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Stir bar sorptive extraction of diclofenac from liquid formulations: A proof of concept study

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ABSTRACT

A new stir bar sorptive extraction (SBSE) technique coupled with HPLC-UV method for quantification of diclofenac in pharmaceutical formulations has been developed and validated as a proof of concept study. Commercially available polydimethylsiloxane stir bars (Twister™) were used for method development and SBSE extraction (pH, phase ratio, stirring speed, temperature, ionic strength and time) and liquid desorption (solvents, desorption method, stirring time etc) procedures were optimised. The method was validated as per ICH guidelines and was successfully applied for the estimation of diclofenac from three liquid formulations viz. Voltarol® Optha single dose eye drops, Voltarol® Ophtha multidose eye drops and Voltarol® ampoules. The developed method was found to be linear ($r = 0.9999$) over 100–2000 ng/ml concentration range with acceptable accuracy and precision (tested over three QC concentrations). The SBSE extraction recovery of the diclofenac was found to be 70% and the LOD and LOQ of the validated method were found to be 16.06 and 48.68 ng/ml, respectively. Furthermore, a forced degradation study of a diclofenac formulation leading to the formation of structurally similar cyclic impurity (indolinone) was carried out. The developed extraction method showed comparable results to that of the reference method, i.e. method was capable of selectively extracting the indolinone and diclofenac from the liquid matrix. Data on inter and intra stir bar accuracy and precision further confirmed robustness of the method, supporting the multiple re-use of the stir bars.

1. Introduction

Pharmaceutical formulations are mixtures of a drug, i.e. the intended therapeutic entity, and various excipients. At any given time, the formulation might also contain impurities from the raw material (drug/excipients) and degradation products resulting from various storage conditions and/or drug–excipient interactions. In recent years, there have been considerable developments in analytical techniques resulting in increased selectivity and sensitivity of analytical methods, however, even with various highly efficient analytical instruments, sample preparation procedures are usually necessary to extract and isolate the analytes of interest from complex matrices as most analytical instruments cannot handle the matrix directly [1].

Conventionally, solid–liquid extraction (SLE) [2–4] and/or liquid–liquid extraction (LLE) [5–7] are used for sample preparation purposes with drug formulations, however, in such a situation, if selectivity is in question (interference from impurities/degradation products etc at the retention time of analyte of interest), then further sample clean up is required. In recent years, Solid phase extraction (SPE) has increasingly been used to extract and estimate drugs [8], excipients [9] or degradation products [10] in pharmaceutical formulations especially, when a method needs to be stability indicating or the extraction involves a complex formulation matrix such as a cream [8]. Despite obvious advantages of SPE, one of the major factors associated with this technique is its cost along with other problems such as clogging/plugging of cartridges, channelling etc [11].

More recently, there have been many developments in the field of sample preparation techniques. In 1990, Pawliszyn and Arthur developed a new sample-preparation technique using a fused-silica fiber coated on the outside with an appropriate stationary phase; this is termed solid phase micro-extraction (SPME) [12]. In contrast to conventional SPE with packed-bed cartridges, the SPME syringe assembly design allows the combination of all the steps of sample preparation into one step and thus reduces sample preparation time, the use of organic solvents and disposal costs. The foremost advantage of the technique is improved detection limits [13]. A development of SPME, stir bar sorptive extraction (SBSE) was introduced as a novel sample preparation technique in 1999 [14]. SBSE is a sorptive and (in general) solventless extraction technique based on the same principles as SPME, but, instead of a polymer-

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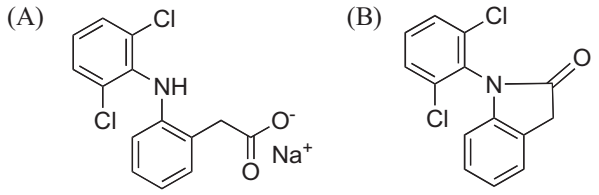

Fig. 1. Chemical structure of diclofenac (A), indolinone derivative of diclofenac (B).

coated fiber, a large amount of the extracting phase is coated on to a stir-bar. Extraction of an analyte from the aqueous phase sample into an extraction medium (e.g. polydimethylsiloxane (PDMS)) is controlled by the partitioning coefficient of the analyte between the silicone phase and the aqueous phase (K_{PDMS}/w) [15]. This partitioning coefficient is well correlated with octanol–water distribution coefficients ($K_{o/w}$) of the drugs. Due to the similarity of K_{PDMS}/w to $K_{o/w}$, chemists can predict extraction efficiencies (SBSE can be efficiently used for hydrophobic compounds with $\log K_{o/w} \geq 2$; and, a high enrichment factor could be obtained for analytes with $\log K_{o/w} > 5$) [15,16]. SBSE based methods have been traditionally used for estimation of various organic analytes such as polycyclic aromatic hydrocarbons (PAH) from aqueous samples/drinking water [17,18], pesticides [19], trace residues and contaminants in foods [20] etc. Most recently, SBSE has been used for extraction of drugs from various biological matrices such as urine [21,22], plasma [23–25] and tissues [26]. Application of SBSE to the extraction and estimation of drugs/impurities in pharmaceutical formulations has not been published in the literature.

The method presented in this paper is a proof of concept study for investigation of the applicability of the SBSE technique in the determination of a drug in liquid formulations. Diclofenac sodium was chosen as model drug. Diclofenac [2-[(2,6-dichlorophenyl)-amino-phenyl]acetic acid] (Fig. 1) is a synthetic non steroidal anti-inflammatory drug (NSAID) usually available as the sodium or potassium salt [27], and is widely used as an analgesic, anti-inflammatory and anti-arthritic agent [28]. Dosage forms for diclofenac (DIC) include tablets, capsules, gels, aerosols, ointments, suppositories, parenteral injections, eye drops (single and multiple use) containing varying amounts of DIC. For the present study estimation of DIC in liquid formulations i.e. eye drops (single and multiple uses) and injection was chosen. A literature survey for analytical methods available for estimation of DIC in formulations revealed many methods utilising a variety of analytical techniques such as UV spectroscopy [29], HPLC coupled with UV detection [29–31], HPLC coupled with fluorescence detection [32], HPLC coupled with mass spectrometry [33,34], capillary electrophoresis [35], potentiometric [36], gravimetric [37], densitometric [38], diffuse reflectance photometry [27], FT-Raman spectroscopy [39]. Further evaluation of the literature revealed sample preparation techniques such as hollow fiber-based liquid phase microextraction (HF-LPME) coupled with HPLC and diode array (DAD)–fluorescence (FLD) detectors (in series) for extraction of DIC from an aqueous matrix [40]. A literature survey for SBSE and DIC estimation revealed one method for the estimation of DIC in environmental water matrices [41].

