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Liquid-liquid extraction by low-temperature partition and high-performance liquid chromatography for determination of flunixin and phenylbutazone in horse hair. Determinação de flunixin e fenilbutazona em pelos de equinos por cromatografia líquida de alta eficiência com extração por partição à baixa temperatura e detecção no ultravioleta

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Abstract

This work proposes the determination of flunixin and phenylbutazone in horse hair using liquid-liquid extraction by low-temperature partition (LLE-LTP) and HPLC-UV detection. The method efficiently extracted the analytes and purified the extract in a single step. The method has never been applied for horse hair and showed to be easy to perform with satisfactory results. Limits of detection and quantification were 0.012; 0.024 ng mg⁻¹ for flunixin and 0.036; 0.073 ng mg⁻¹ for phenylbutazone, respectively. Nine out of 14 samples analyzed showed positive results for at least one analyte. The average recoveries for flunixin and phenylbutazone were above 97.0 and 92.0 %, respectively.

Keywords: Veterinary drugs. NSAID. Sample preparation. Chromatographic separation.

Resumo

Este trabalho propõe a determinação de flunixin e fenilbutazona em pelos de equinos por extração líquido-líquido com partição à baixa temperatura (ELL-PBT) e cromatografia líquida de alta eficiência com detecção no ultravioleta (CLAE-UV). O método foi capaz de extrair os analitos e purificar o extrato em uma única etapa. Os limites de detecção e quantificação foram 0,012; 0,024 ng mg⁻¹ para flunixin e 0,036; 0,073 ng mg⁻¹ para fenilbutazona, respectivamente. A recuperação média para flunixin e fenilbutazona foi superior a 97,0 e 92,0 %, respectivamente. Os analitos foram detectados em 9 amostras entre as 14 amostras analisadas.

Palavras-chave: Fármacos veterinários. AINE. Preparo de amostra. Separação cromatográfica.

Introduction

Usually drug monitoring is executed through blood and urine analyses; however, the drugs in these matrices are not stable for long (e.g., 48 hours). Hair, on the other hand, provides more stability and the drugs accumulated in it can stay protected from degradation for very long periods (SCHLUPP et al., 2004).

Drug analysis for horses using hair as a sample, regardless of the target analytes, is still growing (SCHLUPP et al., 2004; COMIN et al., 2012; DAVIS; STEGELMEIER; HALL, 2014; GRAY et al., 2013). In addition, few studies have been performed on nonsteroidal anti-inflammatory drugs (NSAID) frequently prescribed for horses (DUNNETT; LEES, 2004; MADRY et al., 2016).

The NSAID are a group of compounds which presents anti-inflammatory, analgesic and antipyretic properties and acts by inhibiting the metabolism of arachidonic acid. These drugs are used in veterinary medicine to treat colic and musculoskeletal disorders in horses (LEES; MAY; MCKELLAR, 1991).

The most suitable samples are obtained from the permanent hair of the horses' mane and tail, they grow continuously at relatively constant rates (DUNNETT; LEES, 2004; ANIELSKI, 2008; DUNNETT, 2005; WHITTEM et al., 1998), enabling studies of drug administration history, which are useful for applications in sports, anti-doping control programs, pre-purchase examinations and examination for insurance purposes (DUNNETT; LEES, 2004; BOYER et al., 2007).

Liquid-liquid extraction by low-temperature partition (LLE-LTP) performs both extraction and the clean-up step at the same time based on the difference between the melting point of the organic solvent and the aqueous phase involved in the process. Usually, this technique is executed using water and acetonitrile, the water freezes trapping impurities as the analyte migrates to the acetonitrile phase that remains in the liquid state (GOULART et al., 2010). The strategy of the LLE-LTP has been used for the determination of various analytes in several types of matrices, such as pyrethroids in water (VIEIRA; NEVES; QUEIROZ, 2007), carbamates in water, grape juice and chocolate milk beverages (GOULART et al., 2010; GOULART et al., 2012), benzodiazepines in urine (MAGALHÃES et al., 2012), pesticide residue in honey samples (PINHO et al., 2010), chloramphenicol in milk (REGO et al., 2015), formaldehyde in bovine milk (REZENDE et al., 2017), benzodiazepines in beers (PAULA et al., 2018), and tetracyclines in food products (DESMARCHELIER et al., 2018).

