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Cationic polyelectrolyte copolymer modified polyurethane foam for flow injection preconcentration and separation of trace amounts of β-lactam antibiotics

S.M. Abdel Azeem [a,*], H.M. Kuss [b], M.F. El-Shahat [c]

[a] Chemistry Department, Faculty of Science, Fayoum University, Fayoum City, Egypt
[b] Chemistry Department, Faculty of Science, Duisburg-Essen University, Duisburg, Germany
[c] Chemistry Department, Faculty of Science, Ain-Shams University, Cairo, Egypt

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ABSTRACT

A more sensitive flow injection preconcentration method has been developed for the determination of four β-lactam antibiotics (BLAs) namely cefaclor, cefotaxime, amoxicillin and ampicillin in urine, pharmaceuticals and milk. A mini-column packed with PUF functionalized with the cationic polyelectrolyte, poly(*N*-chloranil *N*,*N*,*N*′,*N*′-tetramethylethylene diammonium dichloride) PCTDD, was utilized for selective preconcentration. The detection limits with this method were 3.3, 3.8, 5.1 and 7.0 ng mL^{-1} and enrichment factors were 38, 21, 39, and 36 for cefaclor, cefotaxime, amoxicillin and ampicillin, respectively with a sample throughput of 12 h^{-1} for all BLAs. Moreover, the BLAs were successfully separated by isocratic elution using a micellar mobile phase. Application of the method developed has resulted in recovery values in the range 95%–109% ($RSD \leq 8.7$), 83%–99% ($RSD \leq 9.7$) and 91%–103% ($RSD \leq 4.0$) for urine, pharmaceuticals and milk samples, respectively.

1. Introduction

β-Lactam antibiotics (BLAs) are the oldest and most widely used group of antibiotics till now. Sub-therapeutic levels of BLAs are given to food producing animals for bulk weight gain and to improve feed efficiency which can lead to the emergence of antibiotic—resistant bacterial strains [1]. On the other hand, the administrated BLAs are not totally absorbed in the body but are excreted unaltered and hence released to the environment [2]. Antibiotic residues in milk or edible tissues are a potential risk for individuals who are hypersensitive to antibiotics. Antibiotics approved for use in food-producing animals have established tolerances for the detection of their residues in milk [1]. The EU regulations 2377/90 set a maximum residue limit (MRL) of 4 μg L^{-1} for ampicillin and amoxicillin in milk [3].

Antibiotics contamination in food due to their use as veterinary drugs and fodder additives requires a stringent drug quality control for BLA determination [4]. Fig. 1 shows the chemical structure of the investigated antibiotics.

The determination of BLAs by liquid chromatography (LC) coupled with fluorescence and ultraviolet detection has been reported [5,6]. Mass-spectrometry has also made impressive progress because of its improved selectivity and high sensitivity. Despite this, a preconcentration step is necessary to reach the low detection limit [7]. Even diode array detectors in HPLC instruments were found to be not completely reliable especially for complex matrices that might clog the column. Nevertheless, the method is quite time consuming and unsuitable for processing a large number of samples. Thus, residual amounts of BLAs in many edible items are not easy to determine as required by the EU Regulations 2377/90 due to a lack of sensitivity [8].

A combination of solid phase extraction (SPE) with simple measuring instruments like spectrophotometry has enabled the development of less expensive analytical methods with relevant efficacy and sensitivity. SPE has also been combined with mass spectrometry [9].

Sample preparation has always been regarded as the bottleneck in chromatographic analysis due to the involvement of several time-consuming manual steps. Furthermore, extensive sample handling increases the possibility of its contamination which could be avoided by direct injection. Flow techniques have drastically reduced or even eliminated the manual sample treatment. SPE technique is a fast, efficient, clean and easily automated procedure, allowing simultaneous analyte preconcentration and removal of matrix [10].

Flow injection-SPE preconcentration has several advantages such as reducing manipulation and size of the sample and consumption of organic solvents, and decreases the analysis time [11]. Moreover, the increased sample throughput of this approach is

* Corresponding author. Tel.: +2 16 960 45 99.
E-mail addresses: samymoh2000@yahoo.com, Sma13@fayoum.edu.eg (S.M. Abdel Azeem).

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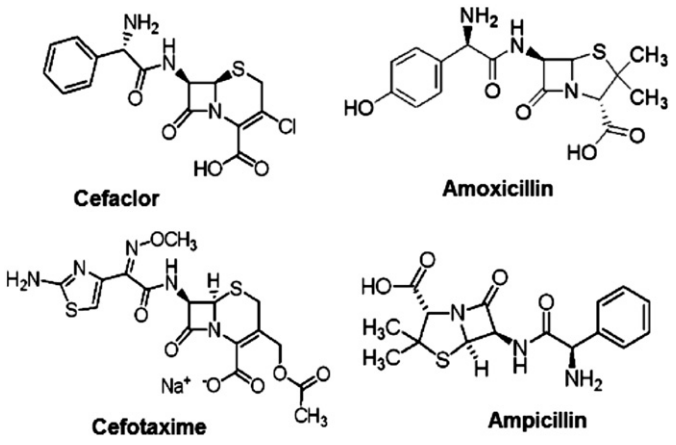

Fig. 1. Chemical structure of cefaclor, amoxicillin, ampicillin and cefotaxime.

helpful for the removal of macromolecules e.g. proteins by a simple and flexible way as well as pharmacokinetic monitoring which requires analysis of several samples [12].

Preconcentration of BLAs has been done by several SPE sorbents. Octadecyl silica (C18) is widely used for off-line preconcentration from milk [13,14], urine [15] and pharmaceuticals [16], and in micro flow system determination in milk [17]. Recently, grafted polyurethane foam (PUF) has been used for the analysis of milk and pharmaceuticals [18].

