Talanta 85 (2011) 1948–1958

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

# Determination of diclofenac from paediatric urine samples by stir bar sorptive extraction (SBSE)–HPLC–UV technique

Prashant Laxman Kole*, Jeff Millership, James C. McElnay

*PKPD Core Facility, School of Pharmacy, Queens University Belfast, 97 Lisburn Road, Belfast BT9 7BL, United Kingdom*

ARTICLE INFO

*Article history:*
Received 3 February 2011
Received in revised form 20 June 2011
Accepted 7 July 2011
Available online 18 July 2011

*Keywords:*
Stir bar sorptive extraction
Sample preparation
Diclofenac
Paediatric
Urine

ABSTRACT

A novel stir bar sorptive extraction (SBSE) method coupled with high performance liquid chromatography (HPLC) and UV detection for the extraction of diclofenac (DIC) from paediatric urine samples has been developed and validated. Selectivity and sensitivity being the prime objectives of the bioanalytical method for clinical samples, an optimised SBSE protocol was developed that selectively extracted DIC from various concurrently administered drugs. The validated assay was found to be linear ($r = 0.9999$) over a concentration range of 100–2000 ng mL$^{-1}$. SBSE showed consistent recoveries (~70%) of DIC across the validated linearity range. Overall, the method exhibited excellent accuracy and precision across all QC concentrations, tested over three days. Calculated LOD and LOQ were found to be 12.03 ng mL$^{-1}$ and 36.37 ng mL$^{-1}$, respectively, however, for the experimental purposes, 100 ng mL$^{-1}$ was considered as the validated LOQ (accuracy and precision at this LQC was <20%). Further, studies on various attributes of the stir bar/SBSE, showed no significant inter- and intra-stir bar variability for DIC extraction. There was no carryover effect with re-use of conditioned stir bars and for the first time, a systematic investigation on the effect of ageing of stir bars on their extraction efficiency was carried out. Results showed that, for the present study, stir bars which were used 150 times were still functional based on in-house acceptance criteria and extraction efficiency. The validated method was successfully applied to the analysis of DIC in paediatric clinical trial samples.



## 1. Introduction

Unlicensed (medicines which do not have product licence/marketing authorisation) and off-label (e.g. prescribed for an unapproved indication) use of medicines for the treatment of various ailments in the paediatric population is prevalent across the United Kingdom [1], Europe [2], United States of America [3] and other countries [4]. This practice raises concerns about the safety and efficacy of the medicines used in paediatric patients [5]. To address this issue, leading regulatory authorities across the world have taken initiatives to stimulate and support paediatric clinical trials to improve the evidence base and allow more medicines to be licensed for paediatric use [6].

Diclofenac [2-[(2,6-dichlorophenyl)-amino-phenyl]acetic acid] (Fig. 1), a synthetic non-steroidal anti-inflammatory drug (NSAID), is widely used as an analgesic, anti-inflammatory and anti-arthritic agent in adults[7]. It is also widely used in an unlicensed fashion to manage acute pain in children across the UK. There is no licensed paediatric oral formulation in the UK and definitive dosing guidelines are yet to be established for its use in children [8]. In 2007, it was included in the European Medicines Agency's (EMEA) priority list of off-patent medicines that require assessment in paediatric patients, noting that data on pharmacokinetics, on long term safety in all the paediatric age groups and on the safety and efficacy in premature infants were required [9].

The sensitive and selective bioanalytical method discussed herein is part of an ongoing research programme which involves the population pharmacokinetic (POPPK) study of diclofenac (DIC) used in an off-label or unlicensed manner in neonates and children, prescribed for their routine care, using opportunistic sampling and sparse data analysis. The research has involved collection of DIC blood concentration data together with complimentary urinary excretion data.

One of the bioanalytical requirements in a POPPK clinical trial involving opportunistic sampling is selectivity, as patients are receiving concurrent drugs which might be excreted in the urine (as such or in the form of metabolites) and thus, the method to be used for quantification should be selective and specific to extract and quantify the analyte of interest. Secondly, as approximate concentrations of the analyte of interest in the target biomatrix are not known, the sensitivity/analytical range of the bioanalytical method should be wide enough to accommodate the variability and avoid further 'over-the curve dilution' validation steps.

* Corresponding author. Tel.: +44 28 90972361; fax: +44 28 90247794.
*E-mail address:* prashantkole@gmail.com (P.L. Kole).

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doi:10.1016/j.talanta.2011.07.016

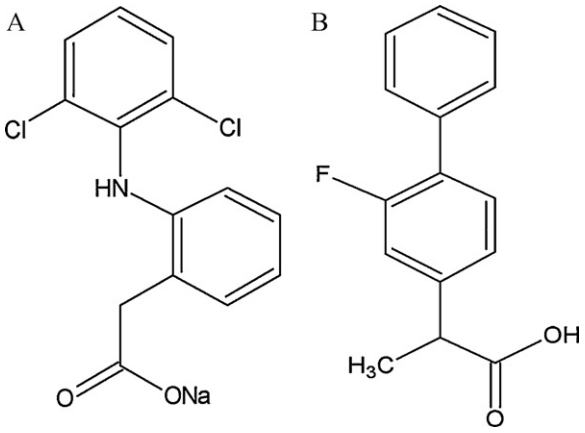


**Fig. 1.** Chemical structures of diclofenac (A) and flurbiprofen (B).

To satisfy the requirements, the sample preparation technique plays an important role. Recently a novel solventless extraction technique called 'stir bar sorptive extraction' (SBSE) has emerged as an eco-friendly sample preparation technique for the extraction of organic analytes from aqueous matrices [10,11]. Based on the principle of 'sorptive equilibrium', SBSE is sensitive to the partition coefficient of the analytes to be extracted [12]. In its simplest form, it consists of an extraction phase/medium (e.g. polydimethylsiloxane (PDMS)) coated on to a stir bar. Extraction of an analyte from the aqueous phase sample into the extraction medium is controlled by the partition coefficient of the analyte between the PDMS phase and the aqueous phase ($K_{PDMS}/w$) [13]. This partition coefficient is well correlated with octanol–water distribution coefficients ($K_{o/w}$) of the analyte of interest. Due to the similarity of $K_{PDMS}/w$ to $K_{o/w}$, is it possible to predict extraction efficiencies (SBSE can be efficiently used for hydrophobic compounds with $\text{Log}\,K_{o/w} \geq 2$; and, a high enrichment factor could be expected for analytes with $\text{Log}\,K_{o/w} > 5$) [12,13]. SBSE based methods have been traditionally used for environmental analysis such as analysis of water samples for polycyclic aromatic hydrocarbons [14,15], pesticides [16], trace residues and contaminants in foods [17]. Most recently, SBSE has been used for extraction of drugs from various biological matrices such as urine [18–20], plasma [21–23] and tissues [20].

