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Determination of oxolinic acid in cow's milk and human urine by means of a single-use phosphorimetric sensor

L.F. Capitán-Vallvey *, Osama M.A. Al-Barbarawi, M.D. Fernández-Ramos, R. Avidad

Department of Analytical Chemistry, University of Granada, Granada 18071, Spain

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Abstract

A single-use phosphorimetric drop plane sensor for the determination of oxolinic acid (OXA) is proposed. The sensor was formed by a 30×16 mm^2 rectangular strip of Mylar type polyester as solid support that contained a circular sensing zone, 6 mm in diameter and 20 μm in thickness, formed by PVC plasticized with tributylphosphate adhered to its surface. When the strip was introduced for 1 hour into a sample solution, the analyte was retained in the sensing zone, making it possible to directly measure the phosphorescence intensity emitted by the OXA in the solid phase, at $\lambda_{exc} = 330$ nm and $\lambda_{em} = 449$ nm. The variables that affect the construction of the sensor have been studied, along with the experimental variables that influence the fixation of the analyte in the sensor. The method's detection limit was 0.01 mg l^{-1} with an applicable concentration range from 0.04 to 1.50 mg l^{-1} and a repeatability of 2.6% at the concentration range of 0.8 mg l^{-1}. The method was applied to samples of human urine and cows' milk, with recovery percentages ranging between 97.6 and 108.7%.

Keywords: Phosphorimetric sensor; Oxolinic acid determination

1. Introduction

One of the current fields of research in Analytical Chemistry is the development of simple and inexpensive analytical tools for the determination of small amounts of analytes which can be handled without analytical preparation in the place where the analytical problem presents itself. One of these new analytical tools is the express test, composed of a reactive strip that works in a single-use or reversible way and operates either when a drop of the sample is placed on the sensing surface or when the strip is immersed in the sample solution. In either case, an analytical signal, usually optical or electrical, is given off by the sensing zone in the presence of the analyte. These sensors have several advantages: their handling is simple and speedy, they are inexpensive and produce analytical results adequate to the goal of the analysis. However, they also have some design and construction problems, mainly due to the need for (1) high selectivity,

* Corresponding author. Tel.: +34-958-248-436; fax: +34-958-243-328.
E-mail address: lcapitan@ugr.es (L.F. Capitán-Vallvey).

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needed to determine the analyte in samples from matrices of complex composition; (2) high sensitivity, needed to determine amounts of the analyte at trace levels; and (3) simplicity of handling, needed to use the sensor in situ, if required. For this study, we selected phosphorescence in solid-phase as the analytical property, because it is a sufficiently selective and sensitive analytical technique for the development of single-use optical sensors to determine intrinsically phosphorescent analytes that can be fixed in the sensing zone.

Phosphorescent sensors, like other optical sensors, can be classified in two groups: (1) intrinsic phosphorescent sensors, which only act as a solid support for fixing an intrinsically phosphorescent analyte [1]; and (2) extrinsic phosphorescent sensors, where the phosphorescence is produced by a chemical process, i.e. usually a complexation process between a reagent, previously fixed in the sensor, and the analyte. Examples of the latter include the determination of aluminium and lead with sulfonic derivatives of the oxine retained in an anion exchange resin [2,3], the determination of europium with thenoyltrifluoroacetone in Chelex 100 resin [4], the use of organosilicon polymer for the analysis of uranyl [5], in the case of inorganic analytes, and the use of Chelex 100-Eu(III) [6] and Amberlite XAD-2-Eu(III) [7] for the determination of tetracyclines and anthracyclines, respectively.

The quenching effect has also been used as a basis for measurement with phosphorescent sensors. Even if intrinsically phosphorescent compounds such as transition metal chelates [8–10] or polycyclic aromatic hydrocarbons [11] suffer in the presence of a quencher analyte, phosphorescence quenching has been used as an analytical signal in some instances, such as with the use of an oxygen sensor for the determination of cholesterol by means of enzymatic oxidation [12] or with the use of a sensor designed to determine nitrogen oxides [13] or sulfite ion [14].