PUF is widely used as a SPE sorbent for the preconcentration of organic compounds such as cationic dyes [19,20], oxalic acid [21], phenol and 1-naphthol [22], gallic acid [23], pesticides [24], polycyclic aromatic hydrocarbons [25], surfactants [26] and carbaryl [27]. This material enables rapid extraction of trace components and/or separation from interfering ions as well as improving the sensitivity of the method [28]. Besides, the existence of several functional groups in PUF provides an opportunity for a wide range of chemical modifications.

Cationic polyelectrolytes are used in water treatment because of their interaction with negatively charged surfaces [29]. The association between polyelectrolytes and oppositely charged surfactants can be understood considering electrostatic and hydrophobic interactions [30,31]. The electrostatically driven interactions between macromolecules have attracted considerable interest in various industrial and environmental applications for the removal of various toxic materials such as acid dyes [32], anionic surfactants [30], and arsenate [33] from water.

In a previous investigation from our group [18], we have used polyurethane foam (PUF) grafted with a basic dye for the flow injection preconcentration of penicillins. Although satisfactory results were obtained, further improvement in the analytical performance should be continued to meet the wide range of applications.

In the present work, we have extended that principle using PUF functionalized with cationic polyelectrolyte, poly(*N*-chloranil *N*,*N*,*N*′,*N*′-tetramethylethylene diammonium dichloride) or PCTDD. It was chosen for the synthesis of the proposed sorbent due to the presence of reactive chlorine atoms which can react with the amino, and hydroxyl groups in PUF under mild conditions. Anionic BLAs would bind to the positively charged immobilized polyelectrolyte via ion–pair associates.

2. Experimental

2.1. Instrumentation

A UV–vis spectrophotometer model UV 1650PC (Shimadzu, Japan) controlled by UV2.10 probe software and equipped with quartz flow cell model Hellma 174.010-OS with a dead volume of 1.5 mL and 10 mm path length was used for the determination of BLAs at 210 nm. A Metrohm model 780 (Herisau, Switzerland) pH meter was utilized for pH adjustments. A peristaltic flow injection pump model FIAS-400 (PerkinElmer, USA), operated with WinLab software was used to propel the solutions. Tygon tubes (1.52 mm i.d.) were used for all the connections and sample loops with volumes ranging from 50 to1000 μL were used to inject definite volumes into the carrier stream. Elix water purification system from Millipore (MA, USA) was used to provide deionized water for sample preparations and washing purposes. The minicolumn was fabricated from a polyethylene tube 30 mm long and 3.0 mm internal diameter. Sorbent plugs (100 mg PCTDD-PUF) were packed manually into the tube and then compressed by a vacuum pump in order to avoid channels. The minicolumn was end-capped with plastic joints and integrated into the flow system. Thereafter, the minicolumn was washed thoroughly by passing 0.5 mol L^{-1} hydrochloric acid followed by deionized water until the effluent was neutral.

2.2. Chemicals

Standard BLA solutions (100 μg mL^{-1}) were prepared from cefaclor (Sigma-Aldrich, USA), cefotaxime (Fluka, Switzerland) amoxicillin (Fluka) and ampicillin (Fluka). Hydrochloric acid (KMF, Germany) solution was prepared by direct dilution with deionized water. Buffer solutions were prepared from 25% ammonium hydroxide (KMF, Germany), and ammonium chloride (Merck, Germany). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Fluka), chloranil (Fluka) and dry toluene (Aldrich, USA) were used in the preparation of the copolymer. Polyurethane foam with density 55 kg m^{-3} was obtained from ContiTech Formpolster GmbH (Lohne, Germany). Cefaclor pharmaceutical samples; Cefaclor acis tablets 500 mg (Arzneimittel GmbH, Germany) and oral suspensions Bacticlor and Cloracef (500 mg each) were purchased from Ranbaxy (Egypt). Ampicillin capsule (Adwic, Egypt) and Amoxicillin acis filmtablette (Arzneimittel GmbH, Germany) were analyzed. Other reagents used in this work were of analytical grade.

2.3. Synthesis of PCTDD–PUF sorbent

The PCTDD copolymer was prepared according to the method reported by Gupta and Prasad [34]. An equimolar amounts of *N*,*N*,*N*′,*N*′-tetramethylethylene diamine (1.23 mL) and chloranil (2.0 g) were dissolved in 50 mL dry toluene and refluxed for 4 h and then kept overnight. The resulting dark brown product was washed with toluene and dried under vacuum. For grafting the PCTDD into the PUF backbone, 0.5 g of PCTDD was dissolved in the least amount of DMF and added to 2.0 g PUF plugs in 100 mL dry toluene and then heated at 90 °C for 72 h (Fig. 2). The brown PCTDD–PUF plugs were washed subsequently with dry toluene and ethanol and then dried in air.

The infrared spectra of PUF and PCTDD–PUF were compared. The spectrum of PCTDD–PUF exhibited two new absorption bands at 1721 and 1659 cm^{-1} which could be assigned to the two C=O groups in the chloranil moiety. Further, the broad band appearing at 3600–3100 cm^{-1} characteristic for the NH_2, OH and NH groups in PUF converted to a narrow band at 3290 cm^{-1} in the PCTDD–PUF spectrum which is characteristic for NH group only. This confirmed the reaction between Cl atoms in the copolymer and NH_2 and OH groups in PUF.