The objective of the present study was to develop and validate a SBSE method for estimation of DIC and its application for determination of DIC in eye drops and injection formulations. Further, it was also intended to apply the developed method for determination of DIC from stressed injection formulations (autoclave and dry heat) and to assess the ability of the methodology to detect and quantify the structurally similar impurity/degradation product 1-(2,6-dichlorophenyl)-indolin-2-one (the indolinone derivative of DIC).

2. Materials and methods

2.1. Chemicals and materials

Diclofenac sodium was purchased from Sigma–Aldrich Ltd (Poole, UK). HPLC grade methanol and acetonitrile were supplied by Fisher Scientific (Loughborough, UK). HPLC grade water was obtained using a Millipore Direct-Q™ 5 Water System (Millipore, Watford, UK). Analytical grade sodium chloride and di-sodium-hydrogen phosphate were purchased from BDH (Poole, UK). Indolinone derivative of the diclofenac was synthesised, purified in-house and assayed for its content in the laboratory. All other reagents were of analytical grade except where otherwise stated. The diclofenac liquid formulations viz. Voltarol® Optha single dose drops (0.1% (w/v)), Voltarol® Ophtha multidose eye drops (0.1% (w/v)) and Voltarol® ampoules (75 mg/3 ml) were obtained from AAH Pharmaceuticals Ltd., Belfast, UK.

2.2. SBSE accessories

Four commercially available stir bars (Twister™) varying in length and thickness of polydimethylsiloxane (PDMS) (0.5 mm thickness and 10 mm length (PDMS volume ~24 μl), 1 mm thickness and 10 mm length (PDMS volume ~63 μl), 0.5 mm thickness and 20 mm length (PDMS volume ~47 μl) and 1 mm thickness and 20 mm length (PDMS volume ~126 μl)) were purchased from Gerstel (Gerstel GmbH, Mulheim Ruhr, Germany). The stir bars were pre-conditioned by sonication in a mixture of dichloromethane and methanol (1:1, v/v) for 10 min and dried with lint-free tissue. The dried stir bars were heated at 200 °C for 15 min before being used for extraction. A 15 position magnetic stirrer (0–1200 RPM) with integrated temperature control plate (IKA® multi position hotplate stirrer RT 15) was purchased from VWR International, UK.

2.3. Preparation of stock solutions, calibration standards and quality control (QC) samples

2.3.1. Stock solutions

A primary stock (PS) solution of DIC was prepared in methanol at 1 mg/ml (1000 μg/ml). The PS solution was diluted with methanol to give a secondary stock (SS) solution of 100 μg/ml. Working standards (WS) at 4, 10, 20, 30, 40, 60 and 80 μg/ml were prepared in methanol from the SS solution. Analytical standards (AS) at 100, 250, 500, 750, 1000, 1500, 2000 ng/ml were prepared in mobile phase by using respective working standards. All the stock solutions PS, SS and WS were stored at refrigerated condition (4 °C).

2.3.2. Aqueous calibration standards (ACS) and QC standards

An aqueous phase (AP) containing 15% (w/v) of sodium chloride was prepared in bulk. The pH of this AP was adjusted to 2.5 using hydrochloric acid. The 5 ml ACS standards were prepared by spiking AP with 25 μl of appropriate WS (so as to give 100, 250, 500, 750, 1000, 1500 and 2000 ng/5 ml). Similarly, three QC standards i.e. 100 ng/5 ml (LQC), 750 ng/5 ml (MQC) and 2000 ng/5 ml (HQC) were also prepared and further used in validation of the method. ACS and QC samples were spiked taking consideration of the final reconstitution volume of 1 ml which will yield 100, 250, 500, 750, 1000, 1500 and 2000 ng/ml concentrations. The total concentration of organic solvent in 5 ml ACS was not more than 0.5% (v/v).

2.4. Chromatographic system

The chromatography was carried out using the Waters® Alliance HPLC system (Waters, Ireland) which consisted of a Waters® 2695 separations module and a Waters® 2487 dual

wavelength absorbance detector which is a two-channel, tunable, ultraviolet/visible (UV/vis) detector. Empower® Software enabled the control of operating parameters, data capture, process and storage. The isocratic separation was performed using a Waters Symmetry® C18 column (5 μm, 3.9 mm × 150 mm) preceded by a Symmetry® guard column of matching chemistry. The mobile phase consisted of acetonitrile:methanol:0.01 M phosphate buffer (pH 4.1) (40:10:50, v/v/v) and was pumped at 1 ml/min. Injection volume was 20 μl and separation was carried out at controlled room temperature (CRT, 20 ± 2 °C). The UV detection wavelength was 281 nm and the analysis run time was 12 min.

2.5. SBSE optimisation

A systematic approach for the optimisation of the SBSE extraction and desorption was adopted to develop the sample preparation method. Assessment of appropriate extraction pH (2–5) was the first step evaluated in the SBSE extraction procedure. The pH which resulted in highest DIC recovery was selected for further optimisation. Suitability of desorption solvents (methanol or acetonitrile) was also assessed at this step. Optimisation of phase ratio was next step assessed. In this step, 5 ml ACS was kept constant and DIC was extracted with four available stir bars (with varying PDMS volume). The stir bar which showed highest recovery of DIC was selected for further optimisation. Furthermore, stirring speed (600, 900 and 1200 RPM), extraction temperature (CRT, 30 and 40 °C) and extraction time (up to 120 min) were optimised considering the highest DIC recovery. Once pH, phase ratio, stirring speed, temperature and extraction time were optimised, the effect of ionic strength on the recovery of DIC was assessed at 5%, 10% and 15% (w/v) concentration of sodium chloride. The salt concentration which resulted in highest DIC recovery was selected for the final extraction method.

Once the extraction conditions were optimised for the DIC, various desorption conditions were assessed. For desorption, two methods i.e. magnetic stirring and sonication were tested. In the magnetic stirring method, the stir bar was stirred at three stirring speeds (600, 900 and 1200 RPM) to determine the optimum desorption stirring speed. Once the stirring speed was optimised, desorption time (up to 40 min) was assessed. The stirring speed and desorption time which resulted in highest DIC recovery were selected. In the case of the sonication method, stir bars were immersed in 1 ml mobile phase/3 ml of acetonitrile and were sonicated for up to 40 min.