The aim of this work was to apply an environmentally friendly extraction method with a single step for both the extraction and the clean-up processes involving the use of less organic solvent and spending less time using LLE-LTP and high-performance liquid chromatography (HPLC) with a UV detector to determine NSAID in horse hair.

Material and methods

Equipment

The chromatographic system consisted of a High-performance Liquid Chromatography System model Prominence LC 20 A series Shimadzu (Kyoto, Japan) equipped with binary pump (model 20AD, Shimadzu Corp.), autosampler (model SIL 20AC, Shimadzu Corp.), oven (model CTO-20A), degasser (model (DGU-20A) and a diode array detector (model SPD-20A). The analytes were separated in a C18 column (Ascentis Express 250 mm × 4.6 mm, 5 µm). Data acquisition was performed using the software Labsolutions LC Real Time Analysis (version 3.41, Shimadzu Corp.).

Reagents and solutions

All solutions were prepared using ultra-pure water from a Milli-Q system (Millipore Inc., Bedford, MA) with resistivity of 18 M Ω cm, and all chemicals were analytical grade and used as purchased without additional purification. Stock solutions of *phenylbutazone* and *flunixin*, 100 mg L⁻¹ in methanol (HPLC grade) were prepared with standards purchased from Sigma-Aldrich (St. Louis, MO, USA). These solutions were stored out of light at 2°C.

Hydrochloric acid (HCl), ammonium acetate (CH₃COONH₄) and acetic acid (CH₃COOH) were purchased from Dinamica (São Paulo, SP, Brazil). Acetonitrile (HPLC grade) was purchased from J.T.Baker (Phillipsburg, NJ).

Sample preparation

Fourteen samples, seven from the mane and seven from the tail, were collected by veterinarians with the owners' consent, in the state of Pernambuco, from horses with no documented administration of the investigated drugs. To evaluate the method, samples completely free of drug residues were collected from a clean horse at Mandro's farm, located in Piracicaba, state of São Paulo (Brazil). The sampling method was adapted from the Society of Hair Testing (COOPER et al., 2012) and consisted of cutting the hair precisely close to the skin, storing it in plastic bags under dry conditions and keeping it out of light.

The samples were first washed with tap water and neutral detergent and then washed with distilled water and methanol (DUNNETT; LEES, 2004). Subsequently, the samples were placed in an oven at 40 °C for drying. The dried samples were chopped into snippets and powdered in a mortar (MIGUEL; GORDO; FERNANDO PESSOA, 2013). 10.0 mg of the hair powder was mixed with 0.5 mL of hydrochloric acid 0.1 mol L⁻¹ in a screw capped glass tube and placed in an oven at 60 °C for 12 hours for digestion (DUNNETT; LEES, 2004; DUNNETT, 2005).

Liquid-liquid extraction by low-temperature partition

The extraction process consisted of the addition of 1.0 mL of acetonitrile to the extract after the digestion step. This mixture was placed in a freezer at -5.0 °C until the separation of the phases occurred and the supernatant could be separated from the aqueous phase (GOULART et al., 2010; VIEIRA; NEVES; QUEIROZ, 2007; GOULART et al., 2008). The supernatant, organic phase, was evaporated in a vacuum oven at 40 °C and the volume was reconstituted to 0.5 mL with acetonitrile. The extract was transferred to a vial for further analysis by HPLC.

HPLC analysis

The maxima wavelengths, 290 nm for flunixin and 260 nm for phenylbutazone, were selected based on the UV-spectra generated in a chromatographic run of a spiked sample at 3.4 ng mg⁻¹ for each analyte.

The mobile phase was composed of 0.05 mol L⁻¹ ammonium acetate buffer solution (pH 3.75, solvent A) and acetonitrile (solvent B). The following gradient was used: 10 to 60 % B from 0 to 5 min, 60 % B held for 2 min, 60 to 80 % B from 7 to 10 min and 80 to 10 % B from 10 to 15 min.

The flow rate was 1.0 mL/min, the column temperature was set at 40 °C and the volume injected was 20.0 µL; the two analytes were eluted within 15 min. The matching retention times were the criteria for positive identification. The absence of interfering peaks was verified for all analytes in blank samples.

