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Research Article

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Luminescence Detection of Fluoroquinolone Antibiotic Contamination in Raw Milk Samples by Lanthanide Probes and its Validation with LC-MS/MS Technique

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Abstract

Fluoroquinolone antibiotics are the most common class of drugs used to treat a wide variety of diseases for both humans and veterinary animals. Their excess use as veterinary drugs results in contamination of common foods (milk, meat, fish, egg, honey) with these antibiotic molecules and their metabolic products. A simple strategy for their detection in raw milk samples is very essential looking into the widespread use of these antibiotics by various unorganized dairy firms and individual farmers in rural India. Thus, in the present work two common lanthanide compounds; one red emissive Eu-C1 and one green emissive Tb-C2 were tested for quantitative detection of four different fluoroquinoloneantibiotics (Ciprofloxacin (CIP), Enrofloxacin (ENR), Difloxacin (DIF) and Flumequine (FMQ)) through luminescence titration method. The Eu-C1 and Tb-C2 complexes showed turn-off and turn-on luminescence responses respectively on incremental addition of antibiotics in the solution phase with high K_{sv} values (up to 5 x 10^3 M^{-1}) and a low detection limit of 25 ppb. A paper-based solid-state test strip impregnated with Eu-C1 complex was also demonstrated to successfully determine the presence of these antibiotics. In parallel, aliquid chromatography-tandem mass spectrometry (LC-MS/MS) based technique was also developed for quantification followed by validation of the detection efficiency using the above lanthanide probes. The concentration dependent intensity graph with standard samples showed good linear regression fitting (R^2~0.99) and detection limit of 1 ppb with recovery efficiency 90 to 105% from spiked raw milk samples.

Keywords

Antibiotic, Fluoroquinolone, LC-MS/MS, Lanthanide, Luminescence, Raw milk

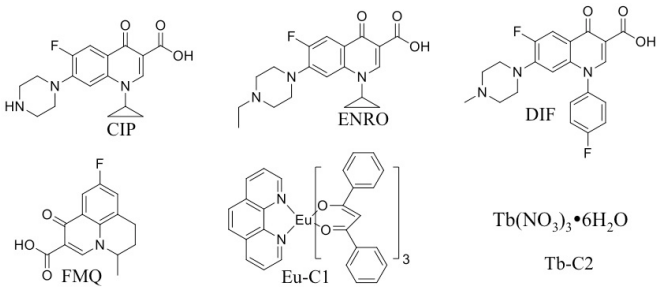
Introduction

Over-use and misuse of antibiotics are contributing to the rising threat of antibiotic resistance which is becoming a serious human health concern throughout the world [1-3]. The consumption of antibiotic-contaminated food (e.g., milk, meat, fish, egg) for a prolonged period is one of the major reasons for antibiotic resistance in humans [4, 5]. To tackle this issue many countries have already taken protective measures to reduce the use of antibiotics in food-producing animals [6, 7]. Amongdifferent antibiotic families, Fluoroquinolone-based antibiotic molecules (Enrofloxacin, Difloxacin and Flumequine), that are active against a high range of pathogenic microorganismsare frequently used ashuman and veterinary drugs for the treatment of various infectious diseases [8]. Although Ciprofloxacin is not commonly used for the veterinary purpose, but ENR is partly de-ethylated to CIP *in vivo* and thus it could also be a

contaminated molecule in those food items [9].The European Union has defined the MRLs for several of these compounds in the different food matrices of animal origin (EC 1990) and according to Commission Regulation (EU) 37/2010 (EC 2010), quinolones range between 100-1900 µg/kg in chicken liver, and 30-100 µg/kg in milk are permissible range. India is presently the second-highest milk-producing country in the world with annual milk production of 100 million tonnes in 2017,thanks to the'White Revolution'program initiated in 1970 by India's National Dairy Development Board [10].The majority of milk in India is produced by individual households and small farms, with limited awareness of the safe use of antibiotics. Thus, proper monitoring of antibiotic levels in raw milk samples through the use of reliable and cost-effective techniques is an urgent necessityin many countries including India.