2.6. Optimised sample preparation protocol and re-conditioning of stir bars

The final sample preparation protocol was as follows; 5 ml aliquot of AP (containing sodium chloride 15% (w/v), adjusted to 2.5 pH) spiked with DIC of known (ACS/QC) or unknown concentration was placed in a glass vial (25 ml capacity) and placed on a magnetic stirrer. A pre-conditioned stir bar was added to this solution and stirred at 600 RPM for 120 min at CRT. After 120 min, the stir bar was removed using forceps and washed with 1 ml of HPLC grade water. The stir bar was dried with lint free tissue paper. 3 ml of acetonitrile was placed in fresh glass vial and the dried stir bar was added to this vial. Desorption was carried out by stirring at 600 RPM for 40 min at CRT. After 40 min, the stir bar was removed and the acetonitrile was transferred to 5 ml glass tube and evaporated to dryness. The dried residue was reconstituted in 1 ml of mobile phase and 20 μl was injected for HPLC analysis.

After each extraction, the stir bar was added to a mixture of dichloromethane and methanol (1:1, v/v) and sonicated for 10 min. After sonication, the stir bar was removed and dried with lint free tissue paper. The dried stir bar was heated at 200 °C for 15 min. After cooling, the stir bar was stored at room temperature for next use.

2.7. Method validation

Once the SBSE extraction and desorption conditions were optimised and consistent results were obtained with final SBSE conditions, the method was taken for validation. All validation experiments were performed according to the ICH guidelines for validation of analytical methods [42].

2.7.1. Specificity

Specificity was determined as non-interference at the retention time of DIC by other impurities after SBSE extraction. 5 ml of blank AP was extracted and analysed with pre-conditioned stir bar (Section 2.6). The chromatogram was checked for any interference at the retention time of DIC.

2.7.2. Absolute recovery of DIC from aqueous QC standards

Recovery of DIC from aqueous QC standards was assessed ($n = 5$) at LQC, MQC and HQC concentrations. The prepared QC samples were extracted and analysed by the procedure described in Section 2.6. Recovery was calculated by using the following formula;

$$\%\text{Recovery} = \frac{\text{Observed concentration}}{\text{Nominal concentration}} \times 100$$

2.7.3. Calibration curve and linearity

Linearity of the proposed method was carried out by analysing ACS (100, 250, 500, 750, 1000, 1500, 2000 ng/ml) on five different days. Seven-point calibration curves were constructed by plotting peak area of DIC vs DIC concentrations. The slope, intercept and correlation coefficient were calculated using the least square regression method. A one-way ANOVA was performed to assess the linearity of the assay based on the values observed for each concentration during the replicate measurement of the ACS.

2.7.4. Accuracy and precision

The accuracy and precision of the developed method was determined by replicate analysis ($n = 5$) of QC samples spiked with DIC at three concentrations i.e. LQC, MQC and HQC on five different days. Within-day accuracy and precision were calculated on a single day using five replicates at each concentration level. Between-day accuracy and precision were calculated using five replicates at each concentration level over five consecutive days. Accuracy was determined by calculating the % bias from the theoretical concentration by using the following formula;

$$\%\text{Bias} = \frac{\text{Observed concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100$$

Day-to-day (inter-day) and within-day (intra-day) precision was calculated in terms of percent coefficient of variation (CV %).

Accuracy of the method was also determined by standard addition method using DIC formulations. Each formulation was diluted with methanol to give 100 and 750 ng/ml concentrations ($n = 3$). These dilutions were analysed and the percentage recovery was calculated. Furthermore, to these pre-analysed concentrations (100 and 750 ng/ml concentrations), known amount of DIC standard was added (150–100 ng/ml concentration and 250–750 mg/ml concentration) and extracted as described. The recoveries of the added concentration were determined and % bias calculated as indicator of accuracy.

2.7.5. Sensitivity (limit of detection (LOD) and limit of quantification (LOQ))

LOD and LOQ values were calculated based on the standard deviation of the response (σ) and the slope (S) of calibration curves prepared for the DIC according to the following equations;

$$\text{LOD} = 3.3\left(\frac{\sigma}{S}\right)$$

$$\text{LOQ} = 10\left(\frac{\sigma}{S}\right)$$

where σ was estimated from the standard deviation of the y-intercepts of the regression lines.

2.7.6. Stability

Stock solution stability was tested at the selected storage condition (4 °C) after a period of one month. The stock solution was considered stable if 95–105% of the nominal concentration was found when compared with a freshly prepared stock solution. Stability of spiked ACS stored at CRT was tested at zero and 180 min.

2.7.7. Robustness

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters. To evaluate method robustness, a few of the parameters were deliberately varied. The acetonitrile/methanol from different lots of the same company and of different companies was used. pH of the mobile phase was changed by ±0.1 unit and its effect on retention time of DIC was also assessed.

In the case of the SBSE method, it was important to assess memory effect/carryover of stir bars. Stir bars (n = 5) after extraction followed by re-conditioning were again desorbed (600 RPM, at CRT for 40 min) in 3 ml of acetonitrile. The acetonitrile was evaporated and residue was reconstituted and analysed.

Furthermore, as each stir bar is used more than once in the whole of method development, validation and application of the method to formulations, inter and intra stir bar accuracy and precision was assessed. To assess this, five stir bars were selected and tagged. QC solutions at LQC, MQC and HQC were prepared (n = 5). In the first instance, one stir bar was used to analyse all the replicates of each QC concentration. In the second instance, five separate stir bars were used to analyse each replicate of three QC concentrations. From the recovery of the DIC, accuracy and precision were determined by formula (see Section 2.7.4).

2.8. Application of the method for analysis of DIC in liquid formulations

The developed and validated SBSE method was further applied for analysis of DIC in three liquid formulations i.e. Voltarol® Optha single dose drops (DIC; 0.1% (w/v)), Voltarol® Ophtha multidose eye drops (DIC; 0.1% (w/v)) and Voltarol® ampoules (DIC; 75 mg/3 ml). These three formulations were selected based on the complexity of their chemical/excipient composition. In addition, to compare the results of the SBSE method, an alternative published method [34] was adopted as a reference method (RM) so as to compare the results of proposed and established method.

2.8.1. Assay

Assay of the three formulations was performed using the SBSE method and the RM. All three formulations were serially diluted with methanol/AP to give a 40 μg/ml concentration of DIC (n = 3). A 25 μl aliquot of this dilution was added to AP (5 ml) and SBSE extraction was carried out (theoretical concentration 1000 ng/ml). The concentration of DIC was measured against a freshly prepared calibration curve and % DIC concentration was calculated and assay concentrations were recorded. Assay concentrations obtained using SBSE and RM were further compared.