Oxolinic acid (OXA), i.e. 1-ethyl-5,8-dihydro-8-oxo-1,3-dioxole[4-5g]quinoline-7-carboxilic acid, is an antibacterial agent used in the treatment of intestinal and urinary system infections produced by gram-negative bacteria such as *Salmonella*. This chemical, whose lethal dose 50 is 2 g kg^{-1} in rats [15], is the active principle in some commercial pharmaceutical preparations such as Urotrato®, Uroxin® or Nidantin®, and along with others, such as nalidixic, piromidic, and pipemidic acids, makes up the group of antibacterial agents known as quinolones. Today, the use of the oxolinic acid as a medical drug is prohibited in several countries, and other similar antibacterial agents called fluoroquinolones, whose action is more effective, are used instead. However, OXA is frequently used in veterinary practice against infections produced by colibacillus in livestock, mainly cows, with a recommended dose of 20 mg kg^{-1}. For this reason, OXA may be present, at trace levels, in milk or derived dairy products.

Several analytical techniques have been used for the determination of OXA. It has been determined in fish, pigs, chickens, honey and human serum and urine samples using HPLC [16–22], liquid chromatography [23] or thin layer chromatography [24].

Electrophoretic and electroanalytical techniques have also been used for the identification and/or determination of OXA [25–27] in pharmaceutical formulations, human serum and urine. Among optical techniques, fluorescence [28] has been used for the determination of OXA along with other quinolones in human urine samples.

The aim of this paper was to establish an analytical methodology that made it possible to determine OXA in complex matrixes using single-use sensors by fixing the analyte in the sensing zone and subsequently measuring its phosphorescence in solid phase. In this way, we could combine the advantages of sensors (simplicity and speed) with the advantages of phosphorescence (selectivity and sensitivity).

2. Experimental

2.1. Instrumentation and software

Phosphorescence measurements were performed with a Perkin–Elmer (Norwalk, CT, USA) LS-50 luminescence spectrometer, interfaced to an IBM PC330-100Dx4 microcomputer through a RS232C connection, and equipped with: a xenon discharge

lamp with a power equivalent to 20 kW during 8 μs and pulse width at half peak height < 10 μs, two monochromators Monk-Gillieson F/3, and a Hammamatsu R298 photomultiplier. In order to check the apparatus, a P1 solid standard ($12.5 \times 12.5 \times 45$ mm^3) containing europium(III) with thenoyltrifluoroacetonate dissolved in a transparent matrix of poly(methylmethacrylate) supplied by Perkin–Elmer, was used. Other apparatus were a digital pH-meter (Crison Instruments, Barcelona, Spain), with combined glass-calomel saturated electrode and a magnetic multi-agitator (SBS Instrument SA, Selecta, Barcelona, Spain). The acquisition, manipulation and mathematical treatment of spectral data were carried out with the software package, Perkin–Elmer FL Data Manager, Statgraphics Plus for Windows 3.1 (Statistical Graphics Corporation, US, 1994-1997), and Microsoft Excel from Microsoft Office 97, v. 8.0, 1997.

2.2. Reagents and materials

A 200 mg l^{-1} stock solution of OXA was prepared by weighing the reagent (Sigma–Aldrich Química S.A., Madrid, Spain) and subsequently dissolving it in ethanol. The solution was stored in an amber glass bottle at $+4$ °C. Daily working solutions were prepared by dilution in water, maintaining in all instances a 4% (v/v) ethanol/water proportion to avoid potential precipitation of the analyte. Stock solutions of different concentrations levels of Pb(II) acetate, Ag(I) nitrate, Tl(I) nitrate, and KI (Merck, Darmstad, Germany) were also prepared. For the preparation of the sensing layer, the following chemicals were tested: polyvinylchloride of high molecular weight (PVC), dioctylphthalate (DOP), 2-nitrophenyloctylether (NPOE), tris(2-ethylhexyl)phosphate (TEHP), bis(2-ethylhexyl) sebacate (DOS), tributylphosphate (TBP) and tetrahydrofuran (THF), all supplied by Sigma-Aldrich Química. As solid support, sheets of Mylar type polyester (Goodfellow, Cambridge, UK) were used. All reagents were of analytical grade unless otherwise stated. Reverse-osmosis type quality water (Milli-RO 12 plus Milli-Q station from Millipore) was used throughout.

2.3. Preparation of the sensor

To prepare the sensing zone, 70 mg of PVC, 0.14 ml of TBP and 1.5 ml of THF were mixed, in that order, in a vial and shaken for 2 min. The membrane was prepared by placing 5 μl of the mixture on a rectangular strip (32×16 mm^2) of Mylar type polyester and drying it in a vacuum dryer for 20 min at room temperature and around 15 mmHg of pressure. The physical characteristics of the sensing zone were as follows: solid and homogeneous 6 mm ∅ circular film, transparent and uncoloured, well-adhered to the solid support. The resulting sensing layer was calculated to have a thickness of about 20 μm.

