The Simultaneous Analytical Method for Determination of Flunixin and Tolfenamic Acid in Animal Tissue with LC-MS/MS

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Abstract

Flunixin and tolfenamic acid are nonsteroid anti-inflammatory drugs (NSAIDs). This study present a QuEChERs extraction method coupled with LC-MS/MS analysis for flunixin and tolfenamic acid in animal tissues. Homogenized sample was hydrolyzed by β-glucuronidase under waterbath at 37°C and then extracted with acetonitrile containing 1% formic acid. The second extraction was salting-out by QuEChERs extraction powder, which is composed of magnesium sulfate, sodium citrate and disodium citrate sesquihydrateacetate. After centrifugation, the supernatant was diluted and analyzed. Quantitative analysis was performed by matrix-matched calibration curve. CORTECS C18 column (2.7 mm, 2.1 × 100 mm) with gradient elution under 0.05% formic acid solution and acetonitrile can reach good sample separation. In validation study, 0.002, 0.005 and 0.01 μg g-1 of flunixin and tolfenamic acid were spiked respectively in all matrices. The recoveries were between 68.2% and 95.9% for both drugs, with RSD values lower than 10%. Limits of quantification (LOQ) was 0.002 μg g-1 for both drugs.

Material and Method

Apparatus

High-speed shaker is a 2010 Geno/Grinder, SPEX SamplePrep (Metuchen, NJ, USA). The homogenizer is a Polytron PT-MR 3100 (Kinematic AG, Littau, CH). LC system comprised an Eksigent ultraLC system (AB SCIEX, Redwood City, CA) equipped with a quaternary pump, an autosampler, a degasser, and a column oven. An CORTECS C18 column (2.7 mm, 2.1 × 100 mm, Waters Corp, MA, USA) was used to separate the analytes. Mass spectrometry was performed using a QTRAP 5500 (AB SCIEX, Framingham, MS, USA) hybrid triple quadrupole mass spectrometer equipped with a Turbo V ion source and TIS (TurboIon Spray) probe operating in ESI-MS-MS positive and negative ion mode.

MRM Parameters

Compound	Precursor ion (m/z) > Product ion (m/z)	DP[b] (V)	CE[c] (eV)
Flunixin	297 > 264[a]	60	32
	297 > 259	60	48
Tolfenamic acid	260 > 216[a]	-60	-23
	262 > 218	-60	-23

[a] Quantification ions; [b] Declustering potential; [c] Collision energy

Sample preparation

Weight 2g frozen homogenized sample

↓

Add 10 mL 0.2M ammonium acetate buffer solution (pH 5.2±0.1) and add β-glucuronidase 100μL, digesting for 1 hr at waterbath at 37℃

↓

Add 10 mL acetonitrile containing 1% formic acid, and extraction powder (4 g of MgSO4 anhydrous, 1 g of NaCl,1 g of NaCitrate and 0.5 g of disodium citrate sesquihydrate) sequentially.

↓

Shake for 1min at 1000 rpm
Centrifuge for 1min at 5000 ×g

↓

Transfer 500 μL of supernatant to eppendorf already contained 500 μL of deionized water, vortex and then centrifuged for 1min at 5000 ×g

↓

Take the supernatant to the vial and then analyzed by LC/MS/MS

LC separation

Time (min)	A	B
0.0->2.0	80->80	20->20
2.0->6.0	80->20	20->80
6.0->6.5	20->0	80->100
6.5->9.5	0->0	100->100
9.5->10.0	0->80	100->20
10.0->13.0	80->80	20->20

