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A B S T R A C T

Phenylbutazone and flunixin meglumine are non-steroidal anti-inflammatory drugs with antiinflammatory and analgesic activities widely used for the treatment of bone and joint inflammations, laminitis and soft tissue inflammation in the horse. The aim of the present study was to develop a new, selective, sensitive and fast analytical approach for phenylbutazone and flunixin quantitative detection in equine plasma. Differential pulse voltammetry experiments were performed with a portable electrochemical transducer by using miniaturized disposable graphite based screen-printed electrodes. The electrochemical detection by differential pulse voltammetry was coupled to prior selective extraction using dedicated molecularly imprinted solid phase extraction (MISPE) columns to reduce/avoid possible interferences present in plasma. Recovery after MISPE for both phenylbutazone and flunixin was >96%, with intra-day values below 5.0% and inter-day values below 6.5%. Method limit of quantification was 0.01 μg/ml for both phenylbutazone and flunixin. The results obtained with DPV method showed a good correlation with those provided by an HPLC reference method. The method can be proposed as a suitable alternative to the existing chromatographic methods for the determination of phenylbutazone and flunixin in equine plasma samples.

1. Introduction

Phenylbutazone (4-butyl-1 ,2-diphenyl-pyrazolidine-3,5-dione) (PBZ) and flunixin (2-[[2-methyl-3-(trifluoromethyl) phenyl]amino]pyridine-3-carboxylic acid) (FXN) are non-steroidal anti-inflammatory drugs (NSAIDs) widely used for the treatment of bone, joint and soft tissue inflammation in the horse [1-5]. In racehorses, despite their significant analgesic and anti-inflammatory effects, PBZ and FXN have the capacity to affect racing performance, thus their administration is regulated to prevent illegitimate uses (doping). However, anti-doping policy should not discourage legitimate use of drugs and medication programs issued by sport regulatory bodies such as United States Equestrian Federation (USEF), Racing Medication and Testing Consortium (RMTC), Association of Racing Commissioners International (ARCI), and Fédération Equestre Internationale (FEI), discriminate between doping and therapeutic use of drugs. On this basis, plasma concentration not exceeding 15 or 2 μg/ml for phenylbutazone and 1.000 or 20ng/ml for flunixin are permitted in samples collected immediately after the race under USEF or RMTC/ARCI medication control programs, respectively. On the contrary, FEI does not allow the presence of NSAIDs in the horse's blood at the time of competition and detection times (indicating the time at which the drug or its breakdown products' concentration cannot be detected by routine or standard methods) of 168 h for phenylbutazone and of 144 for flunixin, have been proposed.

In any case, regardless of the regulation of reference, the detection of phenylbutazone and flunixin in equine plasma represents a relevant analytical problem in veterinary anti-doping controls.

Methods available for the quantification of phenylbutazone and flunixin in equine plasma include high performance liquid chromatography (HPLC), liquid- and gas-chromatography-mass spectrometry (LC-MS and GC-MS) [10-20]. However, most of these methods involve costly investment, long analytical procedure requiring also large amounts of organic solvents. Electroanalytical approaches have been also proposed for phenylbutazone, using a glassy carbon working electrode, a platinum wire counter electrode and a saturated calomel reference electrode, employing both
linear-sweep and differential pulse voltammetry techniques [21]. Electroanalytical detection of FXN has not been reported so far. Electroanalytical methods are well known for their high sensitivity, fast analysis times, thus gaining increasing attention in the analysis of drugs [22]. Very little sample pretreatments are generally required and the use of disposable electrodes, i.e. screen printed electrodes coupled with portable instrumentation, represents an attractive feature for electroanalytical method application in the field of clinical, environmental and pharmaceutical analyses. In analyses sensitive to matrix effects, electroanalytical methods can be coupled with sample purification by solid-phase extraction (SPE) [23,24]. The application of molecularly imprinted polymers (MIPs) to SPE has also improved the selectivity for analytes by extraction from complex matrices. MIPs are a class of highly cross-linked polymer-based molecular recognition elements engineered to bind one target compound or a class of structurally related target compounds with high selectivity [25,26]. Sample-pretreatment by MIP sorbent has been reported for

selective extraction of some NSAIDs (diclofenac, ibuprofen, naproxen and isoxicam) in environmental and clinical matrices [27-30]. In particular MIPs for NSAIDs are divinylbenzene based MIP, which interacts with NSAIDs by non-ionic and hydrophobic interactions [30].