2.4. Pretreatment of the samples

In order to precipitate proteins, 12.5 ml of 1.0 M $ZnSO_4$ and 12.5 ml of 0.23 M $K_4[Fe(CN)_6]$ solutions were added to a volume of 200 ml of the sample, either cow's milk or human urine, after which the solution was filtered through paper. The filtrate was used to prepare the samples as described in Use of the sensor.

2.5. Use of the sensor

50 ml of sample solution containing between 0.04 and 1.5 mg l^{-1} of OXA or 50 ml of filtrate, 1 ml of 1 M lead acetate solution and the necessary amount of 1 M NaOH to adjust the pH to 9.0 were placed in a 100 ml glass vessel. The sensor was then introduced into the solution, hanging from a support, and was magnetically stirred at 30 rpm for 60 min. Then the strip was placed for 20 min in a vacuum dryer at room temperature and around 15 mmHg pressure. The phosphorescence measurement was then carried out as described in the Section 2.6. The relationship between the concentration and the phosphorescence signal was established by the calibration graphs. The membranes were not conditioned before use.

2.6. Phosphorescence measurements

The phosphorescence measurements were performed using the homemade accessory previously

described [29]. The membrane, placed in the accessory, was introduced into the holder of the spectrometer, in such a manner that both excitation and emission beams formed 45° angles with the plane of the sensor. The transmitted phosphorescence emission spectra were recorded with a gate time t_g of 10 ms and a delay time t_d of 0.15 ms, with an excitation and emission slit width of 3 and 15 nm, respectively, at a scan speed of 240 nm min^{-1}. The phosphorescence measurements were carried out at $\lambda_{exc} = 330$ nm and $\lambda_{em} = 449$ nm under a stream of dry nitrogen.

2.7. Validation of the procedures

Calibration function was obtained using ten standards (with three replicate of each one standard) and establishing a linear relationship, by means of linear regression, between relative phosphorescence intensity and analyte concentration in the solution of standard or sample. The linearity of this calibration function was tested applying the lack-of-fit test as suggested by the Analytical Methods Committee [30,31]. The upper linearity level was defined as the concentration level of the standard solution which relative phosphorescence intensity did not exceeding $\pm 5\%$ of the predicted value from the calibration function. Detection and quantification limits, obtained from ten blank solutions, were determined as suggested by the IUPAC [32,33] and the repeatability was obtained from ten standard solutions of OXA which concentration level was 0.8 mg l^{-1} and expressed as relative standard deviation (RSD).

In the other hand, to study the interference that could produce other quinolones, we prepared standard solutions, of 0.1 mg l^{-1} of OXA, that also contained the potentially interfering species at 3.0 mg l^{-1} of concentration level. If the interference occurred this concentration was progressively reduced until interference stopped. The tolerance level was defined as the amount of foreign species that produced an error not exceeding $\pm 5\%$ in the determination of the analyte.

The applicability of the method at the determination of the analyte in real samples was studied with samples of human urine and cow's milk applying the procedure described in Sections 2.4 and 2.5. When the sample did not contained analyte, or the amount contained was lower than the detection limit of the method, a measured volume of standard solution of OXA of known concentration was added into 200 ml of sample (milk or urine). The mixture was shaken for 10 min and next stored for 24 h before its analysis.

3. Results and discussion

3.1. Spectral characteristics

The spectral characteristics of OXA fixed in the strip were: an excitation peak located at 330 nm and an emission peak at 449 nm with a phosphorescence intensity ten times greater than the intensity shown by the blank. Fig. 1 shows the excitation and emission spectra of OXA and blank.

3.2. Experimental parameters

The parameters that may potentially influence the analytical signal of the test strip could be classified in four groups: (1) membrane parameters related to the construction and composition of the sensor; (2) chemical parameters of the fixation process, such as ionic strength and pH; (3) physical parameters that influenced the fixation process such as time, stirring and drying; (4) instrumental parameters that influenced the phosphorescence

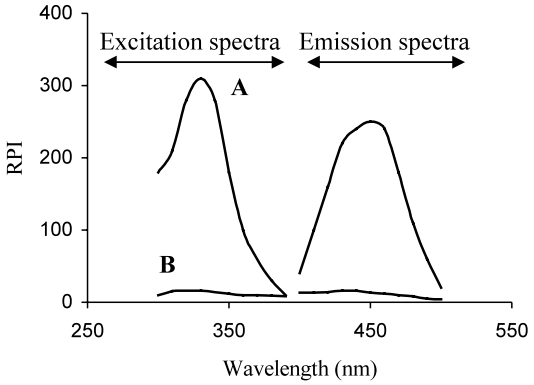

Fig. 1. Excitation and emission spectra of. (A) OXA and (B) blank.

intensity during the measurement of the analytical signal.

