

Development and Validation of a Quantitative UHPLC–MS-MS Method for the Determination of Alpha-Chloralose in Feline Blood and Application on Blood Samples Collected from Cats with Symptoms of Alpha-Chloralose Poisoning

Ulrika Windahl^{1,*}, Sandra Lundgren², Margareta Sprycha¹, Cecilia Tegner², Kristoffer Dreimanis² and Annica Tevell Åberg^{1,3}

¹Swedish National Veterinary Institute (SVA), Uppsala 75189, Sweden

²University Animal Hospital, Swedish University of Agricultural Sciences, Uppsala 75007, Sweden

³Department of Medicinal Chemistry, Analytical Pharmaceutical Chemistry, Uppsala University, Uppsala 75123, Sweden

*Author to whom correspondence should be addressed. Email: ulrika.windahl@sva.se

Abstract

Alpha-chloralose (AC) is used as a rodenticide as well as an anesthetic agent in laboratory animals. It was previously also used as an avicide. Detection of AC in blood samples or in body tissues collected postmortem is key for the diagnosis of clinical cases and a requirement for surveillance of secondary toxicosis, including potential cases in wild animals. Reports on poisoning of humans and non-laboratory animals confirmed by the detection of AC or its metabolites are available, however poisoning of domestic animals are rarely available. Furthermore, reports on clinical cases in domestic animals rarely report quantifications of AC in blood or body tissues. The present study describes the validation of a quantitative ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS-MS) method that can be used in cases of suspected AC poisoning in cats. The validation study showed the method to be fit for purpose. In serum, the limit of quantification was 100 ng/mL and the limit of detection was 30 ng/mL. The new analytical method was applied on blood samples collected from 20 individual cats with a preliminary clinical diagnosis of acute AC poisoning. AC was confirmed in all 20 feline blood samples, and the concentration range of AC was 538–17,500 ng/mL. The quantitative method developed in this study was found to be a fast and selective method for confirmation of AC poisoning using blood samples from cats.

Introduction

Alpha-chloralose (AC) is the alpha isomer formed by the condensation of glucose with trichloroacetaldehyde (chloral), first described in 1893 (1, 2). It has a dose-dependent depressant as well as a stimulant effect on the central nervous system (CNS), and publications on its use as an avicide, a rodenticide and an anesthetic in clinical practice for human, feline and canine patients as well as laboratory animals are available (3–6). The exact mechanisms of action of AC are not well understood. After ingestion or injection, AC is hydrolyzed to chloral. A further reduction into the CNS depressant metabolite trichlorethanol has been described (2, 5). In contrast, others report the excretion of chloralose, either mainly unchanged or metabolized as a glucuronide (3, 5, 7).

AC is no longer used clinically either in veterinary or in human clinical practice. It is however still used as a rodenticide and as an anesthetic agent to sedate laboratory animals

(3, 5, 6, 8, 9), and peer-reviewed case reports summarizing the clinical symptoms of AC poisoning in humans and several animal species, including human cases with lethal outcomes, are available (3–6, 9–11). Together, the data in such reports describe a range of neurological symptoms similar to what are described in older experimental studies evaluating the toxic effects of AC. Most commonly described clinical manifestations of AC poisoning are dose-dependent ataxia, hyperesthesia and muscle tremors as well as lethargy, somnolence, coma and/or stupor. Myoclonic seizures and hyperthermia, increased salivation and symptoms of cranial disorders such as miosis or myosis and impaired pupillary response to light are also frequently described. Behavioral changes are mainly reported in animals, including cats and rodents. In individuals where lethargy is present or consciousness is lost, bradycardia, hypotension, bradypnea and hypothermia can occur and if not treated or reversed in time contribute to a lethal outcome with cardiac failure and respiratory failure (2–6, 9–11).

During the year 2019, a marked increase of suspected cases of AC poisoning in cats admitted to the Swedish Small Animal University Hospital (UDS) at the Swedish University of Agricultural Sciences was noted, as well as at other veterinary hospitals and clinics around the country. In the same year, the UDS and the Swedish National Veterinary Institute (SVA) noted discussions in veterinary internet-based newsgroups and social media on a perceived increased marketing of AC-based rodenticides. The UDS reported these findings to the Swedish Chemicals Agency, which led the latter to restrict the approval of the use of the substance to professional use only. Prior to 17 December 2019, the substance was available for individual use without restrictions (12).