Use of several strategies have been reported for the detection of fluoroquinone antibiotics from milk samples: Microbiological methods [11, 12], Chromatographic methods including liquid chromatography coupled with mass spectrometry (LC-MS) [13-16], High-performance liquid chromatography (HPLC), Optical absorption (UV-Vis spectroscopy) based methods [17-19]and luminescence [20-22]based detection strategies have been demonstrated. Among these methods,the LC-MStechnique is very sensitive and reliable for the detection of trace-level antibiotic residues although it is comparatively expensive and time consuming [23]. Spectrofluorimetric based techniques another popular choice for detection of trace level of any analyse due to its extremely high sensitivity (works even at parts-per-billion level), cheap processing cost, easy portability in distant places and quick response time [24-28]. Analyse specific chemical scaffoldsneed to be designed for this purpose which could show turn-off or turn-on luminescence response on interaction with the target analyses.

Lanthanide ions (Ln^{3+}) show electron-dipole forbidden f-f transition based long lived and sharp emission bands with low quantum yield. Electron rich aromatic ligands have been effectively used to sensitize the lanthanide excited states and thus enhancing the emission intensity which is commonly known as the antenna effect [29, 30]. This antenna effect originates through energy transfer from chromophorecentered triplet state to an excited state of lanthanide ion. Thus,the electron donating ability of the ligands can tune the emission intensity without shifting the emission wavelength and the strategy has been exploited for luminescence-based sensing of a variety of analyses, which have an affinity to make a bond with lanthanide ions [31]. Envisioned by this, we intended to develop lanthanide complex-based luminescence probes to detect trace-level antibiotic molecules. The development of such techniques and proving their effectiveness for the detection of trace level antibiotics with standard techniques like LC-MS/MS is very important. In line with this goal, the present work first demonstrates the standardization ofLC-MS/MS-based protocol for quantitative detection of four different fluoroquinolone-based antibiotics. Then two simple lanthanide probes were utilized to detect the presence of antibiotics through solution phase luminescence titration with similar efficiency (Scheme 1). A simple and ready-to-use paper-based test strip was also demonstrated for the qualitative detection of antibiotic molecules.



Scheme 1: The chemical structures of four different antibiotics and two lanthanide compounds.

Material and Methods

Standard antibiotic molecules (Ciprofloxacin, Enrofloxacin, Difloxacin and Flumequine), Terbium nitrate hexahydrate, Europium chloride, 1,3-diphenyl 1,3-propendione, phenanthroline and Trichloroacetic acid were purchased fromSigma-Aldrich, India and used as received.Ethanol, Methanol, HPLC grade water, Dimethyl sulphide (DMSO), Acetonitrile, phosphoric acid, Formic acid and Potassium hydroxide were procured from Finar Chemicals, India.

The solution phase UV-Vis spectra of the samples were measured by using a Jasco(Model V-670) spectrophotometer at room temperature. Solid and liquid phase luminescence spectra were measured by using a Jascospectrofluorometer (Model FP-6500). FTIR spectra were measured by using a Jasco FTIR spectrophotometer (Model FT-4600) in the Annulated Total Reflection (ATR) mode where a 5 mg sample was directly placedon the sample chamber. Chromatographic analysis by LC was performed by using a Shimadzu prominence LC (Model 1020 Series) which was equipped with a LC-20AD binary capillary two pump system and an auto sampler (SIL-20AC), column oven (CTO-20AC) and a communication bus module 9CBM-20A. Mass spectrometric analysis was carried out by using an ABSCIEX mass spectrometer (QTRAP 4500QuadrupoleLC-MS/MS). The separation of the compounds was carried out under gradient conditions using a KROMASIL column (Dimension of 150 mm x4.6mm). The LC-MS/MS system was equipped with Turbo ion spray interface and analyst® software version 1.6.2. The standard antibiotic molecules were diluted in methanol with five different concentrations (0.5, 1, 2, 3, 4 ppm) and then analysed in LC-MS/MS and concentration vs. intensity correlation graphs were plotted.

