1 % TEA	3	35	50:50	8	1	2.983	1.434	1.346	319948	4933
Potassium dihydrogen phosphate with 0.1 % TEA	3	35	50:50	8	1	2.975	1.383	1.319	331313	5662
Potassium dihydrogen phosphate with 0.1 % TEA	3	25	50:50	8	1	3.075	1.43	1.349	333467	5336
Potassium dihydrogen phosphate with 0.1 % TEA	3	35	45:55	10	1	3.077	1.42	1.339	347013	5516
Potassium dihydrogen phosphate with 0.1 % TEA	3	35	45:55	10	0.8	3.875	1.429	1.35	437708	6793

ACN – acetonitrile, N – number of theoretical plates, TEA – triethylamine

Table II. Risk assessment using failure mode effects analysis approach

Risk factor	Retention time (t_R)	Peak Area (mV-min)	Tailing factor (T_f)	N	Peak height (h)
Flow rate	High	High	High	High	High
Injection volume	Low	High	Medium	Medium	High
Wavelength	Low	High	Low	Medium	High
Oven temperature	Low	High	Low	Low	Low
Mobile phase composition	High	High	High	High	High
Buffer type	High	High	High	High	High
Buffer strength	Low	High	High	High	High
Buffer pH	High	High	High	High	High
Column type	High	High	High	High	High
Column particle size	High	High	High	High	High
Light	Low	High	Low	High	High
Ambient temperature	Low	High	Low	High	High
Contamination	High	High	High	High	High
Humidity	Low	High	Low	Low	Low
Bath sonication of stock solution	Low	High	Low	Low	Low
Bath sonication of buffer	Low	Low	Low	Low	Low
Filtration	Medium	Medium	Medium	Medium	Medium
Sample vials	Low	High	Low	High	High
Analyst	High	High	High	High	High
System calibration	High	High	Medium	Medium	High
Balance	Low	High	Low	Low	High
Water source	High	High	High	High	High
Software	Low	Low	Low	Low	Low

N – number of theoretical plates

Table III. Different levels of risk to rank CMPs and CPPs

Score	Severity (S)	Regularity of occurrence (R)	Detectability (D)
1	Minor	Very unlikely	Normally not detected
2	Moderate	Occasional	Likely detected
3	Major	Regular	Regularly detected

CMPs – critical method parameters, CPPs – critical process parameters

Table IV. Risk priority ranking of CMPs and CPPs

CMPs/CPPs	Failure mode (critical event)	Effect on CAAs (justification of failure mode)	S	R	D	RPN (S*R*D)
Flow rate	Very low and very high	Low flow rate – t_R may increase High flow rate – resolution may decrease and peak may merge with mobile phase peak	3	3	3	27
Injection volume	Very low	Low volume – detectability issue	3	1	3	9
Wavelength	Out of range	Out of range – detection problem	3	3	3	27
Oven temperature	High temperature	High temperature – as the drug is sensitive to the temperature Hence, it may reduce the peak area and can results detection problem.	3	1	2	6
Mobile phase composition	Inappropriate ratio	Inappropriate ratio – may affect t_R, peak area, detection, peak shape, *N*, *etc*.	3	3	3	27
Buffer type	Based upon pK_a values	Wrong selection – affects peak shape, detection problem, *etc*.	3	1	1	3
Buffer concentration	Very low or very high	Low buffer concentration – tailing problem High buffer concentration – precipitation in the column, blockage of channels and line filters	3	3	3	27
Buffer pH	Differences in pK_a values	pH needs to be 2 units less or 2 units more of pK_a value May affect on peak shape and detection	3	3	3	27
Column type	Inappropriate column	Inappropriate column – may affect t_R, peak area, detection, peak shape, *N*, *etc*.	3	1	3	9
Column particle size	Bigger particle size	Number of theoretical plates (efficiency) is inversely proportional to the column particle size Narrow peak widths enhance the resolution of the peak Resolution is inversely proportional to the column particle size Speed of analysis is inversely proportional to the column particle size	3	1	3	9
Light	Drug exposure to the UV light	API degradation due to light exposure may reduce peak height, peak area, *etc*.	3	1	3	9

Table IV. Continued

CMPs/CPPs	Failure mode (critical event)	Effect on CAAs (justification of failure mode)	S	R	D	RPN (S*R*D)
Ambient temperature	Drug exposure to the ambient temperature	API is stable at room temperature If API is unstable at ambient temperature (or at 25 °C), it can result in detection problems, resolution problems, *etc.*	1	1	1	1
Humidity	Drug exposure to the humidity	If API is sensitive to humidity, it can cause an error in results, detection problems, resolution problems, *etc.*	1	1	1	1
Contamination	Improper cleaning of glass-ware, HPLC vials, *etc.*	Reduces the resolution of drug peak Increases the noise	1	1	1	1
Filtration	Filtration of prepared buffer	Help for the removal of impurities	1	1	1	1
Bath sonication of buffer	Bath sonication of buffer	Help in the degassing of the buffer	2	2	2	8
Bath sonication of stock solution	Bath sonication of prepared samples	Improve the dissolution of API in the medium Help in the degassing of prepared samples	1	1	1	1
Sample vials	Construction material of sample (HPLC) vials	Material used for the manufacturing of sample vials may cause leaching or sorption problem	1	1	1	1
Analyst	Change of analyst during sample processing	It may result in variations in the peak area of the same concentration due to the variations in handling	1	1	1	1
System calibration	Not calibrated HPLC system	It may affect the reproducibility/ robustness of the developed method	1	1	1	1
Balance	Not calibrated weighing machine	Improper weighing may affect the concentration buffer, sample concentration, *etc.*	1	1	1	1
Water source	Not purified water	It may produce noise in the chromatograph Resolution may reduce	1	1	1	1
Software	Ancient version	It may affect the integration of peaks It may produce an error during automatic sampling	1	1	1	1

