Research Article

Biomedical Chromatography

Received: 30 October 2008, Accepted: 16 January 2009 Published online 29 April 2009 in Wiley Interscience

(www.interscience.wiley.com) DOI 10.1002/bmc.1215

Quantitative separation of oxytocin, norfloxacin and diclofenac sodium in milk samples using capillary electrophoresis

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ABSTRACT: A simple, sensitive and rapid method has been developed for simultaneous separation and quantification of three different drugs: oxytocin (OT), norfloxacin (NOR) and diclofenac (DIC) sodium in milk samples using capillary electrophoresis (CE) with UV detection at 220 nm. Factors affecting the separation were pH, concentration of buffer and applied voltage. Separation was obtained in less than 9 min with sodium tetraborate buffer of pH 10.0 and applied voltage 30 kV. The separation was carried out from uncoated fused silica capillary with effective length of 50 cm with 75 μm i.d. The carrier electrolyte gave reproducible separation with calibration plots linear over 0.15–4.0 μg/mL for OT, 5–1000 μg/mL for NOR and 3–125 μg/mL for DIC. The lower limits of detection (LOD) were found to be 50 ng/mL for OT, and 1 μg/mL for NOR and DIC. The method was validated for the analysis of drugs in milk samples and pharmaceutical preparations with recovery of drugs within the range 96–100% with RSD 0.9–2.8%. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: oxytocin; norfloxacin; diclofenac sodium; milk analysis; capillary electrophoresis

Introduction

Oxytocin (OT) is a cyclic neurohypophyseal nonapeptide. It possesses a 20-membered cyclic portion that is linked by a disulfide bridge between two-cysteine residues. It possesses uterotonic and galactogenic activity in mammals (Chaibva and Walker, 2007). The main use of OT in clinical practice is the induction and augmentation of labour, control of postpartum hemorrhage and uterine hypotonicity in the third stage of labour. OT is also used to stimulate lactation (Martindale, 2002). Norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid] (NOR) was the first quinolone antibacterial with a fluorine atom substituted at the C-6 position and a piperazine at the C-7. NOR is widely prescribed for the treatment of severe infections in humans as well in animals intended for human consumption (Samanidou *et al*., 2005). Diclofenac (DIC) is a non-steroidal anti-inflammatory drug (NSAID) taken to reduce inflammation and pain in conditions such as arthritis or acute injury. It has been reported that veterinary DIC use in India has caused a crash in the vulture population (Adam, 2006) with major ecological consequences and a sub-cultural Zoroastrian Parsi 'sky burial' crisis (Swan *et al*., 2006). DIC causes kidney failure in vultures that eat treated domestic animals.

Veterinary drugs, when used in food animals, have the potential to generate drug residues in the animals and animal products. The US FDA and other regulatory agencies around the world have set tolerances levels to ensure that residues are not present in excess of the set tolerance levels (Schneider *et al*., 2007). Antibiotics are widely used in dairy cattle management for the treatment of diseases such as mastitis and as dietary supplements. Administration may be performed orally as food additives or directly by injection. The drug residues if present in milk at more than the tolerance limit may cause allergic reactions in sensitive individuals and interference with starter cultures for cheese, butter and other dairy products (Santos *et al*., 2007). The presence of antibiotic residues in milk may also indicate that the milk has been obtained from an animal with a serious infection or with a severe metabolic dysfunction (Bishop and White, 1984), which may be responsible for the promotion of resistant strains of bacteria (Brady *et al*., 1993). It is common practice to give NOR, OT and DIC sodium together to milk-producing animals in countries like Pakistan; therefore it is of great importance to develop a method for simultaneous analysis of drug residues from milk samples.

A number of analytical methods have been published for simultaneous determination of drug residues in milk. HPLC (Moats and Harik-Khan, 1995; Harik-Khan and Moats, 1995; Marazuela and Moreno-Bondi, 2004; Fritz and Zuo, 2007) and GC-MS (Moats, 1990; Wiese and Martin, 1989) are frequently used for drug residue analysis. The recent emergence of CE as a promising, effective and economical approach for the separation of a large variety of substances, as well as availability of automated CE instruments, has promoted the exploration of an increasing number of CE

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Abbreviations used: DIC, diclofenac; NOR, norfloxacin; NSAID, non-steroidal anti-inflammatory drug; OT, oxytocin.

methods for routine analysis. Several methods for the determination of pharmaceutical drugs are presented in the literature using CE, most of them focusing on the quantitation of drugs of the same group (Solangi *et al*., 2007). Only a few papers describe the simultaneous determination of drugs from different groups (Santos *et al*., 2007; Maia *et al*., 2007; Mamani *et al*., 2008). Da Xing and co-workers recently reported an electrophoresis–electrochemiluminescent method for the analysis of enrofloxacin and its metabolite ciprofloxacin in milk samples (Zhou *et al*., 2008). A review has also been published by García-Ruiz and Marin about recent advances of antibiotic analysis by CE (García-Ruiz and Marina, 2006).

