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Analysis of Lidocaine and Its Major Metabolite, Monoethylglycinexylidide, in Elk Velvet Antler by Liquid Chromatography with UV Detection and Confirmation by Electrospray Ionization Tandem Mass Spectrometry

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A sensitive liquid chromatographic (LC) method with UV detection was developed for the determination of residues of lidocaine (LID) and its major metabolite, monoethylglycinexylidide (MEGX), in elk velvet antler. The drugs were extracted from alkaline velvet antler homogenates, cleaned up on a C_{18} solid-phase extraction cartridge, and separated on an Inertsil ODS-3 (3.0 × 250 mm, 5 μm) column using an isocratic mobile phase made up of 0.05 M phosphate buffer (pH 4.0)/acetonitrile (88:12, v/v) at a flow rate of 1.0 mL/min. The limits of quantification for LID and its major metabolite, MEGX, were 10 and 20 ng/g, respectively. The method was validated and used to measure the concentration of residues of LID and MEGX in elk velvet antlers harvested after either LID anesthesia or application of a drug-free control method (electro-anesthesia, EA). No LID or MEGX residues were detected in any of the antlers harvested after EA application. No MEGX residues were detected in any of the velvet antlers harvested after LID application, but residues of LID ranging in concentration from 68 to 4300 ng/g were detected in the three sections of the velvet antlers harvested after LID administration. LC-tandem mass spectrometry was used to confirm the presence of lidocaine detected in the velvet antlers.

KEYWORDS: Lidocaine (LID); monoethylglycinexylidide (MEGX); local anesthetic; elk velvet antler; cervids; liquid chromatography (LC); tandem mass spectrometry (LC-MS/MS)

INTRODUCTION

Velvet antlers are appendages that grow annually from the pedicle structure of the frontal bone of male cervids. Growing antlers are soft, blood-filled tissue covered by skin with a velvet-like texture. They grow very rapidly, often reaching growth rates of 2 cm/day. Antler growth and mineralization is completed in approximately 120 days, reaching its peak commercial value at about 60 or 70 days after which its value decreases due to progressive mineralization and concomitant loss of biological activity. Its composition varies from a soft blood-filled cartilage tissue in the distal portions, through honeycombed cartilage in the mid section, to variably mineralized cartilage and bone tissue at the base. Velvet antler harvesting forms the basis of commercial deer and elk farming industries in China, Korea, Russia, and recently North America, where because of its putative bioactivity and tonic effects there is considerable market potential for its use in medicinal applications and dietary food supplements (*1*).

Lidocaine (2-(diethylamino)-*N*-(2,6-dimethylphenyl) ethanamide, LID) is used as a local anesthetic for harvesting velvet antlers from North American elk (*Cervus elaphus* spp.), red deer (*Cervus elaphus elaphus*), and sika deer (*Cervus nippon*). It has been argued by opponents of the use of lidocaine in the velvet antler industry that this practice, while providing sufficient anesthesia to permit humane harvesting of the antlers, introduces drug residues into the antler and that other, nonchemical, procedures such as electro-anesthesia (EA) and pedicle compression would offer better alternatives for the animal (*2*). Since velvet antlers are processed shortly after harvesting into food supplements, concerns have been expressed about the potential for lidocaine drug residues remaining in these preparations, particularly because it is known that both humans and animals

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metabolize lidocaine to 2-(ethylamino)-*N*-(2, 6-dimethylphenyl) ethanamide [monoethylglycinexylidide (MEGX)], which is also biologically active (see **Figure 1**). It is also known that both LID and MEGX metabolize further into 2, 6-dimethylaniline (2,6-xylidine), suspected to be a potential carcinogen (*3*, *4*). However, there are no published data demonstrating that harvesting velvet antler with lidocaine results in the persistence of residues of LID and MEGX in velvet antler.

Several gas chromatographic (GC) and liquid chromatographic (LC) methods have been published for the determination of LID and MEGX in various biological fluids such as serum and urine (*5*−*19*). There are however, no published methods for the determination of residues of LID and MEGX in velvet antler. This paper describes a sensitive LC-UV method that was developed to determine LID and MEGX at concentrations ≥10 and 20 ng/g, respectively, in velvet antler (*20*). The method was validated and used to measure the concentration of LID and MEGX residues in velvet antlers harvested from 10 animals after LID administration and from 3 animals after EA administration, used as a drug-free control method. Tandem mass spectrometry (MS/MS) was used to confirm that the drug residues detected by LC with UV detection in the harvested velvet antlers were indeed LID.