Preparation of standard solutions

A 100 ppm stock solution for each of the four antibiotics were first prepared by dissolving respective antibiotics molecules in 250 mL methanol. The standard solutions were kept at -4 ^{0}C in dark for up to one month with no degradation. On the day of analysis, the standard solution was further diluted to prepare working standard solutions with 0.5, 1, 2, 3 and 4 ppm concentrations of respective antibiotics in MeOH solvent. These solutions were kept in the dark to avoid photo degradation at room temperature and were used for a period of 24 h and then discarded.

Process of raw milk samples for analysis

1 ml of spiked raw-milk sample was treated with 1 mL of a 20% methanolic solution of trichloroacetic acid and then the mixture was shaken for 2 min followed by centrifugation at 6000 rpm at 25° C for 20 minutes. Then white solid (protein part) was discarded and the clear filtrate was passed through a Whatman 41 filter paper followed by another round of filtration using a syringe filter. The solution was then adjusted to pH ~3.5 with 1 M aqueous sodium hydroxide solution and the final volume was raised to 3 mL with Milli Q water. The samples were then analysed by LC-MS/MS or spectrofluorimetric method for recovery analysis. The spiking was performed by adding minimum volumes (1-10µL) of the stock 5 ppm methanolic solution of standard antibiotic molecules.

Synthesis of lanthanide complex (Eu-C1) and its luminescent paper strip

Europium chloride ($EuCl_3$, 50 mg, 1 mmol), 1, 3-diphenyl, 1,3- propendione (92 mg, 3 mmol) and phenanthrene (150 mg, 1 mmol) were taken in a 50 mL round bottom flask and 15 mL of methanol was added to it.The pH of the solution was adjusted to 6.5 by adding a few drops of aqueous KOH solution and then the solution was stirred at 65 °C for 24 h under a reflux condenser. The solution was cooled to room temperature, filtered and the filtrate was dried under vacuum using a rota evaporator. The final product was purified by silica gel column chromatography using methanol-DCM (1:1) as eluent (Yield, 75 mg, 68 %, m/z 1003) (ESI Figure S1-S4).

For the preparation of a solid-state test strip, A Whatman filter paper was immersed in DMSO solution of EuC1(1 m molar) for 1 minute and then dried under ambient conditions. The lanthanide complex embedded paper was used as a test strip for solid phase luminescence-based detection of antibiotics by employing different concentrations of antibiotic solution.

Luminescence detection of antibiotics in milk samples

The steady state photoluminescence titration experiment of standard antibiotic molecules and spiked milk extract samples with two lanthanide complexes was performed at room temperature. In brief, a 2.5 mL DMSO solution of Eu-C1 or Tb-C2 (1.2 x 10^{-4} M) was taken in a cuvette and the luminescence spectra were recorded with incremental addition of (10 µL at a time) antibiotic samples. The excitation wavelength was fixed at 365 nm for both the complexes and characteristics emission peaks at 614 and 555 nm were observed for Eu and Tb complexes respectively. For solid state paper strip-based luminescence titration, the test strip was exposed to a methanolic solution of antibiotic and respective luminescence spectra were recorded.

