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Confirmation of diminazene diaceturate in bovine plasma using electrospray liquid chromatography–mass spectrometry

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Abstract

Diminazene diaceturate is used as a trypanocide for cattle in tropical regions. This paper describes a LC–MS^n method to confirm the presence of diminazene in bovine plasma. Bound diminazene in plasma samples was freed with dilute phosphoric acid, then concentrated on a bonded C_{18} SPE cartridge. The LC–MS^n method utilized electrospray ionization coupled with an ion trap mass spectrometer. Ions observed in MS^2 and MS^3 product ion spectra, as well as those from the MS^1 spectrum, were monitored. The method was validated with plasma samples fortified with diminazene diaceturate (4–100 ng/mL). Diminazene was confirmed in samples fortified with diminazene diaceturate at levels of 6.4 ng/mL or higher.

Keywords: Diminazene diaceturate; Bovine plasma; Mass spectrometry

1. Introduction

Diminazene diaceturate, 4,4′-(diazoamino)dibenzamidine diaceturate, is an effective antiprotozoal drug marketed under the trade name Berenil™ (Fig. 1). It can be used to treat diseases such as bebesiosis and trypanosomiasis in cattle in tropical regions [1–4]. The drug is usually administered intramuscularly (IM) at a dose of 3–5 mg/kg. While the drug is primarily excreted through the urinary tract, residues of diminazene can remain at levels of 50 ng/mL or higher in plasma for a period of several weeks [5,6]. Quantifiable levels have also been measured in edible tissue (kidney, liver, muscle) for up to 20 days. It is known that the drug can interact with DNA, but the results of mammalian toxicology studies have been mixed. Rats dosed with up to 160 mg/kg body weight/day of the drug for three months did not exhibit any toxicological symptoms. However, dogs given diminazene diaceturate (20–60 mg/kg body weight/day) for an extended period of time did show some ill effects including brain damage, testicular atrophy and death. As a result of these studies, a conservative acceptable daily intake of less than 100 μg/kg/day was set for human consumption [6]. Based on this data, as well as the known depletion rates in bovine tissue, the following maximum residue levels (mg/kg) have been suggested by the Joint FAO/WHO Expert Committee on Food Additives: muscle, 0.5; liver, 1.2; kidney, 6; milk, 0.15 [7].

Diminazene residues in food animals are primarily a concern in tropical regions where the vectors that carry the protozoa are indigenous insects (e.g. tsetse fly or *Boophilus* tick) [1,2]. Diminazene is not approved for use in the United States, and tolerances for drug residues in bovine tissue have not been established. However, with the globalization of food production, it will also be important for other countries to establish programs for the analysis of this drug residue. Diminazene residues are known to persist in bovine plasma [5], therefore collecting and analyzing the plasma of animals for residues at the time of slaughter is an effective method to monitor for possible use of this drug. LC–UV methods are available to quantify diminazene in bovine plasma [8,9] and milk with detection limits in the range of 10–25 ng/mL [10]. For a complete analytical data package, it is also important to obtain complementary MS data

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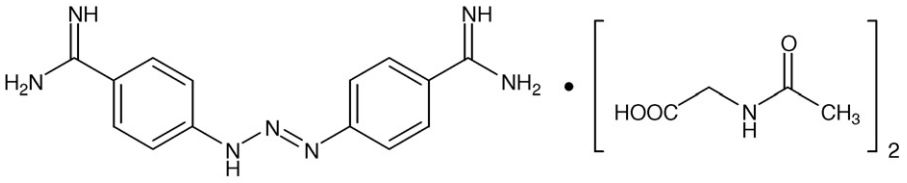

Fig. 1. Structure of diminazene diaceturate (from Merck Version 12 CD).

to confirm the residue's presence in food animals at these levels. While there is one report that characterizes diminazene and its impurities in pharmaceutical formulations by LC–MS [11], a MS confirmatory procedure for residue levels (ng/g) of this drug in animal fluids has not been published. This paper describes an LC–MS method for the positive identification of diminazene in bovine plasma.