EXPERIMENTAL PROCEDURES

Reagents and Materials. Lidocaine was obtained from Sigma Chemical Co. (St. Louis, MO), MEGX HCl was a kind gift from AstraZeneca (Mississauga, ON, Canada), and phenacemide (PHEN) was obtained from Lancaster Synthesis (Pelham, NH). Lidocaine, MEGX and phenacemide were stored at room temperature. All reagents and solvents were of analytical grade. Water was obtained from a Barnstead RO/Nanopure ultra filtration unit. C_{18} solid-phase extraction cartridges (Sep-Pak, 500 mg/3 mL capacity) were purchased from Waters Chromatography (Mississauga, ON, Canada). Negative control velvet antler samples were provided by Dr. Murray Woodbury (Western College of Veterinary Medicine, Saskatoon, SK, Canada).

Stock solutions (100 μg/mL) were prepared by dissolving 25 mg of lidocaine, 25 mg of PHEN, and 29 mg of MEGX HCl into individual 250 mL volumetric flasks with acetonitrile or water. The 2.0 μg/mL working standard solutions of LID, MEGX, and PHEN were prepared by transferring 1 mL of the stock solutions into three individual 50 mL volumetric flasks and diluting to the mark with water. Stock solutions and working standard solutions were kept refrigerated at 2−4 °C. A 0.01 M ammonium formate buffer (pH 4.0) was prepared by dissolving 0.631 g of ammonium formate in about 900 mL of water, adjusting the pH to 4.0 with formic acid, and making it up to 1 L with water. A 0.10 M borate solution was prepared by dissolving 38.0 g of sodium tetraborate decahydrate in a 1 L volumetric flask with water.

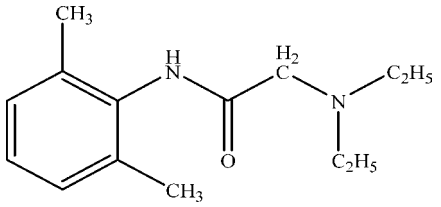
Equipment and Analytical Conditions. A Waters Alliance 2695 separations module, equipped with a Waters 996 photodiode array detector and controlled by Millenium-32 software, was used to separate LID and its major metabolite, MEGX, from matrix components using an Inertsil ODS-3 (3.0 × 250 mm), 5 μm column (GL Sciences, Peterborough, ON, Canada) held at 40 °C, with the UV detector set at 210 nm.

A Quattro Micro triple quadrupole mass spectrometer (MS/MS) interfaced to a Waters Alliance 2695 separations module through a Z-spray electrospray (ESI) interface and controlled by MassLynx version 4.0 software was used for the confirmatory analysis of LID. The analytes, separated on an Inertsil ODS-3 (3.0 × 250 mm, 5 μm) column using a mobile phase composition of ammonium formate (0.01 mol L^{-1}, pH = 4.0):acetonitrile:methanol (70:5:25, v/v/v), were directly introduced into the ESI source (source block and desolvation temperatures were held at 80 and 210 °C, respectively) at a split ratio of 1:10. Nitrogen was used as both drying and nebulizing gas, and argon was used as collision gas. The mass spectrometer was tuned to obtain optimum peak shape and intensity for the protonated molecular ions of LID and MEGX at *m*/*z* 235 and 207, respectively. Collision gas and collision energy were adjusted for the optimized production of the daughter ions of LID at *m*/*z* = 86 and 58 and of MEGX at *m*/*z* = 58.