Results and Discussion

Optimization of LC-MC/MS parameters for quantitative detection of antibiotics

As a starting point for the optimization of the LC-MS/MS conditions, chromatographic separation was realized using gradient elution with 0.2% Formic acid in water (mobile phase A) and Methanol (mobile phase B) in different ratios. The gradient profile was started with 15% of mobile phase B and changed linearly to 90% of mobile phase B within 5minutes and hold there for 20minutes with a column oven temperature of 35°C and the overall run time was 20 min. Data acquisition was performed in multi-reaction monitoring mode (MRM) using positive electrospray ionization (Figure 1) for Ciprofloxacin, Enrofloxacin, Difloxacin and Flumequine. The calibration curve was calculated by line regression of the measured peak areas and the corresponding concentrations of the calibration standard solutions. As can be seen (Figure 2) the calibration curve of each of the four fluoroquinolones showed good linearity with a correlation coefficient (R^2) of more than ~0.990 in the range of 0.5 to 5 ppm. For the linearity validation, relative standard deviations (RSD) of the five-point calibration test of less than 10% were considered to be reliable. The recoveries of the four antibiotics molecules from spiked milk samples were found to be 90%, 94%, 102% and 92% for CIP, FMQ, DIF and ENR respectively. The instrument detection limits for fluoroquinolones were calculated three times the signal noise ratio according to the lowest concentration point in the standard curve with the value ranging from 0.5 to 2 ppb (Table 1 and 2).

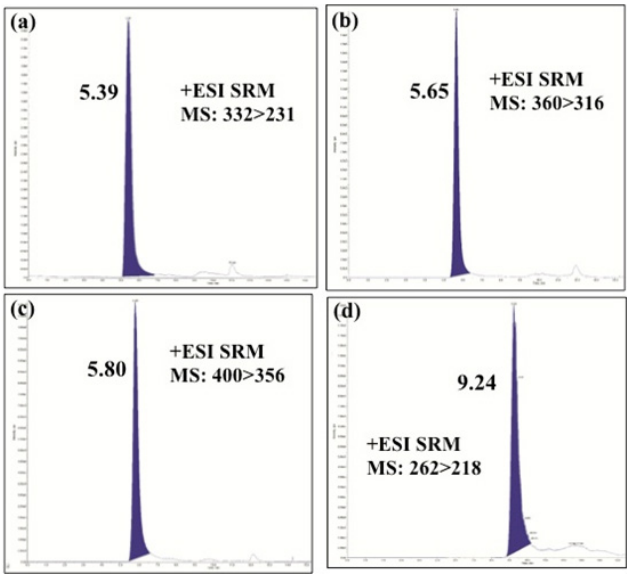


Figure 1: LC-MS/MS chromatogram of four different antibiotics molecules; (a) CIP, (b) ENRO, (c) DIF and (d) FMQ.

Luminescence titration of standard antibiotics with lanthanide complexes

Lanthanide complexes showed characteristic sharp band like emission spectra with long lifetime due to Laporte forbidden f-f transition and have a high co-ordination number with a strong affinity to bind with hard donor ligands [29, 30]. The emission intensity of lanthanide complexes can be tuned by the proper choice of the ligand through 'antenna effect' and thus the development of lanthanide based luminescent sensor has become a promising area [31, 32]. The hard donor oxygen atoms form the 'keto' group of fluoroquinolone antibiotics will have a strong affinity to bind with Lanthanides and luminescence response could be used to quantify these molecules. In this work we have used two lanthanides (III)

Table 1: Summary of data acquisition parametersobtained for quantification offluoroquinolonemolecules using LC-MS/MS.

Antibiotic	Retention Time (min)	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)
Ciprofloxacin	5.39	332.2	231.2	32
Enrofloxacin	5.65	360.1	316.1	30
Difloxacin	5.80	400.2	356.1	32
Flumequine	9.24	262.1	218.0	28

Table 2: Calibration parameters including limits of detection, recovery and quantification parameters obtained for various fluoroquinolone molecules using LC-MS/MS technique.

