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Extraction of Cefquinome from Food by Magnetic Molecularly Imprinted Polymer

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Abstract

Objectives: Cefquinome is a fourth generation cephalosporin with broad spectrum antimicrobial activity. It is commonly used against respiratory tract diseases, acute mastitis, calf septicemia and foot rot in livestock. Clean-up step in sample preparation is invariably required for avoiding interferences in assay from other components. It is envisaged to prepare molecular imprinted based polymer with desired selectivity for cefquinome. Method: Imprinted polymer was synthesized over the surface of magnetite using methacrylate (monomer) acid and ethyleneglycoldimethacrylate (crosslinker) in the presence of cefquinome (imprint molecule). Magnetite was prepared by co-precipitation of 1.98 g $FeCl_2.4H_2O$ and 5.41 g $FeCl_3.6H_2O$. Magnetic non-imprinted polymer was prepared similarly in absence of cefquinome. Selectivity in different solvent was calculated from partition coefficient values in imprinted and non-imprinted polymers. Result: Selectivity of imprinted polymer over non-imprinted polymer was dependent on nature of solvent and pH. Binding of cefquinome was highly selective in acetonitrile. Imprinted polymer lacks selectivity in methanol and 1M NaCl. Ionic interaction between quinolinium ion in cefquinome and carboxylate anion in polymer appears to be major force for recognition of cefquinome by imprinted polymer. Prepared polymer extracted cefquinome from milk, honey, egg and water and extraction efficiency was in the range of 74-93.7%. Conclusion: Selectivity of magnetic imprinted polymer must be determined in different solvents and this will enable selecting binding and elution conditions during extraction of imprint molecule.

Keywords: Cefquinome; Imprinted polymer; Magnetite; Selectivity; Extraction; FTIR; Polymerization

Introduction

Cefquinome is a fourth generation cephalosporin with broad spectrum antimicrobial activity and is resistant to β-lactamase [1,2]. Chemically cefquinome is (6R, 7R)-7-[[(2Z)-2-(2-Amino-1,3-thiazol-4-yl)-2-methoxyiminoacetyl]amino]-8-oxo-3-(5,6,7,8-tetrahydroquinolin-1-ium-1-ylmethyl)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (Figure 1). It is commonly used against respiratory tract diseases, acute mastitis, calf septicemia and foot rot in cattle and also respiratory diseases, Metritis-Mastitis-Agalactia Syndrome in sows. Indiscriminate use of cefquinome in dairy industry leads to retarded milk fermentation, toxicological effects and hypersensitivity, thereby affecting public health. European Union has established a maximum residual limit of cefquinome in milk (20 µg/Kg), fat (50 µg/Kg), liver (100 µg/Kg), kidney (200 µg/Kg) [3,4].

Preparation of sample is usually required before food analysis [5]. Available sample preparation techniques are based on liquid–liquid extraction [6], membrane extraction [7,8], solid-phase extraction [9,10], microwave assisted extraction [11,12] and ultrasonic assisted extraction [13,14]. These methods lack specificity and extracted material may contain large amount of undesired chemical entities which are often removed by another clean up step. Clean up protocols with enhanced specificity is integral part of molecular imprinted polymer (IP) which is synthesized from functional monomer and cross-linking monomer in the presence of target molecule.

Prior interaction of target molecule with monomer enables synthesis of polymer with pre-defined specificity. The target molecules are knocked-off from prepared polymer to create binding sites for target molecule in sample. IP has a molecular recognition site which is chemically and spatially complementary to the target molecules [15]. These polymers are stable at extreme pH and temperature, easy to prepare, involve low cost and are reusable. Such polymers have been prepared against widely different molecules including antibiotics [16-18], pesticides [19,20], heavy metals [21,22], drugs [23], lactate [24] and for different purposes such as artificial antibodies [25], catalyst [26], chemical sensors [27] and solid phase extraction [18]. Use of these materials in solid phase extraction requires negative or positive pressure generating pump or centrifuge machine, limiting its use with ease.