There is also an advantage in quality control to using the same method for determination of several kinds of compounds, without the need to change solvents, analytical columns and procedures. The absence of any of CE methods for determination of NOR, OT and DIC sodium residues in milk was the main driver for the present method. The proposed method may be used as an alternative for screening of antibiotics together with OT and NSAID in the milk of sick lactating animals using capillary zone electrophoresis (CZE) with UV detection.

Experimental

Instrumentation

The capillary electrophoresis (CE) system consists of a Beckman Coultor P/ACE MDQ instrument (Beckman Instruments Inc., Fullerton, CA, USA) equipped with auto sampler, photo-diode array detector and a data handling system comprising an IBM personal computer and P/ACE system MDQ (32 Karat) software. The fused silica capillaries were obtained from Beckman and had the following dimensions: 57 cm total length, 50 cm effective length, 75 μm i.d., 375 μm o.d. The temperature of the capillary and the sample was maintained at 25°C. The capillary was regenerated and conditioned daily with methanol for 1 min, followed by water for 0.5 min., 0.1 M hydrochloric acid for 2 min, water for 0.5 min, 0.1 M sodium hydroxide for 2 min, water for 0.5 min and then running buffer electrolyte for 2 min. Before each of the sample injections, the capillary was washed with 0.1 M sodium hydroxide for 2 min and water for 0.5 min, and then equilibrated with running buffer electrolyte for 2 min. The pH measurement was made with Orion 420A pH meter connected with glass-electrode and internal reference electrodes.

Reagents and Solutions

All reagents were of analytical grade and solvents were of chromatography-grade purity. Pure standards of drugs; OT was obtained from the local market, NOR from Novartis Pharma (Pvt.) Ltd, Jamshoro, Pakistan, and DIC from Abbott Laboratory (Pvt.) Ltd, Karachi, Pakistan. Methanol, sodium tetraborate, sodium hydroxide and boric acid of GR-grade were obtained from E-Merck, Germany. The buffer solutions within the pH range of 1–12 were prepared from the following: 0.1 M hydrochloric acid–potassium chloride (pH 1–2), 0.1 M acetic acid–sodium acetate (pH 3–6), 0.1 M ammonium acetate (pH 7), 0.1 M boric acid–sodium tetraborate (pH 8), 0.1 M sodium bicarbonate sodium carbonate (pH 9), 0.1 M ammonium chloride–ammonia solution (pH 10) and 0.1 M potassium chloride–potassium hydroxide (pH 11–12). The following buffers were also used: 0.1 M sodium dihydrogen phosphate–disodium hydrogen phosphate (pH 8) and sodium tetraborate adjusted with boric or sodium hydroxide (pH 7–10). Buffer electrolyte solutions were prepared fresh daily. All solutions were prepared with deionized double-distilled water.

Standard Stock Solution

Stock solutions were prepared by dissolving 0.1 mg of each of OT and DIC sodium separately in 100 mL of water and NOR in 0.1 M sodium hydroxide and stored at 4°C.

Analytical Procedure

An aliquot of solution containing OT (0.15–4.0 μg/mL), NOR (5–1000 μg/mL) and DIC sodium (3–125 μg/mL), was placed in septum vial (1.5 mL). The sample was injected by auto sampler using the hydrodynamic method. The electropherogram was recorded by using 50 mM sodium tetraborate (pH 10) as a run buffer at an applied voltage 30 kV. The detection was performed by UV at 220 nm.

Analysis of Pharmaceutical Preparation

Eight tablets each of Uritac (Wilson's Pharmaceuticals, Islamabad, Pakistan), Floxin [Hilton Pharma (Pvt) Ltd, Karachi, Pakistan], Urid [Bloom Pharmaceuticals (Pvt) Ltd, Hattar, Pakistan], Noroxin (MSD, Merck Sharp & Dohme of Pakistan Ltd, Karachi) containing 400 mg of active ingredient NOR, and Voltral [Novartis Pharma (Pvt.) Ltd, Jamshoro, Pakistan] containing 50 mg of DIC sodium were separately ground to a fine powder. An amount equivalent to 5 mg of NOR was diluted to 10 mL in 0.1 M sodium hydroxide. An amount equivalent to 1 mg of DIC was diluted in 10 mL water. The solution was shaken thoroughly and sonicated for 10 min. The final solution was filtered through filter paper (Whatman no. 42). An Oxytocin Injection [Star Laboratories (Pvt.) Ltd, Multan, Pakistan] containing 0.02 mg of OT was diluted with water and analytical procedure was followed.