API – active pharmaceutical ingredient, CAAs – critical analytical attributes, D – detectability, *N* – number of theoretical plates, QTMP – quality target method profile, RPN – risk priority number, R – regularity of occurrence, S – severity

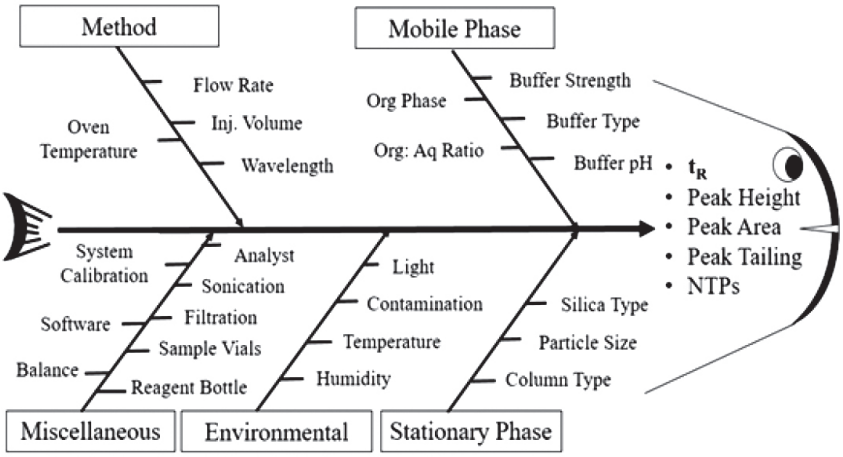

Fig. 1. Ishikawa diagram to review the effect of all the possible factors on CAAs. *N* – number of theoretical plates, t_R – retention time.

Screening of significant variables using 25 full factorial design

Screening design was applied to identify the significant effect of variables such as critical method parameters (CMPs) and critical process parameters (CPPs) on critical analytical attributes (CAAs). The variables such as pH of buffer, ionic concentration of buffer, flow rate, mobile phase composition (concentration of acetonitrile in the mobile phase) and wavelength were selected, based on the risk priority number (RPN), for further screening, with two different levels of each factor, *i.e.*, 2.5 and 3.5, 0.6 and 1.2 mL min^{-1}, 20 and 30 mmol L^{-1}, 35 and 50 %, *V/V*, and 254 and 265 nm, resp., using full factorial design. HyperClone C_{18} column (250 × 4.6 mm id, particle size 5 μm, ODS 130 Å) with a C_{18} Phenomenex®, 4.0 × 3.0 mm id, guard column, and standard solution of IRI (10 μg mL^{-1}) were used as non-variable factors in the study.

All the variables were studied and assessed for their influence on the CAAs. Table V represents the experiments obtained after applying a full factorial design.

Optimization of variables

Box-Behnken design. – Three variables (*i.e.*, flow rate, the concentration of buffer and concentration of acetonitrile) showed a significant effect on responses after accessing full factorial design. Further optimization of these significant variables was performed using response surface methodology (RSM).

A three-level Box-Behnken design (BBD) was implemented to study the interactive effects between the variables. BBD was applied to optimize method variables statistically, with the aim of obtaining a desirable retention time, greater peak area, a larger number of theoretical plates and lower tailing factor (T_f). Each variable such as flow rate, buffer concentration, and concentration of acetonitrile was set at three different levels, *i.e.*, 0.7, 0.8 and

Table V. Suggested experiments with their responses obtained after applying full factorial design