The aim of the present study was to develop a new, selective, sensitive and fast analytical method for phenylbutazone and flunixin quantitative detection in equine plasma, based on electrochemical sensing, using disposable graphite-based screen-printed electrodes and differential pulse voltammetry (DPV) as detection technique. The method was first optimized after the molecularly imprinted solid-phase extraction (MISPE) analyte selective extraction and then applied to phenylbutazone and flunixin spiked equine plasma. The analytical performances of the system were evaluated both in standard solutions (phenylbutazone and flunixin in acetate buffer) and in equine plasma (spiked). Finally, the approach was validated by using an HPLC reference method.

2. Experimental

2.1. Reagents and instruments

Phenylbutazone (PBZ), flunixin (FXN), suxibuzone and ketopro-fen were obtained from Sigma-Aldrich (Milan, Italy). Oxyphenbu-tazone (OPBZ) and ibuprofen were obtained from SALARS (Como, Italy). Acetonitrile, methanol, acetone and water HPLC grade, were obtained from Labscan (Hasselt, Belgium). Acetic acid, formic acid and ammonium formiate were purchased from Fluka (Sigma-Aldrich, Milan, Italy). Molecular imprinted polymer columns (product code: SupelMIP NSAIDs) were obtained from Supelco (Sigma-Aldrich, Milan, Italy). Microprocessor pH 9219 pH-meter was obtained from Hanna Instruments (Italy). The vacuum pump used was a Laboport (KNF Neuberger, Trenton, USA). The SPE vacuum manifold was from J.T. Baker (Deventer, The Netherlands).

2.2. Preparation of solutions

Stock solutions of PBZ and FXN, suxibuzone, ibuprofen, ketopro-fen and OPBZ were prepared as 1 mg/ml solutions in methanol and stored at -20 °C. Working solutions were daily prepared by serial dilutions of the stock solutions in supporting electrolyte.

2.3. Electrochemical measurements

All experiments of cyclic (CV) and differential pulse voltammetry (DPV) were performed with a portable electrochemical transducer Palmsens (Palm Instrument BV, Houten, The Netherlands). The system was connected to a hand-held computer iPaq (Hewlett-Packard Company, Palo Alto, CA, USA) for the acquisition of the analytical data. Miniaturized disposable graphite based screen-printed electrodes (EcoBioServices & Researches s.r.l., Florence, Italy) were used for all the electrochemical assays. They consisted of a round-shaped graphite working electrode (diameter 3 mm), of a graphite counter electrode and of a silver pseudo-reference electrode as reported in [31]. In addition, the silver electrical contacts

were covered by a graphite layer in order to prevent oxidation phenomena. Before use the electrodes were preconditioned by applying CV, scanning 5 cycles at 50 mV/s and 5 mV step potential on the working potential range. This pretreatment eliminates the spurious peaks given by ink impurities, contributing also to the decrease of surface hydrophobicity.

2.3.1. Optimization of the electrochemical procedure

The effect of pH (range 2-7) and of the ionic strength of the supporting electrolyte solution (KCl concentration range 10-200 mM) on the DPV peak current and potentials was investigated to optimize the DPV method. Potential pulse amplitude (E_{pu}lse) ^{was} evaluated in the range of 10-100 mV, with a scan rate of 0.025 V/s, pulse of 0.05 s and a step potential ($E_s$tep) of 5 mV. Step height was evaluated in the range of 2-10 mV with a scan rate of 0.025 V/s, a *t* pulse of 0.05 s and an $E_{pu}l_{se}$ of 50 mV. The influence of the scan rate was examined in the range of 0.01 -0.1 V/s, a *t* pulse of 0.05 s, an $E_s$tep of 5 mV and an $E_{pu}l_{se}$ of 50 mV. Before quantitative measurements voltammetric scans were carried out in the supporting electrolyte until low and stable baseline was achieved (3-4 potential sweeps). Each scan was performed after an equilibrium time of 10 s. All the measurements were performed immerging the sensor in 2.0 ml of solution containing standard or extracted sample in supporting electrolyte solution. Standard addition method was applied for quantitative analysis.