A literature survey for analytical methods available for estimation of DIC in urine revealed several methods utilising a variety of analytical techniques such as liquid chromatography coupled with UV detection [24–30], tandem mass spectrometry [31–33], diode array (DAD) and fluorescence (FLD) spectroscopy [34]. A careful review of all the published methods revealed use of sample preparation techniques such as liquid–liquid extraction (LLE) [30], solid phase extraction (SPE) [28,29,31,33] and fiber-based liquid phase microextraction (HF-LPME) [34]. Few of the methods employed direct injection of the processed biological sample for the estimation of DIC [26,32]. The literature survey also revealed one SBSE extraction method for the estimation of DIC in environmental water matrices [35].

Based on the requirements of the investigation, the objective of the present study was to assess the feasibility of the SBSE approach for the selective extraction of DIC from paediatric urine samples. To define selectivity of the proposed method, the medical notes of children prescribed with DIC in the POPPK research were referred to in order to obtain a comprehensive list of concurrently administered medications. It was hypothesised that the developed SBSE sample preparation technique will selectively extract DIC in presence of concurrently administered drugs and/or their metabolites. In relation to sensitivity, based on available clinical reports, the analytical range of 100–2000 ng mL$^{-1}$ was proposed for linearity studies, and if needed it was also proposed that the SBSE method should be able to be scaled up to 100–10,000 ng mL$^{-1}$, with similar recovery, accuracy and precision. After examining SBSE methods already published for various biomatrices, a further objective of the proposed method was to establish data on inter- and intra-stir bar accuracy and precision along with the effect of ageing of stir bars on their extraction efficiency. Intra- and inter-stir bar accuracy and precision or the effect of ageing has not been evaluated in the published literature.

## 2. Materials and methods

### 2.1. Chemicals and reagents

DIC sodium and flurbiprofen (FLB) were 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$^{TM}$ 5 Water System (Millipore, Watford, UK). Analytical grade sodium chloride and di-sodium-hydrogen phosphate were purchased from BDH (Poole, UK). All other reagents were of analytical grade except where otherwise stated. Drug free urine samples were collected from healthy volunteers (protocol approved by Ethics Committee of the School of Pharmacy, Queen's University Belfast, UK).

### 2.2. SBSE accessories

Commercially available stir bars (Twister$^{TM}$) of 0.5 mm PDMS thickness and 20 mm length (PDMS volume ~47 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, quality control (QC) samples and aqueous phase (AP)

Primary stock (PS) solutions of DIC and FLB (internal standard (IS)) were prepared in methanol at 1 mg mL$^{-1}$ (1000 μg mL$^{-1}$). The PS solution was diluted with methanol to give a secondary stock (SS) solution of 100 μg mL$^{-1}$. Working standards (WS) of DIC at 4, 10, 20, 30, 40, 60 and 80 μg mL$^{-1}$ were prepared in methanol from the SS solution. Analytical standards (AS) containing DIC at 100, 250, 500, 750, 1000, 1500, 2000 ng mL$^{-1}$ and a constant concentration of FLB at 500 ng mL$^{-1}$ were prepared in mobile phase by using respective working standards. All the stock solutions PS, SS and WS were stored at 4 °C. The urine calibration standards (CS) were prepared by spiking 975 μL of blank urine with 25 μL of the appropriate WS solution of DIC to produce final concentrations of 100, 250, 500, 750, 1000, 1500 and 2000 ng mL$^{-1}$. The QC samples at DIC concentrations of 100 (LQC), 750 (MQC), and 2000 (HQC) ng mL$^{-1}$ were prepared in a similar fashion and further used in method validation. An aqueous phase (AP) containing 15% (w/v) of sodium chloride in water was prepared in bulk. The pH of this AP was adjusted to 2.5 using hydrochloric acid. AP was used to dilute the urine samples for extraction of DIC.

### 2.4. Chromatographic system

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

detector which is a two-channel, tuneable, 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 SunFire™ C18 column (5 μm, 4.6 mm × 150 mm) preceded by a 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 $min^{-1}$. Injection volume was 20 μL and separation was carried out at controlled room temperature (CRT, 20 ± 2 °C). The method employed dual wavelength mode detection where in DIC and FLB were monitored at 281 nm and 254 nm, respectively. The run time for each analysis was 10 min.

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

To 1 mL of CS (prepared as per Section 2.3), an appropriate volume (20 μL) of FLB WS was added so as to give 500 ng $mL^{-1}$ concentration. To this, 8.98 mL of AP (containing 15% sodium chloride, w/v, adjusted to 2.5 pH) was added and mixed thoroughly (total volume of CS, IS and AP was 10 mL). The pH of this solution was checked and readjusted to 2.5 by adding concentrated hydrochloric acid (approximately 11.5 μL). A 5 mL aliquot of this solution was transferred to a clean dried glass vial (25 mL capacity) and placed on the 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. For desorption, 3 mL of acetonitrile was placed in a 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 under the gentle stream of nitrogen at 37 °C using a Zymark TurboVap® LV Concentration Workstation (Hopkinton, MA). The dried residue was reconstituted in 0.5 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 the next use.

### 2.6. 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 [36].

#### 2.6.1. Selectivity

The selectivity of the developed method was tested by analysing six independent sources of blank urine samples. Each blank sample was analysed using the proposed sample preparation/extraction procedure. The resultant chromatograms were checked for interfering peaks at the retention times of DIC and FLB at 281 and 254 nm, respectively. Further, among the various concurrently administered drugs, five drugs (viz. ketamine, propofol, paracetamol, clonidine and bupivacaine) were selected (based on their physicochemical properties and urinary excretion properties) to check if they pose any interference to extraction and bioanalysis of DIC and FLB through the SBSE approach (qualitative assessment).

Experimentally, each of the selected drugs was first prepared in mobile phase as AS (1000 ng $mL^{-1}$) and chromatographed so as to assess their retention time and peak properties (i.e. suitability of current chromatographic method). Further, each of the selected concurrent drugs were individually/in combination spiked in the blank urine (1000 ng $mL^{-1}$) in separate experiments and samples were processed as described in Section 2.5. The resultant chromatograms were assessed for extraction of any of these drugs and if they pose any interference at the retention time of the DIC and FLB.

#### 2.6.2. Recovery

The recovery of DIC by the proposed SBSE sample preparation method was assessed using urine QC samples spiked with DIC at three concentrations i.e. LQC (100 ng $mL^{-1}$), MQC (750 ng $mL^{-1}$) and HQC (2000 ng $mL^{-1}$). Five replicates of each QC concentrations were extracted and analysed and the responses compared with those of non-extracted standards (AS), which represent 100% recovery. Further, the effect of concurrent drugs on SBSE extraction efficiency (quantitative assessment)/recovery of DIC and FLB was also carried out by spiking DIC MQC with 1000 ng $mL^{-1}$ of each of the individual concurrent drugs ($n = 5$) and also spiking DIC MQC with 1000 ng $mL^{-1}$ of all the concurrent drugs together ($n = 5$). The objective of this study was to check if any of these concurrent drugs affected recovery of DIC and FLB, either alone or in combination.