pH	Buffer concentration (mmol L^{-1})	Flow rate (mL min^{-1})	Concentration of ACN (%)	Wavelength (nm)	Retention time (min)	Area (mV-min)	N	$T_{f5\%}$	$T_{f10\%}$
2.5	20	0.6	35	254	6.865	1014845	7153	1.629	1.501
3.5	20	0.6	35	254	7.498	433732	7007	1.724	1.556
2.5	30	0.6	35	254	6.651	436733	7812	1.618	1.477
3.5	30	0.6	35	254	7.165	430201	6967	1.703	1.539
2.5	20	1.2	35	254	3.438	504497	4792	1.502	1.395
3.5	20	1.2	35	254	3.678	503442	4386	1.565	1.442
2.5	30	1.2	35	254	3.3	202466	5071	1.485	1.37
3.5	30	1.2	35	254	3.564	215542	4528	1.567	1.428
2.5	20	0.6	50	254	5.083	1022307	7235	1.476	1.379
3.5	20	0.6	50	254	5.315	1021738	6146	1.524	1.418
2.5	30	0.6	50	254	4.904	403822	7554	1.434	1.344
3.5	30	0.6	50	254	5.156	430339	6534	1.498	1.394
2.5	20	1.2	50	254	2.544	508874	4318	1.413	1.339
3.5	20	1.2	50	254	2.662	507613	3994	1.45	1.358
2.5	30	1.2	50	254	2.454	202936	4499	1.431	1.339
3.5	30	1.2	50	254	2.572	218474	4006	1.47	1.374
2.5	20	0.6	35	265	6.826	487675	7259	1.624	1.498
3.5	20	0.6	35	265	7.479	207935	7063	1.72	1.556
2.5	30	0.6	35	265	6.596	195112	8027	1.575	1.442
3.5	30	0.6	35	265	7.137	207523	7029	1.708	1.545
2.5	20	1.2	35	265	3.423	244112	4812	1.498	1.391
3.5	20	1.2	35	265	3.674	243219	4363	1.573	1.445
2.5	30	1.2	35	265	3.297	97084	5092	1.477	1.366
3.5	30	1.2	35	265	3.555	104028	4575	1.577	1.436
2.5	20	0.6	50	265	5.068	483604	7274	1.474	1.379
3.5	20	0.6	50	265	5.311	484442	6246	1.536	1.425
2.5	30	0.6	50	265	4.902	192630	7667	1.44	1.35
3.5	30	0.6	50	265	5.137	205559	6547	1.527	1.419
2.5	20	1.2	50	265	2.539	240126	4330	1.414	1.335
3.5	20	1.2	50	265	2.66	240369	3967	1.454	1.363
2.5	30	1.2	50	265	2.453	98205	4487	1.432	1.341
3.5	30	1.2	50	265	2.568	104061	4089	1.471	1.369

ACN – acetonitrile, N – number of theoretical plates per column length, T_f – tailing factor

0.9 mL min^{-1}; 20, 22.5 and 25 mmol L^{-1}, and 35, 40 and 45 %, *V*/*V*, resp. The software suggested trials (17 experiments) were performed and were assessed for their retention time, peak area, number of theoretical plates and tailing factor 5 % ($T_{f5\%}$) and 10 % ($T_{f10\%}$) as shown in Table VI. From the seventeen experiments, 12 represented the mid-point to each edge of the multidimensional cube. Remaining five were the duplicates of the cube's center point. Desirability was estimated in order to select an optimized method.

Table VI. Method responses for Box-Behnken optimization design

Buffer concentration (mmol L^{-1})	Flow rate (mL min^{-1})	Concentration of ACN (%)	Retention time (min)	Area (mV-min)	*N*	$T_{f5\%}$	$T_{f10\%}$
20	0.7	40	4.687	825291	3114	1.416	1.35
25	0.7	40	4.861	821366	3150	1.442	1.367
20	0.9	40	3.641	641071	3038	1.394	1.326
25	0.9	40	3.761	636219	2984	1.422	1.348
20	0.8	35	4.728	719908	2484	1.417	1.343
25	0.8	35	4.933	720534	2514	1.465	1.371
20	0.8	45	3.779	705633	3711	1.399	1.339
25	0.8	45	3.881	704747	3594	1.413	1.347
22.5	0.7	35	5.773	798098	2694	1.503	1.402
22.5	0.9	35	4.501	633911	2613	1.478	1.377
22.5	0.7	45	4.562	808264	3752	1.438	1.367
22.5	0.9	45	3.534	623966	3454	1.407	1.341
22.5	0.8	40	4.334	714334	3174	1.46	1.376
22.5	0.8	40	4.337	715993	3152	1.46	1.379
22.5	0.8	40	4.336	695975	3185	1.424	1.361
22.5	0.8	40	4.334	713912	3172	1.459	1.375
22.5	0.8	40	4.332	694889	3163	1.427	1.362

ACN – acetonitrile, *N* – number of theoretical plates per column length, T_f – tailing factor

Verification of software-generated optimized solution. – Based on defined conditions software suggested few experiments with predicted solutions. Based on desirability value, a suitable experiment was selected and was performed under suggested conditions. The obtained results were compared with the software predicted solutions for verification. Lesser the residual error the higher is the compatibility between software predicted results and obtained results (15). The final chromatographic conditions of the developed method are reported in Table VII.

Table VII. Chromatographic conditions and parameters of optimized method

Chromatographic conditions	
Column	C_{18} ODS column (250 × 4.6 mm i.d., particle size 5 μm, ODS 130 Å)
Concentration of phosphate buffer (mmol L^{-1}) + 0.1 % TEA	20
pH of phosphate buffer	2.5
ACN/buffer ratio (%, *V/V)*	45:55
Injection volume (μL)	20
Wavelength (nm)	254
Flow rate (mL min^{-1})	0.75
Column oven temperature (°C)	25
Some chromatographic parameters	
Retention time (t_R) (min)	4.09
Number of theoretical plates (per column length)	3763
Tailing factor (5 %)	1.41
Tailing factor (10 %)	1.35

Solutions preparation

Phosphate buffer (20 mmol L^{-1}) was prepared by dissolving the required quantity of potassium dihydrogen *ortho*-phosphate in 1000 mL Milli Q water containing 0.1 % (*V/V*) triethylamine (TEA) and adjusting the pH to 2.5.