Antibiotic	Average Accuracy (%)	Min. Qty Detected	Recovery	Standard Deviation	Linearity
Ciprofloxacin	99.04	1 ppb	90%	1.8×10^{-6}	0.9904
Enrofloxacin	99.43	1 ppb	92%	2.0×10^{-6}	0.9943
Difloxacin	99.89	1 ppb	102%	2.9×10^{-6}	0.9989
Flumequine	99.42	1 ppb	94%	2.3×10^{-6}	0.9942

complexes, the Terbium nitrate hexa hydrate $Tb(NO_3)_3.6H_2O$ (Tb-C2) and a synthesized Europium complex $Eu(phen)(dppm)_3$ (Eu-C1) for luminescence-based detection of antibiotic molecules (Scheme 1).The characteristic absorption, excitation and emission spectra of both complexes are shown in figure 3. As expected, the red luminescent Eu complex showed a sharp emission band at 614 nm ($^5D_0 - {}^7F_2$) and the green luminescent Tb complex showed a prominent emission band at 555nm ($^5D_4 - {}^7F_5$). Next, we have performed solution phase photoemission-based titration of four antibiotic molecules with these two lanthanide probes through incremental addition of analyses to an Eu/Tb solution (1.2 x 10^{-4} M) in DMSO (Figure 4 and 5) at room temperature. As shown in figure 4 (a-d), the steady state photoluminescence spectral intensity of Tb-C2 showed gradual enhancement (turn-on)

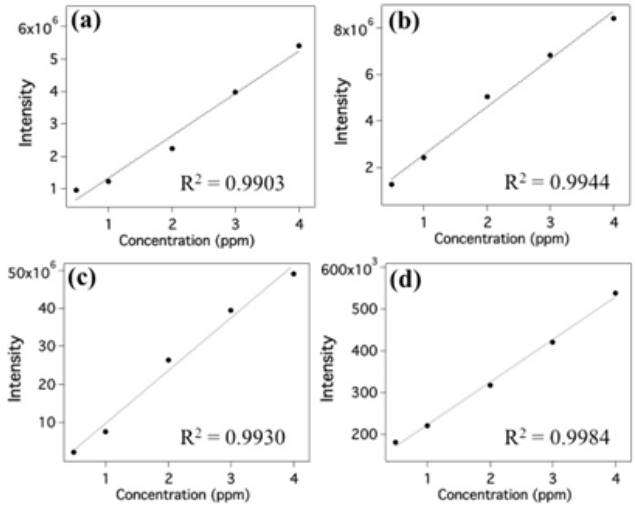


Figure 2: Intensity vs. Concentration correlation curves obtained from LC-MS/MS spectra of four different antibiotics molecules; (a) CIP, (b) ENRO, (c) DIF and (d) FMQ.

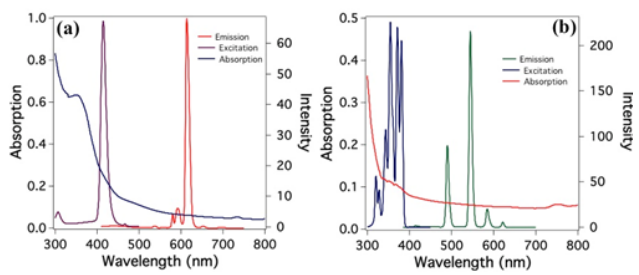


Figure 3: Absorption, Emission and Excitation spectra of two lanthanide compounds (a) Eu-C1 and (b) Tb-C2.

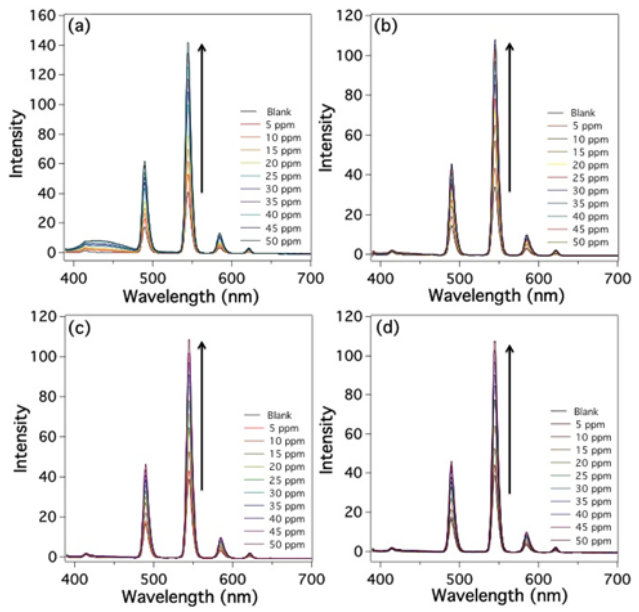


Figure 4: Luminescence titration spectra of four different antibiotics molecules; (a) CIP, (b) ENRO, (c) DIF and (d) FMQ with Tb-C2.