2. Experimental

2.1. Reagents and solutions

Methanol (MeOH) and acetonitrile UV (ACN) were obtained from Burdick and Jackson (Muskegon, MI). Dilute phosphoric acid solution was prepared by diluting *o*-phosphoric acid, 85% (Fisher Scientific, Pittsburg, PA) by 50% (v/v) in water. A 5% methanol in DI water (v/v) was used as a wash for the solid phase extraction (SPE) cartridge. The final diluent for reconstitution of the extracts was 20 mM ammonium formate, pH 4. To prepare this buffer, approximately 0.623 g ammonium formate, 97% (Aldrich Chemical Co., St. Louis, MO) was weighed into 200 mL water. The pH was adjusted to 4 with dilute phosphoric acid. Additional water was added to a final volume of 500 mL. The LC–MS mobile phase consisted of ACN and 20 mM ammonium formate buffer. This buffer was prepared as above except that the pH was adjusted (to pH 4) with formic acid (96%, Aldrich Chemical Co.).

2.2. Standard preparation

The drug which is administered to animals under the trade name Berenil^{TM} is diminazene diaceturate. The active ingredient, diminazene, then dissociates from the counter ion. In this study, diminazene diaceturate was weighed out and concentrations (in ng/mL) represent the entire complex (i.e. a stock solution that is 100 μg/mL of hydrated diminazene diaceturate is equivalent to 54.6 μg/mL of diminazene). Only the diminazene molecule is detected by LC–MS^n.

The diminazene diaceturate standard was purchased through Sigma (Cat # D-7770, 90% minimum purity, St. Louis, MO). A stock solution (100 μg/mL) was prepared by adding 10 mg of diminazene diaceturate standard to a 100 mL polypropylene volumetric flask and diluting to volume with water. The standard was stable for at least one month at 5 °C. It was important to avoid long term storage of diminazene diaceturate in glass. A fortification/intermediate standard (F/I STD, 1000 ng/mL) was prepared by transferring a 1.0 mL aliquot of the diminazene diaceturate stock standard into a 100 mL polypropylene volumetric flask and diluting to volume with water. LC–MS standards were made to 10.0 mL with 20 mM ammonium formate pH 4 reagent in water solution as follows: STD1, 2.0 mL of F/I STD (200 ng/mL); STD2, 1.0 mL of F/I STD (100 ng/mL); STD3, 0.5 mL of F/I STD (50 ng/mL); STD4, 0.25 mL of F/I STD (25 ng/mL). Intermediate standard aliquots were diluted to volume in 10.0 mL glass volumetric flasks, transferred to LC vials, and analyzed immediately. LC–MS standards appeared to degrade in the autosampler at 10 °C within 24 h.

2.3. Preparation and extraction of plasma for LC–MS^n analysis

Bovine plasma was stored frozen (−60 °C or lower). For analysis, two (2.0) mL portions of plasma were thawed and transferred to 13 mm × 100 mm glass test tubes. If needed, samples were then fortified with the F/I STD. As an example, 50 μL of diminazene diaceturate F/I STD (1000 ng/mL) was added to 2.0 mL of plasma for a spike level of 25 ng/mL.

For the initial extraction, phosphoric acid (20 μL of a 50% solution) was added to each plasma sample. The samples were then mixed on a vortex mixer for 10 s. Water (4 mL) was added to each tube and mixed vigorously. If any sample appeared cloudy from obvious particulates, the tube was centrifuged for 2 min at approximately 1000 rpm. OASIS HLB SPE cartridges (3 cc, 60 mg, Cat # WAT094226, Waters Corp., Milford, MA) were used to purify and concentrate the samples. The cartridges were preconditioned with 3 mL of methanol followed by 3 mL of water. A portion of the water was allowed to remain above the resin bed. The samples were then loaded on to the preconditioned SPE cartridges and allowed to elute by gravity flow. The sample test tubes were washed with water, and the water was applied to the SPE. After the water had percolated into the SPE bed, each cartridge was washed with 4 mL of the 5% methanol solution. Vacuum was applied to the SPE cartridges to remove excess water. The residues was eluted from the cartridges with 4 mL methanol into a 12 mm × 75 mm glass test tube. All of the solution was forced from the SPE by positive pressure. Extracts were evaporated to dryness using a nitrogen turbo-evaporator at 50 °C. Each sample was reconstituted with 0.5 mL of 20 mM ammonium formate pH 4 solution and filtered through a polypropylene syringe filter (Whatman 0.45 μm, 13 mm, Cat # 28137-536, VWR Int. Inc., Westchester, PA) into a polypropylene LC vial for analysis.