Standard stock (1000 μg mL^{-1}) solution of IRI was obtained by transferring 10 mg of IRI weighed accurately into a 10-mL volumetric flask and filling up the volume using methanol. Further dilutions were done with the mobile phase to obtain the desired standard solution. Concentrations ranging from 0.5 to 18 μg mL^{-1} were prepared by suitable dilution of the standard solution of IRI.

Preparation of the mobile phase

The salt form chosen in the preparation of the buffer creates a difference in solubility observed. It is suggested in the literature that potassium salt have an edge over sodium salts due to the difference in solubility (24). Hence, a combination of acetonitrile and potassium phosphate buffer containing 0.1 % TEA in the ratio 45:55 %, *V/V,* was used as a mobile phase.

Validation of developed and optimized HPLC method

Developed HPLC method in the present study was validated as per the ICH Q2 (R1) guidelines for linearity, precision, accuracy, limit of detection (*LOD*), limit of quantitation (*LOQ*) and robustness (20).

Specificity. – To ascertain the specificity of the method, a blank, marketed formulation (10 μg mL^{-1}) and IRI (10 μg mL^{-1}) chromatograms were compared and assessed for interference at the retention time of the drug.

Linearity. – A calibration curve was prepared within the range of 0.5 to 18 μg mL^{-1} IRI. The obtained peak area for the respective concentration of the analyte was plotted graphically. Triplicate injections of each concentration were measured. Linear regression and coefficient of determination were calculated from the obtained calibration curve.

Precision. – As a part of intra- and inter-day precision studies, three distinct concentrations, 1, 8 and 16 μg mL^{-1}, were chosen and analysis was performed. Six injections of each concentration were analysed. Intra-day precision studies had to be performed two times in a day (*i.e.*, morning and evening) whereas the inter-day precision on two different days. The peak area was recorded and percent relative standard deviation (RSD) was calculated.

Accuracy. – Three different concentrations (*i.e.*, 2, 4 and 6 μg mL^{-1}) were prepared from the marketed formulation. These dilutions were pre-analysed using the HPLC method. A standard (concentration of 16.00 μg mL^{-1}) was added to the above-prepared dilutions, to obtain final concentrations of 6, 8 and 10 μg mL^{-1}. These samples were analysed and percent recovery was calculated.

Limit of detection and limit of quantitation. – Limit of detection (*LOD*) and limit of quantitation (*LOQ*) were estimated as: $LOD = 3.3\ \sigma/s$ and $LOQ = 10\ \sigma/s$, where, σ is the standard deviation of y-intercepts of the regression line and *s* is the slope of the calibration line (20).

Robustness. – The ability of a method to yield results without variation on subjecting it to deliberate changes in the method parameters determines how robust the developed method is. This was determined by varying parameters such as wavelength (254 ± 1 nm), buffer pH (2.5 ± 0.1), injection volume (20 ± 2 μL) and flow rate (0.75 ± 0.1 mL min^{-1}). The concentration of the IRI solution used for the study was 13 μg mL^{-1}.

Application of method

The proposed method has been applied to an injectable marketed pharmaceutical formulation of CPT-11. A hundred microliters of marketed formula (20 mg mL^{-1}) were withdrawn and diluted ten times using the mobile phase. This was further diluted to obtain three concentrations (*i.e.*, 1, 4 and 10 μg mL^{-1}).

Statistical analysis

All the average values were presented along with their relative/standard deviations. The statistical analyses were performed by applying a two-way analysis of variance (ANOVA). The results were considered to be significant at significance level $p \leq 0.05$.

RESULTS AND DISCUSSION

Risk identification

Preliminary trials. – The nature of the mobile phase affects the nature of the obtained peak. Therefore, it should be chosen in such a way that the drug remains completely ion-

ized or unionized. It is preferred to select a buffer for the mobile phase with pH equaling $pK_{a\ drug} \pm 2$, as this may help to improve the shape of the peak. If the buffer with high pH is selected this may affect the column (solubilization of silica packing) (16, 21). Similarly, the type of buffer may also have an effect on the retention time, peak area, peak shape, tailing factor, peak resolution, *etc*. Hence, during the preliminary trials, different types of buffers (such as ammonium formate, ammonium acetate, sodium acetate and potassium dihydrogen phosphate) with pH 3 and concentration 25 mmol L^{-1} were studied. In the beginning, acetonitrile and buffer with pH 3.0 were studied in 50:50 % (*V*/*V*) ratio. As shown in Table I, peak area and the number of theoretical plates with potassium dihydrogen phosphate buffer was found to be high (*i.e.*, 276628 mV-min and 3102, resp.) in comparison with other buffers. Sodium acetate buffer gave the least peak area and the number of theoretical plates (*i.e.*, 258168 mV-min and 1455, resp.) among all the buffers. Similarly, potassium dihydrogen phosphate gave the shortest retention time value (*i.e.*, 3.509 min) in comparison of all the other buffers when used in 50:50 % (*V*/*V*) ratio with acetonitrile, whereas sodium acetate gave the highest retention time value (*i.e.*, 3.627 min) when used in the same ratio with acetonitrile. Based on the obtained results, potassium dihydrogen phosphate was found to be more appropriate, but none of the buffers was able to reduce the tailing factor < 2.0 when used in 25 mmol L^{-1} concentration.