Method validation

The values for selectivity, linearity, repeatability, limit of detection (LOD), limit of quantification (LQ), accuracy, and precision were calculated to validate the method (ANVISA, 2003; MAPA, 2011). All validation parameters were assessed with a drug-free horse hair sample. The blank samples were spiked with standard solutions to obtain the concentrations required for the calibration process using a linear regression model.

Results and discussion

Liquid-liquid extraction by low-temperature partition (LLE-LTP)

Studies were performed to verify the capability of the method to perform the extraction of the analytes, in acidic digested hair extracts, and the purification of the extract in a single step. Different volumes of the organic and aqueous phases were tested and are shown in Table 1 along with the results obtained, in triplicate, for extraction percentage and coefficient of variation (CV).

Table 1. Volume of acetonitrile (ACN) and volume of hydrochloric acid (HCl), extraction percentage, coefficient of variation (CV) for the LLE-LTP for flunixin (FLU) and phenylbutazone (PHEN).

Test	ACN (mL)	HCl 0.1 mol L ⁻¹ (mL)	Extraction (%)		CV (%)	
			FLU	PHEN	FLU	PHEN
1	0.5	0.5	34.7	27.0	22.5	20.0
2	1.0	1.0	55.0	52.4	11.5	33.4
3	1.5	1.5	59.7	54.0	9.8	27.0
4	0.5	1.0	67.0	49.5	8.4	17.1
5	1.0	0.5	97.5	92.2	1.6	5.6
6	0.5	1.5	43.8	35.5	13.2	11.8
7	1.5	0.5	98.5	91.9	7.8	6.1

As it can be observed, for equal volumes of acetonitrile and HCl the extraction percentage increases with the increase of the respective volumes (tests 1, 2 and 3).

For volumes of acetonitrile lower than HCl volumes the extraction percentage tends to reduce as the proportion of ACN decreases. See tests 4 and 6.

When the volume of acetonitrile is higher than the volume of HCl the extraction percentage increases (tests 5 and 7). Therefore, comparing the test 5 to the test 7, the former was chosen because it requires less organic solvent and presents similar extraction percentage. For lower volumes of ACN it becomes difficult to remove the supernatant.

Most of the works dealing with acidic hair digestion spend more organic solvent and use additional processes for the clean-up step such as solid-phase extraction columns (COMIN et al., 2012; DUNNETT; LEES, 2004).

Chromatographic conditions

A typical chromatogram of a blank sample spiked at 3.4 ng mg^{-1} is shown in figure 1 to ensure about the absence of peak interference.