upon incremental addition of these four analyses (e.g., CIP, ENR, DIF and FMQ). Contrary to that Eu-C1 complex showed turn-off luminescence behaviour when the above four standard antibiotic molecules were added gradually (Figure 5 a-d). Turn on and Turn off luminescence behaviour of Tb-C1 and Eu-C2 complexes can be explained based on the extent of electron donation to the metal centre. The coordination of fluoroquinone antibiotic molecule to Tb-C1 will enhance the electron donation to the metalcentre through antenna effect. Whereas the Eu-C2 complex gets coordinated to antibiotic molecules through replacement of 1,10-Phenanthroline molecule, thus reducing effective electron transfer from ligand to metal centre. The concentration vs. emission intensity plot for four antibiotics against Tb-C2 showed good linearity with a correlation coefficient (R^2) ~0.99when the concentrations of antibiotics were varied between 5 to 50 ppm (Figure 6 a-d). Similarly, concentration vs. emission intensity plots was obtained using Eu-C1 and with a good correlation coefficient by using antibiotic concentration between 5 to 45 ppm (Figure 7 a-d). From the corresponding Stern-Volmer plots for Tb-C2 and Eu-C1 complexes (Figure S5, ESI) the associated K_{sv} values were calculated. The Stern-Volmer constants (K_{sv}) obtained using Eu-C1are 7.4×10^3 M^1 (CIP), 7.2×10^3 M^{-1} (for ENR), 5.1×10^3 M^{-1} (DIF) and 2.3×10^3 M^{-1} (FMQ). Similarly, the Stern-Volmer constants (K_{sv}) obtained using Tb-C2complex are 7.5×10^3 M^{-1} (CIP), 7.3×10^3 M^{-1} (ENR), 4.8×10^3 M^{-1} (DIF) and 2.2×10^3 M^{-1} (FMQ). By considering the minimum concentration of antibiotic molecule causes emission peak intensity to change appreciably, it was found that a minimum detection limit of 25ng/mL was obtained. The analysis of spiked milk samples was also performed to determine the recovery efficiency of the technique and we could find 95% (CIP), 90% (ENR), 96% (DIF) and 89% (FMQ) recovery using Eu-C1 probe. Similarly, the recovery values obtained using Tb-C2 complex are 107% (CIP), 90% (ENR), 92% (DIF) and 104% (FMQ).

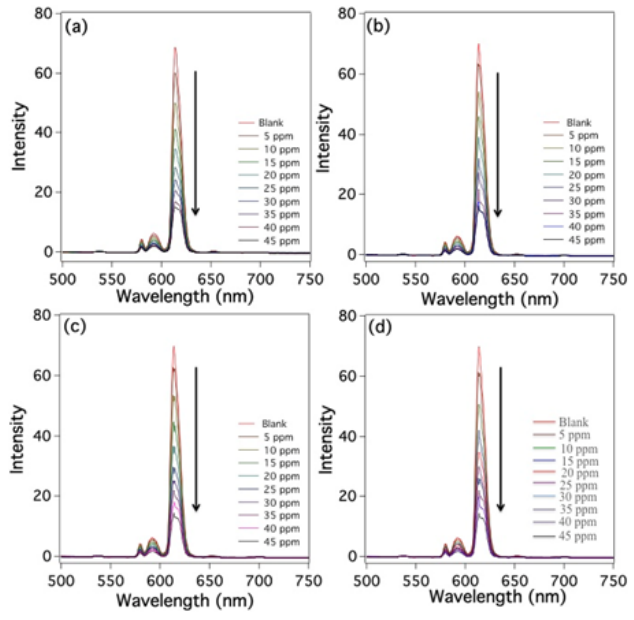


Figure 5: Luminescence titration spectra of four different antibiotics molecules; (a) CIP, (b) ENRO, (c) DIF and (d) FMQ with Eu-C1.