#### 2.6.3. Linearity

The linearity of the developed method was evaluated by analysing CS samples of DIC on three separate days. Each calibration curve consisted of a blank sample (blank urine sample processed without internal standard), a zero sample (blank urine sample processed with internal standard) and was generated from seven non-zero samples covering the expected range (100–2000 ng $mL^{-1}$). Plots of peak area ratio (response) against the respective analyte concentration were used to assess the relationship between the response and concentration. Calibration curves (area ratio with the internal standard versus nominal concentration) were fitted by least-squares linear regression to calculate the slope, intercept and correlation coefficient ($r$).

#### 2.6.4. 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 (100 ng $mL^{-1}$), MQC (750 ng $mL^{-1}$) and HQC (2000 ng $mL^{-1}$) on three different days. The accuracy of the method was calculated by comparing the measured concentrations with the nominal (true) concentration and was expressed as % bias. The precision of the method was expressed as the percent coefficient of variation (% CV).

#### 2.6.5. Sensitivity (limit of detection (LOD) and limit of quantification (LOQ))

LOD and LOQ values were calculated based on the standard deviation of the response ($\sigma$) and the slope ($S$) of calibration curves of DIC according to the following equations:

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

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

where $\sigma$ was estimated from the standard deviation of the $y$-intercepts of the regression lines. For practical purposes, LQC (100 ng $mL^{-1}$) was assessed for acceptable accuracy (<20% bias) and precision (<20% CV) and designated as method LOQ.

#### 2.6.6. Stability studies

Stock solution (PS) stability of DIC and FLB was tested at the selected storage condition (4 °C) for a period of two months. The

stock solution was considered stable if 95–105% of the nominal concentration was found when compared with a freshly prepared stock solution. Further, long term (1 month) and freeze-thaw stability (3 cycles, with at least 24 h interval between each freeze–thaw cycles) studies were carried out using DIC QC samples ($n = 5$) at all three concentration levels (LQC (100 ng mL$^{-1}$), MQC (750 ng mL$^{-1}$) and HQC (2000 ng mL$^{-1}$)). Bench top stability of processed QC samples was carried out at all three QC levels up to 12 h. For samples to be deemed stable the precision (% CV) and accuracy (% bias; when compared to freshly prepared samples) had to be less than 15 for the HQC and MQC and less than 20 for LQC.

#### 2.6.7. Assessment of stir bar and SBSE attributes

To study the robustness and variability of stir bars (within and between), the attributes of SBSE process efficiency were assessed by three approaches.

(i) Inter- and intra-stir bar accuracy and precision:

As each stir bar was used more than once in the method development and validation, it is important to assess if the variation in the assay results is routed through efficiency of individual stir bars. To study this, inter- and intra-stir bar accuracy and precision was assessed. In an experiment, five stir bars were selected and tagged. DIC QC samples 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 (% bias) and precision (% CV) were determined.

(ii) Carry over/memory effect:

As stir bars are re-usable, it was important to assess memory effect/carryover of individual stir bars. Study of carryover is also important to ensure the re-conditioning procedure/protocol is reliable and is efficient to remove any un-desorbed impurities/drug from the stir bar. To study this, re-conditioned stir bars were used. The reconditioned stir bars ($n = 5$) 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.

(iii) Effect of ageing on extraction efficiency of stir bars:

One of the important advantages of the SBSE is its cost-effectiveness through re-use of the stir bars, unlike solid phase extraction (SPE) cartridges. It has been reported in the literature that stir bars can be re-used (with appropriate reconditioning) at least 10 [37] to 100 [38] times. Therefore the effect of ageing on extraction efficiency of DIC and FLB was investigated. To study this, individual stir bars were tagged for number of re-use ($n = 150$). To compare the extraction efficiency of the re-used stir bars ($n = 150$), a fresh lot of stir bars were purchased. DIC QC samples at LQC, MQC and HQC were prepared ($n = 5$) and were analysed with re-used and new stir bars and recoveries of DIC and FLB were compared to establish the comparative efficiency of aged (re-used) and fresh (new) stir bars.

### 2.7. Clinical application

The method that was developed and validated was applied to the analysis of DIC in infant and paediatric urine samples ($n = 20$). The patients (6 months to 12 years) received an oral diclofenac suspension as part of their routine medical treatment. The samples, stored at −20 °C, were allowed to reach room temperature before being extracted and processed for analysis.

## 3. Results and discussion

### 3.1. Analytical method development

For the present method, isocratic reversed phase chromatographic separation using a hydrophobic C18 column was successfully employed for quantitative estimation of DIC from paediatric urine samples. Selection of IS was a critical issue i.e. it was important that the IS should bear very similar physicochemical properties so as to enable SBSE extraction. Based on physicochemical properties of DIC (Fig. 1, Log $P$; 4.5 and p$K_a$; 4.2) [39], FLB (Fig. 1) was found to have structurally and physicochemically (Log $P$; 4.2, p$K_a$; 4.2) similar properties [40].

A wavelength of 281 nm was selected for the determination of DIC as it gave optimum UV sensitivity and selectivity, however, at this wavelength FLB showed low UV sensitivity (resulting in a much smaller signal). Based on FLB UV absorbance characteristics, 254 nm was found to give satisfactory UV response and thus dual wavelength mode was selected for the present method. Various solvent systems were studied for appropriate chromatographic separation and optimisation of analyte's peak properties. The combinations of water, methanol, acetonitrile, phosphate buffers and acetate buffer of a range pH 3–5 were tested. It was observed that, the chromatographic parameters of DIC were sensitive to pH change. A pH of 4.1 yielded optimum peak properties and chromatographic resolution for DIC and FLB. 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 optimised chromatogram with retention times of 6.3 ± 0.1 and 5.5 ± 0.1 min for DIC and FLB, respectively (Fig. 2).

### 3.2. SBSE feasibility analysis and optimisation

The feasibility analysis of DIC SBSE extraction was based on theoretical sorptive recovery which can easily be predicted through the theory of SBSE advanced by Sandra et al. [10]. Theoretical recovery of DIC by SBSE can be calculated using the 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 and FLB have Log $P$ values of 4.5 and 4.2, respectively [39,40]. Based on 5 mL sample extraction volume ($V_w$) and stir bar PDMS phase of 47 μL showed ~100% recovery of DIC and FLB could be expected with SBSE. However, this theoretical recovery depends on various extraction (such as pH of extraction phase, phase ratio, stirring speed, temperature, time and ionic strength of extraction medium) and desorption (method; stirring/sonication, organic solvent strength, stirring speed, temperature, time, etc.) factors.