The increase in feline cases and the accompanying awareness of the risks of secondary poisoning of cats and potentially other carnivores including birds of prey from consuming free-roaming rodents that have consumed AC-containing bait and the lack of access to a quantitative validated analytical method instigated the present study.

Detection of AC in blood samples is key for diagnosis and thereby differentiating AC poisoning from other causes of acute onset of illness, including other intoxications. Readily available laboratory analyses detecting the substance in blood samples and body tissues collected postmortem are also crucial for investigating potential cases of AC poisoning in wild and domestic animals as well as in humans. However, peer-reviewed scientific reports with quantitative laboratory analyses of the substance in blood, urine or other tissues from cases of AC poisoning in animals as well as in humans are scarce.

Segev et al. (5) used Cole's pyridine test, where the presence of chloroform in a sample results in a color change of the solution analyzed, for toxicological analyses of urine samples and/or stomach content from 32 dogs and 12 cats attending the Hebrew University Veterinary Teaching Hospital with symptoms as well as anamnesis leading to suspicion of AC poisoning. The method is not selective enough to confirm the findings, but the results indicated that AC was present in the gastric contents of 34 animals and in the urine of 13. Gas chromatography–mass spectrometry (GC–MS) methods typically involve derivatization reactions of AC to enable detection. Such GC–MS methods have been used to detect AC in fortified pigeon tissue samples by Odam et al. (13), in tissue samples from poisoned ducks by Allender and Keegan (14), in urine from a deceased cat by Grau-Roma et al. (9) and in human urine in intoxication cases by Thomas et al. and Berggrath et al. (3, 11). A headspace GC–MS method described by Kintz and co-workers in a fatal case study involved conversion of AC to chloral before detection in human urine and stomach content samples (15). Savin et al. applied both GC–MS and proton nuclear magnetic resonance methods in the investigation of two human poisoning cases but could not detect AC in the analyzed serum (10). Three more recently published methods involve liquid chromatography–tandem mass spectrometry (LC–MS–MS) for the quantification of AC. This technique is highly selective and sensitive, and AC can be detected and quantified directly, without derivatization. Hunter et al. applied LC–MS–MS to analyze stomach content and tissue samples from poisoned birds of prey (16), and Gerace and co-workers studied the distribution of AC in the human body in a postmortem investigation of a fatal AC intoxication case (6), and Leporati et al. analyzed rodenticides

in human hair (17). However, a quantitative LC–MS–MS method for AC in feline serum is to the best of our knowledge not previously described.

Although ranges of anesthetic and lethal doses have been described for humans as well as various animal species (1, 2, 5, 18), the correlation between levels of either AC or its metabolites in blood samples and the development of specific clinical symptoms including the risk of lethal outcome has not been prospectively investigated in either humans or cats. The objective of this study was to develop and validate a UHPLC–MS–MS method for use in clinical cases of suspected AC poisoning, including not only the confirmation of the presence of AC but also the determination of the concentration of the substance. Such a validated quantitative method would be of use not only to diagnose or exclude AC poisoning in clinical cases but also in further studies on the prevalence of secondary AC poisoning and of potential correlations between detected concentrations of AC in blood samples and prognosis including the risk of lethal outcome. Furthermore, the study aimed to investigate whether AC could be detected in samples collected from 20 individual cats with a clinical preliminary diagnosis of AC poisoning and, if so, quantify the levels and present the range of detected concentrations, as published data using quantitative methods when analyzing feline samples are currently lacking.

Experimental

Chemicals

Chloralose (purity 98.3%, AC 90%) and ammonium formate were purchased from Sigma-Aldrich (Darmstadt, Germany). Acetonitrile, methanol and formic acid were obtained from VWR (Stockholm, Sweden). The water used was purified to >17 M Ω /cm by a Milli-Q system (Millipore, Bedford, MA, USA).