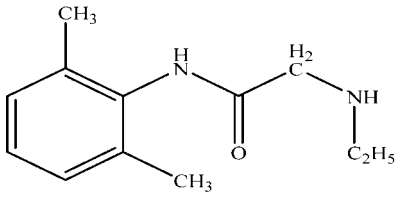
Sample Preparation and Cleanup. The 30 g samples selected from the distal, mid, or base piece of negative control or incurred velvet antlers were cut into thin slices and blended into a homogeneous mixture in a commercial Warring blender. Six, 2.0 ± 0.5 g test portions of the control homogenized velvets were weighed into six individual 50 mL polypropylene centrifuge tubes. Three, 2.0 ± 0.5 g test portions of each incurred velvet were weighed into three separate 50 mL polypropylene centrifuge tubes. Five of the six negative control velvet antler samples were fortified with appropriate volumes of the 2.0 μg/mL working standard solution to prepare samples containing 10, 25, 50, 75, and 100 ng/g of LID and 20, 50, 100, 150, and 200 ng/g of MEGX. The sixth sample was left unfortified and served as control. The control, fortified test samples and incurred velvet samples were allowed to sit for 30 min. Eight milliliters of 0.10 M borate solution was added to each sample and shaken for 6 min on a flat bed rotating mixer (Classic Series C2, New Brunswick Scientific, Edison, NJ) at 250 rpm. The samples were allowed to sit for 10 min. Eight milliliters of extraction solvent (toluene/hexane/2-propanol (78:20:2, v/v/v) was added, and the samples were shaken for 6 min at 250 rpm and centrifuged (Beckman J-6, with 50 mL centrifuge tube carriers) at 4200*g* for 15 min at ambient temperature. Six milliliters of the upper organic layer was transferred into a 10 mL test tube, and the lower aqueous layer was discarded. The extract was evaporated to dryness with nitrogen (Zymark TurboVap LV) at 50 °C, reconstituted with 24 mL of acetonitrile/water (10:90, v/v), and loaded onto a C_{18} cartridge that had been conditioned with 5 mL of methanol and 5 mL of water. After washing the cartridge with 10 mL water, it was evacuated for a further 10 min, and the retained LID and MEGX were eluted with 1 mL of methanol into a 10 mL glass centrifuge tube. Then 75 μL of the 2 μg/mL PHEN working standard solution was added to each sample as external standard. The samples were then evaporated to dryness at 50 °C with nitrogen. The dried residue was reconstituted in 350 μL of mobile phase and filtered for LC-UV and LC-MS/MS analysis.

Validation Study. The selectivity of the analytical method was demonstrated by extracting two sets of five negative control velvet antler samples obtained from each of the three portions of the antler (i.e., the distal, mid, or base pieces) from five different animals and analyzing one set per day using the LC-UV procedure described. The specificity of the method was demonstrated by analyzing negative control velvet samples fortified with xylazine, another anesthetic that could be used in veterinary practice for antler harvesting, to see if it would mask or enhance the ability of the method to detect negative control velvet samples as negative. Absolute recoveries of LID and MEGX added to negative control velvet antler were calculated by comparing the detector signals obtained on extracts of samples fortified at the beginning of the assay with those of negative control extracts to which equivalent concentrations of LID and MEGX standards had been added just prior to analysis. Intra-assay precision and accuracy of the method were determined by fortifying negative control velvet antler samples taken from the distal, mid, or base pieces in triplicate at concentrations of 30, 60, and 90 ppb for LID and of 60, 120, and 180 ppb for MEGX and analyzed using the described procedure. The same experiment was repeated on two more consecutive days to generate data for the inter-assay precision and accuracy. The accuracy of the developed method for estimating the concentrations of LID and MEGX in "unknown velvet antler samples" was verified by analyzing 12 velvet samples from each of the three sections that were prepared, coded, and randomized by a chemist from the Centre for Veterinary Drug Residues Laboratory and given "as blind samples".

Once it was demonstrated that the method was fit for its intended purpose, it was then used to determine the concentrations of LID and MEGX in velvet antlers (left and right antlers) harvested from 13 animals obtained from an elk farm in Winnipeg, MB, Canada. Antlers from 10 of the 13 animals were harvested 4−6 min after a 2% solution of lidocaine was injected subcutaneously around the base of each antler at the rate of 1.2 mL of lidocaine/cm of antler circumference, measured at the pedicle of antler base using the procedure described by Woodbury et al. (*22*). The antlers ranged in sizes from 13 to 17 cm in circumference. Antlers from the remaining 3 animals were harvested



Lidocaine (LID)



Monoethylglycinexylidide (MEGX)

Figure 1. Chemical structures of lidocaine and its major metabolite, monoethylglycinexylidide, MEGX.

using EA rather than lidocaine anesthesia. These served as negative control (drug-free) antlers for the study.