Analysis of Milk Samples

Milk samples (10 mL) were collected 4 h after injecting NOR (1500 mg), DIC (750 mg) and OT (20 μg) from three lactating buffalos. A sample was also collected from local dairy that claimed not to inject buffalo with OT. The samples were deprotienized by adding methanol (10 mL) and the container contents were mixed for 2 min. The samples were centrifuged for 20 min at 3000 rpm and supernatant was collected. Organic solvent was evaporated at 45°C under a nitrogen stream. Subsequently the samples were transferred to volumetric flasks; volume was adjusted to 10 mL with water. The samples were filtered through Whatman no. 42 filter paper. The clear solution was run for recording the electropherogram using the following analytical procedure. The quantitation was made by external calibration curves for all the three drugs.

Recovery of Drugs from Milk Samples

Two milk samples were spiked with drugs: one with 500 μg of NOR and 10 μg of OT and the other with 0.5 and 1.0 μg of OT. The volume was adjusted to 10 mL. The analysis was carried out using the analytical procedure described. The quantitation was carried out from the increase in response with added standards from the calibration curve.

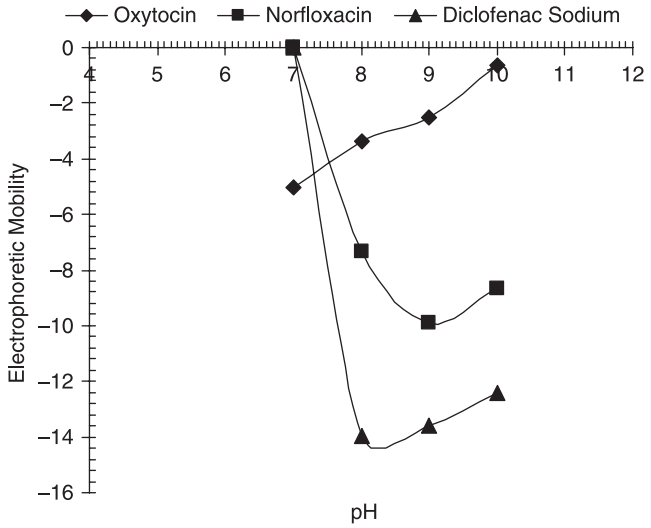

Figure 1. Plot of pH vs electrophoretic mobility.

Results and Discussion

Optimization of CE Conditions

Buffer selection. The electrphoretic mobilities of OT, NOR and DIC were examined in different buffer solutions within the pH range 1–12. However, electrophoretic mobilities were observed above pH 6. Therefore the buffer system comprising sodium phosphate, sodium carbonate, sodium tetraborate, ammonium chloride–ammonia and sodium chloride–sodium hydroxide at the constant concentration of 0.1 M within pH 7–12 was investigated. Among these buffers, better results were obtained using sodium tetraborate in terms of peak shape, sensitivity and selectivity.

Effect of buffer pH. The effect of variation in pH of sodium tetraborate buffer electrolyte on migration times of the analytes was examined within pH 7–10. The electrophoretic mobilities of OT, NOR and DIC were calculated from the observed migration times at different pHs using the equation reported by Solangi *et al*. (2007). The calculated electrophoretic mobilities were plotted vs pH (Fig. 1). It was observed that the effect of pH was not critical. At pH 7 two peaks were identified, one for OT and the other for NOR and DIC, where NOR and DIC indicated the same electrophoretic mobilities. At pH 8, 9 and 10, all three compounds separated completely with different electrophoretic mobilities for each of the compound, but at pH 10 buffer, the peak shape/peak symmetry was observed to have better sensitivity and was selected.

Effect of buffer concentration. The effect of buffer concentration was studied in four selected concentrations of sodium tetraborate (25–100 mM with an interval of 25 mM) under constant instrumentation conditions (pH, voltage, injection time, temperature and wavelength). The optimal separation was obtained with 50 mM sodium tetraborate buffer and was selected.