In the earlier study, we have investigated in detail all the relevant SBSE factors affecting DIC extraction from aqueous samples of pharmaceutical products [41]. The factors investigated were extraction pH (range 2–5), four commercially available stir bars (0.5 mm thickness; 10 mm length, 1 mm thickness; 10 mm length, 0.5 mm thickness; 20 mm length and 1 mm thickness; 20 mm length), stirring speed (600, 900 and 1200 rpm), extraction temperature (CRT, 30 °C and 40 °C), extraction time (up to 120 min) and ionic strength (% sodium chloride in aqueous phase at 5%, 10% and 15%, w/v). In the case of desorption optimisation, two methods i.e. magnetic stirring and sonication were tested. In the magnetic stirring method, factors such as stirring speed (600, 900 and 1200 rpm), desorption time (up to 40 min) and desorption solvent (acetonitrile

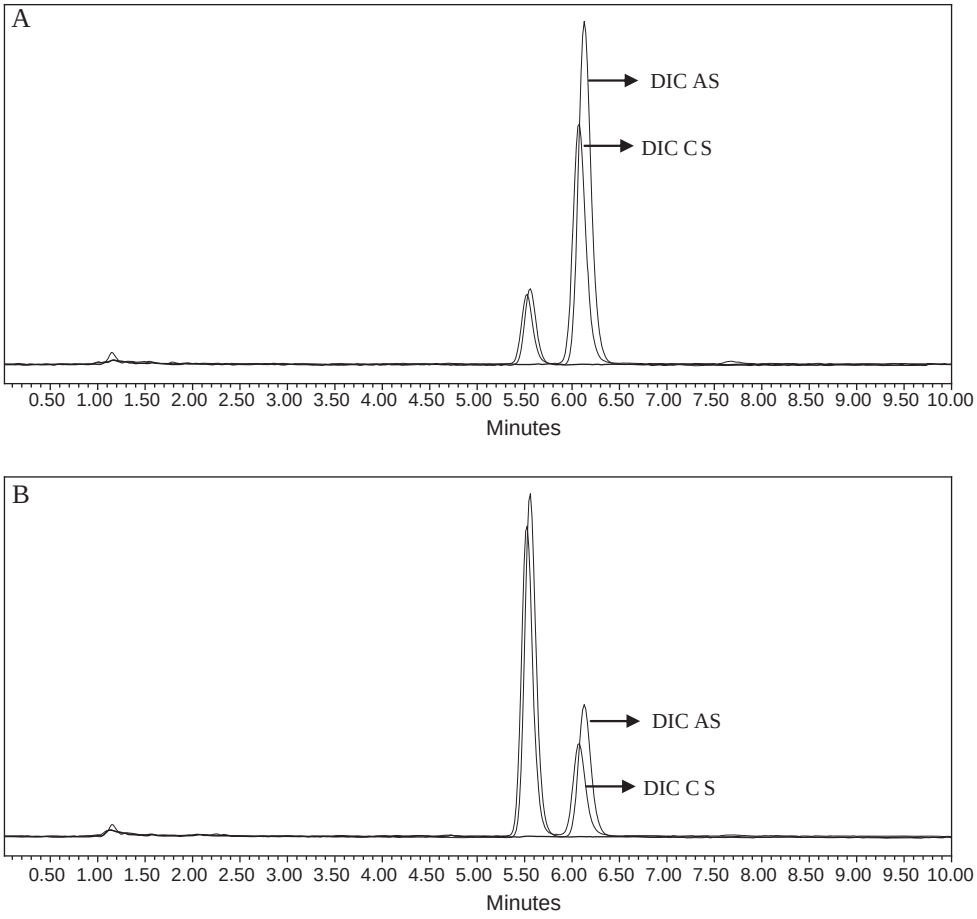


**Fig. 2.** Overlay chromatogram of blank mobile phase, DIC AS (1000 ng mL$^{-1}$), blank urine sample, and DIC CS (1000 ng mL$^{-1}$) at 254 nm (A) and 281 nm (B).

versus mobile phase) were assessed. The results showed that, the optimum conditions for extraction of DIC from aqueous medium were pH 2.5, ionic strength (15% sodium chloride, w/v), stir bar with dimensions 0.5 mm thickness and 20 mm length, stirring speed of 600 rpm, CRT as extraction temperature and 120 min of extraction time. Optimum conditions for desorption were magnetic stirring method, with 600 rpm stirring speed for up to 40 min [41].

Thus based on these conditions, the protocol for extraction of DIC from urine samples was optimised. AP containing 15% (w/v) of sodium chloride adjusted to pH 2.5 was used to dilute (1 in 10 times) spiked urine/patient samples. It was observed that, when urine samples were diluted, the average pH of all CS/QC/patient samples (irrespective of clinical condition) was ~4.5 ± 0.5. The pH of the diluted urine was re-adjusted to 2.5 using hydrochloric acid. In case of all CS/QC/patient samples (irrespective of clinical condition), the average amount of hydrochloric acid added was 11.5 ± 2 μL. The process of dilution and pH adjustment was standardised and resulted in reproducible results (as detailed in method validation results). As FLB has similar structural and physicochemical properties, the optimised conditions for SBSE extraction for DIC were also suitable for the extraction of FLB from urine samples.

### 3.3. Method validation

#### 3.3.1. Selectivity

Selectivity was one of the prime objectives of the proposed bioanalytical method. Specificity of the SBSE sample preparation technique was verified by various means. Firstly, to establish that no interference was derived from matrix components of the urine, six independent blank urine samples were processed as per optimised SBSE method. Fig. 3(A) shows an overlay chromatogram of six blank independent urine samples. It can be seen from the chromatogram that, no interfering peaks were found at the retention times of DIC and FLB.

One of the major challenges for the selectivity of the proposed method was interference that might be posed by concurrently administered drugs and/or their metabolites. Table 1 shows list of concurrent drugs that were administered to patients along with their pharmacological use, chemical nature, p$K_a$, Log $P$ and UV absorption maximum (nm). Based on SBSE theory, it can be claimed that drug/metabolites which have negative Log $P$ values (i.e. hydrophilic molecules), will not be partitioned/extracted by the highly hydrophobic PDMS layer. Secondly, it has been theoretically and practically proven that, SBSE is not efficient in the extraction of analytes/drug/metabolites which have Log $P$ < 2 [12,13]. Thus, drugs viz. neostigmine, glycopyrrolate, cefuroxime, cefotaxime, metronidazole and gentamicin were excluded from the SBSE selectivity assessment. Further drugs vecuronium and rocuronium do not exhibit any UV absorption, thus were excluded from the study. Sevoflurane and isoflurane were inhalation anaesthetics and do not show any considerable urinary excretion (as unchanged drug/metabolites), were also excluded from the selectivity study.