2.8.2. Forced degradation of DIC and formation of cyclic impurity

DIC is manufactured via a stable intermediate, 1-(2,6-dichlorophenyl) indolin-2-one, which is commonly known as the indolinone derivative, by heating in the presence of sodium hydroxide [43]. The impurity level of the indolinone derivative in diclofenac sodium raw material is limited to only 0.2% as detailed in the British Pharmacopoeia (BP). It has been reported that, when DIC injection formulation is either autoclaved or exposed to dry heat conditions, the indolinone derivative of DIC is formed [43]. The selected RM can differentiate and estimate DIC from its indolinone impurity.

The SBSE method was further challenged to selectively extract and estimate DIC from its indolinone impurity. To perform this experiment, DIC ampoules (Voltarol® ampoules (75 mg/3 ml), n = 3) were autoclaved at 121 °C, 1.1 kg/cm^2 pressure for 25 min. Similarly, DIC ampoules (Voltarol® ampoules (75 mg/3 ml), n = 3) were kept at 145 °C in an oven (dry heat) for a period of 60 min. The ampoules from the autoclave and dry heat conditions were further assayed by both the SBSE and RM methods. The results for assay values of DIC acquired from both the methods were compared. In the case of the SBSE method, the chromatograms of the forced degraded samples were also monitored at 254 nm in order to monitor indolinone impurity (as 254 nm is λ_{max} of indolinone). Analytical standard solutions of indolinone were also injected so as to determine the retention time and resolution of indolinone and DIC.

3. Results and discussion

3.1. Analytical method development

In the present method, a C18 column was used successfully for development of the HPLC method for quantitative estimation of the DIC in the pharmaceutical formulations. A wavelength 281 nm was selected for the determination of DIC as it gave less interference from various other excipients and also resulted in a very stable baseline. Various solvent systems studied for optimization of the mobile phase were combinations of water, methanol, acetonitrile, phosphate buffers and acetate buffer of range pH 3–5. It was observed that, chromatographic parameters of the DIC were sensitive to pH change. A lower pH (3–3.5), the DIC peak showed tailing with compromised peak symmetry and pH higher than 4.5 resulted in peak broadening. Also, at lower pH (3–3.5) the retention time of DIC was between 4 and 5 min while at pH 4.5 and above, the retention time for DIC was between 6.5 and 8 min. Among all the pH studied with different buffers, pH 4.1 phosphate buffer showed optimum peak properties. Finally, the combination of acetonitrile, methanol and phosphate buffer (0.01 M, pH 4.1) in the ratio 40:10:50 (v/v/v) % resulted in an optimized DIC chromatogram with a retention time of 6.8 ± 0.1 min, and a total run time of 10 min.

3.2. SBSE optimisation

SBSE with PDMS as the extraction phase follows extraction by partition theory, wherein the analyte with favourable physicochemical properties (such as partition coefficient) is partitioned into the PDMS layer and further diffuses into the bulk coating during the extraction process. When compared to adsorption, SBSE extraction is non-competitive and the extent of analyte extracted from any sample is independent of the matrix components. It is also well known that the retaining capacity of the PDMS for a given analyte is not influenced by other analytes in the bulk of extraction

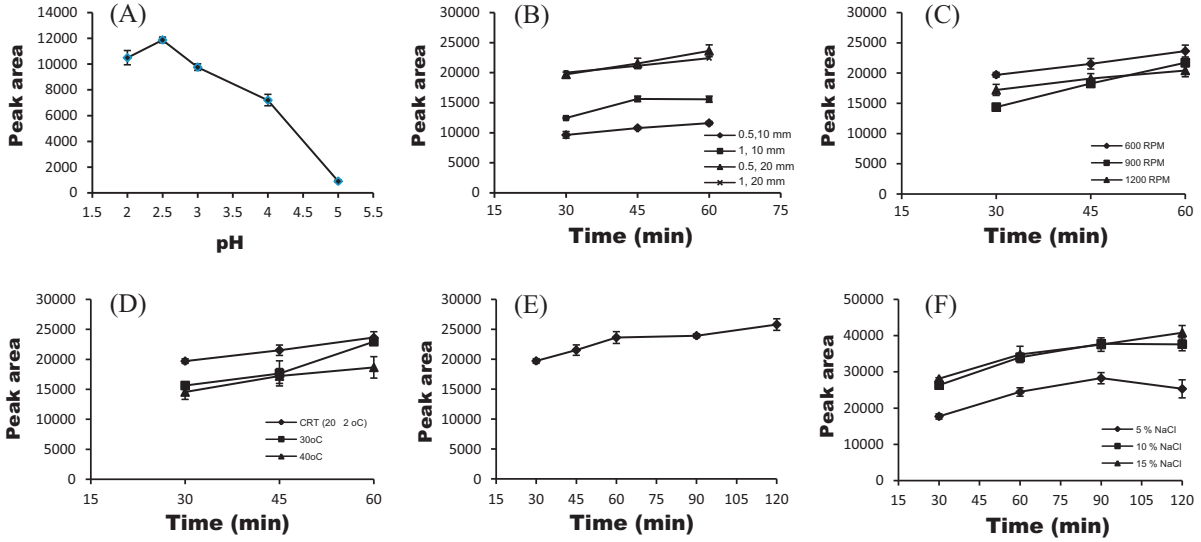

Fig. 2. Results of SBSE extraction optimisation process (A) pH optimisation, (B) phase ratio optimisation, (C) stirring speed optimisation, (D) temperature optimisation, (E) extraction time optimisation, (F) ionic strength optimisation.

phase because each analyte has its own partition equilibrium in the PDMS phase [16]. It is possible to calculate the theoretical recovery of SBSE by employing following formula;

$$\text{Theoretical recovery} = \frac{(K_{o/w}/\beta)}{(1+K_{o/w}/\beta)} = \frac{1}{(\beta/K_{o/w}+1)}$$

where $\beta = V_w/V_{PDMS}$, V_{PDMS} is the volume of PDMS and V_w is the volume of aqueous phase. DIC is a molecule with log P of 4.5 [43]. The calculation of theoretical recoveries for DIC, based on 5 ml sample volume (V_w) and any stir bar (PDMS volume ranging from 24 to 126 μl), showed ~100% recovery of DIC with SBSE. However, this theoretical recovery depends on various factors that affect the efficiency of SBSE.