2.4. LC–MS instrumental operating conditions

The LC–MS^n consisted of a ThermoElectron (San Jose, CA) DECA ion trap mass spectrometer and a ThermoElectron TSP LC instrument with a P4000 quaternary pump, AS3000

Table 1
LC–MS^n acquisition parameters

Scan event	Scan type	Precursor (s)	Collision energy (%)	Isolation width (amu)	Ions monitored
1	MS-SIM	NA	NA	NA	281.5–282.5, 141.1–142.1
2	MS^2-SRM	282	26	2	253.5–254.5
3	MS^3-CRM	282, 254	26, 40	2	236.5–237.5, 219.5–220.5

SIM: selected ion monitoring, SRM: selected reaction monitoring. CRM: consecutive reaction monitoring.

autosampler and SN4000 controller. The instrument was configured using an electrospray (ESI) source in the positive ion mode with a fused silica (0.1 μm I.D.) sample tube. Parameters were optimized by tuning with a solution of diminazene diaceturate (20 μg/mL in ammonium formate buffer) via a syringe pump at 10 μL/min while 250 μL/min of 93:7 (v/v) ammonium formate buffer:acetonitrile was added via a T-union. The combined stream was introduced into the electrospray interface. Source parameters were optimized by monitoring the protonated molecular ion (*m/z* 282, $[MH]^+$) of diminazene. The following parameters were used in the tune file: source voltage = 5000 V; capillary temperature = 325 °C; sheath gas (nitrogen) = 79 arbitrary units; auxiliary gas (nitrogen) = 6 arbitrary units; capillary voltage = 10 V. Automatic gain control was set to 2 prescans and a maximum injection time of 400 ms for all acquisitions. One time segment was used with the three scan events described in Table 1.

The reversed phase LC column was an YMC-AQ (2 mm × 100 mm, 3 μm, Cat # AQ12S031002WT, Waters Corp.). An isocratic LC program (93:7 (v/v, ammonium formate:ACN) was used for the first 8 min of the chromatographic program. The mobile phase was then ramped to 80% ACN over 1 min and allowed to wash the LC column for 2 min. The solvent composition was returned to 93:7 over 2 min, and the column equilibrated for 3 min before the next injection. A divert valve directed the LC effluent into the MS from 0.5 min until 7.9 min. The column was held at room temperature (in an insulated compartment). Sample tray temperature was maintained at 10 °C.

Table 2
Example of relative abundance data

Sample	RT[a]	*m/z* 282 signal s/n[b]=	*m/z* 141.6 signal s/n=	*m/z* 254 signal (RA)[c] s/n=	*m/z* 237 signal (RA) s/n=	*m/z* 220 signal (RA) s/n=	Criteria met?
46 ng/mL standard[d]	5.40	33E6 *24*	42E6 *100*	92E6 (100) *388*	41E6 (45) *392*	5E6 (5) *98*	
184 ng/mL standard	5.48	300E6 *149*	186E6 *269*	568E6 (100) *381*	252E6 (45) *422*	31E6 (5) *302*	
Buffer (solvent blk)	5.28	*ND*	*ND*	3E6 (100) *68*	1.1E6 (36) *11*	0.21E6 (7) *59*	No
Control plasma	ND	*ND*	*ND*	*ND*	*ND*	*ND*	No
Plasma[e] 23 ng/mL (1)	5.75	106E6 *8*	101E6 *70*	190E6 (100) *254*	88E6 (46) *1958*	12E6 (6) *5992*	Yes
Plasma 23 ng/mL (2)	5.31	65E6 *8*	72E6 *99*	164E6 (100) *1308*	73E6 (45) *1910*	9E6 (5) *686*	Yes
Plasma 23 ng/mL (3)	5.50	74E6 *8*	70E6 *53*	146E6 (100) *420*	66E6 (45) *33842*	9E6 (6) *681*	Yes
Plasma 23 ng/mL (4)	5.22	59E6 *17*	65E6 *103*	153E6 (100) *302*	66E6 (43) *593*	9E6 (6) *625*	Yes
Plasma 23 ng/mL (5)	5.47	91E6 *9*	74E6 *75*	191E6 (100) *369*	89E6 (46) *4912*	11E6 (6) *1081*	Yes
Plasma 23 ng/mL (6)	5.17	68E6 *7*	58E6 *93*	152E6 (100) *409*	70E6 (46) *1982*	9E6 (6) *654*	Yes
184 ng/mL standard	5.50	114E6 *52*	96E6 *255*	266E6 (100) *582*	118E6 (44) *441*	15E6 (6) *417*	