It seems that as the concentration of buffer increases, the tailing factor reduces. Hence, it was further planned to study the effect of all the buffers by increasing the concentration from 25 to 35 mmol L^{-1} except for sodium acetate. The sodium acetate was planned to drop out as it was unable to give promising results. The potassium dihydrogen phosphate (35 mmol L^{-1}) with acetonitrile in the 50:50 % (*V*/*V*) ratio was able to reduce the tailing factor to < 2. Even ammonium formate and ammonium acetate buffer in the concentration of 35 mmol L^{-1} were able to reduce the tailing factor compared to their lower concentration (*i.e.*, 25 mmol L^{-1}), but still, it was ≥ 2.

By increasing the concentration of buffers the tailing factor was decreased, but to further decrease the tailing factor, increase the peak area and the number of theoretical plates, 0.1 % (*V*/*V*) triethylamine (0.1 % TEA) was planned to be added to the buffers. When buffers (35 mmol L^{-1} with 0.1 % TEA) were used with acetonitrile in 50:50 % (*V*/*V*), they gave good results with respect to the aforementioned chromatographic parameters, with potassium dihydrogen phosphate being the best. The lower concentration of potassium dihydrogen phosphate (*i.e.*, 25 mmol L^{-1}) with 0.1 % TEA along with acetonitrile in a ratio 50:50 % (*V*/*V*), showed promising results.

Potassium dihydrogen phosphate buffer (25 mmol L^{-1} with 0.1 % TEA) along with acetonitrile in the ratio 50:50 % (*V*/*V*), gave retention time value at 2.975 min for IRI which may affect the resolution of the peak. Hence, to increase the resolution of peak different compositions of the mixtures of potassium dihydrogen phosphate (25 mmol L^{-1} with 0.1 % TEA) with acetonitrile were studied. As shown in Table I, acetonitrile along with potassium dihydrogen phosphate (35 mmol L^{-1} with 0.1 % TEA) in the ratio 45:55 % (*V*/*V*), gave the retention time of 3.077 min. Then, by further decreasing the flow rate (to 0.8 mL min^{-1}) the retention time was found to be 3.875 min which also resulted in an increase of peak area and the number of theoretical plates and the tailing factor was also found within the acceptable criteria.

In the study, acetonitrile was used as an organic solvent as it would help to prevent the build-up of high column internal pressure (15).

Screening of significant variables using 2^5 full factorial design

A full factorial design is an appropriate design to study the influence of all the variables simultaneously on responses, as one can quantify the effect produced by the variables on the responses as well as interactions between variables (25).

All factors with high RPN were studied and assessed for their influence on the CAAs using full factorial design. The variables were coded as A, B, C, D, and E for pH of the buffer, the concentration of the buffer, flow rate, the concentration of ACN and wavelength, resp. Variables that have a significant influence on individual response/selected CAAs were identified by the half-normal plot and Pareto charts. Table VIII presents the analysis of variance (ANOVA) for studying the significant effect of main and interactive variables on responses, variables (main as well as interactive) having a statistically significant effect if p-value < 0.05.

The buffer pH showed a negative influence on all responses except retention time and tailing factor (shown in Table VIII). Since we are aiming to develop a method for rapid analysis of IRI, we prefer a lower pH of the buffer for faster elution of the drug. Simultaneously we observed that with increasing pH the tailing factor increases. Smaller the tailing factor, higher is the peak symmetry or Gaussian distribution pattern. As a result, we opted for a lower buffer pH.

It was observed that the drug exhibited higher sensitivity at 254 nm which was in correlation with the obtained UV-spectrum of IRI. The wavelength for analysis and the pH of the buffer were fixed at 254 nm and pH 2.5 resp. But, the other variables, such as buffer concentration, flow rate and concentration of acetonitrile, were optimized using response surface design (Box-Behnken design) to optimize the variables for the analytical method development.

Optimization of variables

Box-Behnken design. – The response surface design suggested the quadratic model significant for all the method responses considered. Table IX presents the analysis of variance (ANOVA) results of Box-Behnken design for studying the model and coefficient estimate. A process of numerical optimization was applied using the Design Expert® software to get a method with the desired response. For each response, maximum and minimum limit values were set. For the selected composition, an optimum desirability value (0.408) was obtained. ANOVA test was used to identify the significant effect of variables on the responses. The influence of the factors is significant if p-value < 0.05. When the variable has a synergistic effect on the response it is indicated by a positive coefficient estimate and an antagonistic effect is depicted by a negative coefficient estimate. Based on results obtained (Table IX), factor A (concentration of buffer) shows a significant synergistic effect while B (flow rate) and C (concentration of acetonitrile) show a significant antagonistic effect on retention time. This suggests that the retention time increased with increasing concentration of buffer, and at a higher flow rate and concentration of ACN the retention time decreased. Factor B had a significant antagonistic effect on the peak area with a $p < 0.0001$. Factors B and C had a significant effect on the number of theoretical plates. Factor B showed an antagonistic effect while C showed a synergistic