Various experiments investigating parameters that affect extraction (such as extraction pH, phase ratio (β), stirring speed, extraction time, temperature, and ionic strength) and desorption (solvent, method, time) of DIC were investigated. Fig. 2 shows the results of the extraction optimisation process. The effect of sample pH on the extraction efficiency was investigated in the range from 2 to 5. It can be seen from Fig. 2A that, pH 2.5 resulted in highest DIC recovery. The observed effect could be explained on the basis of the pK_a value of DIC (pK_a 4.2) indicating that less than 2% of the DIC will be in the ionised form. It is known that to achieve optimal extractions by SBSE, the compounds should be present in their neutral/unionised form [44]. At pH 2.5, the extent of ionised DIC present in aqueous solution is low leading to higher extraction by the lipophilic PDMS layer of the stir bar.

When all four commercially available stir bars were evaluated for DIC extraction (Fig. 2B), it can be clearly seen that stir bars of 20 mm length showed overall highest recovery of DIC when compared to 10 mm length. When comparing two 20 mm stir bars, both stir bars resulted in comparable DIC recovery. However, comparing DIC peak areas at 45 and 60 min time intervals, stir bars with dimension 20 mm length and 0.5 mm thickness showed higher DIC recovery as compared to 1 mm thickness and were thus selected.

Stirring speed determines the amount of contact time between the analyte and PDMS extraction phase is one of the important factors that affects the efficiency of the SBSE method. Investigation of the effect of speed (Fig. 2C) on extraction efficiency of DIC showed that, a stirring speed of 600 RPM was most optimum resulting in highest recovery of DIC from aqueous solution.

Fig. 2D shows results of the effect of extraction temperature on the DIC recoveries. It can be seen from the profiles that as extraction temperature increased, DIC recoveries dropped. It is well known that temperature usually affects diffusion coefficients of the analytes in the solution state, which directly affects the SBSE recovery. However, by increasing the temperature of the sample solution, the diffusion coefficients of the analytes will increase, but at the same time their partition coefficients may decrease [44], leading to lower SBSE recovery. This phenomenon can best explain the results obtained in this study.

According to the literature, extraction time is one of the most important factors that affect the extraction process [45–47]. The extraction time directly affects the equilibrium time of the analyte with the PDMS extraction phase. Although equilibrium is not essential for analysis, the time of extraction must be carefully controlled to ensure reproducibility, and the extent of the extraction will be lower if the extraction is stopped prior to equilibrium [48]. Fig. 2E shows effect of extraction time on DIC recovery. It can be concluded from the figure that, at 120 min, highest recovery of DIC was achieved. This 120 min was selected as the optimum extraction time for SBSE method.

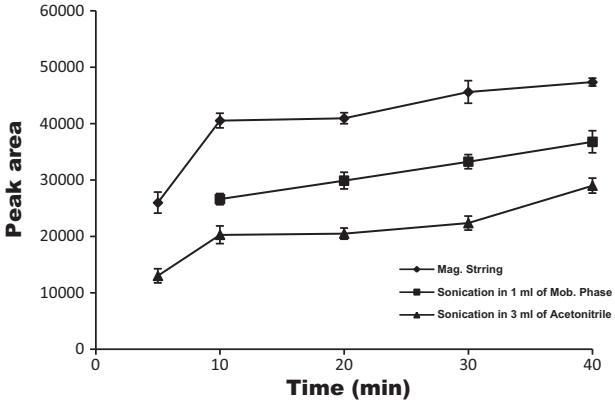

Fig. 3. Results of SBSE desorption conditions.

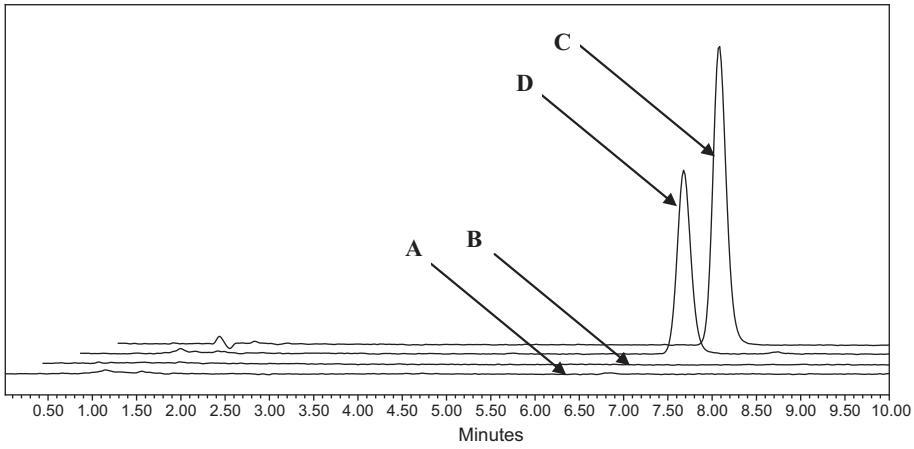

Fig. 4. Overlay chromatograms of blank mobile phase (A), SBSE extracted blank AP (B), DIC analytical standard (1000 ng/ml) (C) and DIC ACS (1000 ng/ml) (D).

Table 1
Absolute recovery of DIC from aqueous QC standards by SBSE method.

Concentration (ng/ml)	Absolute recovery (%)[a]	% R.S.D.
LQC (100)	70.82 ± 2.20	3.11
MQC (750)	70.05 ± 1.40	2.00
HQC (2000)	70.68 ± 0.78	1.10

[a] The values are given as mean ± SD of $n = 5$ determinations.

It is well known that the extraction efficiencies for a large number of compounds can be enhanced by increasing the ionic strength of the sample solution [44]. This is generally phrased as a "salting out" effect. Fig. 2F shows the effect of NaCl concentration on the DIC recovery. The results indicate that as NaCl concentration increased, DIC recovery also increased. However, recovery of DIC from 10% and 15% (w/v) of NaCl were comparable, at 120 min, 15% NaCl resulted in highest DIC recovery and thus was selected as the optimum concentration.

Table 2
Accuracy and precision data of DIC SBSE method ($n = 5$).