Systematic exclusions and probabilities for SBSE extraction based on physicochemical properties, five drugs viz. propofol, paracetamol, clonidine, ketamine and bupivacaine were selected for selectivity assessment. A literature survey was carried out for the urinary excretion profiles of these drugs in paediatric patients and it was found that propofol is excreted in urine intact and along with its metabolites [42], as is the case with paracetamol [43], clonidine [44], ketamine [45] and bupivacaine [46]. It was

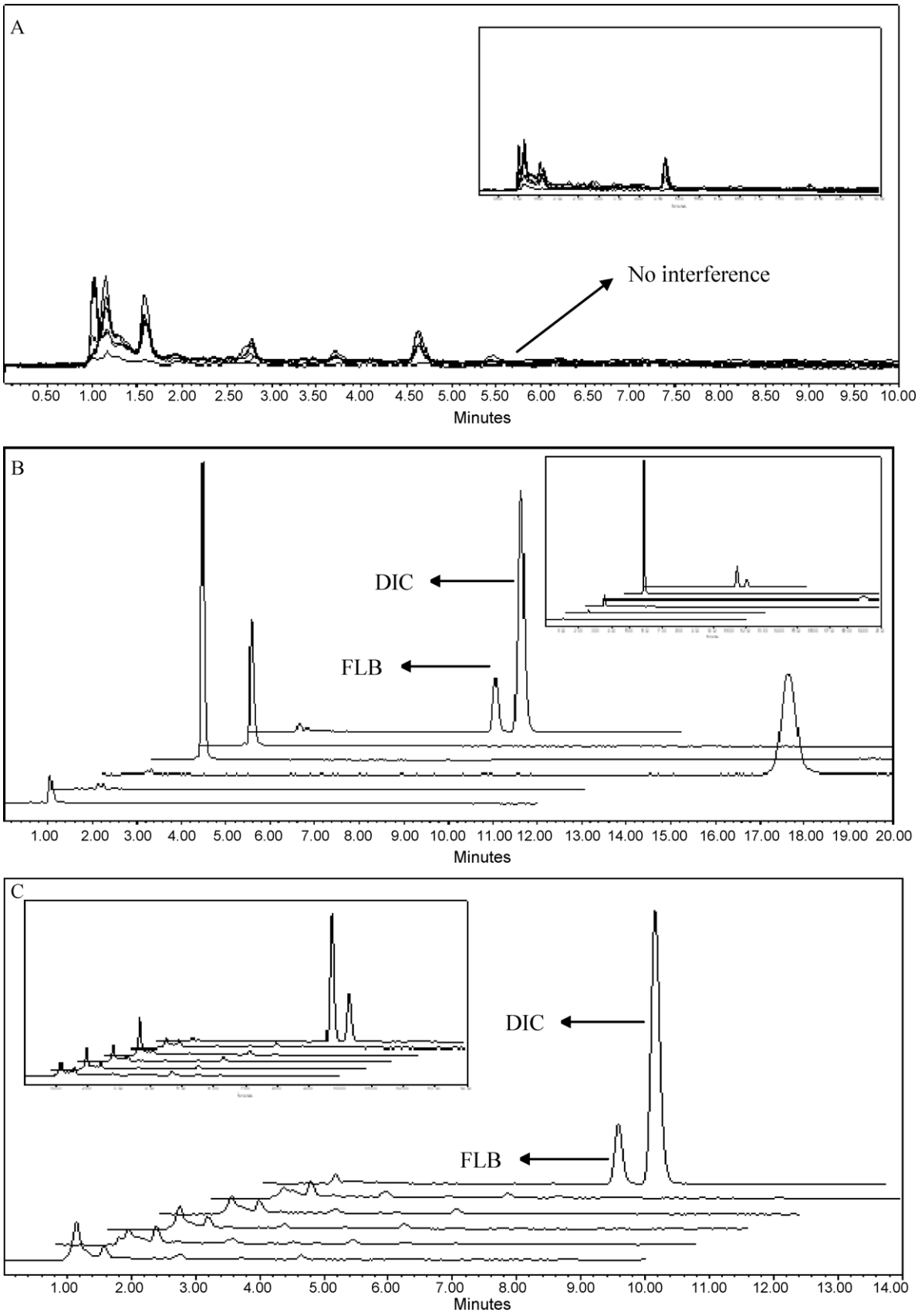


**Fig. 3.** Overlay chromatograms of six independent blank urine samples at 281 nm (inset: chromatograms at 254 nm) (A), overlay chromatogram of AS (1000 ng mL$^{-1}$) of propofol, paracetamol, clonidine, ketamine and bupivacaine and DIC AS with IS FLB (1000 ng mL$^{-1}$ and 500 ng mL$^{-1}$, respectively) at 281 nm (inset: chromatograms at 254 nm) (B), overlay chromatogram of urine spiked with propofol, paracetamol, clonidine, ketamine and bupivacaine and DIC CS with IS FLB (1000 ng mL$^{-1}$ and 500 ng mL$^{-1}$, respectively) at 281 nm (inset: chromatograms at 254 nm) (C).

**Table 1**
List of concurrent drugs administered to patients together with DIC.

| Drug name | Use | Chemical nature | p$K_a$ | Log $P$ | UV absorption maximum (nm) |
|---|---|---|---|---|---|
| Propofol | Intravenous anaesthetic agent | Weakly acidic | 11.1 | 4.1 | 220 280 |
| Sevoflurane | Volatile liquid for inhalation | – | – | 2.4 | 246 |
| Isoflurane | Inhalation anaesthetic | – | – | 2.1 | 205 |
| Paracetamol | Analgesic, anti-pyretic | Weakly acidic | 9.5 | 0.4 | 247 |
| Clonidine | Alpha-2 adrenergic agonist | Weakly basic | 8.05 | 2.7 | 220 |
| Ketamine | Anaesthetic | Weakly basic | 7.5 | 2.9 | 210 |
| Vecuronium | Muscle relaxant | – | 8.9 | 2.09 | No UV absorption |
| Rocuronium | Neuromuscular blocker or muscle relaxant | – | – | 2.71 | No UV absorption |
| Neostigmine | Reversible cholinesterase Inhibitor | Weakly acidic | 12 | −3.03 | 254 |
| Glycopyrrolate | Muscarinic antagonist | Weakly acidic | – | −1.0 | 200 |
| Cefuroxime | Antibiotic | Weakly basic | 2.5 | −0.8 | 280 |
| Cefotaxime | Antibiotic | – | 3.7 | −0.5 | 254 |
| Metronidazole | Antibiotic | Weakly basic | 2.6 | −0.1 | 317 |
| Gentamicin | Antibiotic | Weakly basic | 8.6 | −3.1 | 195 |
| Bupivacaine | Local anaesthetic | Weakly basic | 8.1 | 3.4 | 254 |

hypothesised that, metabolites of these drugs are more hydrophilic than the parent drug and if SBSE could not extract the parent drug then there is less probability that any of the metabolites of these drugs would be extracted in the patient urine samples. Fig. 3(B) shows overlay chromatograms of all five drugs (1000 ng mL$^{-1}$) prepared in mobile phase, together with DIC and FLB AS (1000 ng mL$^{-1}$ and 500 ng mL$^{-1}$, respectively). From the chromatogram, it can be seen that, all the concurrently administered drugs were well resolved from the DIC and FLB peaks. Thus current chromatographic conditions were suitable for the resolution of these drugs form DIC and FLB. The retention times of propofol, paracetamol, clonidine and ketamine were found to be 1.1, 1.2, 1.0 and 15.4 min, respectively and bupivacaine could not be detected up to 30 min, while DIC and FLB were eluted at 6.3 and 5.5 min, respectively.