Effect of voltage and injection time. The effect of applied voltage on the separation was examined within 20–30 kV at an interval of 5 kV. The migration time increased with the decrease in applied voltage, without any improvement in the resolution of peaks. It was therefore the applied voltage of +30 kV was selected to achieve the shortest analysis time and the highest separation efficiency.

Sample injection time (1–5 s) and pressure (0.2–1.0 psi) were varied to achieve a lower detection limit without affecting the quality of peak shape and reproducibility. An injection pressure of 0.5 psi and duration of 4 s injection time offered the best results and were selected (Fig. 2).

Validation of the Method

Linearity. Average peak height/peak area ($n = 3$) was plotted against concentration and linear calibration curves were observed with OT (0.15–4.0 μg/mL), NOR (5–1000 μg/mL) and DIC sodium (3–125 μg/mL) using six calibrates with coefficient of determination (r^2) 0.995 for OT, 0.995 for NOR and 0.998 for DIC. The limits of detection (LOD) measured as signal-to-noise ratio (S/N) = 3:1 were obtained as 50 ng/mL for OT and 1 μg/mL for NOR and DIC. The limits of quantitation (LOQ), measured as S/N = 10:1, were calculated as 0.15 μg/mL for OT and 3 μg/mL for NOR and DIC. The validity of the calibration curves was calculated by the analysis of four test mixtures of OT, NOR and DIC and average values ($n = 3$) were obtained with relative error within ±0.5%.

Precision and accuracy. The intra- and inter-day assay precision was determined by total analysis of six replicates ($n = 6$) samples, under the same conditions, by the same analyst, on the same day and for four days. The repeatability of the separation in terms of migration time and peak area/peak height for all three drugs examined indicated RSD within 0.22–0.54 and 0.8–2.2%, respectively (Table 1).

Selectivity. The responses (migration time and peak height) of the three drugs in test mixtures containing the analytes and common additives (glucose, lactose, sorbitol, gum arabic, starch, magnesium stearate, methylparaben and propylparaben) were compared with the responses of standard solutions containing only the analyte. The additives were added at least twice the concentration of the drug. No peak shape alterations were observed, although new small peaks were present in the electropherograms. The additives did not interfere with the determination with relative error ±1.3%.

Table 1. Linearity and Regression Data of Oxytocin, Norfloxacin and Diclofenac Sodium

Sample no.	Name of drug	Migration time (min)	Mobility mep (cm^2/kVmin)	Calibration range (μg/mL)	LOD (μg/mL)	%RSD ($n = 3$)	
						Peak area	Migration time
1	Oxytocin	4.23	−6.27	0.15–4.0	0.05	0.8	0.22
2	Norfloxacin	6.40	−8.68	5–1000	1.0	2.2	0.40
3	Diclofenac sodium	8.35	−12.39	3–125	1.0	1.0	0.54