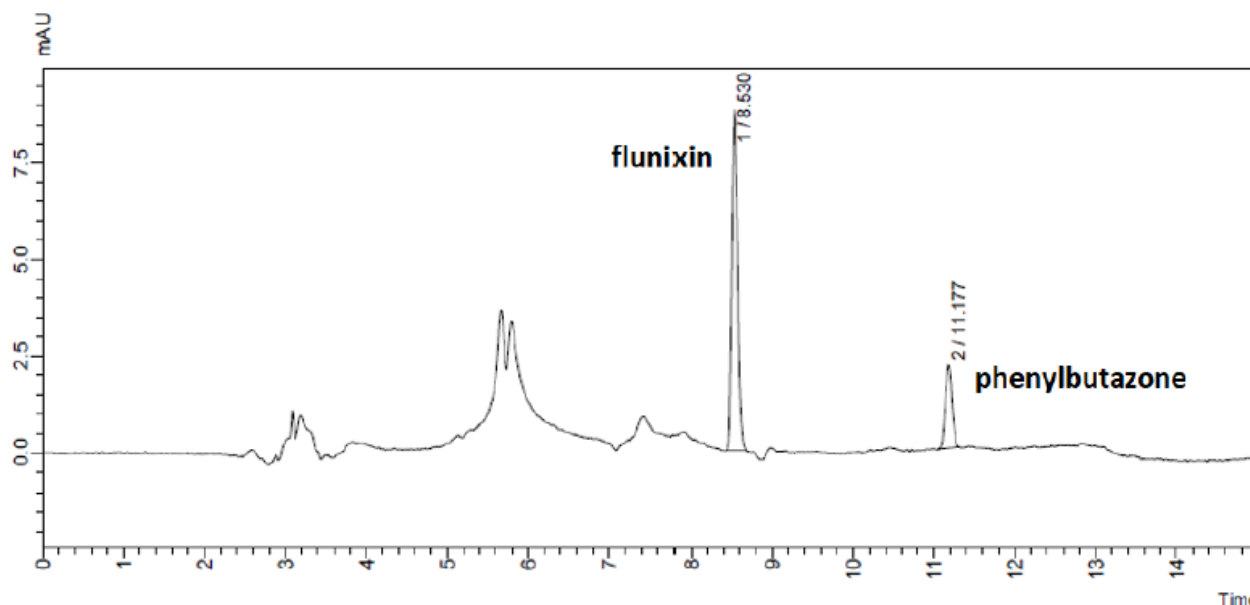


Figure 1. HPLC chromatograms obtained from blank samples spiked at 3.4 ng mg^{-1} for the 290 nm channel.

As it can be seen in Figure 2, flunixin was identified in sample AC3 through comparison of the retention time presented in figure 1, indicating that the analytes are the same.

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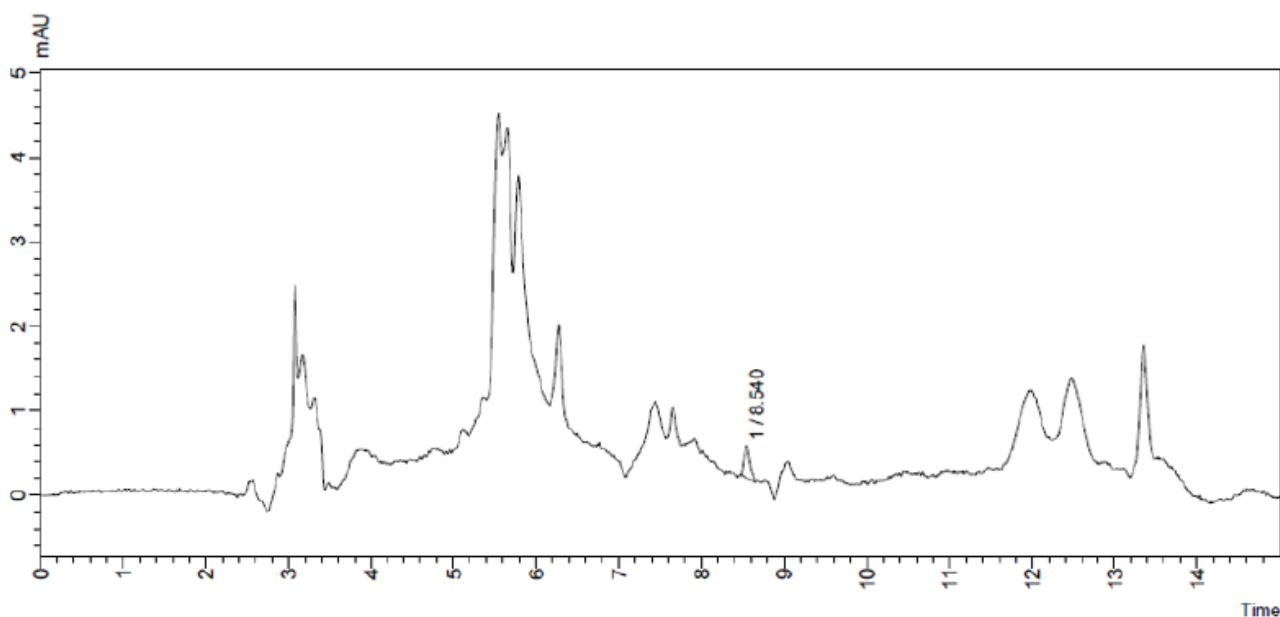


Figure 2. Chromatogram from sample AC3 at the 290 nm channel.

Method validation

The analytical curves were linear from 0.02 to 4.4 ng mg⁻¹ with the following regression equations, $S = 13,4 C - 443,3$ and $S = 36,9 C - 147,5$ for flunixin and phenylbutazone, respectively. Where S is the area of the respective peak analyte and C is its concentration.