Urine samples (blank/MQC) spiked with individual/all the concurrent drugs together were extracted with the proposed SBSE procedure and chromatographed (Fig. 3(C)). It can be seen from the over lay chromatogram that none of the drugs were extracted on to the SBSE stir bar, thus providing selectivity to the proposed method. 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 the extraction phase since each analyte has its own partition equilibrium in the PDMS phase [12].

A literature survey of bioanalytical methods for estimation of DIC in the presence of concurrently administered drugs such as paracetamol revealed one SPE method; however, both the drugs were retained and eluted together [47]. This is indicative that, commonly used stationary phases in SPE can retain these two drugs, however, when the objective of the bioanalytical method is selective extraction of DIC, the present results confirms that SBSE can provide more selectivity than other sample preparation methods such as SPE/protein precipitation. Detailed investigation of the selectivity of the SBSE approach in the proposed method suggested that the chromatographic technique as well as sample preparation were highly selective for separation, resolution and extraction of DIC from other concurrently administered drugs thus achieving the objective of the desired bioanalytical method.

### 3.3.2. Recovery

Absolute recovery of DIC from urine samples was assessed under various experimental conditions at 3 QC concentrations. Table 2 shows data on recoveries of DIC at 3 QC levels in absence and presence of concurrent drugs. At all 3 QC levels, average recovery was found to be in excess of 70%. It can be seen from the table that concurrent drugs (either individual/combined) did not affect the extraction efficiency of DIC and FLB under the optimised SBSE conditions. We earlier reported DIC extraction recovery of ∼70% from aqueous samples of pharmaceuticals [41]. The results obtained in the current study were similar to those reported earlier, suggesting the robustness of the SBSE conditions, and that for given SBSE conditions and matrix components from urine did not affect the sample preparation efficiency. Furthermore, the recovery of DIC using the current SBSE method was much higher than the previously reported SBSE method (34.6 ± 6.9%) for extraction of DIC from environmental aqueous matrices [35].

### 3.3.3. Linearity

The calibration curve for DIC was found to be linear from 100 to 2000 ng mL$^{-1}$. The calibration data was subjected to least square regression analysis and the mean linear regression equation obtained for the selected SBSE method was $Y = 0.0007X + 0.0207$, where $Y$ is peak area ratio (response) and $X$ is the concentration of the analyte in ng mL$^{-1}$. The correlation coefficient value was found to be highly significant ($r = 0.9999$), negating the need for data weighting.

**Table 2**
SBSE recovery of DIC and FLB from urine samples under various conditions.

| Concentration (ng mL$^{-1}$) | | In absence concurrent drugs | % Recovery under various experimental conditions[a] (% CV) In presence of concurrent drugs | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Propofol alone | Paracetamol alone | Clonidine alone | Ketamine alone | Bupivacaine alone | All drugs together |
| DIC | LQC (100) | 70 ± 2 (3) | – | – | – | – | – | – |
| | MQC (750) | 71 ± 3 (4) | 70 ± 5 (7) | 71 ± 3 (4) | 69 ± 1 (2) | 71 ± 3 (4) | 70 ± 5 (8) | 70 ± 4 (5) |
| | HQC (2000) | 70 ± 2 (3) | – | – | – | – | – | – |
| FLB | (500) | 89 ± 3 (3) | 90 ± 2 (2) | 89 ± 3 (3) | 88 ± 3 (3) | 88 ± 5 (5) | 87 ± 3 (4) | 87 ± 3 (4) |

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

**Table 3**
Accuracy and precision data of DIC SBSE method ($n = 5$).

| Concentration ($ng\,mL^{-1}$) | Acceptance limit (%) | Intra-day accuracy (% Bias) | Inter-day accuracy (% Bias) | Intra-day precision (% CV) | Inter-day precision (% CV) |
|---|---|---|---|---|---|
| LQC (100) | ±20 | 6.55 | 5.31 | 5.5 | 4.65 |
| MQC (750) | ±15 | 0.34 | −0.63 | 2.17 | 3.55 |
| HQC (2000) | ±15 | −1.68 | −1.46 | 3.29 | 3.03 |

**Table 4**
Data on inter-stir bar, intra-stir bar accuracy and precision ($n = 5$) for the extraction of DIC.

| Concentration ($ng\,mL^{-1}$) | Inter-stir bar | | Intra-stir bar | |
|---|---|---|---|---|
| | Accuracy (% Bias) | Precision (% CV) | Accuracy (% Bias) | Precision (% CV) |
| LQC (100) | 5.96 | 1.16 | 7.99 | 4.42 |
| MQC (750) | −0.27 | 1.81 | −0.52 | 2.44 |
| HQC (2000) | −0.22 | 2.21 | −1.98 | 3.35 |

One of the secondary objectives of the proposed method was assay scalability (100–10,000 $ng\,mL^{-1}$). Further investigations of the addition of two more concentrations in the linearity range i.e. 5000 and 10,000 $ng\,mL^{-1}$ were carried out (data not shown). The data indicated that, the new concentration range, i.e. 100–10,000 $ng\,mL^{-1}$, was linear with highly significant $r$ value ($r = 0.9998$). Recovery studies of spiked urine samples at 5000 and 10,000 $ng\,mL^{-1}$ concentrations were also carried out and average recoveries ($n = 5$) were found to be $70 \pm 3$ (% CV; 4.9) and $70 \pm 2$ (% CV; 3.1), respectively, indicating that SBSE extraction efficiency for this calibration range is not limited by its capacity. One of the reasons for a highly acceptable linearity range (100–10,000 $ng\,mL^{-1}$) with this sample preparation technique is cleaner samples i.e. absence of interfering matrix components, supported by consistent recoveries at all concentration levels. From Fig. 2, it can be clearly seen that, chromatograms of AS and CS exhibit similar patterns and the chromatogram of CS did not show any significant matrix components (usually elute in first 1–3 min of chromatography).