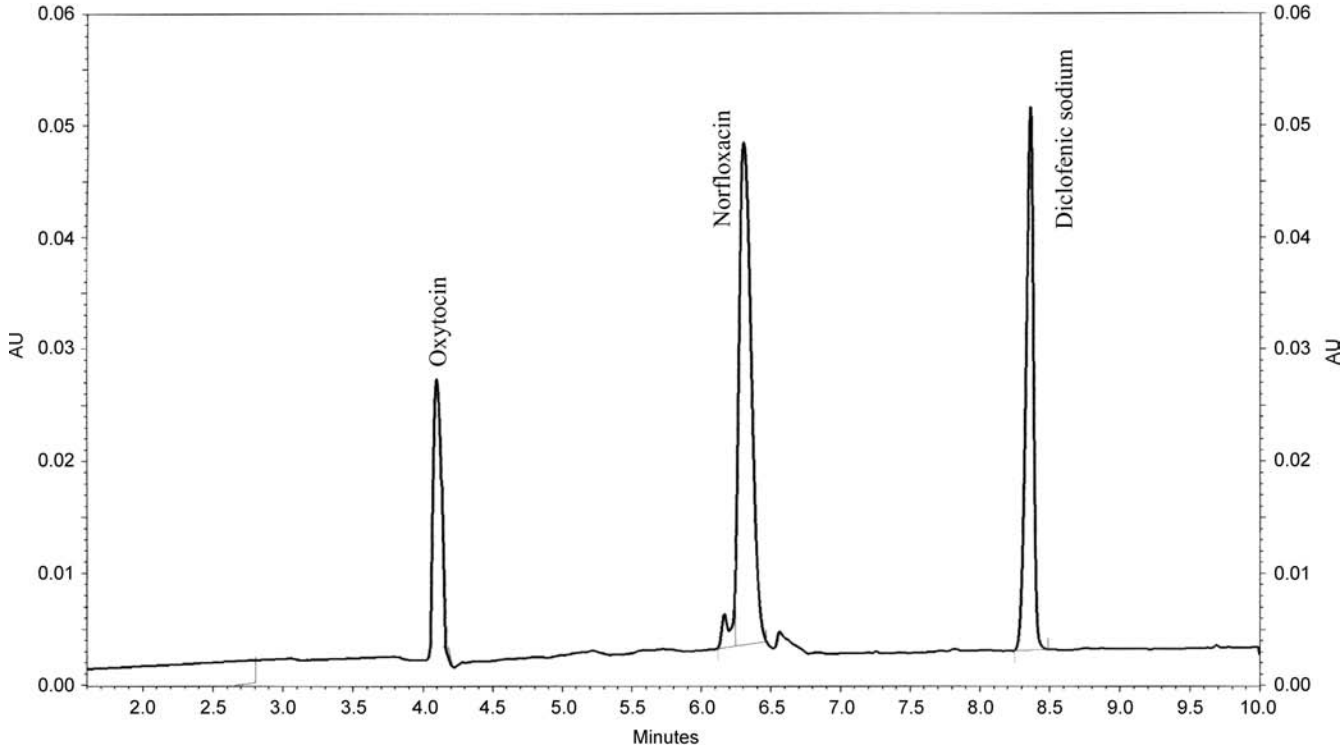

Figure 2. Electropherogram of a mixture containing OT, NOR and DIC sodium. CE conditions: 30 kV, 220 nm; buffer, 50 mM. Sodium tetraborate, pH 10.

Table 2. Assay of drugs in pharmaceutical preparations

Sample no.	Name of compound	Name of tablet/injection	Amount labeled (mg/tablet)	Amount found (mg/tablet)	% RSD
1	Oxytocin	Oxytocin	0.02/injection	0.015/Inj	1.1
2	Norfloxacin	Uritac	400	398	1.5
		Floxin	400	410	1.8
		Urid	400	390	1.4
		Noroxin	400	420	1.6
3	Diclofenac sodium	Voltral	50	45.3	2.1

Sample Analysis of Commercial Pharmaceutical Preparation

The procedure was examined for the analysis of three drugs from six commercial pharmaceutical preparations. A fresh calibration curve was prepared for each day followed by the analysis of the drug from pharmaceutical preparation. The results of analysis for all the drugs agreed with labeled values with RSD ($n = 6$) within 1.1–2.1% (Table 2).

Sample Analyses of Milk

The milk samples were analyzed after deprotinization with methanol. The amount of drugs from milk was observed as 0.319 μg/mL for OT with RSD 1.3%, 130 μg/mL of NOR with RSD 2.1% whereas DIC sodium was not observed below the LOD (Fig. 3, Table 3). The recovery was calculated by spiking the samples with each of the drug and found to be 96.7 and 100%, respectively, for OT and NOR (Fig. 4, Table 3). The amount of OT found from local dairy sample was 0.25 μg/mL with RSD 1.6% (Fig. 5, Table 4) and the recovery calculated from spiked sample was 98% (Fig. 6, Table 4).

Conclusions

A CZE method has been proposed for simultaneous analysis of OT, NOR and DIC sodium in milk samples in short analysis time (less than 9.0 min). The method is equally applicable for the analysis of each of the drug from pharmaceutical formulations. Good results with respect to precision, accuracy and selectivity were obtained in the concentration range 0.15–4 μg/mL for OT, 5–1000 μg/mL for NOR and 3–125 μg/mL for DIC sodium. The lower detection limits were found to be 50 ng/mL for OT, and 1 μg/mL for NOR and DIC sodium. The results shows that the developed CZE method is good alternative to HPLC with shorter analysis time, low operating cost and low solvent consumption, and it is environmentally friendly.