Table VIII. ANOVA results for full factorial design

Coded factors	Retention time (min)		Peak area (mV-min)		N		$T_{f5\%}$		$T_{f10\%}$	
	Coefficient estimate	p-value	Coefficient estimate	p-value	Coefficient estimate	p-value	Coefficient estimate	p-value	Coefficient estimate	p-value
5FI factorial model	4.54000	< 0.0001	0.00004	< 0.0001	5775.63	< 0.0001	1.53000	< 0.0001	1.42000	< 0.0001
A	0.15000	< 0.0001	–24275.34	0.0263	–310.19	< 0.0001	0.03600	< 0.0001	0.02600	< 0.0001
B	–0.08100	< 0.0001	–0.000014	< 0.0001	130.19	< 0.0001	–0.00510	< 0.0001	–0.00772	< 0.0001
C	–1.52000	< 0.0001	–0.000011	< 0.0001	–1318.81	< 0.0001	–0.04500	< 0.0001	–0.03500	< 0.0001
D	–0.71000	< 0.0001	26154.78	0.0182	–219.81	< 0.0001	–0.06600	< 0.0001	–0.04600	< 0.0001
E	–0.00560	0.0589	–0.000013	< 0.0001	26.06	< 0.0001	–	–	–	–
AB	–0.00750	0.0209	29696.53	0.0090	–61.25	< 0.0001	0.00353	0.0001	0.00403	< 0.0001
AC	–0.05800	< 0.0001	26678.34	0.0164	91.88	< 0.0001	–0.00609	< 0.0001	–0.00469	< 0.0001
AD	–0.05900	< 0.0001	28031.03	0.0125	–54.50	< 0.0001	–0.00978	< 0.0001	–0.00603	< 0.0001
AE	–	–	–	–	–	–	0.00372	< 0.0001	0.00284	< 0.0001
BC	0.02800	< 0.0001	28278.22	0.0119	–43.62	< 0.0001	0.00766	< 0.0001	0.00491	< 0.0001
BD	0.02000	0.0002	–28196.22	0.0121	–13.12	0.0097	0.00272	0.0005	0.00359	< 0.0001
BE	0.002000	0.4441	48414.22	0.0002	7.25	0.1089	–	–	–	–
CD	0.25000	< 0.0001	–25763.03	0.0196	–25.75	< 0.0001	0.02100	< 0.0001	0.01700	< 0.0001
CE	0.00290	0.2721	38643.66	0.0015	–18.50	0.0012	–	–	–	–
DE	0.00520	0.0756	–	–	–	–	0.00291	0.0004	0.00203	< 0.0001
ABC	0.00900	0.0094	–26922.78	0.0156	35.69	< 0.0001	–	–	–0.00134	< 0.0001
ABD	–	–	–25847.22	0.0193	47.06	< 0.0001	–0.00091	0.0856	–0.00091	0.0005
ABE	0.00003	0.9900	–	–	–	–	0.00184	0.0047	0.00122	< 0.0001
ACD	0.02500	< 0.0001	-27887.03	0.0129	75.56	< 0.0001	–	–	–0.00141	< 0.0001
ACE	–	–	–	–	9.06	0.0524	–0.00166	0.0081	–0.00153	< 0.0001

Table VIII. Continued

Coded factors	Retention time (min)		Peak area (mV-min)		N		$T_{f5\%}$		$T_{f10\%}$	
	Coefficient estimate	p-value	Coefficient estimate	p-value	Coefficient estimate	p-value	Coefficient estimate	p-value	Coefficient estimate	p-value
ADE	–0.00320	0.2291	–	–	7.69	0.0915	–0.00122	0.0311	–0.00109	0.0002
BCD	–0.01100	0.0041	28373.97	0.0117	–14.44	0.0057	0.00384	< 0.0001	0.00272	< 0.0001
BDE	–	–	–	–	–	–	0.00166	0.0081	0.00128	< 0.0001
CDE	–0.00400	0.1498	–	–	–	–	–0.00284	0.0004	–0.00234	< 0.0001
ABCD	–0.00730	0.0224	25874.97	0.0192	–47.00	< 0.0001	–0.00178	0.0057	–	–
ABCE	–	–	–	–	–13.75	0.0075	–0.00166	0.0081	–0.00191	< 0.0001
ABDE	0.00070	0.7940	–	–	–	–	–0.00147	0.0142	–0.00147	< 0.0001
ACDE	0.00290	0.2721	–	–	–	–	–	–	–	–
BCDE	0.00053	0.8324	–	–	–	–	–0.00172	0.0068	0.00184	< 0.0001
ABCDE	0.00003	0.9900	–	–	–	–	0.00091	0.0856	–	–
R^2	1.0000		0.9805		0.9999		0.9998		1.0000	
Adj R^2	0.9999		0.9534		0.9997		0.9992		0.9998	
Adeq Precision	411.007		20.615		208.559		137.883		298.031	

A – buffer pH, B – buffer concentration (mmol L^{-1}), C – flow rate (mL min^{-1}), D – acetonitrile concentration (%, *V*/*V*), E – UV wavelength (nm), FI – factor interaction
N – number of theoretical plates per column length, $T_{f5\%}$ – tailing factor at 5 % peak width, $T_{f10\%}$ – tailing factor at 10 % peak width, Adj R^2 – adjusted coefficient of determination, Adeq precision – adequate precision
Terms are statistically significant if $p < 0.05$.