2.4. Molecular imprinted solid phase extraction (MISPE)-procedure

MISPE-procedure was performed according to cartridge data sheet and [30] with slight modifications. The cartridge was conditioned with 1 ml acetonitrile, 1 ml methanol and 1 ml buffer solution (10 mM ammonium formiate adjusted to pH 3 by addition of formic acid). Two milliliters of equine plasma was first diluted with 18 ml of 10mM ammonium formiate buffer pH 3 and then applied (final volume 20 ml) to the cartridge. A flow of 1 ml/min was applied, the cartridge was then washed with 1 ml water, completely dried and washed with 1 ml of 40/60 (v/v) acetonitrile/water solution. Finally, the cartridge was fully dried and eluted two times with 1 ml of 1% acetic acid in 20/80 (v/v) acetone/methanol. The extract was evaporated under gentle nitrogen flow to dryness and reconstituted in 100 µl of supporting electrolyte.

The MISPE-procedure was applied to equine plasma samples spiked with PBZ and FXN at 0.05 µg/ml (low concentration level), 0.5 µg/ml (medium concentration level) and 10µg/ml (high concentration level) to obtain the recovery of the extraction.

2.5. *Evaluation of the system analytical parameters*

The proposed quantitative method was validated determining: matrix effect, selectivity, linearity, within-run and between-run precision and accuracy, limit of quantification (LOQ), limit of detection (LOD) in compliance with EU and USA rules [32-34].

2.5.1. *Matrix effect*

Matrix effect was evaluated in equine plasma samples without PBZ and FXN (blank). Samples were extracted with MISPE-procedure and analyzed to evaluate the presence of interfering peaks at the oxidation potential of PBZ and FXN.

2.5.2. Selectivity

The electrochemical behavior of other NSAIDs was studied to check the system selectivity with respect to the PBZ and FXN voltammetric signal. Solutions of suxibuzone, OPBZ (NSAIDs with similar structure of PBZ), ibuprofen and ketoprofen (NSAIDs possibly present in equine plasma) were individually tested at a concentration of 1 μg/ml.

2.5.3. *Linearity*

Linearity test calibration curves with PBZ and FXN working standard solutions at 0.025,0.05,0.1,0.25,0.5,1,2.5,5 and 10 μg/ml in supporting electrolyte were prepared. The measurements were repeated nine times. The acceptance criteria for correlation coefficient (r^2) and the goodness-of-fit coefficient (g) were $r^2 \geq 0.99$ and $g \leq 10\%$.

2.5.4. *Accuracy and precision*

Precision of DPV method was assessed by carrying out 10 replicate analyses on freshly prepared 0.5 μg/ml solutions of PBZ and FXN. Within-day precision and accuracy were evaluated by analyzing blank plasma samples spiked with PBZ and FXN at 0.05 μg/ml (low concentration level, n = 3), 0.5 μg/ml (medium concentration level, n = 3) and 10 μg/ml (high concentration level, n = 3) extracted with the MISPE-procedure and analyzed on the same day. Between-day precision and accuracy were evaluated by analyzing the same samples on 7 days (n = 21 for each concentration level). The acceptance criteria for accuracy were -20% to +10% of the theoretical concentration.

2.5.5. *LOQandLOD*

The sensitivity of the method was expressed as LOQ, which was set at 10 times the standard deviation of the intercepts *(a)* divided by the slope of the calibration curve (S) (LOQ= 10cr/S), and LOD, which was set at 3.3 times the standard deviation of the intercepts *(a)* divided by the slope of the calibration curve (S) (LOD = 3.3cr/S).

2.6.Application to horse plasma samples

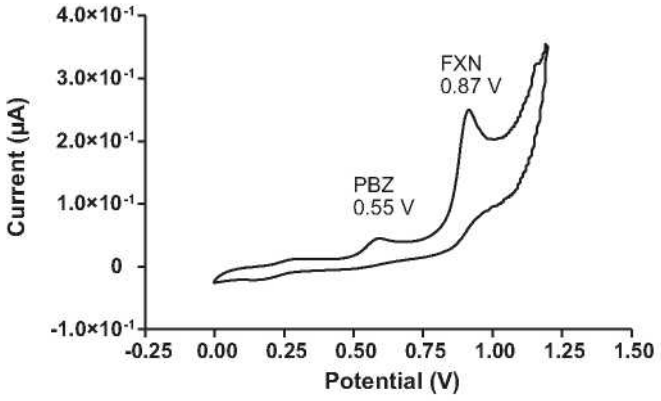
Plasma samples were obtained from 3 horses presented to the Department of Veterinary Clinic of Pisa University for the treatment of musculoskeletal disorders and were included in the study after the owner's

informed consent. One horse was administered intravenously with 4.4 mg/kg of PBZ and sampled at 24 h after treatment. One horse was administered orally with 4.4 mg/kg of PBZ and sampled at 48 h after treatment. One horse was administered intravenously with 1.1 mg/kg of FXN and sampled at 2 h after treatment.