RESULTS AND DISCUSSION

LID and MEGX (**Figure 1**) are basic compounds with pK_a of 7.9 and 8.1, respectively (*21*). In the initial stages of method development, it was decided to adapt the extraction conditions previously reported by Levine and Blanke (*5*) that had been used to extract LID and MEGX from plasma and some unspecified tissue for GC analysis. Our attempts to extract LID and MEGX from velvet antler using that procedure resulted in a very complex extract that was unsuitable for LC analysis. We found that replacing the isoamyl alcohol used in their extraction solvent with 2-propanol (to reduce the interfacial tension) and subjecting the extract to further cleanup on a reversed-phase SPE cartridge gave reasonably clean extracts for LC-UV analysis. **Figure 2A** shows a typical LC-UV chromatogram of a negative control velvet antler (distal piece) that was extracted and analyzed using the procedure described. In **Figure 2B** is displayed a typical LC-UV chromatogram of an extract of a negative control velvet antler (distal piece) that was fortified with 50 ppb LID and MEGX and 75 ppb PHEN (as an external standard) and processed as described. The retention time of the external standard PHEN under these conditions was 16.2 min. MEGX and LID eluted with relative retention times of 0.37 and 0.59, respectively. It can be seen from **Figure 2A**,**B** that LID and MEGX are well-resolved from each other and from other endogenous and/or exogenous velvet tissue components. Additionally, the specificity experiment with xylazine indicated that it would not interfere with the detection and determination of LID residues in the extracts. Absolute recoveries of 68−73, 70−77, and 75−80% of LID added to control velvet antlers were calculated for the distal, mid, or base pieces, respectively. Absolute recoveries of 27−30, 18−22, and 15−19% of MEGX added to velvet antler were also calculated for the distal, mid,

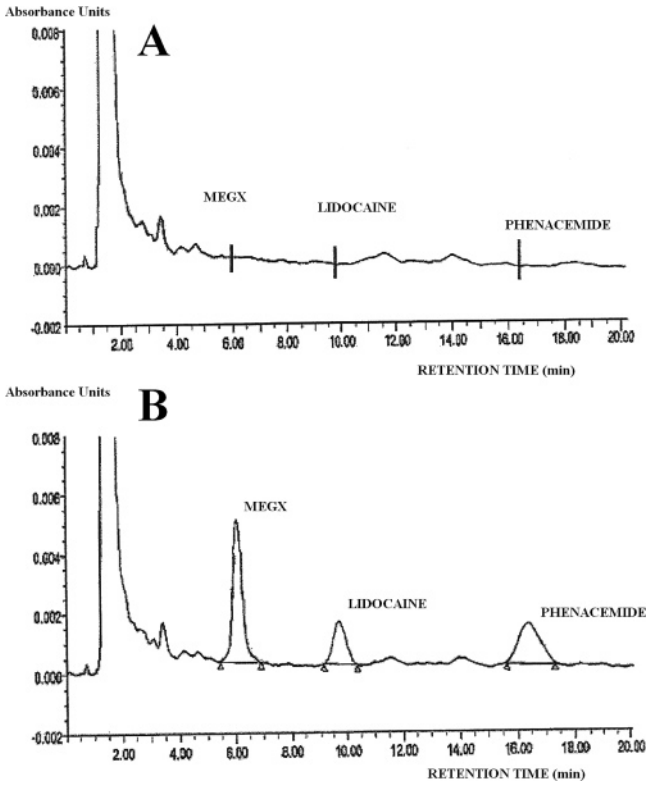


Figure 2. (**A**) Typical LC chromatogram of an extract from blank velvet antler (distal piece) showing the absence of interfering components at the expected retention times for LID, MEGX, and PHEN. (**B**) Typical LC chromatogram of an extract from blank velvet antler (distal piece) fortified with MEGX and LID at a concentration of 50 ng/g each and PHEN at 75 ng/g. Details of the analytical separation are described in the text.

and base pieces, respectively. The variability in recoveries perhaps reflects the differences in the composition of the three sections of the antler. As a result, the three sections of the antler were treated separately, and matrix calibration curves were generated for each of the sections for the quantification of LID and MEGX (**Table 1**). Quantification was based on constructing a calibration curve of the response ratio of LID or MEGX to the external standard PHEN versus the concentration of LID or MEGX added to negative control velvet, derived from the mean response ratio calculated using a minimum of 10 sets of calibration curves. The regression parameters generated for the different sections of velvet are summarized in **Table 1**. Where LID or MEGX was detected in a velvet extract, the response ratio was measured and its concentration interpolated from the appropriate calibration curve.