3.2.1. Study of the components of the sensor

In order to optimise the construction of the sensor, the composition (nature and amount of the components), the volume of mixture deposited on the strip, and the form of drying and drying time of the sensing membrane were all studied. Metacrylate, polyvinylacetate, and ethylene polyterefthalate sheets were tested as solid support. Sheets of polyterefthalate (Mylar type polyester 0.25 mm in thickness) were selected, because they produced a lower phosphorescence background than metacrylate or polyvinylacetate.

The use of polyvinylacetate or polyurethane as polymers in hydro–alcoholic mixtures produced membranes that emitted a phosphorescent background 10% higher than the background emitted by a membrane of PVC when plasticized with TBP. Using PVC as the polymer in THF, the increase in the phosphorescent signal depended on which plasticizer was used. With NPOE or DOP as plasticizers, the increase in the signal was about 10% higher than the blank; with TEHP the increase was about 80% higher; and with TBP the signal was 500% higher. Consequently, we selected PVC as polymer, and TBP as plasticiser dissolved in THF to form the sensing membrane.

3.2.2. Composition of the sensing membrane

To study the influence of PVC (from 10 to 100 mg), TBP (from 0.05 to 0.20 ml) and THF (from 0.5 to 3.0 ml) on the phosphorescence signal, different sensors with different proportions of one component and constant amounts of the other components were prepared and equilibrated with solutions of 1.0 mg l^{-1} of OXA. The higher phosphorescence intensity was obtained with a mixture composition of 70 mg of PVC, 0.14 ml of TBP and 1.5 ml of THF, because this signal was 15% superior to membranes that contained other compositions. Consequently, this composition was selected for the subsequent experiments.

The influence of the volume of the reagent mixture used for membrane construction was tested by preparing different membranes and equilibrating them with 1.0 mg l^{-1} OXA solution. Five microliters of mixture was the minimum amount needed to produce the higher signal (Fig. 2).

Since the sensor must be totally dried before use, the influence of both the method and time of drying were studied at room pressure and under vacuum. It was found that, at room pressure, the time needed to dry the sensor was 60 min, while using a vacuum desiccator, at 15 mmHg pressure, 20 min were sufficient.

3.2.3. Fixation process of the oxolinic acid in the sensor

The experimental parameters involved in the equilibration process are: pH and ionic strength of the sample solution, speed and shaking time of the sample during the process, and drying time of the membrane after the equilibration process.

Phosphorescence intensity increased when the equilibration time increases from 5 to 60 min (Fig. 3); that is why we selected 60 min as the optimal value for the study of the rest of the experimental parameters.

Several sensors were then equilibrated for 60 min with different solutions of OXA at 1.0 mg l^{-1}, at shaking speeds ranging between 30 and 90 rpm. The signal increased up to 60 rpm, while at 90 rpm the emitted intensity decreased to 10%.

It was observed that the RTP signal increased if the sensor was dried after equilibration with the

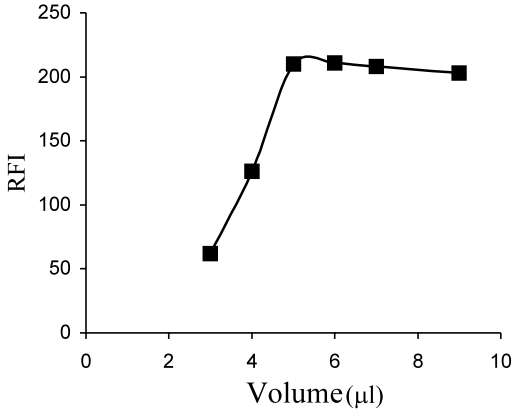

Fig. 2. Influence of volume (μl) of reagent mixture (PVC, TBP and THF), used for membrane construction, on the relative phosphorescence intensity (RPI) emitted by the sensor after its equilibration with 1.0 mg l^{-1} of OXA, for 60 min.