The limits of detection and quantification were 0.012; 0.024 ng mg⁻¹ for flunixin and 0.036; 0.073 ng mg⁻¹ for phenylbutazone.

The accuracy of the proposed method was measured by spiking the samples with the analyte standards and the recovery rates are shown in Table 2.

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Sample	Spiking (ng mg ⁻¹)	Flunixin % recovery	Phenylbutazone % recovery
A1	0.08	115.7 ± 5.1	102.1 ± 3.1
	3.4	101.1 ± 1.6	73.3 ± 2.4
A2	0.08	107.5 ± 7.1	93.9 ± 6.5
	3.4	104.4 ± 2.1	76.0 ± 3.7
A3	0.08	116.5 ± 1.4	103.3 ± 4.3
	3.4	102.3 ± 2.5	94.0 ± 1.9
A4	0.08	102.0 ± 5.0	100.1 ± 6.9
	3.4	102.7 ± 0.5	92.0 ± 0.7
A5	0.08	118.8 ± 4.5	101.8 ± 8.5
	3.4	104.9 ± 1.3	90.3 ± 0.7
A6	0.08	101.0 ± 11.9	108.0 ± 10.5
	3.4	112.1 ± 5.5	95.1 ± 7.0
A7	0.08	103.9 ± 1.3	92.3 ± 1.1
	3.4	112.9 ± 1.9	88.4 ± 1.4
AC1	0.08	106.0 ± 1.5	89.9 ± 9.6
	3.4	99.7 ± 0.4	84.1 ± 1.6
AC2	0.08	101.2 ± 5.4	94.8 ± 3.3
	3.4	103.5 ± 0.9	80.3 ± 4.2
AC3	0.08	105.2 ± 2.9	88.8 ± 8.2
	3.4	98.4 ± 3.2	83.5 ± 6.8
AC4	0.08	104.8 ± 7.2	95.2 ± 0.2
	3.4	106.0 ± 6.8	89.6 ± 6.2
AC5	0.08	97.5 ± 2.4	74.5 ± 1.2
	3.4	117.9 ± 3.6	86.5 ± 4.2
AC6	0.08	104.9 ± 7.0	77.9 ± 5.6
	3.4	103.5 ± 7.2	84.2 ± 9.2
AC7	0.08	101.4 ± 8.5	77.5 ± 8.6
	3.4	108.4 ± 6.8	73.7 ± 4.4

A= mane samples; AC= tail samples

The values presented in Table 2 show higher recovery rates for the lower concentrations, which can be related to the smaller amount of analyte that has to migrate from the aqueous phase to the organic phase, thus favoring trace analysis.

The recovery rates varied from 97.5 ± 2.4 to 118.8 ± 4.5% and from 73.3 ± 2.4 to 108.0 ± 10.5 % for flunixin and phenylbutazone, respectively.

Works dealing with extraction of flunixin and phenylbutazone in horse hair are scarce, moreover the ones found do not deal with acidic hair digestion and do not even report the recovery

rates of the analytes (MADRY et al., 2016; RICHARDS et al., 2011), limiting measurement comparison of the efficiency of the method.

Table 3 shows some references that deal with either the analytes or the matrix treated in this work and can be useful for some considerations.

Table 3. Comparison of studies evaluating detection of NSAID in biological samples, including hair samples.