[a] RT: retention time (min).
[b] s/n: signal to noise.
[c] RA: relative abundance.
[d] Concentration is for diminazene diaceturate.
[e] Samples were concentrated 4× during the procedure, with the final extract from a plasma fortified at 23 ng/mL equivalent to a 92 ng/mL standard solution.

Injections of 20 μL were made. The syringe was flushed with 400 μl of water between chromatographic runs to reduce carry-over between samples.

2.5. Data treatment and confirmation criteria

For qualitative assessment, individual ion transition chromatograms were generated and the resulting chromatographic peaks integrated A smoothing function (7 point Gaussian) was used. Relative abundances were calculated from these peak areas and compared to contemporary standards.

Several specific criteria were required for the qualitative confirmation of diminazene in plasma. The MS^2 transition (to *m/z* 254) and both MS^3 transitions (to *m/z* 237, 220) need to produce chromatographic peaks with a signal to noise ratio (s/n) greater than 40:1 when using the *Genesis* integration program. The relative abundance of these three transitions should match an external standard within ±20%. When the MS^1 selected ion chromatograms were evaluated, the peak for *m/z* 141.6 should be present with a s/n greater than or equal to 3:1. Finally, the retention time of the residue in plasma extract must match at least one external standard analyzed on the same day within ±5%; all of the ion chromatograms monitored also should co-elute within a window of 5%.

3. Results and discussion

3.1. Extraction of diminazene from plasma

Initially the same extracts that were prepared for the LC–UV determination of diminazene in plasma were analyzed by LC–MS^n [8]. While the recoveries of diminazene from these samples as measured by LC–UV were acceptable, the LC–MS^n signal obtained for the residue in plasma extracts was significantly lower than corresponding standards. An end spike (spiking control plasma extract with a known amount of diminazene diaceturate standard) also had significantly reduced signal, less than 10% of what was observed with a corresponding standard, indicating that matrix suppression of the analyte response was an issue. Most likely the ion-pair reagent (heptane sulfonic acid) utilized in the LC–UV extraction method caused the observed signal suppression. While it was initially hoped that a volatile ion-pair reagent such as propylfluoropropionic acid could be used for both extraction and chromatographic separation of diminazene in conjunction with LC–MS analysis [10], this compound caused ion suppression and long-term instrument contamination.

Rabanal et al. [12] have developed a method for the analysis of diminazene and other antiprotozoals in human serum and urine by capillary electrophoresis that does not require ion-pair reagents. A simple extraction procedure with a copolymeric (OASIS) solid phase extraction column was described in that study. In the current work, that extraction is utilized to isolate the residue from bovine plasma for LC–MS. Because the primary intent of this method is to provide qualitative confirmatory data, it was not necessary to quantitatively evaluate the efficiency of the extraction. Issues such as short term compound stability and ion suppression, though not as significant as with the ion-pair extraction method, still complicate the ability to determine recoveries. The signals for the ion transitions monitored were sufficient and stable enough at the fortification levels of interest (10–100 ng/mL diminazene diaceturate) to meet the criteria for residue confirmation.

3.2. Optimization of chromatographic parameters

Avoiding the use of ion-pair reagents was also an important consideration when optimizing the chromatographic analysis of diminazene in plasma extracts. An LC–MS method developed to characterize pharmaceutical preparations of diminazene utilized a reversed phase LC column with an ammonium formate:acetonitrile mobile phase [11]. These chromatographic conditions were used as a starting point to develop and validate the LC–MS^n method reported here. Chromatographic separation from matrix components was obtained using a 20 mM ammonium formate (pH 4):acetonitrile (93:7) isocratic program with an aqueous type C18 LC analytical column. Although the use of ion pair reagents was avoided, the stability of the chromatographic retention time was somewhat variable with the elution time of diminazene in both standards and extracts varying on occasion by more than 5% throughout a day's analysis. Confirmation criteria, however, could still be met by bracketing the sample extracts with standards.