Confirmation of the two forms of PCTDD–PUF was done by measuring the number of NH_2 groups in PUF before and after reaction with PCTDD using Griess titration. The polymer was mixed with 0.1 mol L^{-1} HCl solution and standard $NaNO_2$ solution was then gradually added. The end-point was detected by

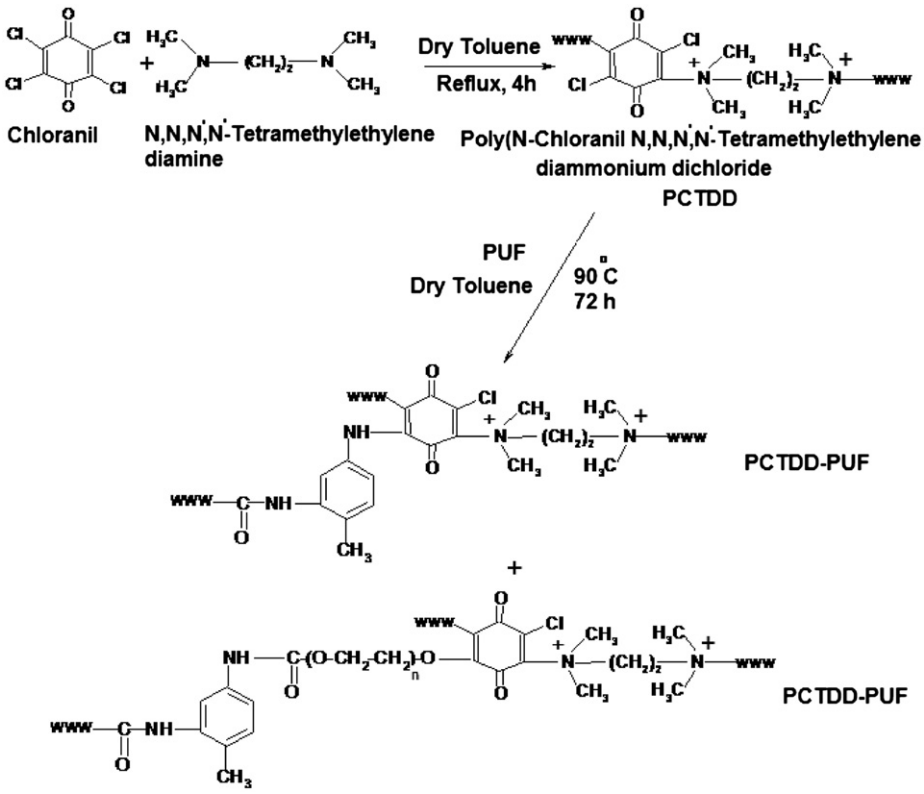

Fig. 2. Schematic diagram for the chemical synthesis of PCTDD–PUF sorbent.

spotting on starch–iodide paper. Results indicated that, 0.0694 mol $g^{-1}$of NH_2 groups in PUF was bonded to PCTDD. Also, the elemental analysis of PCTDD–PUF indicated an increase in the nitrogen content by 2.9% which is equivalent to 0.1035 mol g^{-1} of PCTDD repetitive unit. Therefore, the remaining 0.0341 mol $g^{-1}$of PCTDD should be attached via reaction to OH groups in PUF.

2.4. Sample preparation

2.4.1. Cow milk

The standard addition method was applied for the analysis of commercial cow milk. For this purpose, five portions (10 mL each) from commercial cow milk were spiked up to 100, 200, 300 and 400 ng mL^{-1} level of individual BLA. To each spiked portion, 5 mL of 20% acetic acid was added in order to promote protein precipitation. The mixture was centrifuged at 4000 rpm for 10 min. The supernatant was withdrawn, filtered through a 0.2 μm cellulose acetate membrane filter, adjusted to pH 9.0 and analyzed by the developed method.

2.4.2. Urine

Human urine is a complex matrix containing high levels of urea, uric acid, proteins, fats, sodium, potassium, bicarbonate and chloride ions. Therefore, it was analyzed to evaluate the matrix effects on the quantification of the studied BLAs. The sample was taken from healthy volunteer, divided into 10 mL portions and then spiked to 100 or 200 ng mL^{-1} level by an antibiotic compound. The spiked sample was adjusted to the optimum pH value by ammonia buffer, filtered through a 0.2 μm membrane filter and analyzed.

2.4.3. Pharmaceuticals

A weighed portion from the pharmaceutical sample, equivalent to 50 mg according to the reported BLA content, was dissolved in the least amount of methanol and diluted to 50 mL with deionized water. Aliquots equivalent to 2.5 or 5 μg antibiotic was injected into the preconcentration system under the optimized conditions.

2.5. Flow injection (FI) preconcentration

The FI preconcentration manifold is presented in Fig. 3. In the preconcentration step (A), a 6.0 mL sample containing 250 ng mL^{-1} BLA at pH 9.0 was pumped through the PCTDD–PUF minicolumn at a flow rate of 3.0 mL min^{-1}. The analyte was retained as an ion pair complex and the effluent was discharged to the waste. Meanwhile, the eluent loop (200 μL) was filled up with 0.1 mol L^{-1} HCl solution and the baseline was set by the flow of deionized water carrier into the spectrophotometer. In the elution step (B), the valve was switched to the inject position where the solution in the eluent loop was discharged into the carrier stream under a flow rate of 1.5 mL min^{-1}. The analyte was desorbed from the minicolumn and driven into the spectrophotometer where the peak height absorbance was recorded at 210 nm and the average of triplicate recordings was used for quantification [35,36].