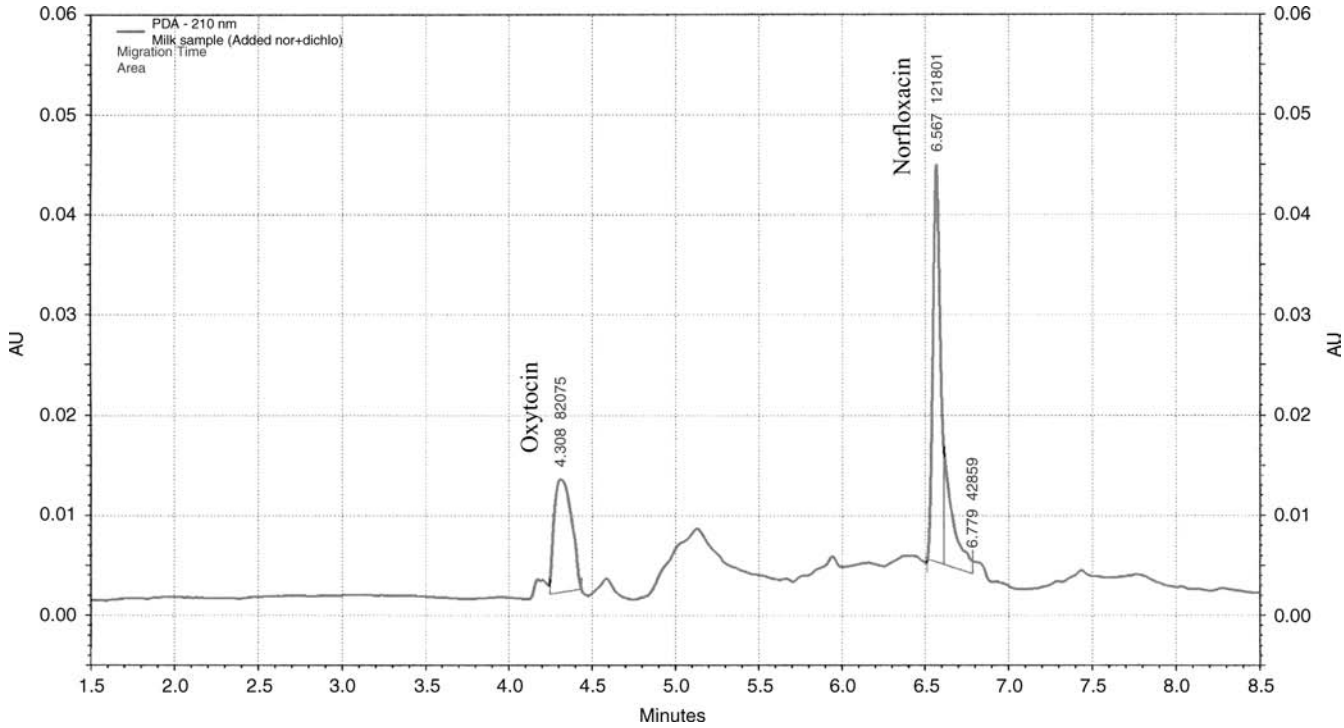

Figure 3. Electropherogram of milk sample of sick buffallo after chemotherapy with OT, NOR and DIC sodium; CE conditions same as in Fig. 2.

Table 3. Assay of drugs in milk samples

Sample no.	Name of compound	Amount taken	Amount added (μg)	Amount found (μg)	Recovery (%)	%RSD
1	Oxytocin	20 μg	0	0.319	—	1.3
			10	9.988	96.7	1.6
2	Norfloxacin	1500 mg	0	130.2	—	2.1
			500	633.0	100.4	2.8
3	Diclofenac Sodium	750 mg	—	ND[a]	—	—

[a] ND, not detected.