### 3.3.4. Accuracy and precision

The results obtained for intra- and inter-day accuracy and precision analyses are summarized in Table 3. Accuracy and precision of the method was evident from low values of % bias and % CV, indicating that the developed method is highly repeatable and reproducible. Overall, accuracy and precision values for within and between-day were <20% at LQC and <15% at all other

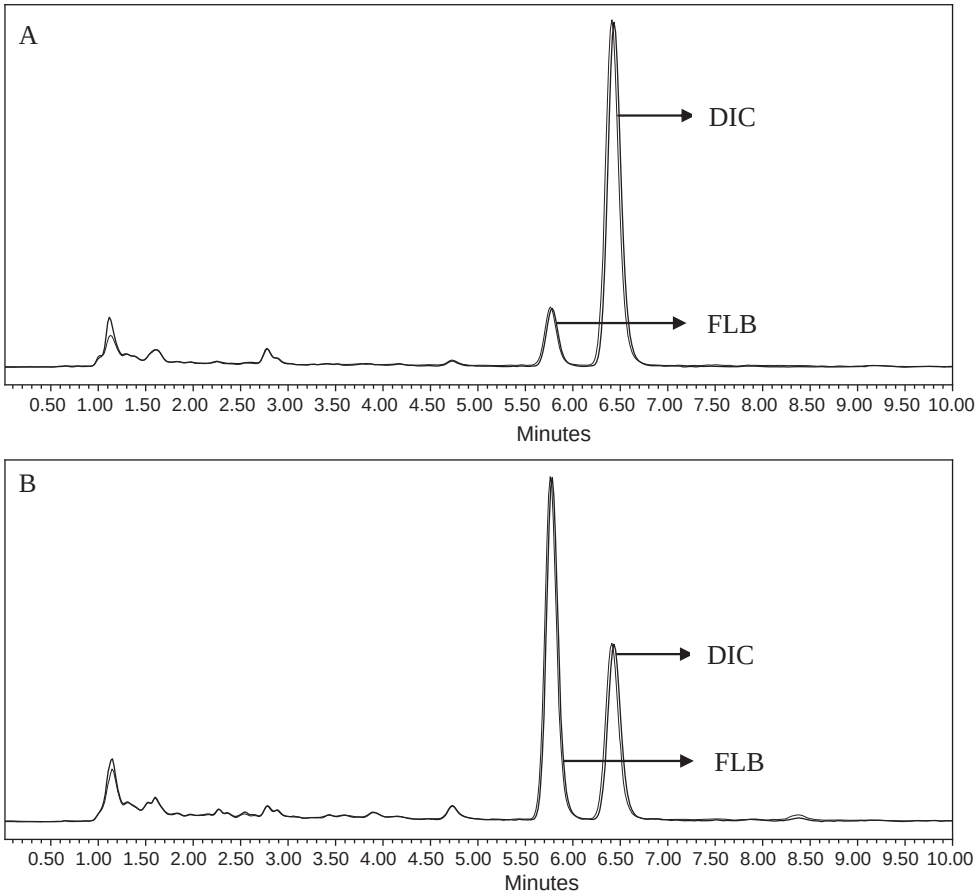


**Fig. 4.** Overlay chromatogram of DIC HQC (2000 $ng\,mL^{-1}$) extracted with aged and new stir bar at 281 nm (A) and 254 nm (B).

concentrations tested. Thus it can be claimed that, the method possessed good accuracy and precision.

*3.3.5. Sensitivity*

Calculated LOD and LOQ of the developed method were found to be 12 ng mL$^{-1}$ and 36 ng mL$^{-1}$, respectively. However, for the experimental purposes, 100 ng mL$^{-1}$ was considered as the validated LOQ as accuracy and precision at this concentration was <±20%.

*3.3.6. Stability studies*

Stock solution stability for PS was tested at refrigerated storage (4 °C) for a period of two months. The concentrations of DIC and FLB PS were found be within 95–105% of the nominal concentration when compared with a freshly prepared stock solutions.

In case of long term stability study, DIC QC samples (at three concentrations) were analysed on the day of preparation (0 day) and after one month of storage at −20 °C. Results indicated that, the stability of samples stored at −20 °C were well within acceptable limits of accuracy and precision when compared with 0 day results. The freeze–thaw stability of urine QC standards was investigated by comparing the measured DIC concentration in thawed spiked samples over three freeze–thaw cycles (with up to 24 h intervals between cycles) with those not frozen (fresh QC standards). Results of the analysis did not show any significant reduction/deviation in the measured concentrations of DIC, indicating that, DIC is stable within the matrix for up to three freeze–thaw cycles. Accuracy and precision data for all 3 QC concentrations were well within the limits of acceptance. Bench top stability of processed DIC samples was carried out for up to 12 h. The duration of stability was based on the number of samples to be analysed per day (maximum $n = 45$) and analysis time per injection (10 min). The results of the study indicated that DIC (at all 3 QC concentrations) was stable in the processed samples (i.e. mobile phase) for up to 12 h and did not show any significant deviation between time zero and 12 h assay readings.

*3.3.7. Assessment of stir bar and SBSE attributes*

Several advantages of SBSE approach have been reported in the published literature; however, for a few of the claims, there is a lack of technical evidence. In the proposed method, an attempt was made to investigate (quantitatively) some the factors associated with the stir bars that may cause assay variability. One of the important attributes tested was variability that may arise within or between stir bars. Results of inter- and intra-stir bar accuracy and precision are summarized in Table 4. Low values of % bias and % CV at all QC levels for both intra-stir bar and inter-stir were highly significant.

Samples of desorption carried out on reconditioned stir bars, did not show any peak at the retention times of DIC and FLB. Thus it can be claimed with confidence that, there was no carryover/memory effect within the stir bars and this observation also supports effectiveness of the reconditioning protocol developed for this study.

In the published literature on SBSE methods, authors have reported that, stir bars can be re-used a number of times; however, there is lack of actual quantitative data on ageing of stir bars and its effect on extraction efficiency.