Table IX. ANOVA results for Box-Behnken design

Factor	Retention time (min)		Peak area (mV-min)		N		$T_{f5\%}$		$T_{f10\%}$	
	Coefficient estimate	p-value	Coefficient estimate	p-value	Coefficient estimate	p-value	Coefficient estimate	p-value	Coefficient estimate	p-value
A	0.07500	0.0003	–1129.62	0.7459	–13.13	0.5492	0.016	0.0344	0.0094	0.0187
B	–0.56000	< 0.0001	–89731.50	< 0.0001	–77.63	0.0074	–0.011	0.1099	–0.0120	0.0066
C	–0.52000	< 0.0001	–3730.13	0.3024	525.75	< 0.0001	–0.026	0.0036	–0.0120	0.0051
AB	–0.01300	0.4188	–231.75	0.9624	–22.50	0.4705	–0.002	0.8207	0.0013	0.7823
AC	–0.02600	0.1454	–378.00	0.9387	–36.75	0.2529	–0.009	0.3506	–0.0050	0.2884
BC	0.06100	0.0060	–5027.75	0.3240	–54.25	0.1085	–0.002	0.8649	–0.0003	0.9558
A^2	–0.18000	< 0.0001	10305.95	0.0609	–75.10	0.0348	–0.031	0.0067	–0.0220	0.0012
B^2	0.08300	0.0010	13660.20	0.0212	–22.60	0.4576	0.002	0.8615	–0.0006	0.8951
C^2	0.18000	< 0.0001	–4621.05	0.3504	–18.35	0.5436	0.009	0.3133	0.0017	0.7056
R^2	0.9986		0.9906		0.9896		0.8651		0.9083	
Adj R^2	0.9968		0.9784		0.9761		0.6917		0.7903	
Adeq Precision	89.437		28.069		27.835		7.766		10.521	

A – concentration of buffer (mmol L^{-1}), B – flow rate (mL min^{-1}), C – concentration of acetonitrile (%), N – number of theoretical plates per column length, $T_{f5\%}$ – tailing factor at 5 % peak width, $T_{f10\%}$ – tailing factor at 10 % peak width, Adj R^2 – adjusted coefficient of determination, Adeq precision – adequate precision
Terms are statistically significant if $p < 0.05$.

effect on the number of theoretical plates. Factor C exhibited an antagonistic effect on the tailing factor 5 % with p = 0.0036, which was significant. Factor A showed significant synergistic effect while B and C showed a significant antagonistic effect on tailing factor 10 % with p-values of 0.0187, 0.0066 and 0.0051, resp. Furthermore, all the responses except peak area showed a significant effect due to the quadratic term A^2; regarding retention time, the number of theoretical plates, tailing factor 5 % and 10 % this effect was found to be antagonistic with p-value < 0.0001, 0.0348, 0.0067 and 0.0012, resp. Quadratic term B^2 exhibited a higher synergistic effect on the retention time compared to the peak area with a p-value of 0.001 and 0.0212, resp.

Verification of software-generated optimized solutions. – Results obtained by following the Box-Behnken design suggested trials were assessed for retention time, peak area, number of theoretical plates and tailing factors 5 % and 10 %. Criteria for method optimization were fixed based on CAAs for optimization and maximum and minimum levels of response parameters were adjusted. Then a list of solutions provided by the software was assessed.

The variables were optimized using Design Expert® software as shown in Fig. 2. A solution shown in Fig. 2 was selected as it was matching with the defined targets of QbD. Then the experiment was executed as per the optimized conditions. Then the same was performed for verification. The output was verified by comparing the results predicted by the software with the observed results. A difference of ± 3 % was found between the predicted and observed results as shown in Table X.

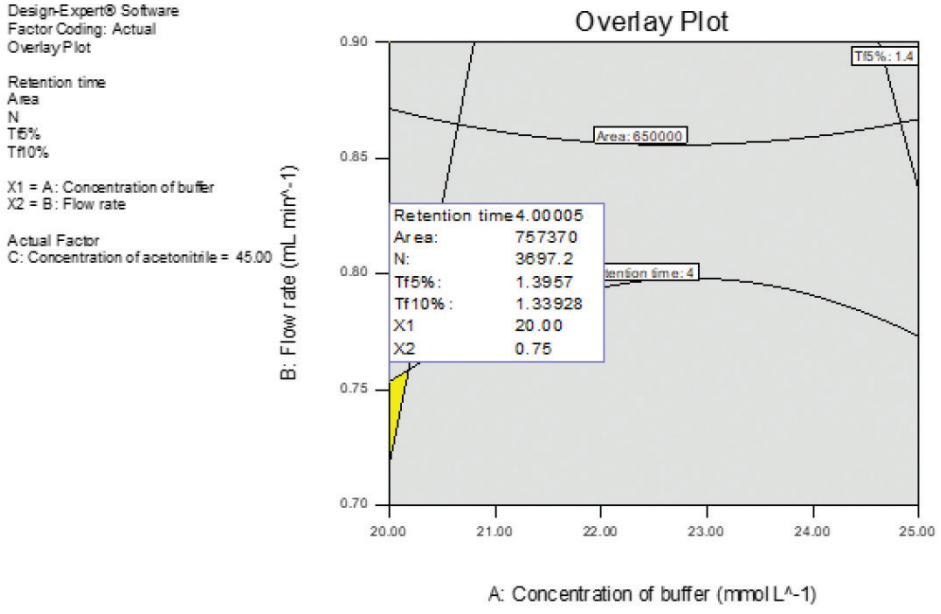

Fig. 2. Overlay of the selected variables representing the optimized analytical conditions. A – concentration of buffer (mmol L^{-1}), B – flow rate (mL min^{-1}), C – concentration of acetonitrile (%, *V*/*V*).