The results of the intra-assay and inter-assay precision and accuracy experiments conducted for LID and MEGX at three defined concentrations covering the analytical range (i.e., close to the LOQ, midpoint of the calibration curve, upper point of the calibration curve), which are reported in **Table 2**, indicate that LID and MEGX can be estimated with accuracies of ≤15%

Table 1. Regression Parameters Generated for the Quantification of LID and MEGX in Velvet Antler

	lidocaine		MEGX	
section	slope ± SD[a]	intercept ± SD	slope ± SD	intercept ± SD
distal 40	0.0146 ± 0.0009	−0.0164 ± 0.0010	0.0056 ± 0.0003	−0.0002 ± 0.00
mid	0.0134 ± 0.0050	−0.0229 ± 0.0060	0.0053 ± 0.0003	−0.0007 ± 0.0010
base	0.0131 ± 0.0020	−0.0052 ± 0.0070	0.0050 ± 0.0006	−0.0096 ± 0.0060

[a] SD, standard deviation of a minimum of 10 sets of calibration curves.

Table 2. Intra-assay and Inter-assay Precision and Accuracy of the Analytical Method

	mean concentration ± SD in ng/g found					
	LID concentration added (ng/g)			MEGX concentration added (ng/g)		
	30	60	90	60	120	180
			Distal Piece			
within day 1 ($n = 3$)	30 ± 2	62 ± 2	92 ± 1	59 ± 5	120 ± 4	177 ± 8
between days 2, 3, and 4 ($n = 9$)	29 ± 2	62 ± 2	90 ± 3	59 ± 3	122 ± 6	176 ± 5
accuracy[a] (%)		102 ± 2			99 ± 1	
			Mid Piece			
within day 1 ($n = 3$)	30 ± 1	61 ± 1	89 ± 3	63 ± 2	127 ± 1	177 ± 4
between days 2, 3, and 4 ($n = 9$)	30 ± 1	59 ± 4	90 ± 5	62 ± 1	122 ± 6	176 ± 2
accuracy (%)		100 ± 2			103 ± 4	
			Base Piece			
within day 1 ($n = 3$)	32 ± 1	64 ± 2	94 ± 4	61 ± 5	119 ± 5	185 ± 9
between days 2, 3, and 4 ($n = 9$)	31 ± 2	63 ± 2	92 ± 4	61 ± 3	119 ± 6	184 ± 8
accuracy (%)		106 ± 2			101 ± 2	

[a] Accuracy = 100 × (concentration added − concentration found)/concentration added.

Table 3. Verification of the Accuracy of the Analytical Method Using Blind-Fortified Velvet Antler Samples

	lidocaine				MEGX			
			Base Piece					
concn added (ng/g)	15	25	45	80	45	75	100	150
mean concentration found ($n = 3$)	14 ± 1	26 ± 3	46 ± 3	78 ± 5	46 ± 5	76 ± 4	100 ± 2	156 ± 4
mean % accuracy ($n = 3$)	95 ± 4	101 ± 8	103 ± 6	98 ± 6	102 ± 11	101 ± 6	100 ± 2	104 ± 3
			Blood Piece					
concentration added (ng/g)	15	35	70	85	60	90	120	180
mean concentration found ($n = 3$)	14 ± 1	36 ± 1	69 ± 2	89 ± 2	62 ± 7	86 ± 6	123 ± 1	184 ± 9
mean % accuracy ($n = 3$)	93 ± 0	102 ± 2	99 ± 2	105 ± 1	104 ± 11	96 ± 7	103 ± 9	102 ± 6
			Wax Piece					
concentration added (ng/g)	0	12	50	90	0	70	90	170
average concentration found ($n = 2$)	ND[a]	13	47	89	ND	68	90	174
average % accuracy ($n = 2$)	100	105	94	98	100	98	99	103

[a] ND, no detectable concentration found.