Concentration (ng/ml)	Intra-day accuracy (% Bias)	Inter-day accuracy (% Bias)	Intra-day precision (% R.S.D.)	Inter-day precision (% R.S.D.)
LQC (100)	−2.76	−1.55	3.83	1.98
MQC (750)	2.12	0.20	0.93	2.97
HQC (2000)	−1.59	0.63	0.41	1.34

Fig. 3 shows results of the SBSE desorption optimisation. Overall, it can be seen from the figure that, the magnetic stirring method resulted in the highest recovery of DIC as compared to sonication method (1 ml mobile phase/3 ml acetonitrile). Among the solvents tested for desorption of DIC, higher recoveries of DIC were obtained with acetonitrile as compared to methanol. When the effect of RPM was assessed for desorption, 600 was found to be the optimal stirring speed. Assessment of desorption time showed highest recovery of DIC at 40 min. Thus the final optimised SBSE conditions were; for extraction—pH of 2.5, 0.5 mm thickness and 20 mm length stir bar, 600 RPM, CRT, 120 min extraction time and 15% (w/v) of NaCl concentration followed by desorption in 3 ml of acetonitrile at 600 RPM at CRT for 40 min.

3.3. Method validation

3.3.1. Specificity

Specificity of the SBSE method was assessed by analysing blank AP. Fig. 4A shows chromatograms of blank and DIC analytical standard. Fig. 4B shows chromatograms of the SBSE extracted blank AP and DIC ACS. It can be seen from the chromatogram of blank AP that, no interfering peaks were observed at the retention time of DIC.

3.3.2. Absolute recovery of DIC from aqueous QC standards

Absolute recovery of DIC was assessed at three QC concentration of DIC. Table 1 shows data on recoveries of DIC at three QC levels. At all three QC levels average recovery was found to be

Table 3
Accuracy of the method by standard addition method.

Formulation	Concentration (ng/ml)	Amount added (ng/ml)	Theoretical amount (ng/ml)	Amount found (ng/ml)[a]	Recovery (%)[a]	% Bias
Voltarol® Optha single dose drops	100	–	100	101.39 ± 4.51	–	–
	100	150	250	257.26 ± 9.60	102.90 ± 3.84	+2.90
	750	–	750	754.64 ± 8.56	–	–
	750	250	1000	1013.90 ± 40.57	101.39 ± 4.06	+1.39
Voltarol® Ophtha multidose eye drops	100	–	100	105.25 ± 4.77	–	–
	100	150	250	259.24 ± 3.51	103.70 ± 1.40	+3.70
	750	–	750	764.65 ± 7.85	–	–
	750	250	1000	980.41 ± 47.02	98.04 ± 4.70	−1.96
Voltarol® ampoules	100	–	100	100.68 ± 2.20	–	–
	100	150	250	249.52 ± 13.53	99.81 ± 5.41	−0.19
	750	–	750	763.07 ± 6.82	–	–
	750	250	1000	960.82 ± 34.82	96.08 ± 3.48	−3.92

[a] The values are given as mean ± SD of $n = 3$ determinations.

Table 4
Inter-stirbar–intra-stirbar accuracy and precision ($n = 5$).

Concentration (ng/ml)	Inter-stir bar		Intra-stir bar	
	Accuracy (% Bias)	Precision (% R.S.D.)	Accuracy (% Bias)	Precision (% R.S.D.)
LQC (100)	−3.86	3.38	−1.55	1.98
MQC (750)	−0.67	1.72	0.20	2.54
HQC (2000)	0.92	1.28	−0.52	2.14

in excess of 70%. The recovery of DIC found using the developed SBSE method was much higher than the previously reported SBSE method (34.6 ± 6.9%) for extraction of DIC from environmental aqueous matrices [41].

3.3.3. Calibration curve and linearity

The calibration curve (peak area (μV s) vs concentration (ng/ml)) of DIC was found to be linear from 100 to 2000 ng/ml. The calibration data was subjected to least square regression analysis and the mean linear regression equation obtained for the proposed SBSE method was $Y = 35.04X - 198.24$ where Y is the area under the peak in μV s and X is the concentration of analyte in ng/ml. The correlation coefficient value was highly significant ($r = 0.9999$). Further, one-way ANOVA was performed to assess the linearity of the assay based on the values observed for each drug concentration during the replicate measurement of the standard solutions during construction of calibration curve. The calculated F-value ($F_{calc} = -0.00453$) was found to be less than the critical F-value ($F_{crit} = 2.69$) at 5% level of significance, indicating that there was no significant variation among all the replicate measurements of all the calibration sets.

3.3.4. Accuracy and precision

The results obtained for intra and inter day accuracy and precision analyses are summarized in Table 2. Results of accuracy determination by standard addition method employing DIC formulation are summarised in Table 3. Accuracy and precision of the method was evident from low values of % bias and % R.S.D., indicating that the developed method is highly repeatable and reproducible. From Table 3, it can be seen that, almost 100% of externally added DIC was recovered by the SBSE method. Obtained recovery (amount found) and % bias values further confirms the accuracy of method.

Table 5
Assay values of various formulations by SBSE and reference method.

Formulation	Assay by SBSE method (%)[a]	Assay by reference method (%)[a]
Voltarol® Optha single dose drops	96.99 ± 1.56	99.46 ± 0.81
Voltarol® Ophtha multidose eye drops	100.93 ± 3.10	102.86 ± 1.69
Voltarol® ampoules	103.37 ± 1.40	104.15 ± 0.40

[a] The values are given as mean ± SD of $n = 3$ determinations.

3.3.5. Sensitivity (limit of detection (LOD) and limit of quantification (LOQ))

The LOD and LOQ of the developed method were found to be 16.06 and 48.68 ng/ml respectively for DIC, which indicates the sensitivity of the developed method.

3.3.6. Stability

Stock solution stability for PS was tested at refrigerated storage (4 °C) for a period of one month. The concentration of DIC PS was found be within 95–105% of the nominal concentration when compared with a freshly prepared stock solution. Spiked ACS (at three QC levels) samples were found to stable at CRT for up to 180 min (assay found within ±3% of the assay at zero time (freshly prepared standards).

3.3.7. Robustness

In robustness testing, it was observed that acetonitrile/methanol of different lots from the same manufacturer or different sources of acetonitrile/methanol had no significant influence on the assay of the DIC. No significant differences in peak parameters such as retention time, peak area, asymmetry factor, capacity factor etc were observed. For the developed method, varying the pH of the phosphate buffer by ±0.1 did not significantly affect the peak parameters and sensitivity of the method. Slight changes to percentage (±1) of aqueous or organic components of mobile phase did not alter the chromatographic parameters significantly.

Results of the assessment of the memory effect/carry over by stir bars did not show any significant carry over effect. This confirms that, the conditioning protocol (after and before each extraction) was efficient in removing any residual impurities adsorbed on to the stir bars.

