Development of an LC-MS/MS Method for the Determination of 20-Hydroxyecdysone and Its Metabolites in Calf Urine

Application to the Control of Its Potential Misuse in Cattle

Application Note

Food Safety

Authors

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Abstract

Ecdysteroids, which are steroid hormones present in invertebrates and in plants, could be potentially used as anabolic agents in food-producing animals. The control of ecdysteroid misuse in cattle relies on the development of an efficient method for their detection in biological matrices at trace levels ($\mu g.L^{-1}$). In this context, an analytical procedure dedicated to the identification of 20-hydroxy-ecdysone and its metabolites in urine samples, based on purification on two solid-phase extraction cartridges (SPE C_{18} and SPE SiOH) and LC-(ESI+)-MS/MS measurements has been developed. The performance of tandem quadrupole MS/MS, in terms of sensitivity and specificity, allowed measurements at trace levels in both spiked and incurred samples. Good linearity was observed for all analytes from 0.12 ng to 12 ng on column.

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Introduction

Ecdysteroids are steroid hormones present both in invertebrate species (mainly Arthropods) and plants (belonging to *Asteraceae, Caryophyllaceae,* or *Polypodiaceae*). In arthropods, ecdysteroids act as moulting hormones, whereas these molecules are thought to protect plants against nonadapted phytophagous insects. The archetypal ecdysteroid in both kingdoms is 20-hydroxyecdysone (20E), and several studies have underlined its possible growth-promoting effects in various animal species (rats, mice, and Japanese quail), including humans and cattle [1–3]. Clinical studies demonstrated that 20E is more anabolic than methandrostanolone (dianabol), with no androgenic or other undesirable side effects usually observed with classical steroids [4]. However, despite its growth-promoting properties, only a few methods have been reported for its detection in biological matrices, and no information is available concerning its metabolism in cattle [5]. In this application, the development of a method able to detect and identify 20E and its main metabolites at trace levels (ppb) in calf urine is described [6]. This method was applied to the analysis of calf urine samples after 20E oral administration and used to assess the kinetic of elimination of these substances.

Experimental

Compound Standards

Standard reference 22S,23S-homobrassinolide (belonging to brassinosteroids, vegetable steroid hormones) was from Sigma-Aldrich (St. Quentin Fallavier, France); 20-hydroxyecdysone, 14-deoxy, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone were a kind gift from Pr. Lafont.

Sample Preparation

Twenty-five nanograms of 22S,23S-homobrassinolide were added as internal standard (IS) to 5 mL of calf urine, centrifuged at 3,500 g for 15 min, then purified on SPE C18. The C18-SPE cartridges were conditioned with 10 mL methanol, then 10 mL water, following which the urine samples were applied. The columns were then washed with 6 mL of a water/methanol (80/20) mixture, and the ecdysteroids were subsequently eluted with 10 mL methanol. The eluant was then evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 50 µL ethanol and 150 µL cyclohexane before loading onto a SPE SiOH, previously activated with 25 mL cyclohexane. The phase was washed with 6 mL ethyl acetate/cyclohexane (80/20) and the compounds of interest were then eluted with 10 mL of a mixture of chloroform/methanol/acetone (6/2/1). The solvent was evaporated to dryness under nitrogen and the final extract was redissolved in 50 µL of methanol/water (30/70) containing 0.5% acetic acid. From this extract 10 µL was injected onto the HPLC column.

Instrumentation

LC:

Column:	GEMINI C_{18}, Phenomenex (3 µm, 110 Å, 50 × 2 mm)/Agilent equivalent: ZORBAX Extend-C18 3.5 µm, 2.1 mm × 50 mm (p/n 735700-902)	
Column temperature:	40 °C	
Mobile phases:	A: MeOH B: 0.5% acetic acid in water	
Flow rate:	0.3 mL/min	
Gradient:	Time (min)	%B
	0	90
	8	0
	10	0
	12	90
	16	90
Injection volume:	10 µL	
MS:	G 6410A QQQ, Agilent Technologies	
Ionization:	ESI (+)	
Fragmentor:	120 V	
Mass range:	100–500 amu	
Scan time:	300 ms	
Capillary:	4000 V	
Nebulizer:	35 psi	
Drying gas:	11 L/min	
Gas temperature:	325 °C	

The monitored transitions for each target compound are reported in Table 1. The first transition corresponds to the most sensitive signal.