Magnetic imprinted polymers offer distinct advantage over non-magnetic polymers in their ease of separation under magnetic field after binding to their target molecules. These particles are prepared by encapsulating inorganic magnetic particles in molecular imprinted polymers and thus retain magnetic properties and specificity for target analyte.

This has encouraged many workers to prepare magnetic imprinted polymers having specificity towards chloramphenicol [28], sulfonylurea [29], oxytetracycline [30], β-lactam antibiotic [31], protocatechuic acid [32], macrolide antibiotics [33], metronidazole [34], levofloxacin [35], polychlorinated biphenyls [36], erythromycin [37]. The present work focuses on synthesis of cefquinome specific magnetic polymer and its use in extraction of the antibiotic from food matrix.
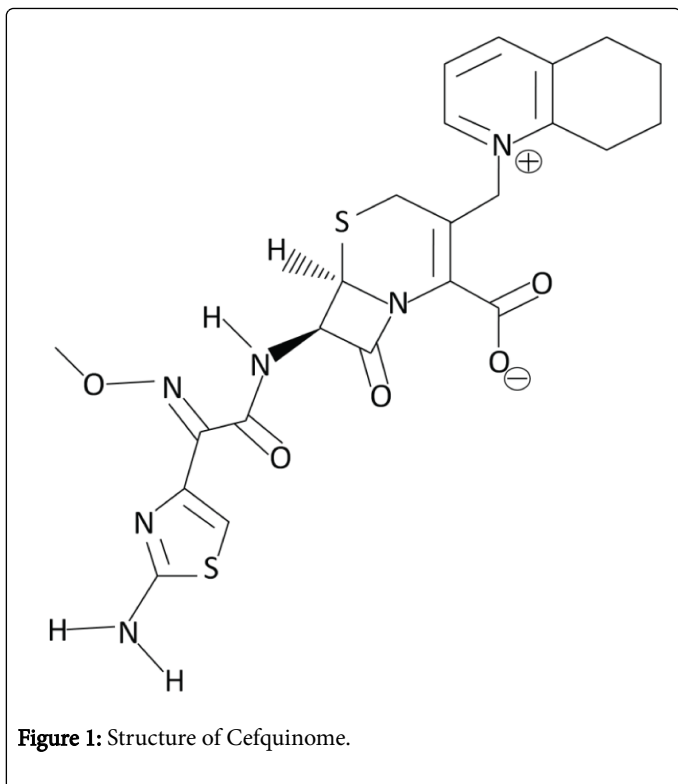
Figure 1: Structure of Cefquinome.

Materials and methods

Materials

Cefquinome sulphate, tetracycline, cefalexin, gentamicin, penicillin, ampicillin, methacrylic acid (MAA), ethyleneglycol dimethacrylate (EGDMA), oleic acid, iron (II) chloride ($FeCl_2.4H_2O$), iron (III) chloride ($FeCl_3.6H_2O$), polyvinylpyrrolidone (PVP), azobisisobutyronitrile (AIBN) were purchased from Sigma Aldrich, USA. Methanol (HPLC grade), ethanol, acetic acid glacial and acetonitrile (HPLC grade) were procured from Hi-media, India.

Food samples

Milk samples were obtained from Institute cattle yard (NDRI). Honey was obtained from Dabar India, Ltd. Eggs were obtained from local market. These food samples were spiked with cefquinome stock solution (100 μg/mL) prepared in water.

Preparation of magnetic imprinted polymer (MagIP)

Preparation of Fe_3O_4 magnetite-oleic acid-EGDMA mix: Magnetite was prepared by co-precipitation method [38]. In brief, 1.98 g $FeCl_2.4H_2O$ and 5.41 g $FeCl_3.6H_2O$ were dissolved in 100 mL deaerated water. The mixture was purged with nitrogen gas and stirred vigorously at temperature 80°C. Then, 40 mL NaOH solution (2 N) was added drop wise. After 1 h, magnetic precipitates were separated with help of external magnet and washed several times with deaerated water. After washing, precipitates of magnetite were dried at 60°C. One gram Fe_3O_4 magnetite (freshly prepared) and 1.0 mL oleic acid (99% pure) were mixed together for 10 minutes. Then 3.77 mL EGDMA (98% pure) was added and contents were mixed.