Preparation of solutions and AC standards

Ammonium formate, 1 M in water, was prepared by dissolving 0.63 g of ammonium formate in 10 mL of Milli-Q water. The mobile phases used for UHPLC were prepared by mixing water, methanol, 1 M ammonium formate and formic acid 90:10:0.1:0.05 or 5:95:0.1:0.05 (v/v/v/v) for mobile phase A and B, respectively. The stock standard solution of AC was prepared in methanol at a concentration of 1.0 mg/mL. From the stock standard solution, medium standard solutions of AC at \sim 150 and \sim 12 μ g/mL, respectively, were prepared in water/methanol (60:40, v/v). The AC calibration solutions were prepared in acetonitrile from one of the medium standards approximately at the following concentrations: 100, 450, 770, 1,000, 1,500, 2,000, 2,500 and 3,500 ng/mL. Quality control (QC) solutions of AC were diluted in acetonitrile from the other medium standard at \sim 100 ng/mL (limit of quantification (LOQ)), 400 ng/mL (low; QCL), 1,300 ng/mL (medium; QCM) and 2,000 ng/mL (high; QCH).

Instrumentation

The quantification of AC was performed by UHPLC–MS–MS on a Waters Acquity UPLC™ system with a binary pump, a sample organizer with a flow-through-needle injection configuration and a column manager. The LC system was coupled to a Xevo TQ-S micro triple quadrupole mass spectrometer

Table I. MRM Transitions for the Detection of AC

MRM	[M-H] ⁻ <i>m/z</i> precursor	[M-H] ⁻ <i>m/z</i> fragment	Collision energy (V)
Quantitative	307.1	70.80	26
Qualitative 1	307.1	84.90	26
Qualitative 2	307.1	100.8	24
Qualitative 3	307.1	116.8	24

from Waters (Milford, MA, USA). The chromatographic column was an Acquity UPLC BEH C₁₈, 1.7 μm, 2.1 × 50 mm equipped with a VanGuard pre-column 1.7 μm, 2.1 × 5 mm, both from Waters (Milford, MA, USA). The sample volume injected was 10 μL, and the column temperature was set to 65°C. The mobile phase was delivered as a gradient at a flow rate of 0.35 mL/min. The gradient started at 15% B for 0.5 minutes, elevated to 60% B from 0.5 to 1.5 minutes and then to 90% B from 1.5 to 3 minutes. At 3.8 minutes, the composition was back at 15% B for equilibration. The electrospray ion source was set to negative potential with a capillary and cone voltage of 0.8 kV and 40 V, respectively, and a desolvation temperature of 500°C. The ionization was assisted by a nitrogen desolvation gas flow at 1,000 L/h. AC was deprotonated to the [M-H]⁻ precursor ion of *m/z* 307.1. Selected fragment ions and collision energies are presented in [Table I](#).

Cases

Leftover serum (*n* = 15) and whole blood (*n* = 5) from samples collected at the intensive care unit at UDS as part of the clinical investigation of 20 cats admitted to UDS with suspected AC poisoning were for research purposes cataloged and stored in -20°C. The inclusion criteria were sudden onset of clinical symptoms consistent with previously published cases of AC poisoning including two or more relevant neurological symptoms, in previously healthy cats allowed to roam free outdoors (i.e., cats that could have access to wild rodents).

Sample preparation

Serum, plasma or whole blood (100 μL) was transferred to a 96-well plate, and 300 μL of acetonitrile was added to precipitate the proteins. For the calibration curve or QC samples, 200 μL of acetonitrile and 100 μL of AC standard solution in acetonitrile were added. A cap mat was attached to the plate, and the samples were vortexed for 10 minutes. After 10 minutes of centrifugation at 2,860 g, 100 μL of the supernatant was transferred to a new 96-well plate and 100 μL of Milli-Q water was added. A new cap mat was attached and the samples vortexed for 2 minutes before UHPLC–MS–MS analysis.

Method validation

The feline blood samples used for method validation were serum donated by the serology department of SVA after use for other laboratory diagnostic purposes. The method validation followed the guideline on the bioanalytical method validation issued by the European Medicines Agency and included selectivity, limit of detection (LOD), LOQ, linearity, system carry-over, accuracy, precision, matrix effects and stability

of AC in matrix, as well as in extract after sample preparation. The method was developed without the use of an internal standard (IS) since isotopic labeled AC was not available. In some published methods, other rodenticide substances have been applied as IS, but since this method is intended for analysis of cat serum and cats eat rodents and thereby are at risk to be exposed to rodenticides, that was not an alternative in this case.