The criteria for how many times the stir bar can be re-used for a given method should be based on its extraction efficiency over a period of time/number of times re-used and limit of

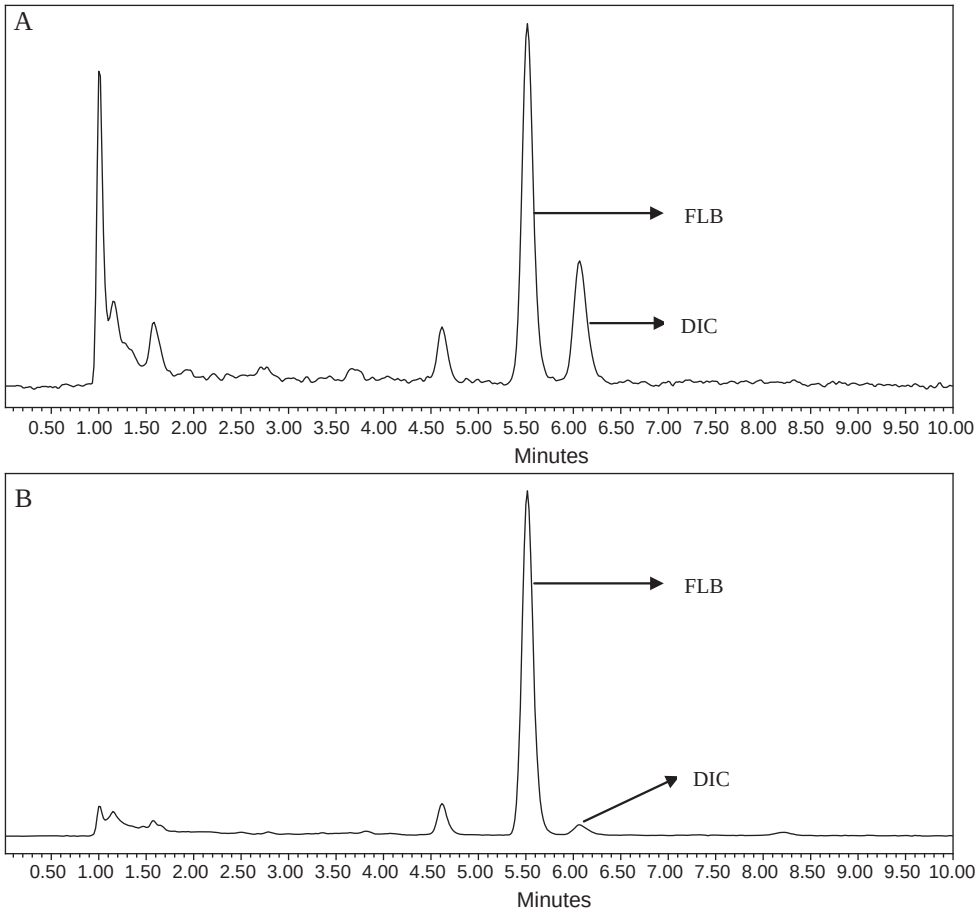


**Fig. 5.** Chromatogram of DIC extracted from patient urine sample at 281 and 254 nm.

detection/quantification of the method. In the present study, we devised in-house criteria for quality control of stir bars based on (i) chromatographic profile, (ii) periodical extraction efficiency (after $n = 50$ times use) assessment of re-used stir bars and its comparison with extraction efficiency of new stir bar (assuming 100% efficiency) and (iii) physical deterioration/damage to PDMS layer.

In the case of extraction efficiency, our in-house criterion was that a re-usable stir bar should have at least 80% of the extraction efficiency when compared with extraction efficiency of a new stir bar. To investigate this factor, stir bars were tagged for number of times they were used ($n = 150$). Fig. 4 shows overlay chromatograms of extracted DIC QC standard (HQC) using aged (re-used, $n = 150$) and new stir bars. It could be seen from the figure that, there was little difference between the chromatographic profiles of re-used and new stir bars. To visualise the quantitative aspect of the study, DIC QC samples at three concentrations were prepared with re-used and new stir bars and recoveries were calculated. The results of the study were well within the standard bioanalytical accuracy and precision acceptance criteria (<±20% at LQC and <±15% at MQC and HQC).

### 3.4. Clinical application

The selective and sensitive method thus developed and validated was clinically validated by application of the method to actual paediatric urine samples. No major difficulties were identified during analysis of the patient samples. The concentration range for all patient samples analysed by the proposed method was found to be 109–1529 ng mL$^{-1}$. The lowest concentration found with patient samples was well above the LOQ (100 ng mL$^{-1}$) of the method, similarly, none of the patient samples were above the highest calibration point (2000 ng mL$^{-1}$) of the method. Fig. 5 shows a chromatogram of DIC extracted from a patient urine sample. It could be seen from the figure that, no interfering peaks from concurrent drugs and/or metabolites were found, justifying the selectivity of the developed method.

## 4. Conclusions

Sample preparation plays an important role in achieving desired selectivity and sensitivity of bioanalytical methods. This is the first report describing the quantitative estimation of DIC by SBSE in paediatric clinical research samples. At the outset of this clinical study, we decided to assess the selectivity of the sample preparation technique rather than use of selective detection technique such as mass spectrometry. Although much emphasis is given to selective and sensitive analytical instrumentation, conventional analytical instrumentation such as HPLC–UV can still be effectively used if selective sample preparation techniques can be employed for analyte extraction.

It was observed that, SBSE extraction conditions optimised in the developed method were easy to operate and more importantly was highly reproducible. Based on results of the method validation it can be concluded that, the method was selective, specific, linear, accurate, precise and sensitive. Due to unique extraction conditions that were optimised for DIC and FLB, the concurrently administered drugs did not get extracted, imparting selectivity to the method. The linearity assessment indicated that, it was possible to scale up the linearity range from 100 to 10,000 ng mL$^{-1}$. This was possible because of lack of saturation in the PDMS phase and consistent recoveries of DIC and FLB across the linearity range.

Further, studies carried out on the assessment of various attributes of SBSE confirmed that, there is low inter–intra stir bar extraction variability, imparting robustness to the bioanalytical method. Lack of carryover/memory effect showed the efficiency of the re-conditioning protocol. Furthermore, studies on extraction efficiency of aged/re-used stir bars when compared with new stir bars also showed excellent results. This study is the first reported in which systematic investigations on these attributes were carried out.

The SBSE based bioanalytical method described here offers several advantages over the existing methods such as, higher linearity range (due to lack of saturation in PDMS phase), selectivity, reusability of the stir bars leading to reduced sample preparation cost, lack of need for sophisticated analytical instrumentation such as mass spectroscopy. Although the extraction and desorption processes are slightly long; with the use of two fifteen position stirrers (IKA® multi position hotplate stirrer RT 15) as described in the experimental section, thirty samples can be extracted within approximately 3 h which gives a reasonable overall extraction time, offering a selective, sensitive, precise and cost effective alternative for the determination of DIC in a biomatrix such as urine.

## Acknowledgements

The authors would like to thank; Dr. Neil Morton (Chief Investigator), Dr. Julia Wolfe-Barry and Dr. Angela Baker (research registrars), and Emma Scobie (research nurse) for patient recruitment and collection of study samples. The authors also thank Mr. Jeff Rothwell, Rosemont Pharmaceuticals Ltd. for the provision of the diclofenac IMP and Dr Jennifer Bell as study co-ordinator. The authors would like to acknowledge the Knowledge Transfer Partnership between Rosemont Pharmaceuticals Ltd. (Leeds, UK) and Queen's University Belfast for financial and academic assistance.

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