Preparation of preassembly solution: 1.0 mmol cefquinome and 344 μL MAA (98.5% pure) were added to 10 mL deaerated water to allow the interaction between MAA and cefquinome. The mixture was stirred for 10 min.

Preparation of pre-polymerization mix: Magnetite–oleic acid–EGDMA mix was mixed with preassembly solution and then the contents were sonicated for 30 min.

Polymerization reaction: 0.4 g PVP was added to 100 mL 80% aqueous ethanol solution and the contents were heated to 60°C while purging nitrogen gas. Then, pre-polymerization mix and 3 mL AIBN (98% pure) were added. The contents were maintained at 60°C for 24 h under continuous stirring at 300 rpm. The prepared polymer was separated with the help of external magnet and washed several times with methanol: acetic acid (8:2 v/v) mixture, till washings were free from cefquinome. The polymer was further washed three times with deaerated water and dried at 60°C. Magnetic non-imprinted polymer was prepared similarly as MagIP, except cefquinome was omitted from pre-assembly solution.

Characterization

MagIP was characterized by scanning electron microscope (SEM; Carl Zeiss, Germany) and Fourier–transform infrared spectrometry (FT-IR Shimadzu, IR affinity, Japan).

Selectivity of imprinted polymer

Selectivity of prepared polymer was calculated by ratio of partition coefficient of cefquinome for imprinted and non-imprinted polymer in water, acetonitrile, methanol, 1 M NaCl and at pH range from 4 to 7 in aqueous buffer [39]. Polymers (20 mg) were incubated with 2 mL cefquinome (40 μg) prepared in different solvent for 24 h at 30°C. Unbound cefquinome and other studied antibiotics were assayed in supernatant by measuring absorbance at 221 nm. Bound antibiotic was calculated by subtracting unbound antibiotic from total antibiotic added.

Cross-reactivity of cefquinome imprinted polymer

40 μg each of antibiotics was incubated with 20 mg polymer in 2 mL, 20 mM phosphate buffer (pH 7.0) for 24 h at 25°C. Bound antibiotic was calculated by subtracting unbound antibiotic from total antibiotic.

Extraction of cefquinome from water, milk, honey and egg-white

40 mg imprinted or non-imprinted polymer was conditioned in sequence with 3 mL methanol and 3 mL water. 2 mL water (degassed) or 2 mL of milk or 2 g honey or 2 mL egg-white was diluted to 16 mL with H_2O. Then, these diluted food samples were spiked with 200 μg cefquinome (solubilised in 2 mL acetonitrile) and mixed with conditioned imprinted or non-imprinted polymer for 10 min. Unbound cefquinome was collected with ten successive washings with 3 mL acetonitrile. The bound cefquinome was then eluted by successive elution with 3 mL 0.2 M NaCl.

Results and discussion

Cefquinome imprinted polymer coated over magnetite was well furnished with magnetic property which leads to the easy separation of