2.6. Chromatographic separation

For separation of the BLAs, an aliquot from a mixture of BLAs (250 ng mL^{-1} each) was preconcentrated and eluted according to the method mentioned in Section 2.4. After desorption by 0.1 mol L^{-1} HCl solution, the BLAs were separated by passing the eluate into another separation column of 25 cm length and 3 mm internal diameter packed with 1.0 g PCTDD–PUF. This column was end-capped with polyethylene joints and implemented to the FI system so that the eluate emerged from the preconcentration minicolumn was mixed with the mobile phase before entering into the separation column. Isocratic separation

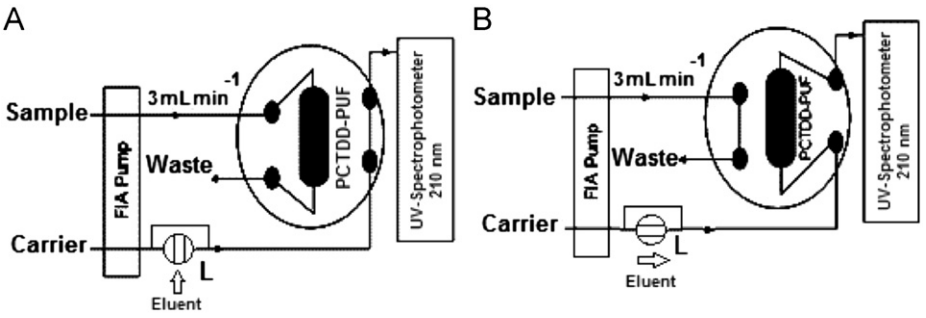

Fig. 3. Schematic diagram of the FI-manifold for the determination of BLAs: 250 ng mL^{-1} antibiotic sample, 3.0 mL min^{-1} flow rate, 120 s preconcentration time, 0.1 mol L^{-1} HCl eluent at 1.5 mL min^{-1}, 100 mg PCTDD–PUF minicolumn and eluent loop L. Preconcentration mode (A) and elution mode (B).

required a mobile phase composed of ammonia buffer at pH 8.0 and the cationic surfactant cetyltetramethylammonium bromide (CTAB) at a concentration of 1×10^{-3} mol L^{-1} ($CMC=3\times10^{-4}$ mol L^{-1}). Absorbance peaks for all BLAs were recorded continuously with a 0.15 mL min^{-1} flow rate of the mobile phase at a fixed wavelength of 210 nm.

3. Results and discussions

3.1. FI-preconcentration

3.1.1. Sample pH

The optimum chemical and hydrodynamic conditions were determined using a 4.0 mL solution containing 250 ng mL^{-1} of BLA compound. The correct adjustment of sample pH is necessary to improve the formation of the ion–pair complex between the BLAs and the PCTDD leading to better sorption. The influence of this parameter on the recovery was studied in the pH range 7–10. Buffer solutions containing KH_2PO_4/NaOH (pH 7) and ammonia/ammonium (pH 8–10) were used to adjust the sample pH. The recovery reached a maximum value in the narrow pH ranges 8.5–9.0, 9.0–10.0, 9.0–9.5 and 8.5–9.0 for cefaclor, amoxicillin, ampicillin and cefotaxime, respectively. As can be seen in Fig. 4, when the sample pH was decreased to pH 7.0, the recovery significantly decreased, probably due to the low availability of dissociated antibiotics which drastically reduced the complex formation. Decreasing the pH below 7.0 resulted in low and constant recovery which suggested no effect due to the immobilized copolymer. On the other hand, at pH ≥ 9.5 the absorbance signal showed a slight decrease, except for cefotaxime for which the decline was large. This observation might be attributed to the existence of negatively charged hydroxide ions in large amounts so that the probability of their attractive interaction with the positive sites in the sorbent increased. Consequently, a reduction in cefotaxime adsorption would occur. Therefore, in the subsequent experiments, the samples were adjusted to pH 9.0.

3.1.2. Sample flow rate and volume

The sample flow rate was optimized carefully to increase analytical throughput and avoid backpressure inside the minicolumn. The flow rate was studied in the range 0.5–6.0 mL min^{-1}. The recovery of analytes was approximately constant and maximum within the range from 0.5 to 3.0 mL min^{-1}. This implied quantitative retention and sufficient contact time as well as good kinetic properties of the sorbent. Flow rates less than 0.50 mL min^{-1} were not studied as the analysis would have been time consuming. At flow rates higher than 3.0 mL min^{-1}, there was a steady decrease in recovery because the passage of BLAs through the minicolumn was too fast and hence the contact time was not sufficient for complete retention into the solid phase.

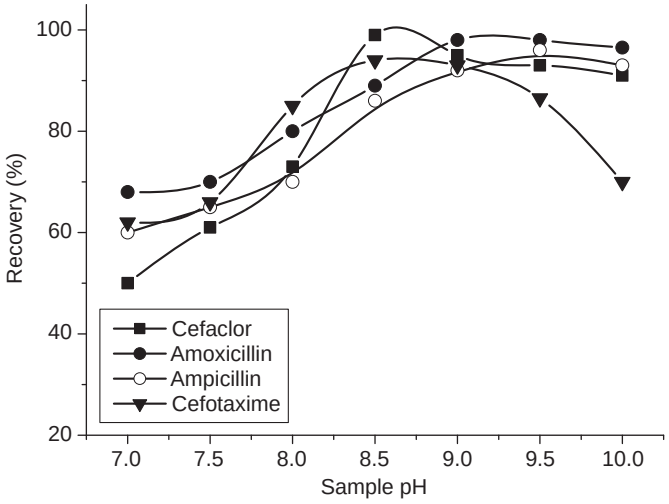

Fig. 4. Influence of sample pH on the recovery of BLAs (4 mL solution, 250 ng mL^{-1}).

Accordingly, a flow rate of 3.0 mL min^{-1} was recommended for subsequent experiments as a compromise between sensitivity and efficiency and to achieve high enrichment factors. The influence of physical shape of the sorbent was studied using both the plug and blended forms keeping a fixed amount of sorbent in the minicolumn. The plug form showed better results than the blended one since the latter resulted in increased backpressure at high flow rates without noticeable improvement in retention efficiency. However, the two physical forms caused very low overpressure in the closed system compared to resin or silica gel based sorbents.