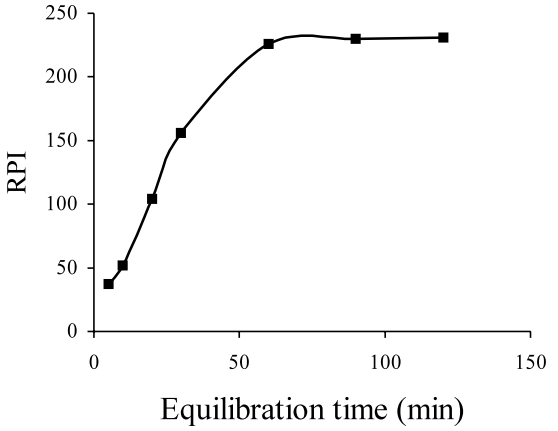

Fig. 3. Influence of the equilibration time on the relative phosphorescence intensity (RPI) emitted by a sensor equilibrated with a (1.0 mg l^{-1}) solution of OXA.

analyte before the measurement. This fact was probably related to the quenching of phosphorescence produced by the water which was freely dissolved in the membrane of PVC and to the uptake from the aqueous solution by an initial rapid process [34,35]. In order to dry the sensor after the equilibration process, two different methods were tested: desiccation with and without vacuum. The best results were obtained with drying for 20 min in a desiccator over $CaCl_2$ under vacuum (15 mm). An increase in the drying time did not produce any improvement in the results.

Fig. 4 shows that the phosphorescence intensity increased linearly when the pH of the OXA solution increases from 1.9 to 8.0 (pK_a 6.78 [36]).

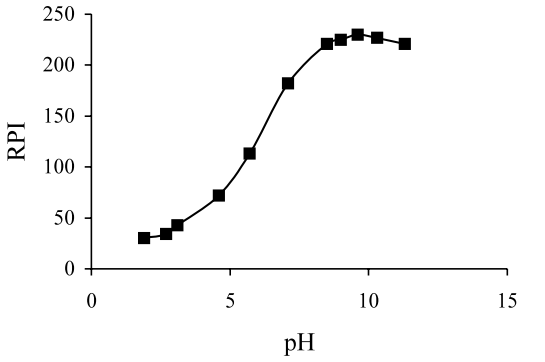

Fig. 4. Influence of the pH of the solution on the relative phosphorescence intensity (RPI). Concentration of OXA solution 1.0 mg l^{-1}.

Between 8.0 and 10.3 this phosphorescence intensity remained constant, decreasing slowly from pH 11.0. So the working pH was 9.0.

The influence of ionic strength on the phosphorescence of OXA was studied using NaCl. Experimental results showed that the RPI was independent of the NaCl concentration up to 0.1 M, decreasing at higher values. Thus, when the ionic strength increased from 0.1 to 0.5 M, the signal decreased about 65% with respect to the initial value. For higher values the decrease in phosphorescence was slower, being 85% of the initial value at 1.8 M in NaCl.

The role of heavy atom in the RTP emission was studied by placing it both in the solution during the uptake of OXA, and in the membrane in the form of a lipophilic compound. $Pb(CH_3CH_2)_4$ and CH_3CH_2Br were incorporated into the PVC matrix in different percentages, but in all cases the RTP signal did not change. In solution we tested: Ag(I), Pb(II), Tl(I), Hg(I), Cl^-, Br^-, and I^-, observing that only Pb(II) enhances the RTP emitted by the analyte (Fig. 5). Consequently, the equilibration processes were performed in the presence of 0.02 M $Pb(NO_3)_2$.

3.2.4. Instrumental parameters

The instrumental parameters studied were: excitation and emission width of slits and delay and gate times. The highest RPI was obtained using the following values: 3 nm for excitation, 15 nm for

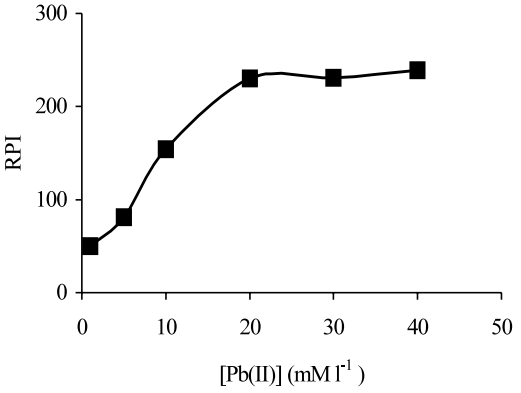

Fig. 5. Influence of lead on the relative phosphorescence intensity (RPI) of OXA at 1.0 mg l^{-1} concentration level.

emission slits, 0.15 ms for delay time and 10 ms for gate time.