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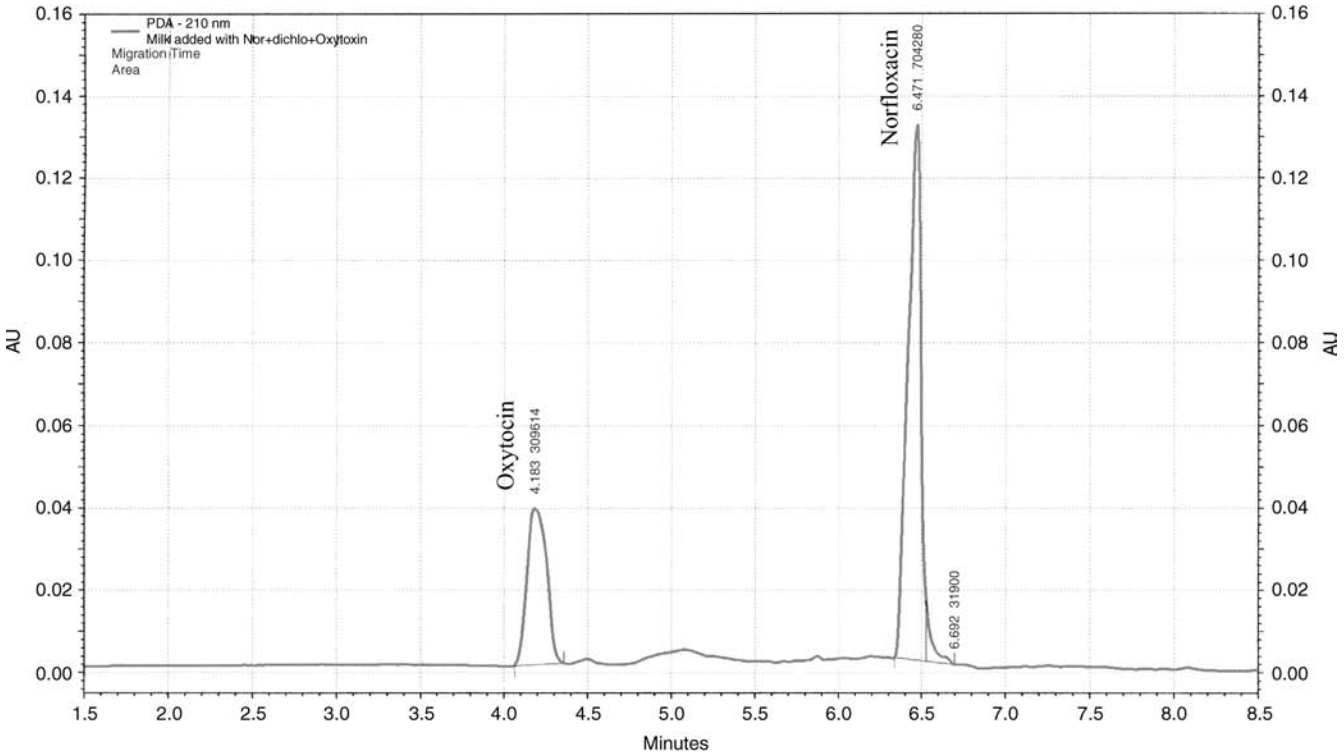

Figure 4. Electropherogram of milk sample of sick buffallo spiked with NOR (500 μg) and OT (10 μg); CE conditions same as in Fig. 2.