Results and Discussion

Standard solutions of target compounds were analyzed according to the LC-MS/MS parameters described in the Experimental section, which allowed us to obtain the ion chromatograms of 20E, M1, M2, and IS, each at 5 ng on column (Figure 1). All the compounds are eluted within less than 10 min with very good chromatographic resolution and peak shape.

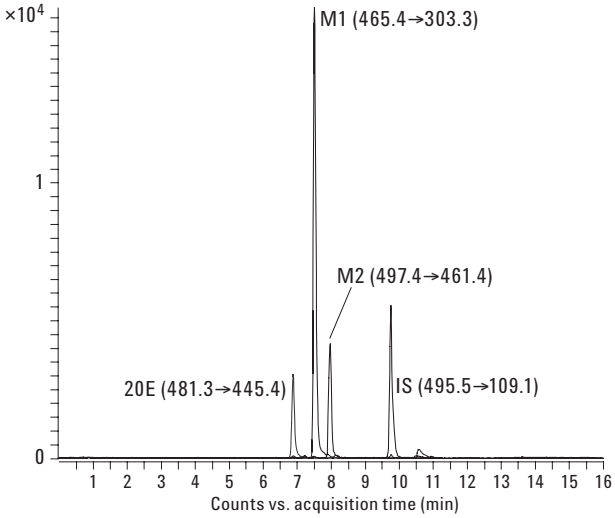

Figure 1. Overlaid extracted ion chromatograms (EICs) for the most sensitive transitions monitored for 20E and its metabolites in positive ion mode.

Table 1. Monitored SRM Transitions for 20E and Its Main Urinary Metabolites and Parameters of Acquisition for Their Analysis by LC-MS/MS (QQQ)

Analytes	Transition 1	Collision energy (eV)	Transition 2	Collision energy (eV)	Transition 3	Collision energy (eV)	RT (min ± 0.2)
22S,23S-homobrassinolide (IS)	495.5→109.1	20	495.5→127.1	10	495.5→459.1	5	9.8
20-hydroxyecdysone	481.3→445.4	10	481.3→371.4	10	481.3→165.1	20	7.5
14-deoxy,20-hydroxyecdysone (M1)	465.4→303.3	20	465.4→285.3	25	–	–	7.9
20,26-dihydroxyecdysone (M2)	497.3→461.4	5	497.3→351.1	15	497.3→371.2	20	6.8

To assess the specificity of the method, a blank urine and a urine sample fortified with 20E (1 $\mu g.L^{-1}$) were analyzed. Figure 2 shows the blank traces without any interference at the expected retention time for 20E, demonstrating the good selectivity of the monitored signals. The target analyte 20E was identified in the spiked urine sample with three SRM transitions. The monitored signals are detected with good sensitivity and show high signal-to-noise (s/n) ratios. These results were in accordance with Decision 2002/657/EC criteria, which require more than four identification points [7] in order to validate an identified compound.

The linearity and the repeatability of the method were assessed with the analysis of a pool of urine samples fortified at different concentration levels: the calibration curve was established with five concentration points (0.2, 0.5, 1, 5, and 20 $ng.mL^{-1}$). The calibration curve correlation coefficients (R^2) were better than 0.99, thus demonstrating the good linearity of the method for 20E.

The method has been successfully applied to incurred calf urine samples after 20E oral administration over four days. 20-hydroxyecdysone was detected in urine as rapidly as 30 minutes after its administration and up until 24 hours after the last administration. 20E metabolism was investigated and two main metabolites, 14-deoxy,20-hydroxyecdysone (M1) and 20,26-dihydroxyecdysone (M2), could be identified [8]. Both M1 and M2 were monitored by LC-MS/MS (Table 1). Figure 3 presents the ion chromatograms for M1 in the urine samples collected before and two days after the last 20E administration.