Results

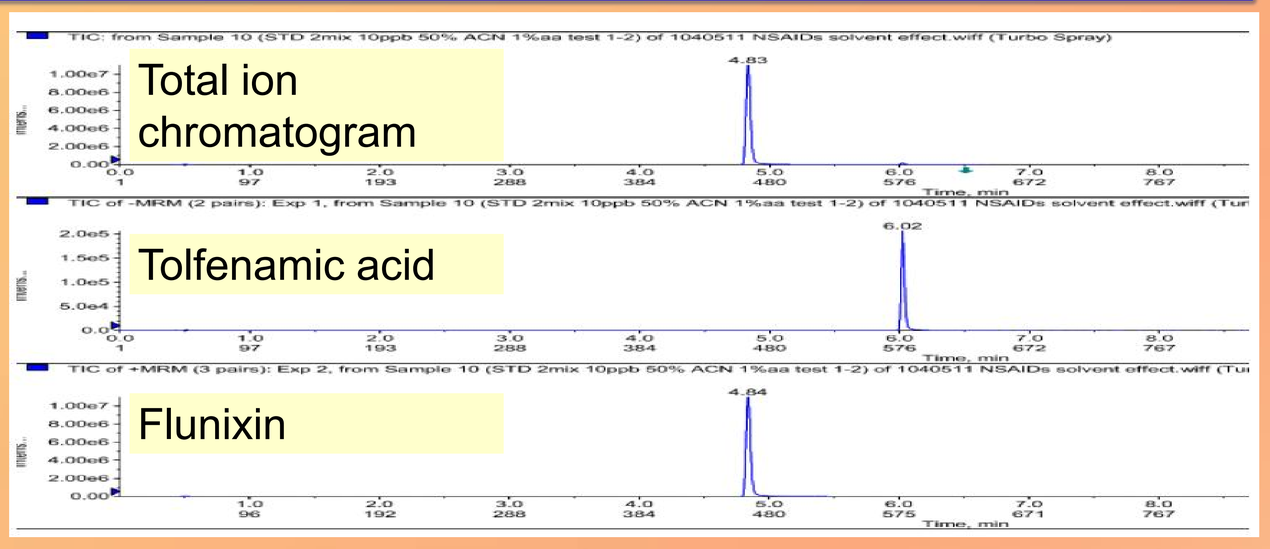

Figure 1. MRM chromatograms of eugenol and tricaine methanesulfonate at concentration of 10 ng/mL.

Table 1. Recoveries, CV and LOQ of flunixin in pork, bovine muscle, milk, pig liver, pig kidney and pig fat

Matrix	Intra-day precision[a] 0.002 μg/g Recovery (%)	CV (%)	0.005 μg/g Recovery (%)	CV (%)	0.01 μg/g Recovery (%)	CV (%)	Inter-day precision[b] 0.005 μg/g CV (%)	LOQ (μg/g)
Pork	95.9	2.1	88.9	3.1	87.1	2.4	3.1	0.002
Bovine muscle	95.3	8.1	84.5	9.4	94.5	4.1	4.8	0.002
Milk	87.2	3.1	91.3	1.6	94.3	1.4	2.4	0.002
Pig liver	86.2	1.7	91.9	2.9	94.0	2.5	3.4	0.002
Pig kidney	84.3	2.6	85.0	1.4	86.0	1.8	1.2	0.002
Pig fat	76.2	5.1	78.5	0.9	81.5	1.5	3.1	0.002

Table 2. Recoveries, CV and LOQ of tolfenamic acid in pork, bovine muscle, milk, pig liver, pig kidney and pig fat

Matrix	Intra-day precision[a] 0.002 μg/g Recovery (%)	CV (%)	0.005 μg/g Recovery (%)	CV (%)	0.01 μg/g Recovery (%)	CV (%)	Inter-day precision[b] 0.005 μg/g CV (%)	LOQ (μg/g)
Pork	92.7	4.1	83.8	8.8	87.5	9.1	7.7	0.002
Bovine muscle	88.2	5.9	95.1	3.1	90.3	1.0	9.0	0.002
Milk	78.0	3.7	85.0	2.4	87.0	2.1	2.6	0.002
Pig liver	89.7	5.0	86.3	5.3	81.0	3.7	5.0	0.002
Pig kidney	83.0	3.6	82.7	4.2	82.1	3.3	6.0	0.002
Pig fat	68.2	4.8	69.8	4.7	71.7	2.5	4.2	0.002

[a] N =5; [b] N =15

Conclusion

Flunixin and tolfenamic acid are two commonly used NSAIDs in husbandry. In Taiwan, MRL for this two drugs has been stipulated but without related method can be used on routine monitoring program. The method we provided based on QuEChERs extraction, and the results shows good recoveries, low variance and high sensitivity when analyzing these drugs. Now, the method has become Taiwanese official method and can be used for large-scale market surveillance.