3.3. Ion trap MS analysis of diminazene

Ions observed in the ESI MS^1 spectrum of diminazene were *m/z* 282, $[MH]^+$, 254, $[MH–N_2]^+$ and 141.6, $[MH_2]^{2+}$. In addition, a second chromatographic peak with a predominant ion at *m/z* 136 could also be observed in the full scan analysis of standards or samples that were not freshly prepared. This is indicative of *p*-aminobenzamidine, a decomposition product of diminazene. These data are consistent with the LC–MS analysis of pharmaceutical preparation of diminazene [11]. However, for the purposes of this study, additional diagnostic ions were required in order to meet established confirmation criteria [13]. Thus, MS^2 and MS^3 product ion spectra were also obtained. The only abundant ion in the product ion spectra of *m/z* 282 was *m/z* 254, $[MH–N_2]^+$. This ion was further dissociated to yield a MS^3 spectra consisting of *m/z* 237 (*m/z* 254–NH_3) and 220 (*m/z* 254–2NH_3) product ions. The MS, MS^2 and MS^3 spectra obtained from a standard of diminazene are shown in Fig. 2.

Because only a few structurally significant ions were observed in each MS^n spectra, selected monitoring was performed (instead of obtaining full product ion scans) to increase the signal to noise (s/n) for the resulting chromatographic peaks. Qualitative identification was primarily accomplished by evaluating the MS^n ion transitions. However, the doubly charged ion ($[MH_2]^{2+}$, $m/z = (283.1)/2 = 141.6$) observed in the MS^1 mode was unique to diminazene and was also used as an indication of confirmation. The protonated molecular ion itself (*m/z* 282) was monitored, but a distinct peak was not always visible above background when analyzing plasma extracts at low levels; there was no s/n requirement for this peak.