As can be observed, M1 was not detected in the urine collected before 20E administration, whereas it was throughout the four-day administration period. Furthermore, it could still be detected and identified (in accordance with the four identification points required) two days after the last administration of 20E. This result is of prime interest in the context of potential misuse of ecdysteroids since it offers the longest period for detection, following administration, and therefore enables a more efficient control mechanism.

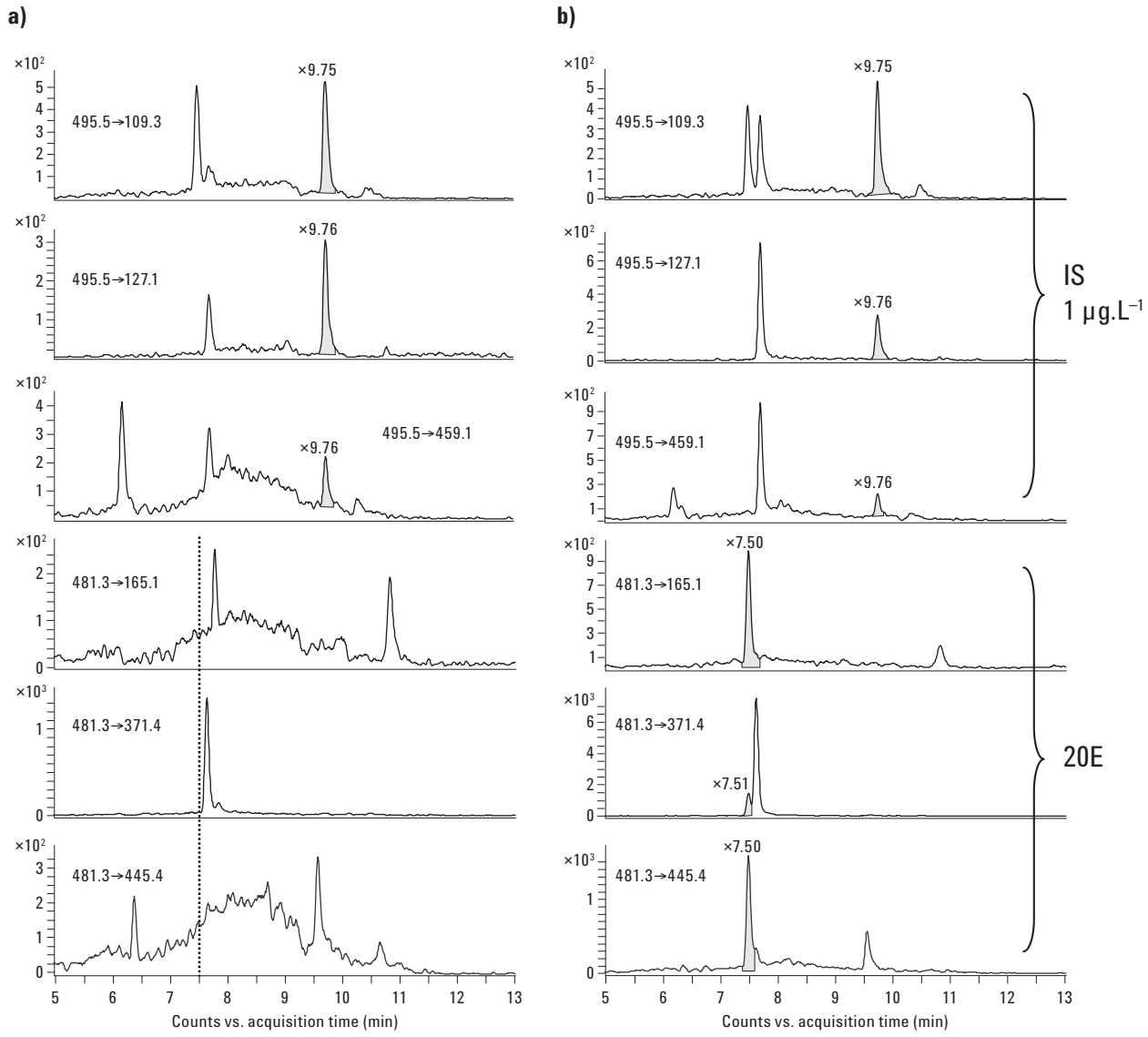

Figure 2. SRM ion chromatograms for a) the blank urine sample and b) the spiked urine sample (1 µg.L-1). LC-(ESI+)-MS/MS measurements.

