Quantitative analysis of an anti-viral immune escape compound ML-7 in feline plasma using ultra performance liquid chromatography/electrospray ionization mass spectrometry

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ABSTRACT

An analytical method for the quantitative measurement of ML-7, a product with possible anti-immune escape activity for feline infectious peritonitis virus (FIPV), in feline plasma was developed and validated. The sample preparation consists of a solid-phase extraction step on an MCX cartridge. ML-7 and ML-9, used as the internal standard for the analysis, were separated on an ACQUITY UPLC^{TM} BEH C$_{18}$ reversed-phase column (1.7 μm, 50 mm × 2.1 mm I.D.), using isocratic elution with acetonitrile and 0.1% formic acid in water as the mobile phase. Both compounds were subsequently quantified in MRM mode on a Micromass® Quattro Premier^{TM} XE triple quadrupole mass spectrometer. The use of a Thermo Scientific® Exactive^{TM} orbitrap mass spectrometer made it possible to confirm the proposed fragmentation pattern of both ML-7 and ML-9. A validation study according to EC requirements was carried out, in which the method showed good performance. Linear behaviour was observed in the 1–2500 ng ml^{-1} range, which is relevant for real sample analysis. Accuracy and precision were within the criteria requested by the EC requirements throughout this concentration range. Extraction recovery of ML-7 was 72%. Matrix effect for ML-7 was not higher than 8%. The method was successfully used for the monitoring of ML-7 in feline plasma after intravenous, subcutaneous or oral administration of an ML-7 formulation, for the determination of pharmacokinetic parameters, with a limit of quantification of 1 ng ml^{-1} and a limit of detection of 0.4 ng ml^{-1}. The proposed method also shows good characteristics for the analysis of ML-7 in plasma of other animal species and human plasma.

1. Introduction

Infection of cats with feline coronavirus (FCoV) can have two different outcomes. Cats infected with avirulent FCoV-strains usually develop mild enteritis, while virulent FCoV-strains can cause progressive and usually fatal vasculitis known as feline infectious peritonitis (FIP) [1]. FIP is one of the most common infectious causes of death in young cats because neither effective treatment nor vaccine is available. It is an enigmatic disease and its pathogenesis is not fully understood. Recent work of our research group gave new insights into the interaction of infected monocytes (the *in vivo* target cell of the virus) with the immune system. We demonstrated that the virus has developed multiple mechanisms to evade the host's humoral immune system. Once the virus is replicating in a monocyte, it will ensure that it is protected from the omnipresent antibodies by inhibition of viral protein expression in the plasma membrane. This is achieved by intracellular retention of viral proteins or antibody-induced internalization of proteins that reached the plasma membrane [2,3]. Both mechanisms offer protection from recognition by antibodies and thus from antibody-dependent complement-mediated lysis (ADCML) and cell-mediated cytotoxicity (ADCC) [4,5]. ADCML can occur either through the formation of membrane attack complexes or through effector cells which carry a complement receptor (neutrophils, monocytes or macrophages). ADCC is executed by neutrophils, monocytes, macrophages or natural killer cells.

These immune-evasion mechanisms could explain why FIPV infected cells are not eliminated despite high antibody titres found in most infected cats. The internalization pathway was fully characterized and was found to be dependent on (among others) myosin light chain kinase (MLCK) [6]. ML-7, in full 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride, a potent and selective MLCK inhibitor was found to be a good candidate for *in vivo* blocking of the immune-evasive internalization and might

thus form the basis of a new and innovative anti-immune evasion therapy. With ML-7, the immune-evasion mechanisms of FIPV can be blocked and by doing so, the cat will be enabled to overcome the infection. The anti-immune evasion therapy is a completely new approach for the control of chronic viral diseases. It offers the following potential advantages, compared to other existing antiviral therapies: (1) continuous administration of the chemical is not required; (2) there are no associated problems related to resistance; (3) it can lead to elimination of the virus; (4) it can be applied for different viruses using this immuno-evasion process. ML-7 is a chemical that has been developed in 1987 at the Mie University in Japan to study the function of MLCK. It is the most active of a family of naphthalene derivatives, as a selective MLCK inhibitor [7]. Its structure is shown in Fig. 1, as well as that of ML-9, a related MLCK inhibitor. Up till now, no patents were filed on the compound, which is currently commercially available at most large chemical companies such as Sigma–Aldrich, Calbiochem, Alexxis and VWR.