Sample	Analyte	Method	Digestion	LOD	LOQ	Recovery range	Reference
Horse hair	flunixin	LE- LC/MS	none	0.05 pg mg ⁻¹	0.1 pg mg ⁻¹	---	C
Horse hair	phenylbutazone	LE- LC/MS	none	10 pg mg ⁻¹	25 pg mg ⁻¹	---	C
Horse hair	ciprofloxacin	SPE- HPLC/UV	acidic	0.12 ng mg ⁻¹	0.25 ng mg ⁻¹	82.9 -93.1 %	B
Horse hair	enrofloxacin	SPE- HPLC/UV	acidic	0.06 ng mg ⁻¹	0.12 ng mg ⁻¹	71.7-74.6 %	B
Cow's milk	flunixin	LLE- HPLC-UV	none	0.5 µg kg ⁻¹	0.7 µg kg ⁻¹	79.9-90.3 %	A
Cow's milk	phenylbutazone	LLE- HPLC-UV	none	5.0 µg kg ⁻¹	10 µg kg ⁻¹	66.0-66.9 %	A
Bovine muscle tissue	flunixin	LLE- HPLC-UV	β- glucuronidase	6.0 ng g ⁻¹	15.0 ng g ⁻¹	62.8 -74.0 %	D
Bovine plasma	flunixin	LLE- HPLC-UV	none	0.03 µg mL ⁻¹	0.06 µg mL ⁻¹	69.0-85.0 %	E
Horse hair	flunixin	LLE-LTP HPLC-UV	acidic	0.012 ng mg ⁻¹	0.024 ng mg ⁻¹	97.5-117.9 %	This work
Horse hair	phenylbutazone	LLE-LTP HPLC-UV	acidic	0.036 ng mg ⁻¹	0.073 ng mg ⁻¹	74.5-108.0 %	This work

LE: liquid extraction; SPE: solid phase extraction; LLE: liquid-liquid extraction; LLE-LTP: liquid-liquid extraction by low temperature partition. A=(JEDZINIAK et al, 2009); B= (DUNNETT et al., 2004) ; C=(MADRY et al., 2016); D= (ASEA et al., 2001); E= (JEDZINIAK et al., 2007).

Table 3 shows that most of the previous studies on flunixin and phenylbutazone determination adopted liquid-liquid extraction (LLE) or liquid extraction (LE) for sample preparation, requiring an additional step for sample clean-up and using more organic solvent to perform the analyses.

The evaluation of the method proposed here becomes difficult with the absence of works dealing with horse hair and the same analytes, however one can consider the recovery rate, the easy of performing the method and the economy of organic solvent and time very important advantages regardless the analytes or matrices.

The procedure presented here is efficient for the analysis of flunixin and phenylbutazone in horse hair samples from different regions with reliable, precise and accurate results.

Horse hair sample analyses

Fourteen samples, seven from the mane and seven from the tail, from seven random horses were analyzed to quantitate flunixin and phenylbutazone, the results are shown in Table 4.

Table 4. Concentrations of flunixin and phenylbutazone found in the samples of mane and tail.

Samples	Flunixin (ng mg ⁻¹)	Phenylbutazone (ng mg ⁻¹)
A1	< 0.012	< 0.036
A2	< 0.012	3.8±0.2
A3	< 0.012	< 0.036
A4	2.8±0.1	< 0.036
A5	< 0.012	< 0.036
A6	2.1±0.1	< 0.036
A7	2.6±0.2	< 0.036
AC1	4.2±0.7	< 0.036
AC2	3.4±0.4	< 0.036
AC3	3.8±0.6	< 0.036
AC4	< 0.012	1.3±0.1
AC5	< 0.012	< 0.036
AC6	22.6±1.6	< 0.036
AC7	< 0.012	< 0.036

A= mane samples; AC= tail samples

Nine out of the 14 samples showed positive results for at least one analyte. The least concentration of 1.3±0.1ng mg⁻¹ was found for phenylbutazone in a tail sample and the maximum concentration was 22.6±1.6 ng mg⁻¹ from a tail sample for flunixin.

Conclusion

The liquid-liquid extraction by partition at low temperature showed an excellent capacity to extract the analytes and purify the extract in a single step, saving solvent and time. Recovery ranged from 97.5 to 118.8% and from 73.3 to 108.0 % for flunixin and phenylbutazone, respectively.

The limits of detection (0.012 and 0.024 ng mg⁻¹) and quantification (0.036 and 0.073 ng mg⁻¹) for flunixin and phenylbutazone, respectively, permitted the confirmation of the analytes with great reliability.

It was possible to detect at least one of the analytes in 9 out of 14 samples, the least concentration of 1.3±0.1 ng mg⁻¹ was found for phenylbutazone in tail hair.

The extraction method, which is new for horse hair analyses, proved to be an excellent and new alternative for extraction of anti-inflammatory drugs in horse hair. Future work intends to deal with the influence of hair color on extraction, and drug accumulation in horse hair.

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