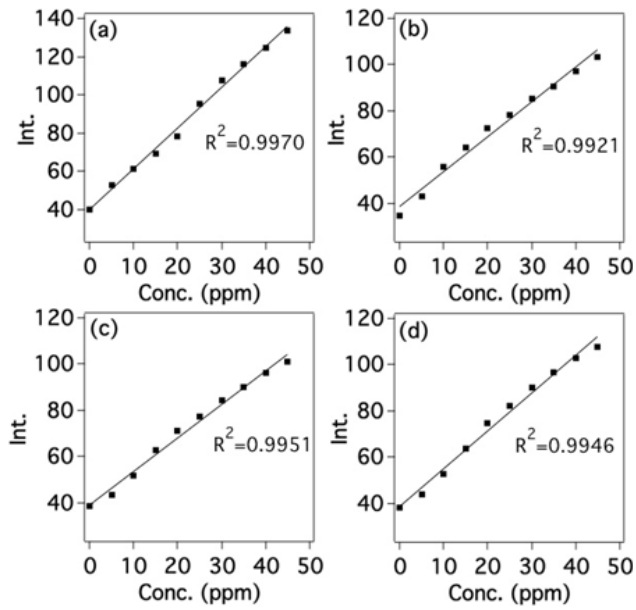


Figure 6: Intensity vs. Concentration corelation curves obtained from luminecence titration spectra of four different antibiotics molecules; (a) CIP, (b) ENRO, (c) DIF and (d) FMQ with Tb-C2.

A paper-based test strip for the detection of antibiotics

To demonstrate a simple, rapid and cost-effective approach, which could be used onsite for qualitative analysis, we have also prepared a paper-based test strip by impregnating a filter paper with luminescent Eu-C1 complex by dip coating the paper in a DMSO solution of the complex. These strips were prepared by immersing Whatman filter papers (5 cm × 2 cm) in a DMSO solution of the Eu-C1(1×10^{-3}M) for 5 minutes followed by drying under vacuum for 24h. In the first experiment, a 20µL solution (concentration 50 ppm) of

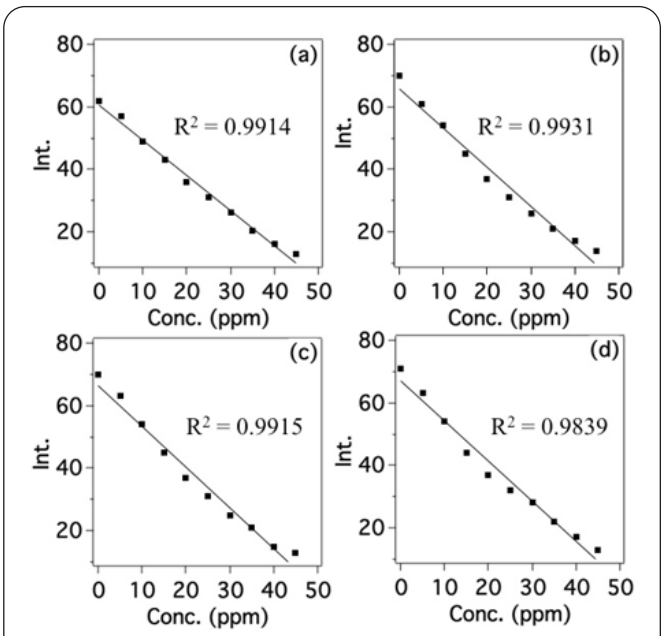


Figure 7: Intensity vs. Concentration corelation curves obtained from luminecence titration spectra of four different antibiotics molecules; (a) CIP, (b) ENRO, (c) DIF and (d) FMQ with Eu-C1.