2.7.Liquid chromatography

The results obtained with the electrochemical sensor and MISPE-procedure were compared to those obtained with an HPLC-UV method previously developed and validated in our laboratory. The chromatographic system consisted of a binary gradient pump SpectraSYSTEM® P2000 Thermo Finnigan, with a UV-VIS SpectraSYSTEM® 3000 Thermo Finnigan detector and an autosam-pler SpectraSYSTEM® AS3000 Thermo Finnigan (Waltham, MA, USA). Chromquest® (Thermo Finnigan, Waltham, MA, USA) software was used for data processing. The column was a SunFire^{T}M C18 (4.6 mm x 250 mm, Sμ!!! particle size) (Waters, Milford, MA, USA).The detection of PBZ and FXN was done at 254 nm. Quantification was performed using internal calibration. The separation was done under isocratic conditions at a flow-rate of 1.5 ml/min. Mobile phase was acetonitrile-methanol mixture (75-25%) and acetic acid (0.01 M, 75-25%, v/v). The LOD is 0.25 μg/ml for both PBZ and FXN.

Fig. 1. Cyclic voltammogram of PBZ (1 μg/ml) and FXN (1 μg/ml) on screen-printed graphite electrodes in acetate buffer 0.25 M pH 4.7 with KCl100 mM, 50 mV/s.



3. Results and discussion

In the present study electrochemical-based detection was applied for detecting simultaneously PBZ and FNX, belonging to NSAIDs in equine plasma. Selective detection was achieved by coupling the electrochemical detection to MISPE-procedure. PBZ detection by voltammetric techniques has been reported [21], in standard solutions and in pharmaceutical formulations using a glassy carbon working electrode, a platinum wire counter electrode and a saturated calomel reference electrode, employing both linear-sweep and differential pulse voltammetry techniques. In the case of FXN no data are available, to our knowledge, about its electrochemical detection.

3.1. *DPV method optimization*

The electrochemical behavior of PBZ and FXN at the screen-printed graphite electrode was studied by cyclic voltammetry. The analysis showed an irreversible peak corresponding to a product of oxidation for both PBZ and FXN at +0.55 and +0.87 V, respectively (Fig.1).

The effect of pH, the ionic strength of the supporting electrolyte solution on the DPV peak current and potentials was first investigated. The peak current of both PBZ and FXN increased with decreasing pH (from 4.0 to 2.0) and decreased with increasing pH (from 5.0 to 7.0). Thus pH of 4.0 for the supporting electrolyte solution was chosen (Fig. 2). Concentrations of KCl in the supporting electrolyte ranging from 10 up to 200 mM were tested, with 100 mM resulting in higher peak current magnitude; the latter was then selected for further experiments. At lower concentration (10mM) poor peak resolution was found while at higher concentration (200 mM) high noise effects in currents response were observed.

Finally the effect of the differential pulse parameters on PBZ and FNX oxidation peaks was studied. Potential pulse amplitude (Epulse) was investigated within the range 10-100 mV, using a scan rate of 0.025 V/s, a *t* pulse of 0.05 s and a step potential ($E_s$tep) of 5 mV. An increase in the net peak current with increasing $E_{pu}l_{se}$ was observed and the resolution of response showed a maximum for an Epulse of 50 mV, which was selected for the analysis. The variation of the step height in the 2-1 0mV range had little effect on net current peak height and produced a slight increase of this response. A step height of 5 mV was chosen as a reasonable value to give well-defined voltammograms. In these experiments a scan rate of 0.025 V/s, a *t* pulse of 0.05 s and an E_{pu}lse of 50 mV were used. The influence of the scan rate was examined in the 0.01-0.1 V/s range, using a *t* pulse of 0.05 s, an E_st_ep of 5 mV and an $E_{pu}l_{se}$ of 50 mV. The net current was observed to increase with scan rate in the applied range. However, both background current and noise also increased with increasing scan rate. Taking into account the best signal-to-noise ratio and peak resolution, a scan rate of 0.025 V/s was chosen for further experiments. On the base of this behavior the following DPV parameters were selected: $E_{pu}l_{se}$ of 50 mV, E_st_ep of 5 mV, scan rate of 0.025 V/s and pulse of 0.05 s in acetate buffer 0.25 M pH 4.7 withKCl100mM.