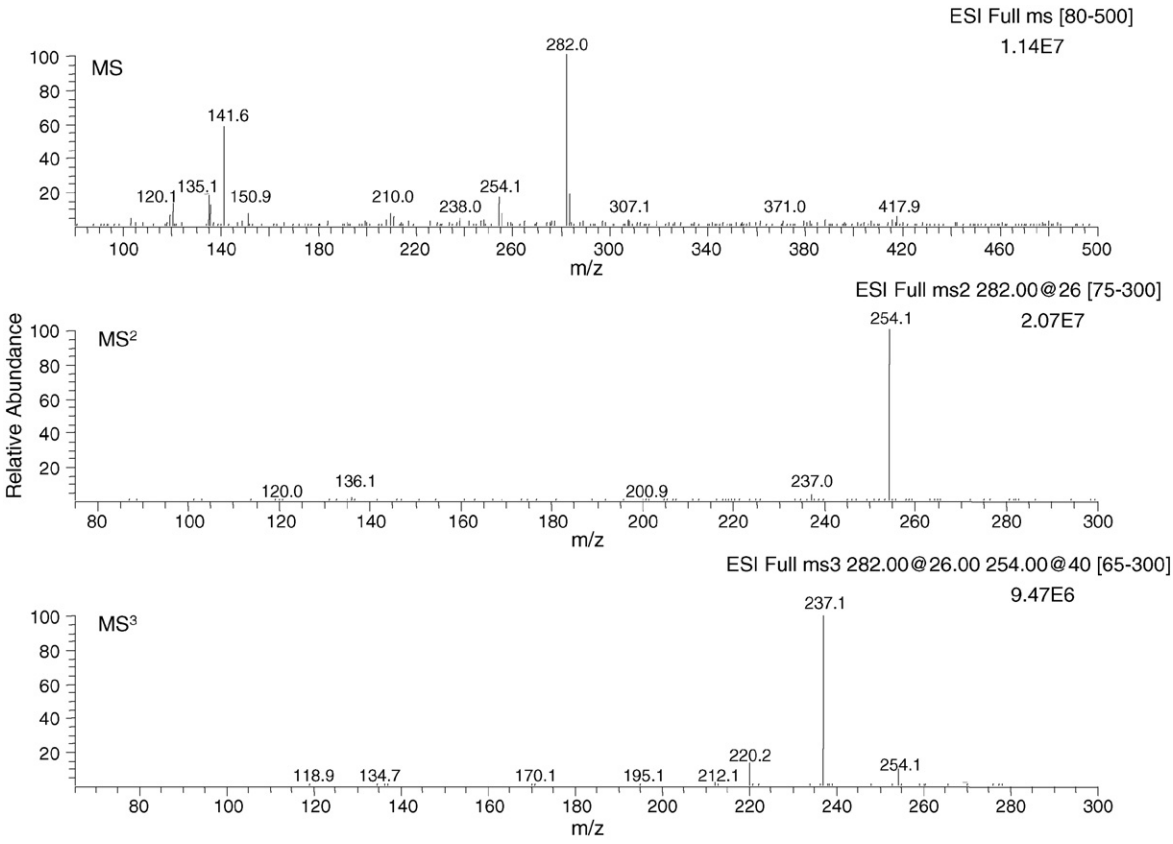

Fig. 2. Electrospray MS, MS^2, and MS^3 spectra of diminazene (25 ng/mL diminazene diaceturate injected).

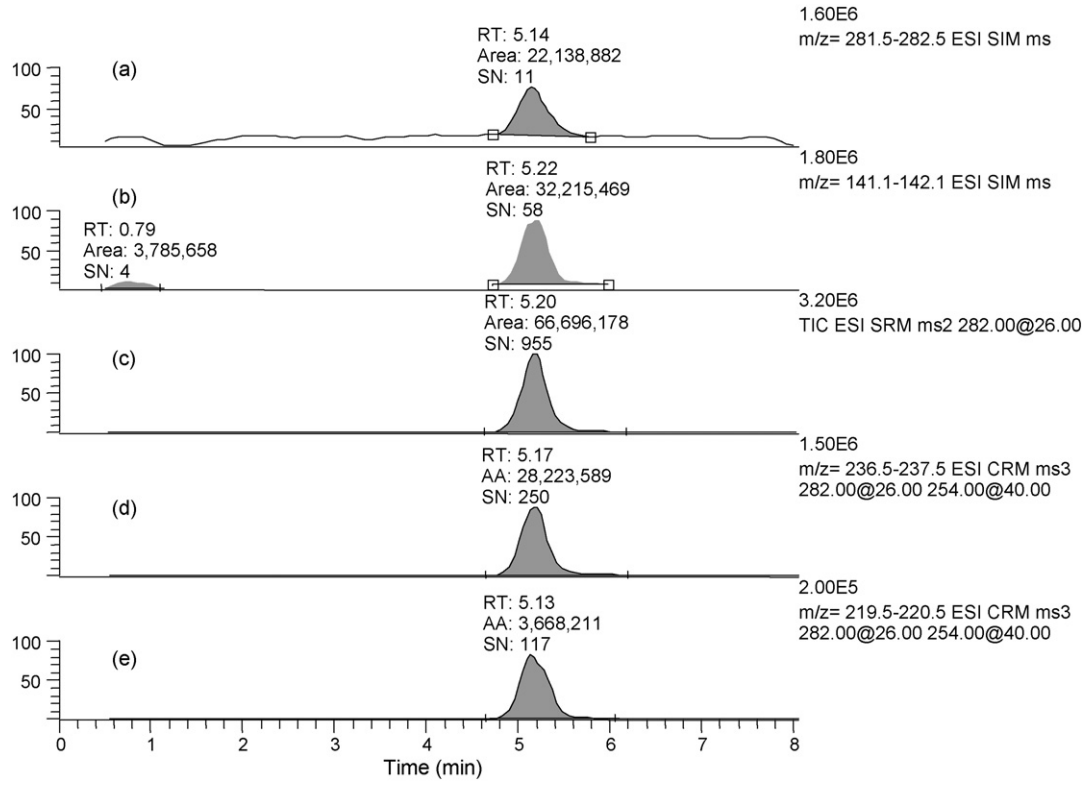

Fig. 3. Ion chromatograms of diminazene diaceturate standard (46 ng/mL):(a) Selected ion monitoring (SIM) of m/z 282; (b) SIM of *m/z* 141.6; (c) Selected reaction monitoring (SRM) of *m/z* 282 to 254; (d) Consecutive reaction monitoring (CRM) of *m/z* 282 to 254 to 237 (e) CRM of *m/z* 282 to 254 to 220. The *y*-scale (intensity) for the ion transitions has been set to a fixed number in Figs. 3–5 to better compare the chromatograms.