ENR was added to a test strip and the applied volume was multiplied in successive strips. The strips were then kept under 365 nm UV light and the optical images were recorded which show diminished red intensity with incremental addition of antibiotic molecules (Figure 8). In a similar experiment a 50 µL Eu-C1 (1×10^{-3}M) solution was dropped on the paper and then 20 µL of ENR solution was added above it. Similar masking of red illumination was observed when the paper was kept under 365 nm UV light under repeated experiments (Figure 8). The solid-state photoluminescence spectra also showed diminished intensity at 614 nm on the gradual addition of ENR over the paper-based test strip (Figure S6, ESI). Thus, we could demonstrate that the presence of trace level fluoroquinone antibiotics could be qualitatively determined by using paper-based test strips using simple techniques.

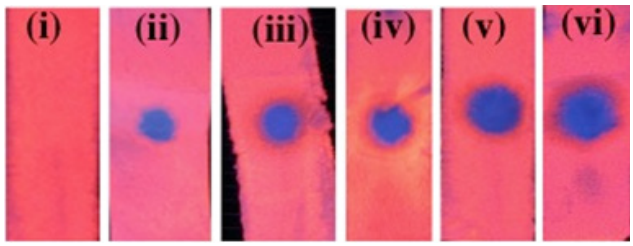


Figure 8: The images of paper-based test strips with an incremental addition of ENR solution (i to vi).

The comparative detection performance of LC-MS/MS and luminescence techniques

If we compare the results obtained from LC-MS/MS and Luminescence based detection of antibiotics, then two aspects need to be discussed. The mass spectrometric method is obviously more sensitive with a detection limit of up to 1ng/mL and more importantly, it can identify every fluoroquinolone molecule through its retention time and mass parameters. The luminescence-based technique also allows the detection of these molecules with a detection limit of 25 ng/mL which is much below the limit set by most of the agencies. However, identification of molecules through luminescence titration is not straightforward as lanthanide emission peak position (wavelength) does not get affected through coordination with a chromophore. The simplicity of its operation, cost-effectiveness, ready availability and the son-the-spot testing option is the advantages of luminescent titration-based detection techniques as compared to LC-MS/MS-based techniques. After standardization of parameters, both techniques were tested for detection of antibiotics present in spiked milk samples through protein separation and purification steps of milk as mentioned in the experimental section and found to be effective equally with a recovery efficiency of more than 90%. The luminescence technique will be more user-friendly and affordable for all individual farmers and small farm owners involved in the production of milk.

Conclusion

In summary, first, we have developed and standardized an LC-MS/MS methodology for quantitative detection of four quinolones-based antibiotics(CIP, ENR, DIF, FMQ) with a detection limit of 1 ng/mL and linear regression fitting close to unity (R^2~0.99). Next, we have demonstrated two simple luminescent lanthanide probes for the detection of these antibiotic molecules. One Europium complex and one terbium compound showed turn-off and turn-on emission responses respectively through interaction with quinolone molecules. The luminescence change of these two probes with standard antibiotic molecules showed linear concentration-dependent correlation fitting with Stern Volmer constant up to 7.5×10^3 M^{-1}andminimum detection limit of 25 ppb. We have also demonstrated a cost-effective technique through a solid state test strip-based luminescence detection kit for these antibiotics. While the mass spectrometric technique has the advantages of the ultra-low detection limit of every individual antibiotic molecule, the luminescence-based techniques developed here stand for simple, on the site and cost-effective operation for detection of the quinolone-based molecules at the nanogram level. Through the use of the LC-MS/MS technique, we have also validated the efficacy of these cost effective lanthanide probes and want to propose them as cost effective luminescent probes for onsite testing of antibiotic contamination in raw milk.

Acknowledgements

None.

Conflict of Interest

Authors declares that they have no conflict of interest.

Ethical Approval

This study does not contain any studies with human or animal subjects.

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