Table 1

Accuracy and precision of the DP voltammetric-sensor for the detection of PBZ and FXN in spiked equine plasma samples, previously treated by MISPE.

Nominal concentrations (μg/ml)	Measured concentrations (mean ± SD, μg/ml)	Accuracy (%)	Intra-day precision (CV%; $n = 3$)	Inter-day precision (CV%; $n = 21$)
PBZ				
0.05	0.048 ± 0.001	95.2	1.5	3.8
0.5	0.473 ± 0.015	94.7	2.5	3.2
5	4.80 ± 0.10	96.0	4.0	6.5
FXN				
0.05	0.049 ± 0.002	97.3	1.8	2.6
0.5	0.500 ± 0.015	100.6	2.0	3.0
5	4.93 ± 0.15	98.7	5.0	4.0

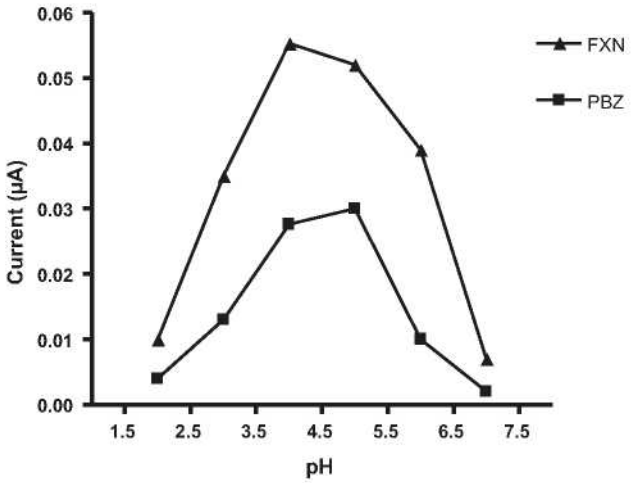


Fig. 2. Effect of pH on peak current for 0.5 μg/ml of PBZ and FXN in acetate buffer 0.25 M pH 2-7 with KCl100 mM. DPV conditions: E_{pulse} 50 mV, E_{step} 5 mV, scan rate of 0.025 V/s and pulse of 0.05 s.

DPV for both PBZ and FXN is based on oxidation at graphite based screen-printed electrodes in a supporting electrolyte consisting of acetate buffer 0.25 M pH 4.7 with KCl 100 mM. According to Chan and Fogg [21] the voltammetric behavior of PBZ under the above conditions depends on oxidation, probably associated with a carbonyl group in the pyrazolone ring, whereas the mechanism for the electrochemical oxidation of FXN may involve an electron abstraction, which leads to a nitrogen centered radical cation that undergoes rearrangement and deprotonation leading to a carbon centered radical para to the amino group [35,36].

3.2. Evaluation of the system analytical parameters

3.2.1. Matrix effect

Matrix effect was also assayed by analyzing equine blank plasma samples after its extraction on MISPE. No interfering peaks at the oxidation potential of PBZ and FXN, +0.55 and +0.87 V, respectively, were found in the relative voltammetric analysis.

Typical DPV voltammograms for PBZ and FXN are shown in Fig. 3. The peak potential obtained for PBZ and FNX are +0.55 and +0.87 V, respectively.

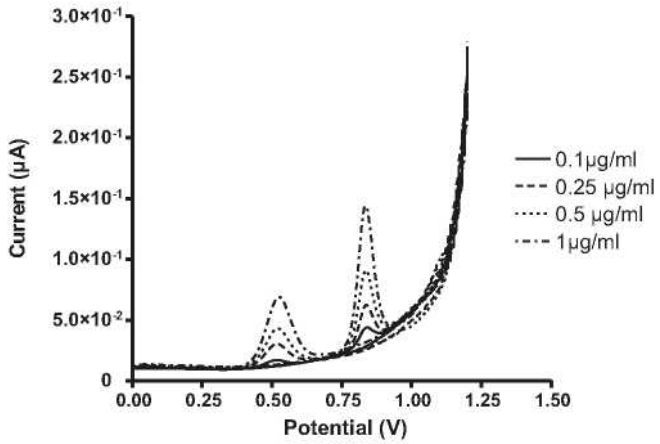


Fig. 3. DP voltammograms of different concentrations (0.1, 0.25, 0.5, and 1 µg/ml) of PBZ and FXN in acetate buffer 0.25 M pH 4.7 with KCl100 mM. DPV conditions: Epulse 50 mV, $E_s$tep 5 mV, scan rate of 0.025 V/s and pulse of 0.05 s.