polymer from sample matrix after binding to target molecules. Magnetite and polymer act as a core and coat of magnetic imprinted polymer respectively. 4.2 g polymer was obtained from 4 mmol MAA and 20 mmol EGDMA. Cefquinome imprinted polymer was spherical in shape, porous and was 630 ± 143 nm in size (Figure 2). Polymer exhibited peak at 540 cm^{-1} (characteristic of Fe-O bond stretching), 1150 cm^{-1} (C-O stretch), 1370 cm^{-1} (C-H rock), 1450 cm^{-1} (C-H bend), 1724 cm^{-1} (C=O stretch) and 2350 cm^{-1} (C≡N) in FTIR (Figure 3). C=O stretch and C-O stretch peaks imply that carboxyl groups are present on the surface of polymerized matrix and will be available for interaction with cefquinome. The peak at 2350 appears of nitrile group (−C≡N). AIBN is a nitrile containing compound and has been used as initiator in polymer formation. This compound is thermally decomposed and generates free radical which subsequently acts on monomer and cross-linker. In this process, nitrile group get attached to monomer and cross-linker and hence to the finished polymer.
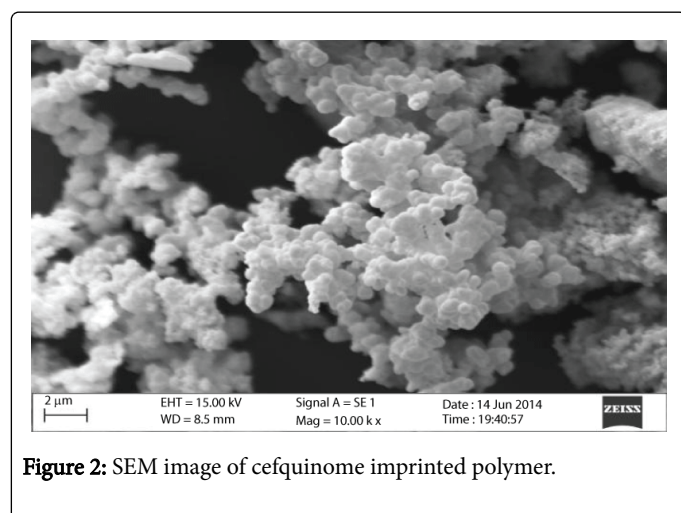

Figure 2: SEM image of cefquinome imprinted polymer.

Effect of solvent on binding of cefquinome: Binding capacity of imprinted and non-imprinted polymers was evaluated in H$_2$O, methanol, acetonitrile and 1M NaCl (Table 1). The binding of cefquinome to imprinted polymer in acetonitrile was 62% which was distinctly higher than in water (17%), methanol (15%) and 1M NaCl (5%). In general, binding of cefquinome to non-imprinted polymer was low and was in the range of 5-15 % (Table 1).

Selectivity of imprinted polymer over non-imprinted polymer was strikingly high in acetonitrile (selectivity 31.01) in contrast to low selectivity in water (selectivity 1.84). Cefquinome failed to exhibit selectivity in methanol and 1 M NaCl. Non-polar solvents such as acetonitrile and methanol facilitate non-covalent interactions.

Dipole moment of acetonitrile is 3.54 and thus it can easily facilitate conversion of carboxylic group present in polymer into dissociated state. β-Lactum nucleus in cefquinome is surrounded by quaternary quinolinium, an amino thiazolyl moiety and O-alkylated oxime. It exists as zwitter ion. Quaternary quinolinium cation of cefquinome can interact with carboxylate anion of polymerized matrix through ionic interaction.

Further, hydrogen bonds between carboxylic group of polymerized matrix and amino and carbonyl group of cefquinome can be easily formed and these may assist in orienting cefquinome to allow ionic interaction between quinolinium and carboxylate. Methanol is protic non-polar solvent and can form hydrogen bonds both with cefquinome and polymer.