The selectivity of the method was evaluated by the analysis of blank serum samples (*n* = 10). The LOD was defined as the lowest concentration where the signal-to-noise (S/N) ratio of all the multiple reaction monitoring (MRM) transitions was at least 3. The LOQ was defined as the lowest concentration where the S/N ratio of the MRM transition used for quantification was at least 10. The linearity was evaluated for the eight non-zero calibration levels in the range of 100–3,500 ng/mL used in the study. The calibration solutions were injected twice in each validation run—first and last in each sequence. Accuracy and precision were calculated at four concentration levels: at LOQ, QCL (low), QCM (medium) and QCH (high). Six replicates were analyzed at each level, and the analysis was repeated on three different days. The relative measurement uncertainty was calculated as twice the inter-day precision with a confidence interval of 95% (i.e., a coverage factor of 2). The matrix effect in serum was evaluated by extraction of AC at QCL and QCH levels from six different blank serum samples. The concentration was compared to QCL and QCH spiked in acetonitrile (*n* = 2). Dilution integrity was evaluated by experiments where serum was spiked with AC at concentrations outside the range of the calibration curve. These samples were diluted 5 or 10 times in blank feline serum before the analysis. The stability of AC was evaluated both in serum, spiked at QCL and QCH levels (*n* = 6) that went through three freeze/thaw cycles and in five naturally contaminated samples, that is, serum from cats with suspected AC poisoning. The samples were analyzed three times, at *t* = 0 and after 3 and 7 months. The stability of AC in extract after sample preparation was evaluated by keeping the 96-well plate in the autosampler at 10°C for 6 days before the second analysis.

Results

Ten blank feline serum samples were analyzed to evaluate the selectivity of the method. No interfering peaks were detected in any of the four MRM transitions for AC ([Figure 1a](#)); hence, the selectivity was satisfying. The LOD was calculated to be 30 ng/mL (S/N = 3), and the LOQ was calculated to be 100 ng/mL (S/N = 10; see [Figure 1b](#)). The accuracy and precision were calculated from six replicates of each of the four concentration levels ([Table II](#)). The experiments were repeated on three different days. For low, medium and high concentrations, the precision varied from 2.7% to 7.8%, and the accuracy was between 91.8 and 108. At the LOQ, the precision was 15% and the accuracy was 117%, showing satisfactory accuracy and precision for the method. The relative measurement uncertainty was calculated to be 22% from the interday precision (RSD^R).

The linearity of the calibration curves from the validation study was $\geq R^2$ 0.99, and the system carryover, calculated from the highest calibration concentration, was below 0.5%. At low and high QC concentrations, the matrix effect