Results of inter- and intra stir bar accuracy and precision are presented in Table 4. Low values of % bias and % R.S.D. at all QC levels for both intra stir bar and inter stir were highly significant. It can be concluded from the results that, each stir bar used in this

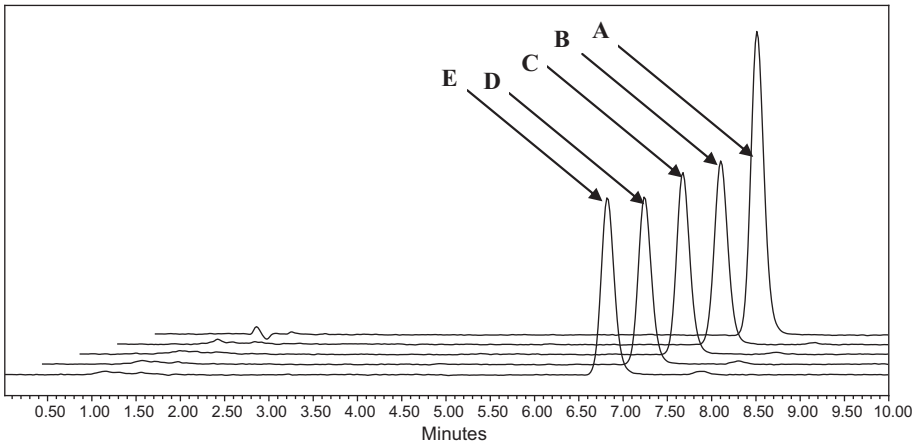

Fig. 5. Overlay chromatogram of DIC analytical standard (1000 ng/ml) (A), DIC ACS (1000 ng/ml) (B), DIC extracted from Voltarol® Optha single dose eye drops (C), Voltarol® Ophtha multidose eye drops (D), and Voltarol® ampoules (E).

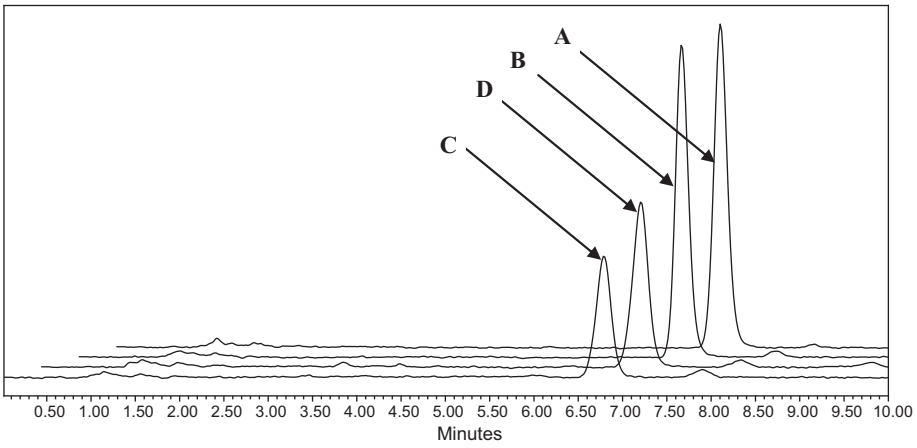

Fig. 6. Chromatograms of DIC ACS (1000 ng/ml) (A), DIC extracted from Voltarol® ampoules (B), DIC extracted from dry heat degradation (C) and DIC extracted from wet heat (autoclave) degradation (D).

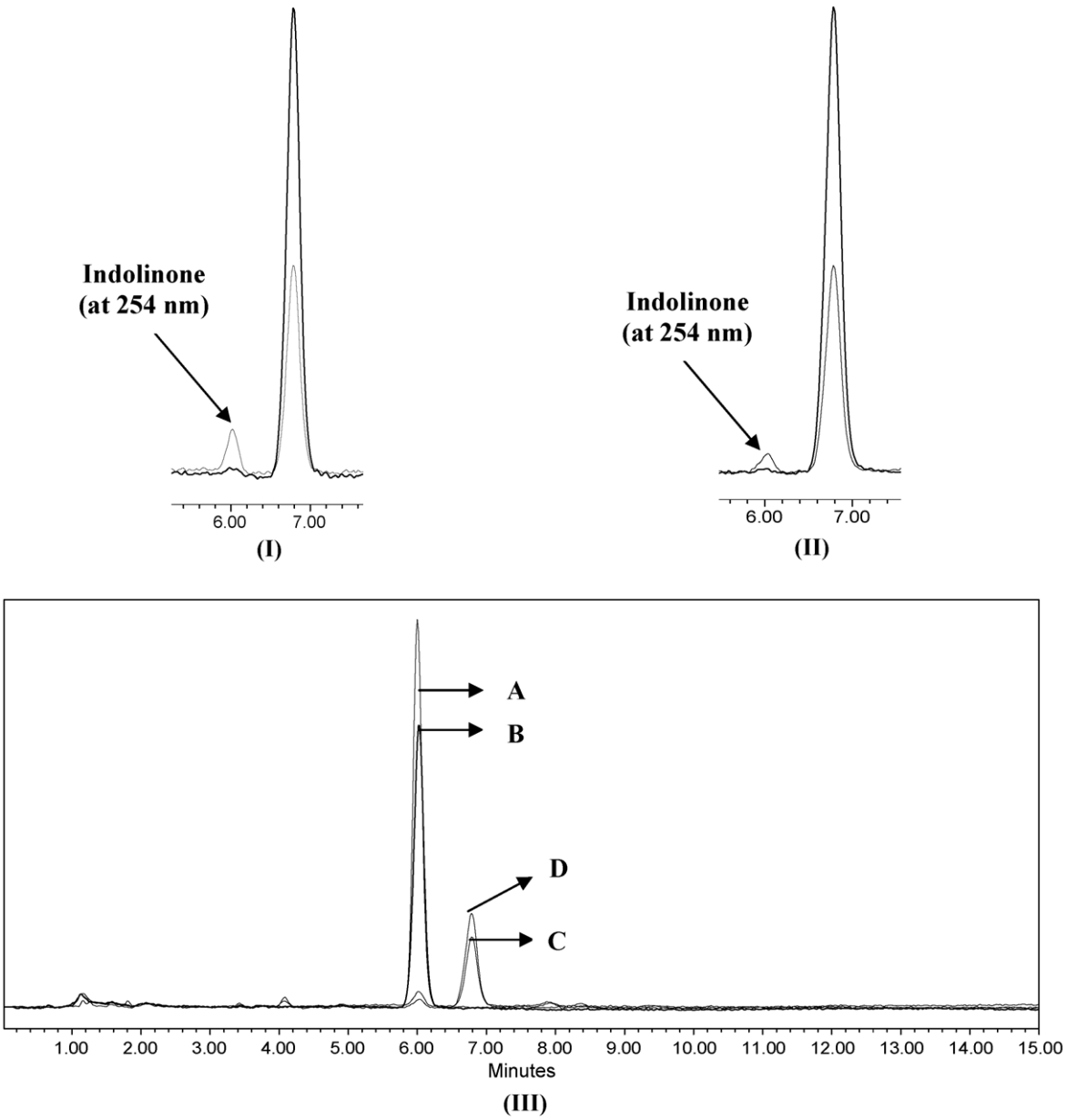

Fig. 7. Overlay chromatograms of DIC extracted from Voltarol® ampoules after dry heat (I) and wet heat (II) degradation at 254 and 281 nm, overlay chromatograms (III) of indolinone analytical standard (1000 ng/ml) (A), Indolinone ACS (1000 ng/ml) (B), DIC extracted from dry heat degradation (C) and DIC extracted from wet heat (autoclave) degradation (D) at 254 nm.