The sample volume was studied within the range of 0.5–15.0 mL at a flow rate of 3.0 mL min^{-1}. The sample volume was calculated by multiplying the preconcentration time and the flow rate. It was found that the height of the absorbance signal increased linearly up to a volume of 9.0 mL implying quantitative preconcentration of the analyte. At higher volumes, the increase in the absorbance becomes nonlinear because the retention capacity of the minicolumn was extrapolated, leading to the deviation from linearity. Thus, a 6.0 mL sample volume was selected in this study to achieve relatively high sample throughput and moderate sample consumption. The results compiled in Table 1 show the variation of absorbance signal with the preconcentrated sample volume.

3.1.3. Eluent study

Elution of BLAs with hydrochloric acid from the PCTDD–PUF minicolumn was investigated. Other acids (nitric, sulfuric and acetic) were tested as eluents but they resulted in strong

Table 1
Effect of the preconcentrated sample volume on the peak height absorbance of the studied BLAs.

Sample volume (mL)	Peak height absorbance			
	Cefaclor	Amoxicillin	Ampicillin	Cefotaxime
1.5	0.011	0.012	0.0078	0.019
3.0	0.047	0.029	0.023	0.042
6.0	0.075	0.052	0.035	0.055
9.0	0.119	0.089	0.073	0.092
12.0	0.122	0.106	0.075	0.110
15.0	0.124	0.115	0.079	0.113

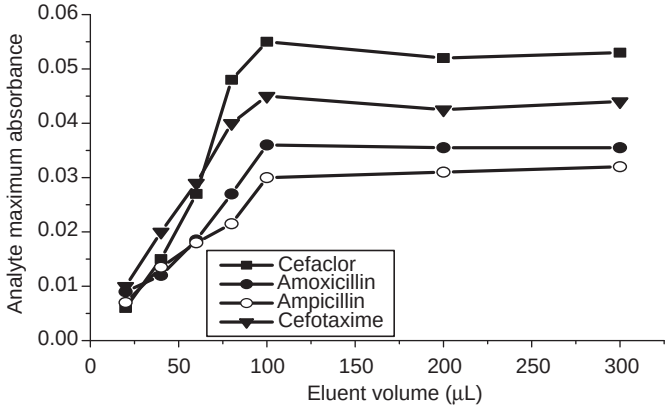

Fig. 5. Influence of the eluent volume on the absorbance of 4 mL BLA solution at concentration 250 ng mL^{-1}, and sample flow rate 3 mL min^{-1}.

background signals. Desorption with acid was fast, since it rapidly decreases the pH inside the minicolumn which in turn changes the ionic form of the antibiotic, resulting in the predominant existence of uncharged forms in solution. This decreases the retention as the formation of the ion–pairs depends on the concentration of the charged species. Moreover, the chloride ion can also occupy positively charged centers of the solid-phase, making retention of the anionic form of the antibiotics difficult. Hydrochloric acid solutions at varying concentrations from 0.025 to 0.175 mol L^{-1} were examined using a 200 μL loop. The absorbance was maximum and nearly constant at concentrations $\geq$0.15 mol L^{-1}. At higher concentrations, a slight increase in the absorbance was observed which might be due to the background absorbance. Therefore, 0.15 mol L^{-1} was chosen as the appropriate eluent concentration. The use of eluent as the carrier was examined but the appearance of peak tailing due to background signal necessitated more time to restore the base line.

3.1.4. Volume of the eluent loop

The influence of the eluent volume was tested in order to ensure complete desorption of the BLAs. Several eluent loops in the range 20–300 μL were applied. The derived results showed regular increase in absorbance within the range 20–100 μL as shown in Fig. 5. Maximum absorbance peaks were obtained for volumes ranging from 100 up to 300 μL. Thus, the 100 μL loop was enough to achieve complete recovery; however, the 200 μL loop was chosen in order to assess a relatively large volume. The variation of eluent volume and concentration with absorbance for cefaclor is shown in Fig. 6. The maximum absorbance occurred at volume of 200 μL and concentration of 0.15 mol L^{-1}.

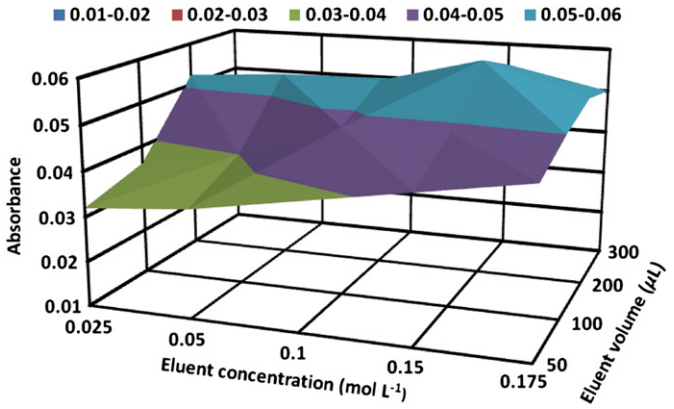

Fig. 6. Influence of the eluent volume and concentration at a time on the absorbance of cefaclor sample of concentration 250 ng mL-1, volume 6 mL, and sample flow rate of 3 mL min^{-1}.