Table 4. Concentrations of LID Found in the Distal, Mid, and Base Pieces of Harvested Velvet Antlers[a]

		mean lidocaine concentration ± SD ($n = 3$) found in					
		distal piece		mid piece		base piece	
animal	preharvest treatment	left	right	left	right	left	right
animal 1	LID	1230 ± 60	1380 ± 7	433 ± 18	387 ± 11	541 ± 31	442 ± 39
animal 2	LID	423 ± 12	387 ± 7	224 ± 15	211 ± 12	199 ± 25	152 ± 8
animal 3	LID	1022 ± 17	160 ± 2	224 ± 27	1100 ± 60	538 ± 9	674 ± 7
animal 4	LID	899 ± 22	1070 ± 20	270 ± 8	279 ± 7	385 ± 8	618 ± 44
animal 5	LID	628 ± 18	811 ± 11	270 ± 6	261 ± 11	199 ± 7	219 ± 4
animal 6	LID	771 ± 58	1054 ± 88	238 ± 13	281 ± 10	278 ± 3	360 ± 25
animal 7	LID	148 ± 1	542 ± 26	136 ± 4	249 ± 28	294 ± 13	275 ± 22
animal 8	LID	1072 ± 44	1118 ± 80	247 ± 25	317 ± 67	428 ± 13	318 ± 19
animal 9	LID	1661 ± 80	802 ± 15	701 ± 15	252 ± 2	457 ± 24	262 ± 11
animal 10	LID	123 ± 15	–	68 ± 7	217 ± 67	856 ± 65	4342 ± 41
animal 11	EA	ND	ND	ND	ND	ND	ND
animal 12	EA	ND	ND	ND	ND	ND	ND
animal 13	EA	ND	ND	ND	ND	ND	ND

[a] There were no detectable concentrations of MEGX in any of the antlers tested. –, unavailable for analysis; EA, electro-anesthesia; ND, not detectable.

and precisions (RSD) of ≤10%. The results of the analysis conducted on the "blind-fortified" samples reported in **Table 3** demonstrate clearly that the method is fit for its purpose. The results of the analysis of the harvested velvet antlers from 13 elk are reported in **Table 4**. The results clearly show that significant concentrations of LID were detected in all the antlers obtained from the 10 animals whose antlers had been harvested after LID administration with concentrations ranging from 123 to 1230 ng/g, from 68 to 1100 ng/g, and from 152 to 4342 ng/g in the distal, mid, and base pieces, respectively. There were no detectable concentrations of MEGX in any of the antlers from the treated animals. Additionally, there were no detectable concentrations of LID and/or its major metabolite, MEGX, in any of the velvet antlers harvested by EA.

Confirmation by LC-MS/MS. To confirm the identity of LID detected in the harvested velvet antlers, the UV detector was replaced with a triple quadrupole mass spectrometer that has the ability to provide unequivocal identification of LID by its ability to detect its protonated molecular ion $[M + H]^+$ at $m/z = 235$ and its daughter ions at $m/z = 86$ and 58, representing

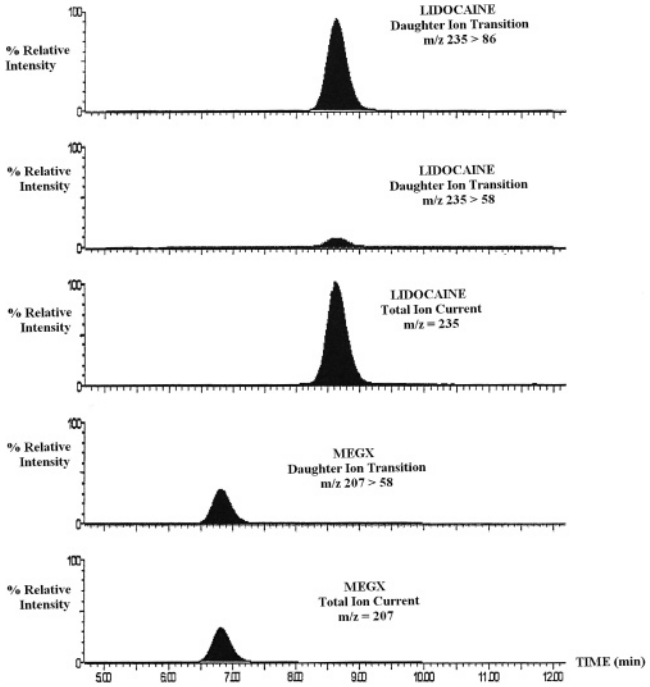


Figure 3. Typical LC-MS/MS chromatogram of an extract of a negative control distal piece fortified with LID and MEGX at a concentration of 100 and 200 ng/g showing the transition ions required for confirmation (LID: 235 > 86, 235 > 58, MEGX: 207 > 58).