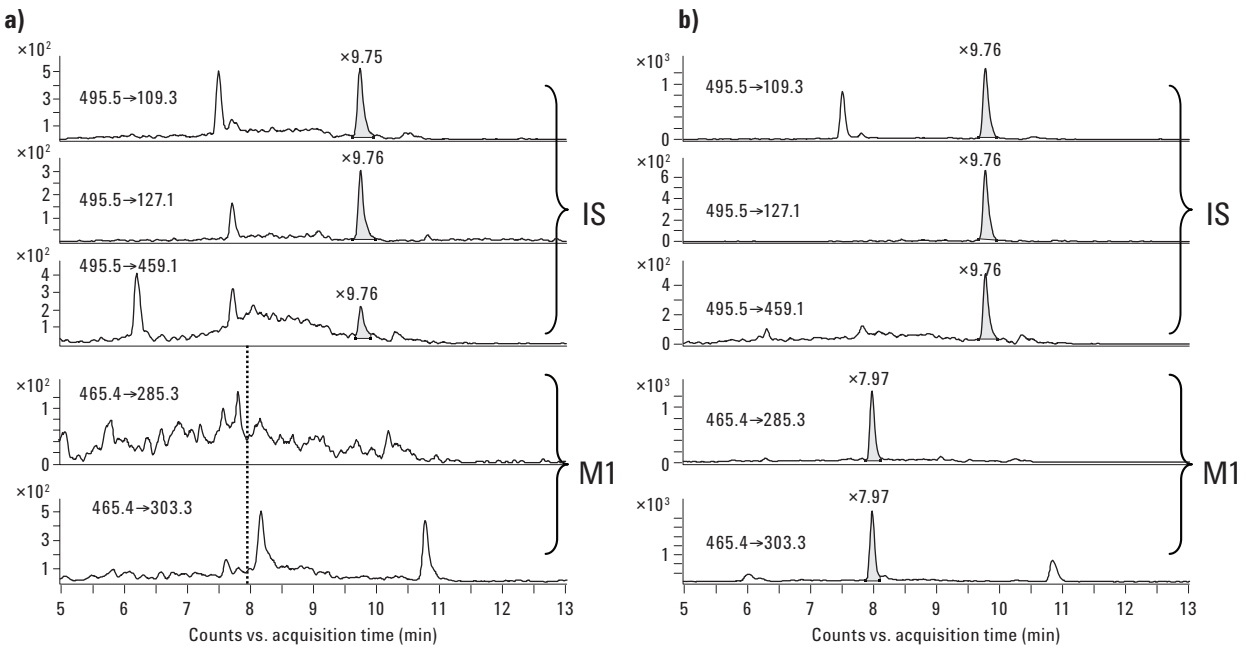

Figure 3. SRM ion chromatograms of IS and M1 in urine sample collected a) before 20E administration and b) two days after the last 20E administration. LC-(ESI+)-MS/MS measurements.

Conclusions

This work demonstrates the performance of LC-MS/MS, which provides efficient identification of 20E and its main metabolites in calf urine. The monitoring of these compounds facilitates the control of the potential misuse of 20E in meat-producing animals. Tandem quadrupole MS/MS is an analytical technique very well suited to this purpose, since it increases confidence in the unambiguous identification of the target compounds, in accordance to the criteria fixed by Decision 2002/657/EC. The successful analysis of the calf urine samples proved the robustness of the developed protocol. Application of this methodology also enabled the determination of the first elimination kinetics and the main metabolites of 20E in calf urine.

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