Finally, we studied the influence of the atmospheric oxygen on phosphorescence intensity during the measurement process. We observed a strong quenching if measurements were made in the presence of atmospheric oxygen; namely, the phosphorescence was reduced to 8% on average with respect to measurement under dry nitrogen. Placing the reagent strip between two quartz sheets in the holder cell, which reduces the oxygen dependence when using filter paper as solid support [37,38], did not produce good results in this case.

3.3. Analytical figures of merit

With this type of disposable sensor, which could be called a preconcentration sensor, the equilibration time necessary for the development of the analytical signal varied depending on the concentration level of the analyte.

In this case it was reasonable to consider the fixation process of OXA in the sensing zone as a solid phase extraction process, in which the amount of analyte absorbed in the sensor was a function of two factors: concentration C of analyte in the sample solution and equilibration time t of the strip with the sample solution, because the fixation process showed a slow kinetics. As a consequence, the phosphorescence intensity I emitted by the analyte was expressed as a function of two variables: $I=f(C,t)$. In a previous article [29] we used a spline function in the two variables, $s(C,t)$, continuing and strictly increasing in each one of the two variables, to define the calibration function. In the current case, we established the relationship between the analytical signal and the concentration of analyte by linear regression, working at different equilibration times, from ten standards, and three replicates of each one standard.

Table 1 contains the linear models obtained and their analytical parameters. As we observed in Table 1, an equilibration time higher than 60 min did not improve the results obtained, because the detection and quantification limits and the applicable concentration ranges were the same for 60, 90 and 120 min.

3.4. Interferences

In order to study the effect of the presence of the quinolones nalidíxico acid, piromidic acid, pipemidic acid and flumequine was studied as described in Section 2.7. The experimental data showed that the species nalidixic acid, pipemidic acid and flumequine did not interfere at concentration level ≤ 3.0 mg l^{-1}, whereas piromidic acid interfered at concentration level ≥ 0.1 mg l^{-1}.

4. Applications

To check the applicability of the method to the determination of OXA, samples of human urine and cows' milk were selected and tested. As the samples did not contain OXA at levels higher than the detection limit, a recovery study was carried out after the addition of the adequate amounts of the analyte. The results obtained are summarised in Table 2. The recovery percentage, as an average of three independent determinations, ranged from 97.6 to 108.7%.

5. Conclusions

A test strip based on intrinsic phosphorescence measurements was designed and offers sufficiently good repeatability at a low cost. The proposed test strip made it possible to determine OXA in urine and milk, with the only pre-treatment needed being the addition of lead nitrate and adjustment of the pH. The results obtained indicated good accuracy and precision. Using different calibration graphs, we could adapt the analytical method to the needs of the problem since it is possible to fit the method to the concentration level of the current analytical problem by selecting an appropriate equilibration time. The method was simple and inexpensive and made it possible to obtain analytical information about pollutants at trace levels in samples of milk and human urine. The disadvantage of the test strip was its relatively long

Table 1
Analytical figures of merit at different equilibration times

Parameters	Equilibration time (min)				
	15	30	60	90	120
Intercept (a) (mg l^{-1})	52.1	95	24	58	67
Slope (b) (mg l^{-1})$^{-1}$	194.4	284.5	478.9	478.5	527.6
Lack-of-fit test (P-value)	0.65	0.48	0.27	0.90	0.81
Linear range (mg l^{-1})	0.06–3.0	0.04–2.0	0.02–1.5	0.02–1.5	0.02–1.5
Detection limit (mg l^{-1})	0.02	0.02	0.01	0.01	0.01
Quantification limit (mg l^{-1})	0.06	0.04	0.02	0.02	0.02
Repeatability (RSD)%	2.9	2.3	2.9	3.1	3.4

Equation of the linear regression: $I = bC$ (mg l^{-1})$+a$.

Table 2
Recovery study of OXA in actual samples

	Added (mg l^{-1})	Found (mg l^{-1})[a]	Recovery (%)	RSD (%)
Human	0.300	0.305	102.0	3.9
Urine	1.000	0.976	97.6	2.6
	1.500	1.516	101.1	3.4
Milk (cow)	0.300	0.326	108.7	1.5
	1.000	0.993	99.3	1.9
	1.500	1.495	99.7	2.4

[a] These data were the average of three independent determinations using an equilibration time of 60 min.

response time of 60 min when the OXA concentration is low.

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