Thus, in contrast to acetonitrile, methanol will disrupt hydrogen bond formation between cefquinome and carboxylate. Dipole moment of acetonitrile (3.54) is distinctly higher than either methanol (1.7) or water (1.85) and therefore, in comparison to methanol, acetonitrile has better capability to polarize carboxyl group available in polymerized matrix. This explains the selectivity of imprinted polymer towards cefquinome in acetonitrile, not in methanol.
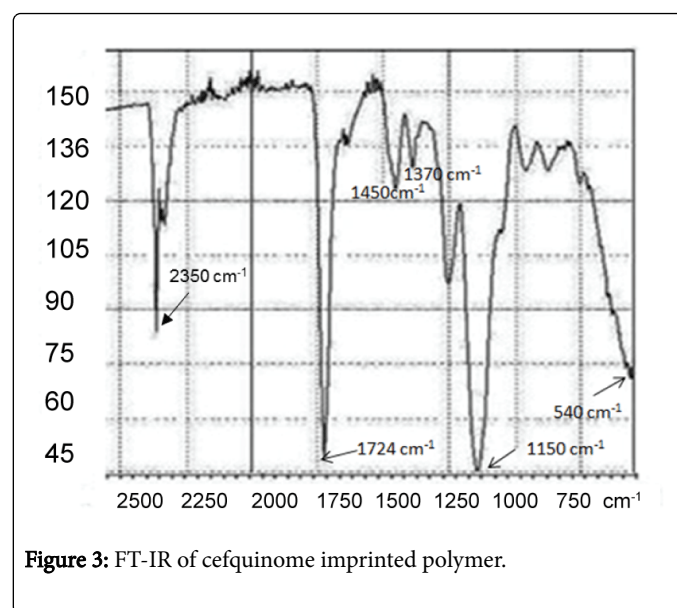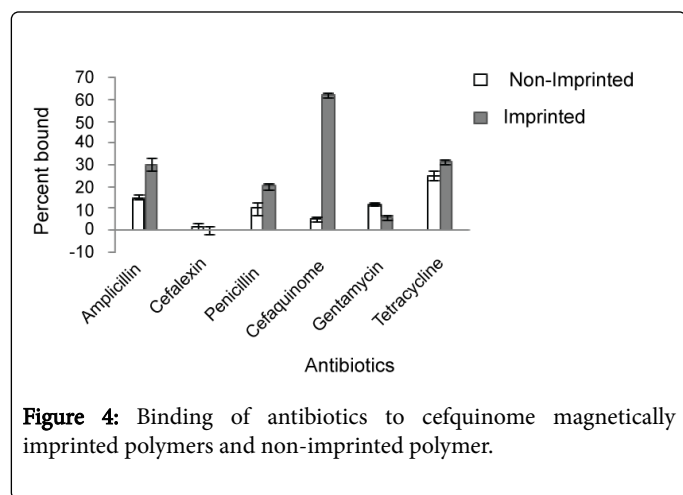

Figure 3: FT-IR of cefquinome imprinted polymer.

Effect of pH on binding of cefquinome: The second factor that influences the binding efficiency of cefquinome to imprinted polymer was pH. At pH 7.0 and at pH 6.0, the binding of cefquinome was 53 and 46% respectively. Comparatively, at lower pH (pH 5.0 and 4.0), the binding was 3 to 7.5-fold lower (Table 1). pH influences dissociation of carboxylic group. pKa of polymerized methacrylic acid is 5.5 [40] and thus at increased pH, more number of carboxylic group will exist as carboxylate anion which can interact with quinolinium cation. At lower pH, the number of carboxylate anion in polymer will be limited and will result in low binding. The ionic interaction between cefquinome and carboxylate anion is further supported by very low cefquinome binding (5%) in presence of 1M NaCl (Table 1).

Cross reactivity of cefquinome imprinted polymer: The specificity of cefquinome imprinted polymer was checked by studying the binding of three β-lactam group antibiotics (ampicillin, cefalexin, penicillin), gentamycin and tetracycline. The binding pattern of these antibiotics to imprinted and non-imprinted polymers suggests that binding of cefquinome to imprinted polymer is highly specific (Figure 4). Further, the binding of cefalexin and gentamycin to imprinted polymer was less than 7%. However binding of penicillin was 20%, and that of ampicillin and tetracycline about 30%. The high selectivity of imprinted polymer towards cefquinome will be advantageous in separation methods.