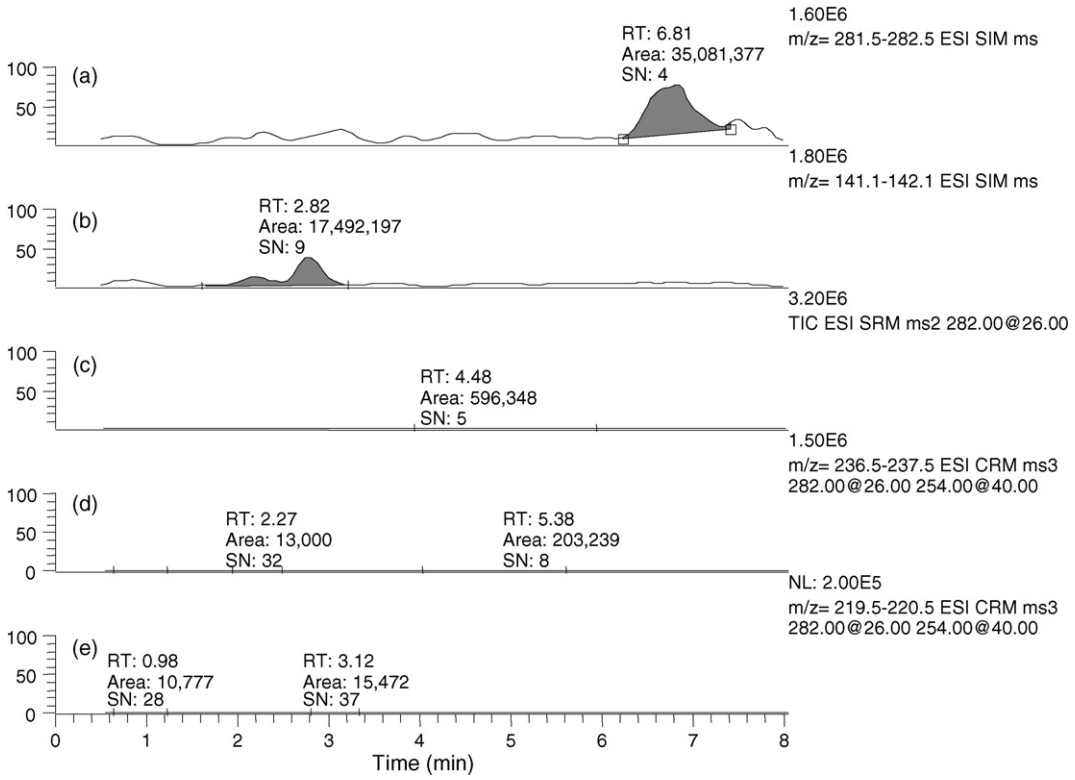

Fig. 4. Ion chromatograms from plasma control: (a) SIM of *m/z* 282; (b) SIM of *m/z* 141.6; (c) SRM of *m/z* 282 to 254; (d) CRM of *m/z* 282 to 254 to 237; (e) CRM of *m/z* 282 to 254 to 220.