Although ML-7 has never been used *in vivo* as an antiviral compound, it has been administered to rats and mice to preserve lung or intestinal epithelial barriers during stress- or lipopolysaccharide-induced injury [8–11]. MLCK is a possible target for drugs intended for relief of lung or bowel injury during sepsis because it controls paracellular permeability [12,13].

To design an effective dosage regimen of the compound ML-7 in the treatment of FIP viral infections in cats, determination of major pharmacokinetic parameters, such as C_{max} (peak plasma concentration), T_{max} (time to reach peak concentration), $t_{1/2}$ (half-life of elimination) and AUC (area under the plasma concentration versus time curve) must be achieved. These parameters can be evaluated on plasma concentration versus time profiles of the drug after administration to a sufficient number of animals. For this purpose, a reliable and sensitive analysis method of the drug in plasma must be available. The development, and subsequent validation, of such a method for the determination of ML-7 in feline plasma is presented and discussed in this paper. To our knowledge, the determination of ML-7 – or a related component – in animal plasma, has not been published yet. Also the determination of ML-7 in other body fluids and/or tissues of human or animal origin has not been described yet, demonstrating the novelty of our approach. For the clean-up of the plasma samples, two major strategies were followed and evaluated. The first one is based on a liquid–liquid extraction with ethyl acetate, while the second one involves a solid-phase extraction (SPE) step on an Oasis® MCX (mixed-mode cation exchange sorbent) cartridge. Extracts were then injected onto an UPLC (ultra performance liquid chromatography) instrument and components were measured by ESI-MS/MS (electrospray ionization tandem mass spectrometry). Since the extraction with ethyl acetate was shown to be very prone to a variable matrix effect, the method with clean-up on an MCX cartridge was retained for further validation experiments. It showed thereby good performance characteristics. It was sensitive enough with a limit of quantification (LOQ) of 1 ng ml^{-1} and a limit of detection (LOD) of 0.4 ng ml^{-1} that could be established. Finally, it was shown to be of practical use for the monitoring of plasma concentration time profiles of ML-7 after administration (intravenous, subcutaneous, oral) of a drug formulation to cats, used for pharmacokinetic analysis. The method also showed potential for the quantitative analysis of ML-7 in plasma of other animal species, and in human plasma.

2. Material and methods

2.1. Standards and chemicals

ML-7 and ML-9 were purchased from Sigma (Bornem, Belgium). Stock solutions of ML-7 and ML-9 at a concentration of 1 mg ml^{-1}

were prepared in methanol and stored at −20 °C. They were stable for at least 9 months. Further dilution of these stock solutions in methanol resulted in working solutions of 0.01–10 µg ml^{-1} for ML-7, and of 1 µg ml^{-1} for ML-9. The working solutions were stored at 4 °C, and were stable for at least 9 months.

The solvents used for the mobile phase – water and acetonitrile – were both of UPLC grade (Biosolve, Valkenswaard, The Netherlands). The additive to the mobile phase, formic acid, was of LC–MS grade (Biosolve). Methanol and water – used in sample clean-up – were of HPLC grade (VWR, Leuven, Belgium). The other reagents used in sample preparation – ammonia solution 25% and hydrochloric acid fuming 37%, were of *pro analysi* grade and purchased from Merck (Darmstadt, Germany).

Trichloroacetic acid (TCA), ethyl acetate, sodium hydroxide pellets (NaOH), dichloromethane – used in preliminary experiments of sample clean-up evaluation – were all of *pro analysi* grade and purchased from Merck.