3.1.5. Carrier flow rate

The carrier transfers the eluent into the minicolumn to desorb the antibiotics. It has a remarkable influence on the analytical throughput; however ideally, it should not participate in the chemistry of the chromatographic process. In this work, deionized water was selected as the recommended carrier solvent since it shows no absorbance signal at the wavelength of measurement and it does not have the ability to disrupt the bound BLAs from the sorbent. The eluent was injected into the carrier solvent passing at varying flow rates between 1.0 and 6.0 mL min^{-1}. Maximum analytical signals were observed for flow rates between 1 and 2 mL min^{-1}. The absorbance decreased gradually when the flow rate was increased from 2 to 4 mL min^{-1} followed by a strong decrease at elevated flow rates which may be due to the dispersion of the analyte. Finally, the carrier flow rate was set at 1.5 mL min^{-1} for further desorption studies.

3.2. Sorbent capacity

The sorbent capacity is a very important parameter since it indicates the maximum amount of analyte that can be quantitatively retained onto the sorbent. It was found to be 30 μg g^{-1} (0.82 μmol g^{-1}), 29 μg g^{-1} (0.60 μmol g^{-1}), 32 μg g^{-1} (0.88 μmol g^{-1}) and 31 μg g^{-1} (0.88 μmol g^{-1}) for cefaclor, cefotaxime, amoxicillin and ampicillin, respectively. The sorbent showed equal adsorption capacities for the two penicillins (amoxicillin and ampicillin) which were slightly greater than those for the two cephalosporins (cefaclor and cefotaxime). For cephalosporins, the sorbent had superior adsorption capacity for cefaclor than cefotaxime. The sorbent capacity thus follows the order: amoxicillin = ampicillin > cefaclor > cefotaxime. This observation might be due to the nature of side groups in the BLAs. The presence of similar side groups (phenyl) in case of amoxicillin, ampicillin and cefaclor might explain their closer capacities than cefotaxime which contains a five member heterocyclic ring (thiazole) (cf. Fig. 1). As we expect from our previous investigations, PUF has a greater affinity to adsorb hydrophobic aromatic compounds and phenols than heterocyclic ones.

3.3. Matrix effect

To assess the possible application of the proposed methodology to real samples, the influence of several interfering substances was investigated; especially those commonly existing along with the antibiotic compounds. Solutions containing individual BLA (250 ng mL^{-1}) and the interfering substance at varying concentrations were prepared and the developed procedure

Table 2
Interference effect on BLAs determination.

Foreign substance	Maximum tolerance ratio (mol L^{-1})			
	Cefaclor	Amoxicillin	Ampicillin	Cefotaxime
Glycine	1:60	1:80	1:110	1:80
Tyrosine	1:60	1:60	1:90	1:80
Valine	1:70	1:60	1:80	1:90
Glutamic acid	1:70	1:50	1:100	1:60
Tryptophan	1:60	1:40	1:90	1:50
Glucose	1:80	1:70	1:70	1:70
Barbaturic acid	1:100	1:30	1:80	1:70
Acetylsalicylic acid	1:70	1:50	1:100	1:60
Aspartic acid	1:50	1:40	1:60	1:60
Paracetamol	1:100	1:30	1:90	1:90
Caffaeine	1:90	1:40	1:80	1:70
Oxalic acid	1:100	1:30	1:90	1:60
Citric acid	1:100	1:40	1:90	1:130
Hippuric acid	1:60	1:40	1:90	1:100
Uric acid	1:90	1:50	1:70	1:100
Humic acids	1:80	1:40	1:100	1:90

was applied. The foreign substance was considered interfering when it resulted in a deviation in the recovery by $\geq \pm 5\%$. The obtained data are compiled in Table 2. Selectivity of the sorbent was observed to a varying extent towards the majority of the studied foreign substances. The strongest interference on the cefaclor recovery was induced by the presence of aspartic acid. Ampicillin showed the least interference from all the added interfering substances except for glucose, aspartic acid, caffeine and uric acid. The tolerated ratios were always higher than those commonly found in the majority of real samples which confirm good selectivity of the proposed procedure.

3.4. Sorbent reuse

Intra-day use of sorbent was found to be sustainable after more than 15 cycles of sorption–desorption for about six months. No significant change in the retention capacity was observed which indicates minimum loss in analytical sensitivity. This extended use of the minicolumn could be due to minimum leaching of the chemically anchored copolymer. Another factor which contributes to the long lifetime of the minicolumn is the system design which permits washing with water before each preconcentration cycle.

3.5. Analytical performance

The calibration lines obtained without preconcentration can be represented by the regression equations: $A=(0.00667)C-0.00347$ ($r=0.9963$), $A=(0.00594)C-0.00356$ ($r=0.9981$), $A=(0.00420)C-0.00283$ ($r=0.9972$) and $A=(0.00458)C-0.00161$ ($r=0.9959$) for cefaclor, amoxicillin, cefotaxime and ampicillin, respectively. Employing the FI-preconcentration manifold, the regression lines obtained were: $A=(8.92156E-5)C+0.00157$ ($r=0.9951$), $A=(5.87530E-5)C-0.00334$ ($r=0.9932$), $A=(7.5914E-5)C-0.00228$ ($r=0.9942$) and $A=(4.1675E-5)C-0.00158$ ($r=0.9918$), respectively within the corresponding analytical ranges 40–600, 50–500, 30–500 and 25–400 ng mL^{-1} after 120 s preconcentration time. The limits of detection (LOD), estimated as three times the standard deviation of the blank ($n=5$), were found to be 3.3, 5.1, 7.0 and 3.8 ng mL^{-1} for cefaclor, amoxicillin, ampicillin and cefotaxime respectively. Similarly, the limits of quantification were 11.0, 17.1, 23.6 and 12.8 ng mL^{-1}, respectively. The analytical performance data are compiled in Table 3.

The experimental enrichment factor (EF) [37] was calculated as the ratio of the slopes of the calibration graphs obtained with and without preconcentration. The EF values were found to be 38, 21, 39 and 36 for cefaclor, cefotaxime, amoxicillin and ampicillin, respectively. Obviously, the largest EF value was obtained for amoxicillin which is comparable to that for cefaclor followed by ampicillin. On the other hand, cefotaxime showed the lowest EF value. It is noteworthy that, the order of EF values is directly proportional to the sorption affinity.