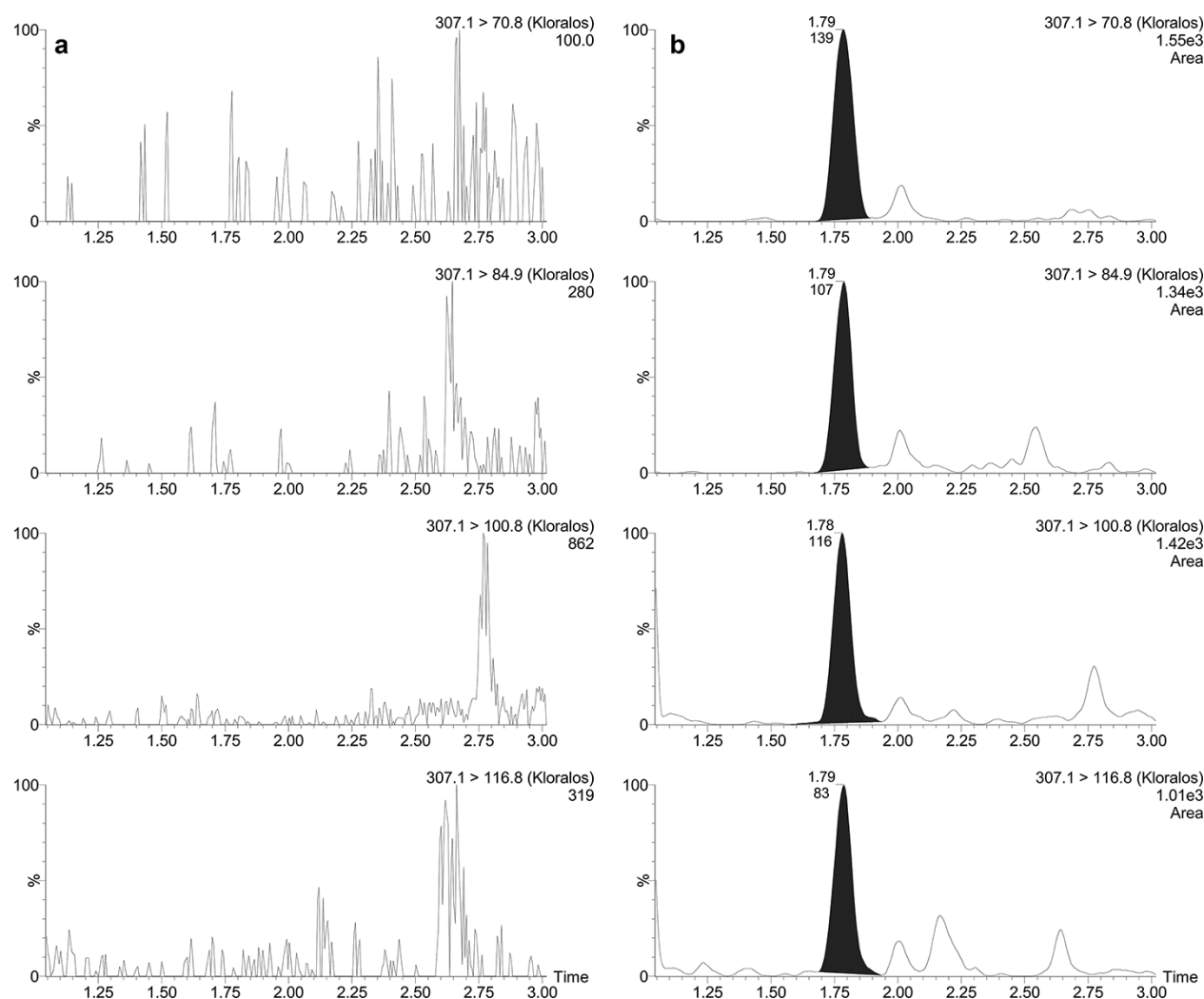


Figure 1. Chromatograms of the four MRM transitions for AC in blank feline serum samples analyzed (a) and feline serum samples spiked at LOQ, i.e., 100 ng/mL (b).

Table II. Summary of the Validation Data*

Validation round		LLOQ (92 ng/mL)	QCL (417 ng/mL)	QCM (1,282 ng/mL)	QCH (1,923 ng/mL)
1	Mean (ng/mL)	107.7	405.2	1 280	1 962
	SD (ng/mL)	7.2	32	55	80
	RSD ^R (%)	6.7	7.8	4.3	4.1
	Accuracy (%)	117	97.2	100	102
2	Mean (ng/mL)	85.8	382.8	1 345	2 082
	SD (ng/mL)	13	22	46	73
	RSD ^R (%)	15	5.6	3.4	3.5
	Accuracy (%)	93.2	91.8	105	108
3	Mean (ng/mL)	93.5	386.8	1 220	1 887
	SD (ng/mL)	8.6	27	33	79
	RSD ^R (%)	9.2	7.1	2.7	4.2
	Accuracy (%)	102	92.8	95.3	98.3
	RSD ^R (%)	11	7.0	4.4	4.6

* $n = 6$ at each concentration. The experiment was repeated on three different days.

was 111% (RSD 3.7%) and 98% (RSD 5.0%), respectively, which was considered acceptable for the method. The dilution integrity was 98% at both 5 and 10 times of dilution. The freeze/thaw stability study resulted in a 93% recovery at QCL and 107% at QCH. The stability study of AC in extract in the

sample manager showed a 0.1% decrease of the QCL concentration after 6 days. Hence, AC was stable both in the extract after sample preparation and in the matrix that was frozen and thawed repeatedly. However, when five of the cat serum samples collected from cats with suspected AC poisoning were

Table III. Summary of the In-matrix Stability Data*

	$t = 0$	$t = 0 + 3$ months	$t = 0 + 7$ months	RSD (%)	Area ratio alpha/beta chloralose
Sample A (ng/mL)	1,120	1,370	1,650	19	2.4/2.6/5.4
Sample B (ng/mL)	2,700	2,860	2,980	5.0	3.9/4.2/4.8
Sample C (ng/mL)	5,000	6,150	5,650	10	4.9/5.0/5.6
Sample D (ng/mL)	8,300	7,660	7,350	6.2	4.0/4.2/4.9
Sample E (ng/mL)	8,950	9,700	9,850	5.0	5.2/5.4/7.0

*The samples were analyzed on three different days.