Table 6
Study of forced degradation of DIC from Voltarol® ampoules.

Condition	% Assay SBSE method[a]	Reference method[a]
Initial assay (no stress)	103.37 ± 1.40	104.15 ± 0.40
Dry heat	59.04 ± 3.79	58.93 ± 0.84
Autoclave	84.61 ± 8.94	89.01 ± 1.13

[a] The values are given as mean ± SD of $n = 3$ determinations.

method gave reproducible results. This can be attributed to proper conditioning of the stir bars. In this study, each stir bar was used more than 125 times.

3.4. Application of the method for analysis of DIC in liquid formulations

The developed and validated SBSE method was successfully applied for the assay of DIC from liquid formulations. The selected DIC formulations represent a simple to moderately complex formulation matrix. The Summary of Product Characteristics (SPC) of Voltarol® Optha single dose drops indicated list of excipients such as boric acid, Polyoxyl 35 castor oil, tromethamine and water for injection. The SPC of Voltarol® Ophtha multidose eye drops indicated excipients such as benzalkonium chloride, disodium edentate, hydroxypropyl γ-cyclodextrin, hydrochloric acid, propylene glycol, trometamol, tyloxapol, and water for injection and the SPC of Voltarol® ampoules indicated excipients such as mannitol, sodium metabisulphite (E.223), benzyl alcohol, propylene glycol, sodium hydroxide and water.

Results of assay values of all three selected formulations by both the SBSE and the RM methods are given in Table 5. It can be seen from the table that the assay values determined by the SBSE method of all three formulations are comparable to that of RM method. The standard deviation values of the SBSE method also indicated precision of the method. Fig. 5 shows chromatograms of DIC analytical standard, DIC ACS, DIC extracted from Voltarol® Optha single dose drops, Voltarol® Ophtha multidose eye drops, and Voltarol® ampoules. It can be seen from the figure that, no additional peaks were observed in the chromatograms of DIC extracted from all three formulations. No interference or major difficulties were found in the analysis of any formulation. This study also confirms the selectivity of the proposed method.

In addition, the ability of SBSE method for selective extraction and quantitation of DIC from its structurally similar impurity indolinone was also assessed. Voltarol® ampoules were exposed to dry and wet heat conditions. Table 6 shows results of the assay of DIC from non-stressed and stressed Voltarol® ampoules. It can be seen from the table that, dry heat resulted in more extensive degradation of the DIC as compared to wet heat (autoclave). The assay values obtained by the SBSE method for all stressed and non-stressed formulations were comparable to that RM method. Fig. 6 shows chromatograms of DIC ACS (A), DIC extracted from Voltarol® ampoules (B), DIC extracted from dry heat degradation (C) and DIC extracted from wet heat (autoclave) degradation (D). It can be seen from the chromatograms that, an additional peak was observed before the retention time of DIC. To confirm the identity of this peak, an analytical standard of indolinone was analysed under present chromatographic conditions. Based on retention time and literature reference, this additional peak was confirmed as indolinone (Fig. 7, I and II). As λ_{max} of indolinone is around 254 nm, the samples were reinjected and monitored at 254 nm. Fig. 7, III shows chromatograms of indolinone analytical standard (A), indolinone ACS (B), DIC extracted from dry heat degradation (C) and DIC extracted from wet heat (autoclave) degradation (D) at 254 nm. Thus it can be inferred that indolinone formation occurred in both the stress conditions. It can also be concluded from this study that, the SBSE method can also selectively extract the indolinone impurity along with DIC. The chromatographic resolution of indolinone and DIC further improves the efficiency of the proposed method. The quantitative estimation of indolinone was not carried out using the proposed method as it was not developed and validated for the determination of indolinone. However, the SBSE method has the potential to be used as standard method for impurity profiling of DIC in bulk and in pharmaceutical formulations the same way as the RM/pharmacopoeial method.

4. Conclusions

A SBSE method for estimation of DIC from liquid formulations was developed and validated. Isocratic chromatographic separation of DIC showed retention time of 6.2 min and overall sample analysis time was less than 12 min. Optimisation of the SBSE protocol showed that factors such as matrix pH, phase ratio, extraction time have significant effects on the recovery of DIC. The ionic strength (% NaCl) was one of the most significant factors that resulted in higher recoveries of DIC from the AP. Similarly, magnetic stirring was found to be the most efficient method for DIC desorption. Desorption time was one of the most significant factors that contributed to higher recoveries of DIC. As temperature had a negative impact on the recovery of DIC, extraction at CRT made the method simple and easy to perform. Over all, the whole of SBSE protocol was found to be easy to operate and more importantly was highly reproducible.

Results of method validation suggests that, the developed method was specific, linear accurate, precise and suitable for estimation of DIC from formulations (having a simple to moderately complex matrix). Inter and intra stir bar accuracy and precision confirmed the robustness of the method. The stir bar conditioning protocol was found to efficient indicating no carryover of DIC from run to run. The, results obtained using the SBSE method were comparable to that of the reference method. It was also confirmed that, the SBSE method can selectively extract the indolinone from the formulation matrix (probably due to favourable physicochemical properties) and chromatographic conditions can resolve it from DIC. The method could be further developed and validated for simultaneous quantitative estimation of DIC and indolinone.

The authors believe that this is the first application of SBSE to the analysis of drugs in formulated products. This proof of concept study was conducted to explore the efficiency of SBSE in analysis of DIC (and impurity) in formulated samples. We have now initiated studies aimed at the application of this methodology to more complex formulations of DIC e.g. gels, suppositories etc and we are also looking at the determination of DIC and metabolites in biological matrices such as urine.

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