Table 3
Analytical performance data of the developed FI-preconcentration procedure.

Parameter	Cefaclor	Amoxicillin	Ampicillin	Cefotaxime
Enrichment factor	38	39	36	21
Preconcentration time (s)	120	120	120	120
Sample volume (mL)	6	6	6	6
Sample frequency (h^{-1})	12	12	12	12
Linear range (ng mL^{-1})	40–600	50–500	25–400	30–500
LOD (ng mL^{-1})	3.3	5.1	7.0	3.8
LOQ (ng mL^{-1})	11.0	17.1	23.6	12.8
Precision (*RSD%*, $n=15$)	2.3		4.5	

Table 4
Retention time (R_t) and recovery (%) values for the isocratic separation of BLAs.

Parameter	Amoxicillin	Cefotaxime	Cefaclor	Ampicillin
R_t (s), Reference	272	308	370	449
R_t (s), Mixture[a]	270 ± 7	300 ± 10	380 ± 15	450 ± 5
Recovery (%)[b]	90	86	92	96

[a] Mean value for three replicates measurements.
[b] Recovery percentage was calculated from the ratio of peak areas.

3.6. Separation of the BLAs by the developed FIA system

Although liquid chromatography is the most widely used technique to separate antibiotics, the utilization of such a simple set-up for this purpose is not yet known till now. Micellar mobile phases have some advantages e.g. they possess unique separation selectivity, leads to stable detector responses and enables direct injection of samples containing very complex matrices, such as urine and milk. Several organic compounds have been separated using micellar mobile phases such as phenols [38], sugars [39] and amino acids [40].

Herein, we have attempted to achieve an isocratic separation pattern that can distribute these compounds along the analytical column as all the BLAs are intended to be detected at a single wavelength. In this context, a 25 cm long and 3 mm i.d column packed with PCTDD–PUF sorbent was employed. A micellar mobile phase composed of ammonia/ammonium buffer with pH 8.0 and CTAB surfactant was passed at a flow rate of 0.15 mL min^{-1}. A constant flow of the mobile phase could be obtained due to the high porosity of the sorbent, too low flow rate of the mobile phase and the existence of two rolling pumps in the FIAS-400 instrument. The BLAs were separated based on their relative attractive interaction forces towards the positively charged stationary phase (sorbent) and the cationic surfactant. Both the solution pH and CTAB concentration in the mobile phase were examined. It was found that, a pH of 8 for the mobile phase and a CTAB concentration of 1×10^{-3} mol L^{-1} gave the best results. The retention time (R_t) was found to be reproducible and it was used for the identification of BLAs by matching its value obtained in the mixture to that of a reference chromatogram (Table 4). The recovery was calculated from the ratio of peak areas for the antibiotic compound in the mixture with that of the reference peak.

The separation pattern showed some overlap between the amoxicillin and cefotaxime signals which affected the recovery values. On the other hand, cefaclor and ampicillin were well separated from each other and from the other two BLAs.

The value of peak areas for the two overlapping peaks corresponding to cefotaxime and amoxicillin were calculated based on the similarity in both their peak heights and band widths. A similar overlapping area can be expected on both sides of a vertical line drawn at the point of intersection of the peaks. This point simultaneously recognizes the end point for the cefotaxime signal and the starting point for the amoxicillin signal [41]. The base line has a constant trend in the region of BLA peaks but due to some technical reasons it was not quite stable. However, it approximately fits the base line for the signals of cefaclor and ampicillin.

3.7. Accuracy and precision

The accuracy and precision were validated by measuring the relative reproducibility intra-day standard deviation (RSD_{intra}), inter-day relative standard deviation within 5 day (RSD_{inter}) and recovery for 6.0 mL spiked commercial milk sample. Two spiked levels at 50 and 400 ng mL^{-1} were tested which represent the limits of the linear range. The results for four replicate measurements are summarized in Table 5. The precision indicated by the values of RSD_{intra} and RSD_{inter} were found to be less than 9.4% and 11.0%, respectively. Accuracy data represented by the mean recoveries were in the range 81%–104% with standard deviations not exceeding 10.5%. Therefore, the method can be considered accurate and precise which confirm its validity for real applications.

Table 5
The relative intra-day standard deviation (RSD_{intra}), inter-day standard deviation (RSD_{inter}) and recovery values for spiked commercial milk sample after four replicate measurements.

BLAs	Spiked level (ng mL^{-1})	RSD_{intra}	RSD_{inter}	Recovery (%) (mean ± S.D)
Cefaclor	50	6.3	5.7	94 ± 4.6
	400	7.1	2.5	94 ± 4.6
Amoxicillin	50	8.1	6.0	94 ± 4.6
	400	9.4	3.5	101 ± 5.2
Ampicillin	50	1.1	3.8	97 ± 2.8
	400	5.8	9.9	104 ± 5.0
Cefotaxime	50	4.4	8.5	98 ± 7.3
	400	9.0	11.0	81 ± 10.5

Table 6
Determination of BLAs in spiked human urine using the proposed procedure.