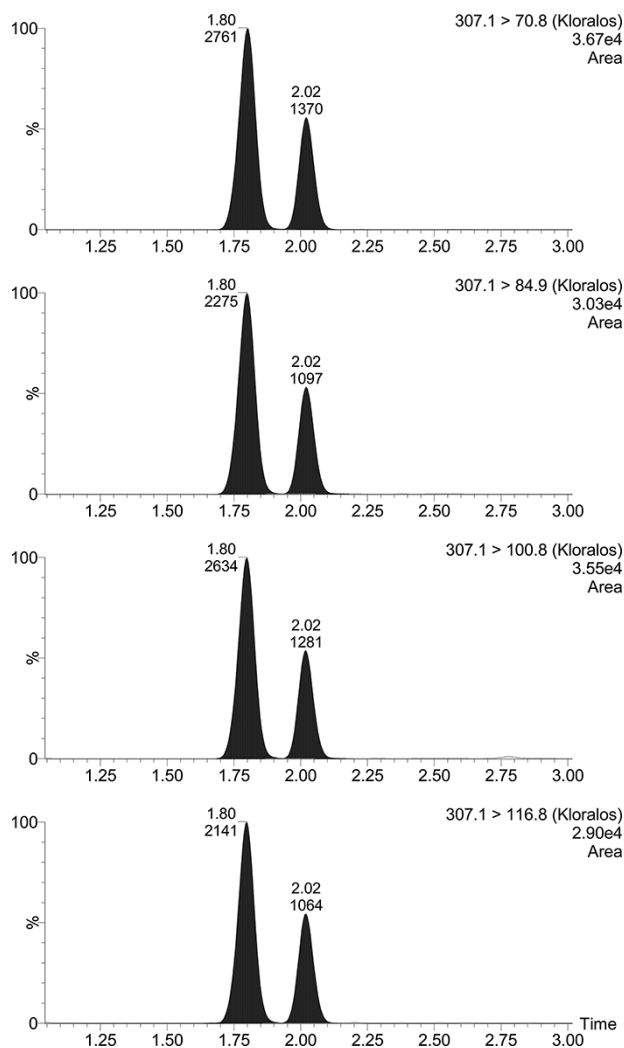


Figure 2. Chromatograms illustrating the alpha and beta peaks from chloralose at 1.80 and 2.02 minutes, respectively. The sample is feline serum from one of the cats with suspected AC poisoning; the concentration of AC in the sample was 3,140 ng/mL.

analyzed at three occasions, at time zero, after 3 months and after 7 months, the concentration of AC slightly increased with time in four of the five samples (Table III). When looking at the area of the peaks of alpha- and beta-chloralose, there was a slight increase in the ratio between alpha and beta forms; chromatograms of alpha- and beta-chloralose are shown in Figure 2.

AC was detected in all of the analyzed samples from the 20 individual cats with clinical symptoms leading to AC poisoning being suspected by the attending veterinarians at UDS. The results are presented in Figure 3. The lowest concentration

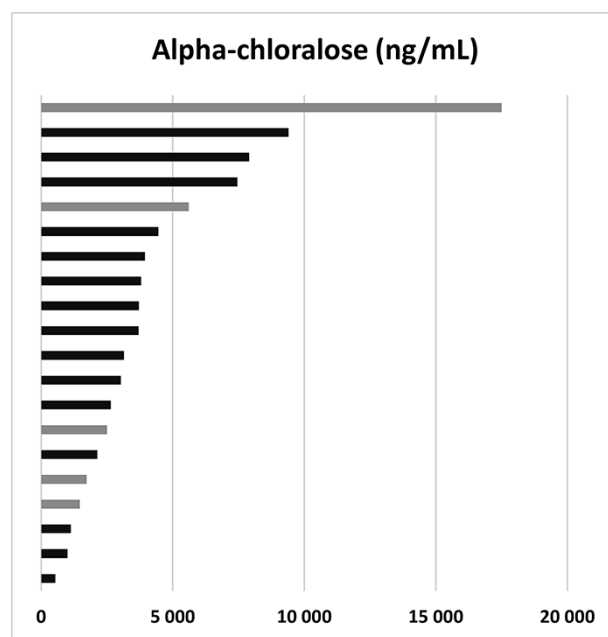


Figure 3. Concentration of AC in the 20 blood samples; serum ($n = 15$, black bars) and whole blood ($n = 5$, gray bars) collected from 20 individual cats with suspected AC poisoning.

was 538 ng/mL and the highest 17,500 ng/mL. The mean and median concentrations were 4,335 and 3,420 ng/mL, respectively. In half of the samples ($n = 10$), the AC concentration was higher than the highest concentration of calibration curve, and these 10 samples were diluted in blank feline serum before reanalysis in order to fit the interval of the calibration curve.