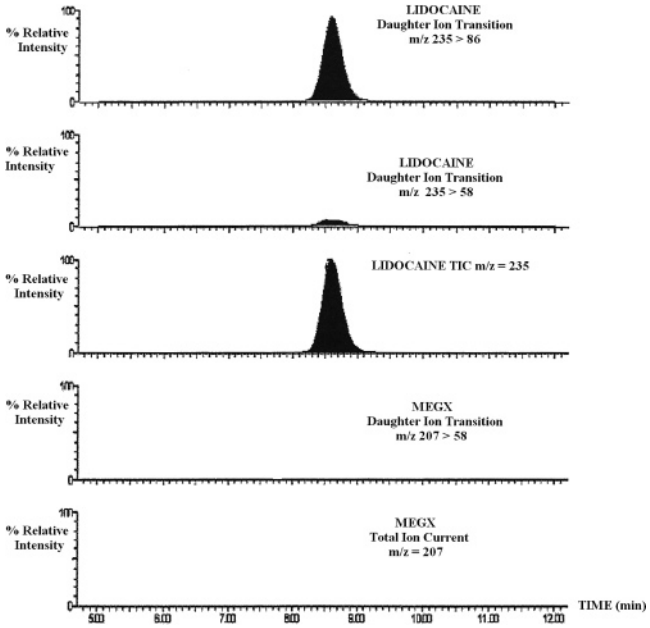


Figure 4. LC-MS/MS chromatogram of an extract from the distal piece of a harvested antler (animal 2) confirming the presence of LID in this sample that had previously been determined to contain 387 ± 7 ng/g of LID and no detectable residues of MEGX by LC-UV analysis.

the structurally significant ions $[CH_2N(CH_2CH_3)_2]^+$ and $[CH_2NH(CH_2CH_3)]^+$, respectively. For completeness, the mass spectrometer was also tuned to detect the $[M + H]^+$ ion for MEGX at $m/z = 207$ and its daughter ion at $m/z = 58$. Confirmation was deemed to have been achieved if all of the following conditions were met: (a) the retention times of the "unknown component peak" in the velvet antler extract matched the retention time of chemical standards of LID or MEGX to within ±2.5%; (b) the presence of $[M + H]^+$ ions for LID or MEGX at $m/z = 235$ and 207, respectively, under selected ion monitoring (SIM) conditions; (c) the presence of the daughter ions of LID at $m/z = 86$ and 58 and for MEGX at $m/z = 58$ under multiple reaction monitoring (MRM) conditions; (d) the ion abundance ratios of the daughter ions (transition ions) from the protonated molecular ions for LID and MEGX in the velvet extract matched those of the pure standards (to within a specified tolerance) under the same experimental conditions.

Figure 3 shows a typical LC-MS/MS chromatogram of an extract of negative control velvet antler (distal piece) fortified with LID and MEGX at a concentration of 100 and 200 ng/g, respectively, under the described experimental conditions. MEGX and LID eluted with retention times of 6.8 and 8.6 min, respectively, showing the ion transitions 235 > 86 and 235 > 58 for LID and 207 > 58 for MEGX. **Figure 4** is the LC-MS/MS chromatogram of an extract (distal piece); this confirms the previous results obtained by LC-UV (i.e., that this sample contained LID at a concentration of 387 ± 7 ng/g of LID but no detectable concentration of MEGX). Note that the LC-MS/MS system was used only to provide qualitative confirmation of the presence or absence of LID in those velvet samples that had been analyzed by LC-UV; it was not used in any way to quantify how much LID was present.

It has been demonstrated that the addition of SPE cleanup to the liquid−liquid extraction allowed us to develop and validate a simple, selective, and sensitive method for the analysis of LID and its metabolite, MEGX, in velvet antler. To the best of our knowledge, this is the first published analytical method for LID and MEGX in velvet antler that provides data to indicate beyond any shadow of doubt that the practice of using LID in velvet antler harvesting does leave residues of the drug in the velvet.

While the objective of this study was not to determine the toxicological impact of the presence of LID in harvested velvet antler, it is hoped that the data presented in this paper will help regulatory agencies to determine whether the demonstrated presence of LID residues in velvet should cause any potential public health and safety concerns for the consumer.

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