3.2.2. Selectivity

The selectivity of the system was studied by testing four different NSAIDs of potential use in equine medicine. Suxibuzone, ibuprofen and ketoprofen did not show any electrochemical behavior in the investigated potential range. On the contrary OPBZ showed oxidation peaks around +0.40 V (Fig. 4). The voltammo-gram of a mixture of PBZ and FNX shows three distinct peaks. The shape of the PBZ oxidation peak does not change appreciably, even in the presence of high concentrations of its metabolite.

3.2.3. Linearity

Linearity was assessed in the range 0.025-10 μg/ml in the supporting electrolyte. The regression lines were described by the following equations: $y = 0.04x + 0.01$, $r^2 = 0.99$ and g 3.7% for PBZ and $y = 0.85x + 0.01$, $r^2 = 0.99$ and $g = 5.6\%$ for FXN. The calibration plots are reported in Fig. 5.

Table 2

DP voltammetric-sensor and HPLC analysis for the detection PBZ and FXN content in spiked equine samples, previously treated by MISPE; *n* of samples for each concentration level=3.

Nominal concentrations (μg/ml)	Observed concentrations with DPV method (mean ± SD, μg/ml)	Observed concentrations with HPLC method (mean ± SD, μg/ml)
PBZ		
0.05	0.048 ± 0.001	0.047 ± 0.001
0.5	0.473 ± 0.015	0.473 ± 0.008
5	4.80 ± 0.10	4.80 ± 0.050
FXN		
0.05	0.049 ± 0.002	0.049 ± 0.003
0.5	0.500 ± 0.015	0.505 ± 0.013
5	4.93 ± 0.15	4.95 ± 0.·087

Table 3

DP voltammetric-sensor and HPLC analysis for the detection PBZ and FXN content in horse plasma samples. Plasma samples were previously treated by MISPE. Horse treatment by i.v. intravenous injection and per os, oral administration.

Treatment	DPV method (mean, μg/ml)		HPLC method (mean, μg/ml)	
	PBZ	FXN	PBZ	FXN
i.v. 4.4 mg/kg of PBZ, 24 h	1.05	–	1.00	–
os 4.4 mg/kg of PBZ, 48 h	0.065	–	<LOQ	–
i.v. 1.1 mg/kg of FXN, 2 h	–	1.3	–	1.5

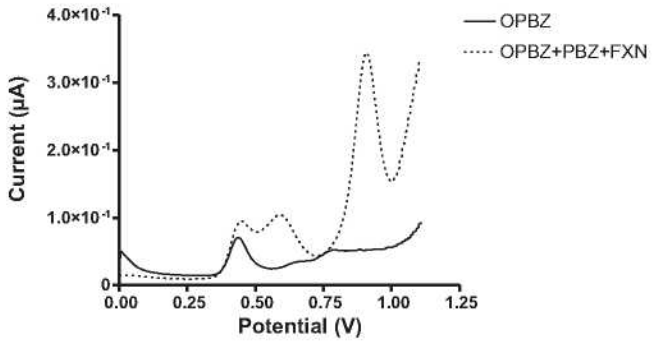


Fig. 4. Selectivity of the sensor. Voltammetric scans of potentially interfering compounds added to equimolar amount of PBZ and FXN. OPBZ alone (1 µg/ml) - straight line and PBZ, FXN and OPBZ together (1 µg/ml) - dotted line in acetate buffer 0.25 M pH 4.7 with KCl100 mM.

3.2.4. Accuracy and precision

Accuracy of estimated PBZ and FXN concentration was more than 94% at three concentrations used as quality control samples. The precision expressed as inter-day coefficient of variation (CV%) ranged from 2.6% to 6.5% and the intra-day CV% ranged from 1.5% to 5.0% (Table 2). The coefficients of variation (CV%) of 10 replicate analyses on freshly prepared 0.5 µg/ml solutions were 1.5% and 0.9% for PBZ and FNX, respectively.