Table X. Verification of optimized analytical conditions

Response	Predicted results	Observed results	Residual values (%)
Retention time (min)	4.00	4.09	–2.3
Peak area (mV-min)	757370	752970	0.6
Number of theoretical plates (per column length)	3697.20	3763	–1.8
Tailing factor (5 %)	1.40	1.41	–0.7
Tailing factor (10 %)	1.34	1.35	–0.8

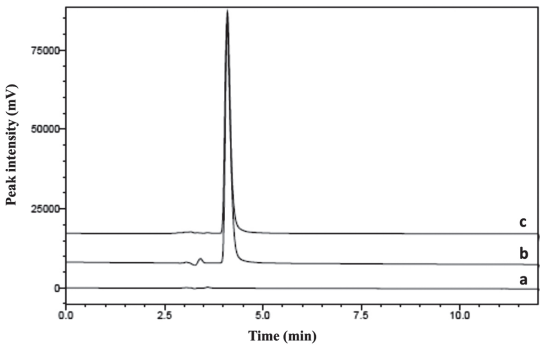

Fig. 3. Chromatogram of: a) blank (mobile phase), b) IRI standard stock solution, 10 μg mL^{-1}, c) injectable marketed formulation, 10 μg mL^{-1}.

Preliminary validation

Developed and optimized HPLC analytical method was validated in accordance with ICH Q2 (R1) guidelines (20).

As reported in Fig. 3 retention time of IRI was found to be 4.09 min.

Linearity. – Standard solutions were prepared and injected into the HPLC system in a range of 0.5 to 18 μg mL^{-1}. The linear regression equation was found to be $y = 72060x + 6056.6$. The coefficient of determination (R^2) was found to be 0.9993.

Precision. – Three distinct concentrations, *i.e.*, 1, 8 and 16 μg mL^{-1} were chosen, and analysis was carried out for the intra- and inter-day precision studies. Table XI shows RSDs of peak area of intra-day and inter-day precision results which were found in the range of 0.1 to 0.4 %.

Accuracy. – Standard addition to the samples gave recovery of 101.4, 100.8 and 100.3 %, resp., which was within the official limits (26). The obtained results are given in Table XII.

Robustness. – The RSD values with deliberate changes in wavelength, buffer pH, injection volume and flow rate ranged 0 to 0.1 % (Table XIII).

Table XI. Intra-day and inter-day precision

Intra-day precision		Inter-day precision	
IRI mean concentration (μg mL^{-1})	RSD (%)	IRI mean concentration (μg mL^{-1})	RSD (%)
0.90	0.1	0.90	0.4
8.28	0.4	8.27	0.2
16.85	0.4	16.82	0.4

Table XII. Accuracy evaluation

Expected concentration of marketed formulation (μg mL^{-1})	Back calculated concentration (μg mL^{-1})	Compliance with the label claim (%)[a]	Concentration of standard (μg mL^{-1})	Expected concentration (standard + marketed formulation) (μg mL^{-1})	Back calculated concentration (μg mL^{-1})	Recovery (%)[a]
2	1.99	99.3 ± 0.6	16.00	6	6.09	101.4 ± 0.2
4	3.93	98.3 ± 0.2		8	8.07	100.8 ± 0.6
6	6.03	100.6 ± 1.0		10	10.03	100.3 ± 0.3

[a] Mean ± SD (n = 6).

Table XIII. Robustness of the method

Chromatographic condition		IRI concentration (μg mL^{-1})[a]	
		Mean	RSD (%)
Wavelength (nm)	252	13.72	0.1
	254	14.18	0.0_0
	256	14.54	0.1
Buffer pH	2.4	14.36	0.1
	2.5	14.18	0.0_0
	2.6	14.26	0.1
Injection volume (μL)	18	12.78	0.1
	20	14.18	0.0_0
	22	15.63	0.1
Flow rate (mL min^{-1})	0.74	14.47	0.0_0
	0.75	14.18	0.0_0
	0.76	14.09	0.0_0

[a] n = 3.

Limit of detection and limit of quantitation. – The limit of detection and limit of quantitation were determined and found to be 4.87 and 14.75 ng mL^{-1}, resp.

Method applicability

The method might be useful for analysis of IRI in the injectable formulation; 99.1–102.0 % compliance with the label claim as found in the formulation.

CONCLUSIONS

In this article, the HPLC method with UV detection for the estimation of IRI was proposed. The method was successfully developed and optimized with "quality by design" (QbD) approach. Various variables were screened and optimized to obtain a suitable HPLC method within the design space; it posed a few advantages such as short run time and the use of a UV detector which makes it cost-effective. The method was preliminarily validated. It might be applied to the analysis of IRI in injectable marketed formulation.

Acronyms, abbreviations, symbols. – AQbD – analytical quality by design, ATP – analytical target profile, BBB – Box-Behnken design, CAA – critical analytical attributes, CMPs – critical method parameters, CPPs – critical process parameters, DoE – design of experiments, FMEA – failure mode effects analysis, IRI – irinotecan, OFAT – one factor at a time, QbD – quality by design, QTMP – quality target method profile, RPN – risk priority number.

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