| Solvent	Polymer	Percentage bound	Binding Capacity (μg/mg beads)	Partition Coefficient	Selectivity
Water	Imprinted	17	1.7	20.48	1.84
	Non-imprinted	10	1.0	11.11	
Methanol	Imprinted	15	1.5	17.64	1.0
	Non-imprinted	15	1.5	17.64	
Acetonitrile	Imprinted	62	6.2	136.15	31.01
	Non-imprinted	5	0.5	5.26	
1M NaCl	Imprinted	5	0.5	5.26	1.0
	Non-imprinted	5	0.5	5.26	
20 mM Acetate buffer pH 4.0	Imprinted	7	0.7	7.52	1.42
	Non-imprinted	5	0.5	5.26	
20 mM Acetate buffer pH 5.0	Imprinted	15	1.5	17.62	1.58
	Non-imprinted	10	1	11.11	
20 mM Phosphate buffer pH 6	Imprinted	46	4.6	85.15	7.66
	Non-imprinted	10	1	11.11	
20 mM Phosphate buffer pH 7	Imprinted	53	5.3	112.76	21.43
	Non-imprinted	5	0.5	5.26	

Table 1: Effect of solvent on binding, partition coefficient and selectivity of cefquinome magnetically imprinted polymer.

Figure 4: Binding of antibiotics to cefquinome magnetically imprinted polymers and non-imprinted polymer.

Extraction of cefquinome from food: The extraction capability of cefquinome imprinted polymer was evaluated for extraction of cefquinome from water, milk, honey and egg-white. For this purpose water, milk, honey and egg-white spiked with cefquinome were 10-folds diluted with water to allow electrostatic interaction between cefquinome and imprinted polymer. The unbound cefquinome was washed with acetonitrile (Eluent 2-10) and bound cefquinome was eluted with 0.2 M NaCl (Eluent 11 onwards). The binding and elution profile indicates that cefquinome did bind to imprinted polymer in water or diluted food matrices and the bound cefquinome could be eluted with 0.2 M NaCl (Figure 5). Also, there was very little binding of cefquinome to non-imprinted polymer. The recovery from water, milk, honey and egg white was 80, 93.7, 92.8 and 74%, respectively.
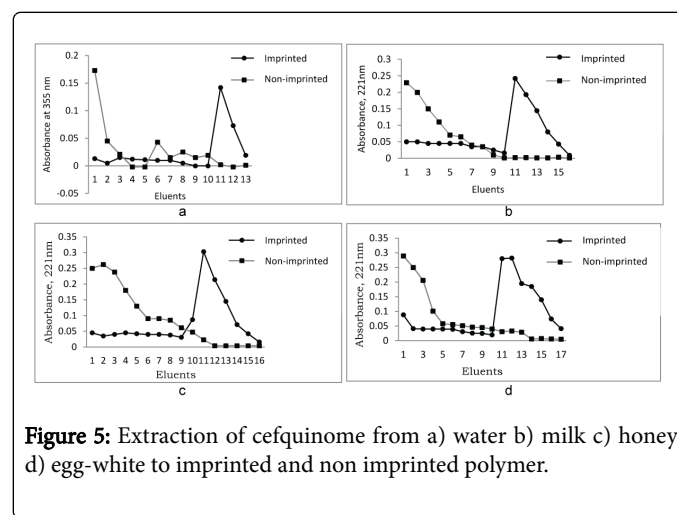
Figure 5: Extraction of cefquinome from a) water b) milk c) honey d) egg-white to imprinted and non imprinted polymer.

Conclusion

It is essential that binding of analyte is evaluated under different solvent conditions before concluding presence or absence of specific binding sites in imprinted polymer. This also helps in selecting appropriate conditions for analyte binding to imprinted polymer and its elution conditions. MagIP particles exist in colloidal state in absence of magnetic field and thus it is advantageous for quick interaction

between analyte and dispersed particle. Also, MagIP can be easily separated by application of external magnetic field. Cefquinome imprinted magnetic polymer can be used for extraction of cefquinome from water and food matrices.

Acknowledgment

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