2.2. Plasma extraction procedure

A 250 µl plasma sample was transferred into a capped 1.5 ml microcentrifugation cup and spiked with 25 µl of the working solution of 1 µg ml^{-1} of the internal standard ML-9. Blank samples (225 µl) used for the preparation of the calibration curve were also spiked with 25 µl of the different working solutions of ML-7. After vortex mixing for 15 s, 100 µl of a 1 M HCl solution were added. After vortex mixing for 15 s, the sample was centrifuged at 13,000 rpm for 10 min. Further clean-up of the supernatant was performed by solid-phase extraction over an Oasis® MCX cartridge (60 mg, 3 ml) (Waters, Milford, MA, USA). The cartridge was conditioned subsequently with 3 ml methanol, 3 ml water and 3 ml 0.1 M HCl solution, before loading of the sample. After passage of the last sample drops, the cartridge was rinsed with 3 ml water and 3 ml 0.1 M HCl solution. After drying under vacuum, ML-7 and ML-9 were eluted with 3 ml of an ammonia 25% solution in methanol (5/95, v/v). The eluate was evaporated to dryness at 40 °C, under a gentle stream of nitrogen gas. The dry residue was redissolved in 250 µl of a methanol/water (1/1, v/v) mixture, vortexed for 15 s and transferred over a 0.20 µm nylon filter (Millipore, Billerica, MA, USA) into an autosampler vial, and a 5 µl aliquot was injected onto the UPLC column.

2.3. Chromatography

The LC system consisted of an ACQUITY UPLC^{TM} Binary Solvent Manager and Sample Manager, from Waters. Chromatographic separation was achieved using an ACQUITY UPLC^{TM} BEH C_{18} reversed-phase column (1.7 µm, 50 mm × 2.1 mm I.D.) from Waters. The column was maintained at a temperature of 30 °C. Mobile phase A was acetonitrile, while mobile phase B was a solution of 0.1% formic acid in water. Mobile phase was delivered to the UPLC column at a flow rate of 0.3 ml min^{-1} for a total run time of 10 min, using the following gradient program: 0–3.50 min: 27.5% A, 72.5% B; 3.50–3.60 min: linear to 90% A, 10% B; 3.60–7.00 min: 90% A, 10% B; 7.00–7.10 min: linear to 27.5% A, 72.5% B; 7.10–10.00 min: 27.5% A, 72.5% B. Remark that ML-7 and ML-9 elute during the isocratic part of the UPLC run, i.e. the first 3.60 min. The gradient part is just functional to sufficiently rinse the column between two sample injections.

2.4. Mass spectrometry

The UPLC column effluent was interfaced with a Micromass® Quattro Premier^{TM} XE mass spectrometer instrument (Waters), equipped with an ESI ion source, which was used in the positive ion mode. A divert valve was used to send the UPLC effluent directly
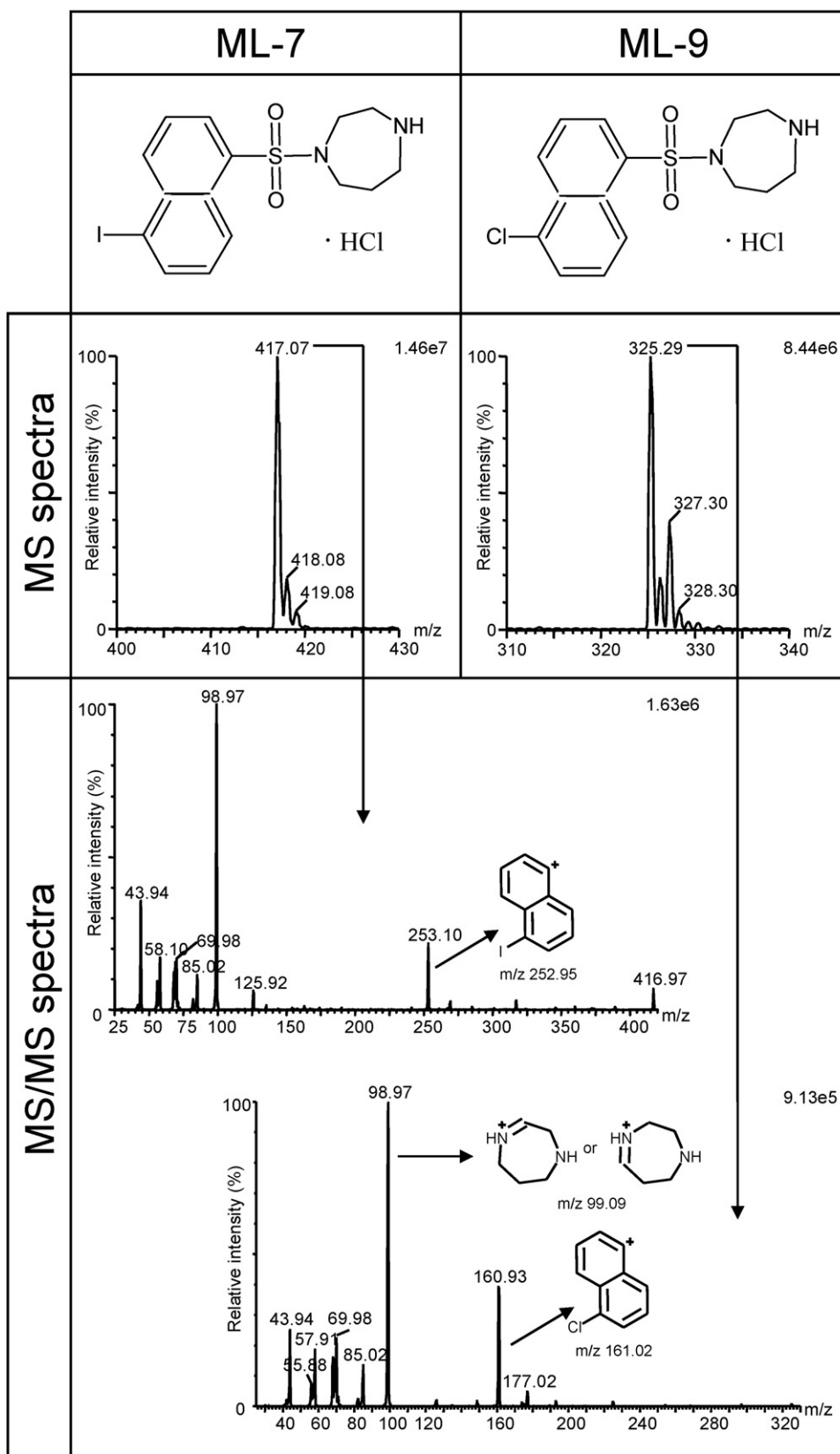
Fig. 1. Chemical structures, MS and MS/MS spectra of ML-7 and ML-9.