3.2.5. *LOQandLOD*

The LOQ and LOD for both PBZ and FNX were 0.01 µg/ml and 0.005 µg/ml, respectively. LOQ found in this study using voltammetric-based sensing are lower than those obtained with HPLC (LOQ *0.5µg|m\)* and GC/MS (LOQ 0.05 µg/ml) analyses [10-12,14-19]. The present results compared favorably with those obtained with LC/MS/MS (LOQ 0.01 µg/ml) analysis, being more sensitive and less expensive,

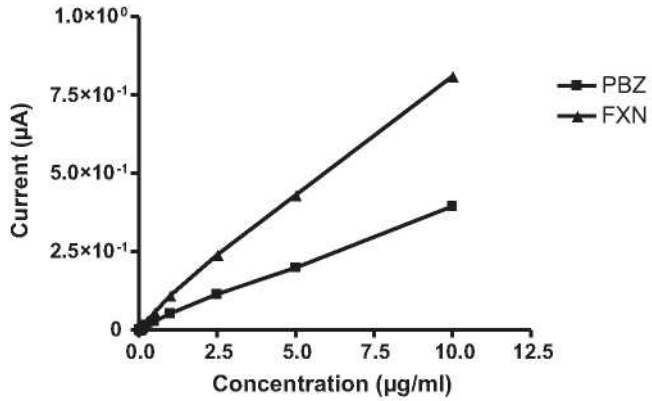


Fig. 5. Calibration curves of PBZ and FXN using

both in terms of analysis time and instrumentation costs [13,20].

3.3. MISPE-procedure recovery

The MISPE-procedure was applied to equine plasma samples spiked with PBZ and FXN to evaluate the field application of DPV-based detection to the analysis of real matrices. An average recovery ranging from 95.2 ± 1.1% to 96.0 ± 2.0% for low to high PBZ spiked samples was observed, while FXN showed an average recovery ranging from 97.3 ± 4.2% to 98.7 ± 3.0%.

3.4. Method validation by HPLC analysis

Electrochemical-based sensing was compared with HPLC reference analysis, previously developed and validated in our laboratory. PBZ and FXN content of spiked equine samples was evaluated with the two methods after sample extraction with MISPE procedure. The findings obtained with voltammetric-based sensing are in very good agreement with results obtained by HPLC analysis (Table 3). Statistical tests on the data at the 95% significance level generated a correlation line with the following equation and correlation coefficient: $y = 0.9997 \pm 0.0043x + 0.00072 \pm 0.012$, $r^2 = 0.9998$ for PBZ and$y = 1.003 \pm 0.0062x + 0.0014 \pm 0.018$, $r^2 = 0.9997$.Thus an excellent correlation was found between the here developed approach and the reference HPLC analysis. Thus the proposed DPV-based method coupled to MISPE resulted very suitable forthe quantitative analysis of PBZ and FXN in equine plasma as target matrices.

3.5. *Detection of PBZ and FNX in horse plasma samples*

The sensor was further applied to equine plasma samples obtained from 3 horses treated with PBZ or FXN and the relative results were also compared with HPLC analysis.

As shown in Tables 1 and 2 the DPV based-sensing showed an excellent accuracy and precision (both intra- and inter-day) in the analysis of the drugs also in plasma samples. Furthermore a very good agreement between the sensor-based and HPLC analyses was found also on real horse plasma sample (Table 3).

Thus by this analytical set-up, based on the combination of MISPE with disposable screen-printed electrodes, accurate, precise, cheap and fast analysis of PBZ and FXN can be achieved in equine plasma samples.

4. Conclusion

A simultaneous sensitive, selective detection of PBZ and FXN, which belong to NSAIDs and are widely used for their anti-inflammatory activity, is achieved in equine plasma by electrochemical-based sensing, coupled to selective extraction with molecularly imprinted polymers.

The sensor was successfully applied to the determination of PBZ and FXN both in spiked and real equine plasma samples. The sensor performances were compared to the reference method (i.e. HPLC analysis) and excellent correlation was found ($r^2 > 0.999$). The developed approach requires very simple and fast sample pre-treatment with very low solvent consumption, reducing the impact on the environment

as well as the analysis cost. The sensitivity of the method is very good (LOQ=0.01 μg/ml). We believe that the method can be considered as a suitable alternative to the existing chromatographic methods for the determination of PBZ and FXN in equine plasma samples.

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