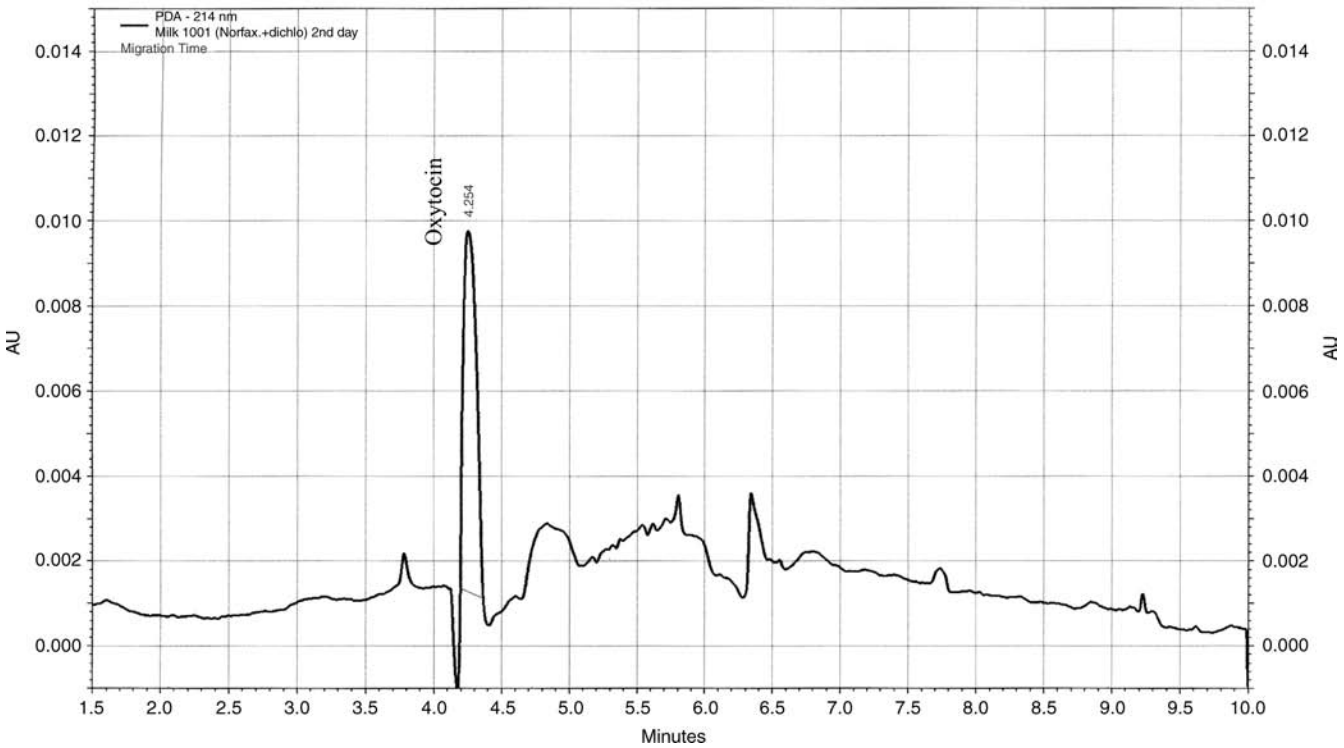

Figure 5. Electropherogram of milk sample from a local dairy who claimed for not injecting OT; CE conditions same as Fig. 2.

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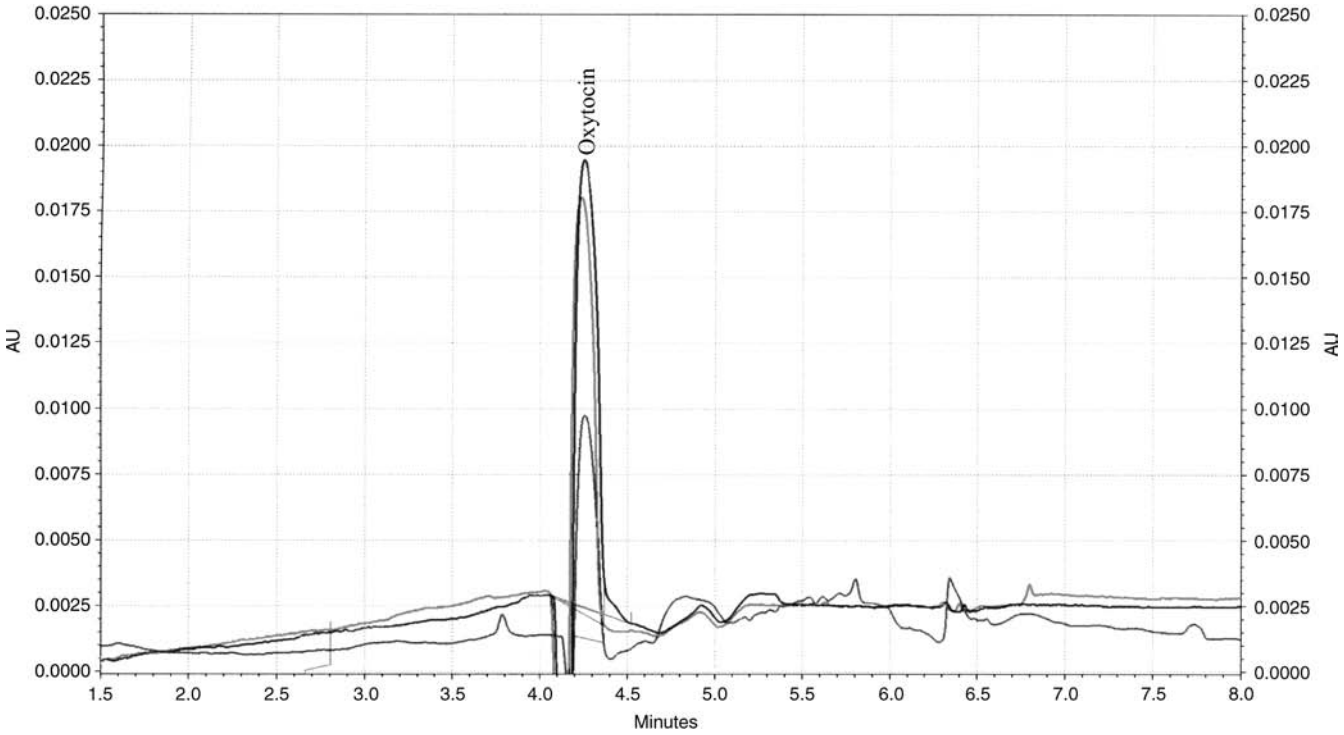

Figure 6. Electropherogram of milk sample from a local dairy spiked with OT (1 and 0.5 μg); CE conditions the same as in Fig. 2.

Table 4. Results of milk sample spiked with oxytocin

Sample no.	Sample	Amount of OT added (μg)	Amount found (μg)	Recovery (%)	% RSD
1	Milk	0	0.25	—	1.6
2		1	1.23	98.4	0.9
3		0.5	1.70	98.2	1.3

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