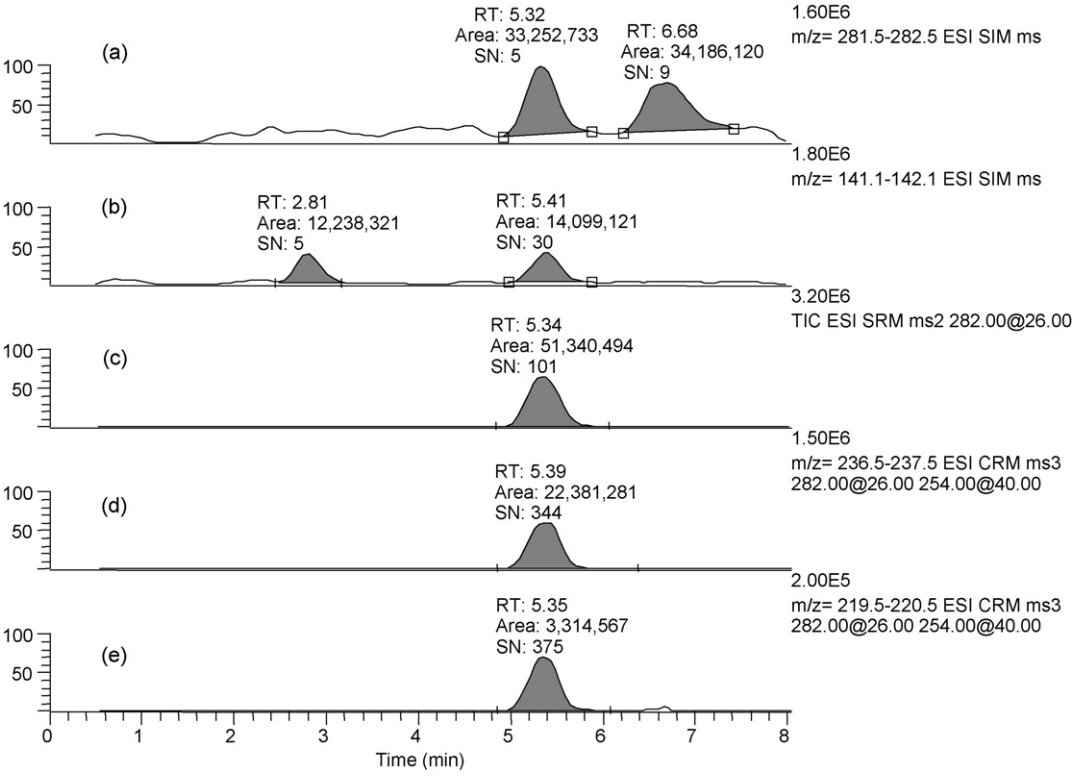

Fig. 5. Ion chromatograms from plasma fortified with diminazene diaceturate at 10 ng/mL:(a) SIM of *m/z* 282; (b) SIM of *m/z* 141.6; (c) SRM of *m/z* 282 to 254; (d) CRM of *m/z* 282 to 254 to 237; (e) CRM of *m/z* 282 to 254 to 220.

Table 3
Summary of method validation

Day	Reagent blank	Control	Samples fortified with diminazene diaceturate (ng/mL)					
			4.6	6.4	9.2	23	46	92
	Number of samples confirmed/number of samples analyzed							
1	0/2	0/6				6/6	6/6	6/6
2	0/2	0/1			5/6	6/6		
3		0/6	2/6					
4							6/6	
5		0/1		3/3			2/2	
Total	0/4	0/14	2/6	3/3	5/6	12/12	14/14	6/6

3.4. Method validation

This confirmation method was validated with several sets of plasma controls and fortified plasma samples. An example of one day's validation data, with relative abundance and signal to noise calculations, is shown in Table 2. Figs. 3–5 show the selected ion chromatograms obtained from a standard of diminazene diaceturate (Fig. 3), a plasma control extract (Fig. 4), and an extract from plasma that had been fortified at approximately 10 ng/mL (Fig. 5). A summary of the method validation is given in Table 3. In general, diminazene was confirmed in all of the plasma fortified at the levels of interest (10–100 ng/mL diminazene diaceturate to correspond with the LC–UV method). One of the 9.2 ng/mL extracts was not confirmed because the chromatographic retention time varied by more than 5% from that of any standard ran on that day, but this was considered an anomaly. The confirmation criteria described above were also met for samples that had been fortified at 6.4 ng/mL with diminazene diaceturate. A set of six extracts that had been fortified at 4.6 ng/mL with the complex was also analyzed. Although most of the MS^n ions were detected in all of these samples, only two met all the confirmation criteria. Thus, the confirmation limit for this method was determined to be 6.4 ng/mL diminazene diaceturate (which corresponds to 3.5 ng/mL diminazene). Diminazene was not confirmed in any of the control extracts or the reagent blanks that were analyzed as part of the validation.

In conclusion, we have developed an LC–MS^n method capable of confirming diminazene in bovine plasma extracts that have been fortified with the diaceturate complex at levels below 10 ng/mL. The use of ion pair reagents in the extraction and chromatographic separation was avoided, and the MS parameters allowed for selective analysis of unique and structurally significant ions. This LC–MS^n confirmatory method will be an effective tool for the monitoring and regulation of diminazene diaceturate use in food animals.

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