BLAs	Spiked (ng mL^{-1})	Found (ng mL^{-1}) mean ± S.D, n=3	Recovery (%)	*RSD* (%)
Cefaclor	100	95 ± 5.1	95	5.4
	200	198 ± 5.0	99	2.6
Amoxicillin	100	109 ± 2.5	109	2.3
	200	194 ± 4.5	97	2.1
Ampicillin	100	104 ± 6.0	104	4.1
	200	197 ± 7.1	99	3.6
Cefotaxime	100	106 ± 3.5	106	3.3
	200	191 ± 8.9	96	8.7

3.8. Application to real samples

The preconcentration system developed was able to determine these antibiotics in pharmaceutical, urine and cow milk samples. The results for the analysis of a urine sample are listed in Table 6. They were found to be satisfactory as the recovery varied from 95% to 109% with *RSD* values of 2.1%–8.7%. For the analysis of pharmaceuticals, the amount of sorbent was increased to a one gram column for efficient recovery as the sample concentration was too high. From Table 7, the recovery was found to be between 83% and 99% and the *RSD* varied from 0.1% to 9.7%. Although a high *RSD*% was obtained in the analysis of Bacticlor, it was still less than the permitted value for the analysis of real samples (10%).

3.9. Analysis of cow milk

For the analysis of cow milk, spiked samples were subjected to analysis according to the recommended procedure. Since the sample might contain a mixture of antibiotics, a separation step was necessary before the detection of all the analytes at a single wavelength. Accordingly, the BLA was first preconcentrated and separated from the matrix with a 3 cm long minicolumn followed by separation from possible co-existing BLAs on a 25 cm long column according to the setup illustrated in Fig. 7. The effluent from the sample was not allowed to pass through the separation column (C2) or the flow cell. By switching the valve (V), the effluent was discharged to the waste route (1) to avoid additional time for cleaning. Otherwise, the carrier solvent was passed for more than one hour in order to restore the absorbance base line. First, the sample was preconcentrated on the minicolumn C1. After this, the eluent E was injected at a flow rate suitable for separation on the separation column C2 using the micellar mobile phase and the signal was recorded. Standard addition curves

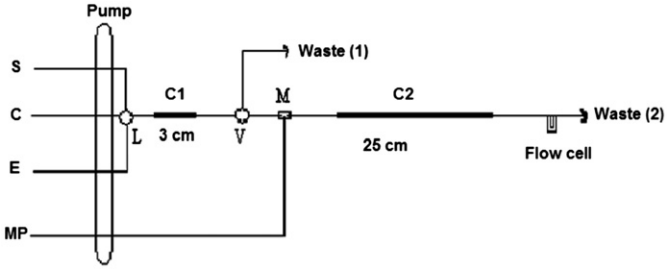

Fig. 7. Preconcentration/separation setup used for the analysis of spiked milk: Sample pH 9.0 (S), carrier (C), eluent (E), mobile phase (MP), preconcentration minicolumn (C1), separation column (C2), eluent loop (L), three way Teflon valve (V), and mixing coil (M).

Table 7
Analysis of pharmaceutical samples using the FI-preconcentration procedure with 1.0 g sorbent column.

Pharmaceutical sample	Amount reported (mg)	Amount injected (μg)	Found (μg) Mean ± SD	Recovery (%)	*RSD* (%)
Ampicillin	500	5	4.94 ± 0.40	99	8.7
Bacticlor	250	2.5	2.32 ± 1.20	93	9.7
Clorocef	250	2.5	2.10 ± 0.25	83	2.3
Cefaclor acis	500	5	4.82 ± 0.05	96	0.1
Amoxicillin acis	500	5	4.61 ± 0.06	92	1.4

Table 8
Standard addition lines obtained for analysis of BLAs in milk.

BLAs	Regression equation	R^2
Cefaclor	$A=(0.00115)C+0.0326$	0.9740
Amoxicillin	$A=(0.00255)C+0.0159$	0.9645
Ampicillin	$A=(0.00183)C+0.0290$	0.9867
Cefotaxime	$A=(0.00128)C+0.0123$	0.9877

Table 9
Recovery of the investigated BLAs from spiked commercial milk sample.

BLAs	Fortified (ng mL^{-1})	Found (ng mL^{-1}) (mean ± S.D, $n=3$)	Recovery (%)	*RSD* (%)
Cefaclor	200	195 ± 3.1	97.5	1.6
	400	393 ± 9.6	98.2	2.2
Amoxicillin	200	210 ± 4.7	105.0	5.2
	400	384 ± 8.5	96.0	2.2
Ampicillin	200	182 ± 6.1	91.0	3.4
	400	375 ± 11.5	93.7	3.1
Cefotaxime	200	188 ± 5.2	94.0	2.8
	400	372 ± 14.9	93.0	4.0

were obtained and the recovery percentages were calculated. The results obtained are reported in Table 8.

Indeed, strong background signals were observed due to the complex matrices in milk which led to an increase in the apparent absorbance. Spiking the milk with increasing concentrations of BLAs (100–400 ng mL^{-1}) resulted in a regular increase in absorbance but it remained small compared to the matrix signal. However, satisfactory correlation coefficients were obtained for the standard addition lines. The regression equations are listed in Table 8. Despite this, the method can be considered satisfactory since a separation step was carried out prior to the determination to remove most of the interfering substances. The recovery values obtained for the spiked milk samples are varied between 91% and 105% and the corresponding *RSD* values were from 1.6% to 4.0% (Table 9).

Finally, the method under consideration was applicable for the determination of these antibiotics in urine, pharmaceutical and milk samples where their concentrations were within the allowed LOD of the procedure.

4. Conclusions

In this work, a fast and simple FI-preconcentration procedure was developed for the determination of BLAs. The developed procedure is very simple, sensitive, inexpensive and eco-friendly and it shows high tolerance to interfering ions. The adequate accuracy of the procedure makes it an alternative to conventional methods for the determination of BLAs in pharmaceutical and biological samples. The results for the analysis of urine, pharmaceuticals and milk samples have confirmed the reliability of the method. The PCTDD–PUF minicolumn was recycled several times without significant effect on its retention efficiency. Finally, the separation of the BLAs studied could be applied for simple analysis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.05.007.

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