Discussion

The quantitative UHPLC–MS–MS method developed and validated in the present study was found to be fast, selective and fit for purpose for confirmation of AC poisoning using blood samples. The LOD and LOQ for the method were 30 and 100 ng/mL serum, respectively, while the range of AC in the 20 investigated feline blood samples in the study was between 538 and 17,500 ng/mL. Hence, the LOD and LOQ were good enough using the dilution approach applied, where the proteins in the blood samples were precipitated with acetonitrile, and then, the supernatants were diluted with water before the UHPLC–MS–MS detection. Adding extra evaporation and redissolution steps are classical ways to concentrate sample extracts, making the analytical method more sensitive, but it also makes the method significantly slower, and the additional steps increase the measurement uncertainty of

the method. The method described herein successfully distinguishes the active alpha isomer of chloralose responsible for the toxic effects of the compound from the inactive beta form (see Figure 2 and Table III) (19). Reports on both the beta and the alpha isomers are rare, but the beta isomer was also reported by Thomas et al. in a human AC poisoning case where 10–38% of the chloralose detected in urine was beta-chloralose (3).

When acute poisoning is suspected, the availability of rapid analyses able to accurately detect the toxic substance is key for the diagnosis and guidance of treatment choices. For example, rodenticides often contain derivatives of vitamin K antagonists, but the administration of vitamin K and other relevant treatments for poisoning with such rodenticides does not affect the course of diseases due to AC (11). Blood is a readily available sample material antemortem in both animals and humans; samples can be repeatedly collected with minimally invasive methods. The analysis of blood also enables the detection of unchanged substances, as opposed to urine, where toxic compounds often are excreted as metabolites. For example, Savin et al. analyzed urine in the investigation of two human poisoning cases (10) and found that the AC concentration was significantly higher after hydrolysis, suggesting that the conjugated form of AC is the predominant state in urine.

All of the 20 cats selected for the present study had multiple neurological symptoms that according to descriptions of toxic effects of AC poisoning described in general, non-species-specific reports on the chemical as well as in the reports by Segev et al. and Grau-Roma et al. on feline cases can be attributed to AC poisoning (2–6, 9–11). As AC was detected in all blood samples from the 20 cats, the diagnosis was confirmed. As described above, reports on clinical cases of AC poisoning in cats are rare. Segev et al. (5) described 12 confirmed cases and 1 suspected case and Grau-Roma et al. described 1 case (9). With so few scientific publications available, knowledge and awareness can be expected to be low in veterinary practitioners. Further studies and publications of typical and atypical symptoms of confirmed AC poisoning in cats are warranted to increase knowledge and awareness in the small animal veterinary community of when suspicion of AC poisoning is relevant. Cat owners often admit their animals for diagnosis and care, which enables samples to be collected and analyzed to confirm AC poisoning. Thus, cats might also be considered sentinels for an increased presence of AC in a country or region and the possible risk of secondary poisoning of pets and wildlife. Availability of a stable, selective, fit-for-purpose analyses is a requirement for surveillance of toxicosis in wild animals.

Furthermore, results from quantitative analyses of blood samples collected on single or multiple occasions from clinical cases of AC poisoning could be used in prospective studies as well as in retrospective reviews of clinical cases with the aim to increase the knowledge on the disease progression, uptake and distribution of AC. Possible clinically relevant correlations between levels of AC or its metabolites in blood and development and the progression of clinical symptoms including the risk of lethal outcome have not yet been prospectively investigated.

Conclusions

The method described herein is a fast and selective method for confirmation of AC poisoning using blood samples from cats. It is a quantitative method with an LOQ of 100 ng/mL and an LOD of 30 ng/mL. The results from the validation study showed the method to be fit for purpose both for clinical use and for research purposes.

AC could be confirmed in all blood samples collected from 20 cats treated for suspected AC poisoning at the intensive care unit of the UDS. The concentration range of AC in the samples was between 538 and 17,500 ng/mL.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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