to the waste during the first 1.2 min and the last 6 min of the chromatographic run. During the rest of the LC run, when ML-7 and ML-9 were eluted from the column, the UPLC effluent was sent to the detector. The instrument was calibrated with a solution of 1 mg ml^{-1} of NaI, according to manufacturers' instructions. Thereafter, the instrument was tuned by direct infusion of a solution of 5 μg ml^{-1} of ML-7 and ML-9 in the ESI source at 20 μl min^{-1}, in combination with the LC mobile phase using a T-union. The following tune parameters were found to give optimal detection of ML-7 and ML-9: capillary voltage, 3 kV; cone voltage, 45 V; extractor voltage, 3 V; RF lens, 0 V; source temperature, 120 °C; desolvation temperature, 250 °C; desolvation gas, 750 l h^{-1}; cone gas, 20 l h^{-1}. Optimal collision energy in MS/MS mode, corresponding to a (nearly) 100% fragmentation of the protonated molecule (=collision-induced dissociation (CID)), was found to be 35 eV for both ML-7 and ML-9. The following MRM transitions – using the most prominent product ions – 417.07 > 98.97 and 325.29 > 98.97, were retained for the quantification of ML-7 and ML-9 respectively, using the MassLynx® software (Waters).

For the study of the fragmentation pattern, a Thermo Scientific® (Waltham, MA, USA) Exactive™ orbitrap mass spectrometer was used. A solution of 10 μg ml^{-1} of ML-7 and ML-9 was therefore infused in the hESI source at 5 μl min^{-1}, using the following tune parameters: spray voltage, 4.5 kV; capillary voltage, 25 V; tube lens voltage, 60 V; skimmer voltage, 20 V; capillary temperature, 250 °C; sheath gas, 26 (arbitrary units); auxiliary gas, 4 (arbitrary units); spare gas, 1 (arbitrary units). Fragmentation was obtained by a combination of in-source CID at a collision energy 25 eV, and fragmentation in the HCD (Higher Energy Collisional Dissociation) collision cell at a collision energy of 30 eV. The instrument was before all infusion experiments calibrated in positive and negative ionization mode, according to manufacturers' instructions, to ensure the mass accuracy of the results.

2.5. Validation

Three blank plasma samples of different origin were used for validation experiments: blank 1 = in-house blank at the Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, that looks "very dirty", confirmed by the consistent pellet after the ultracentrifugation step following the addition of 100 μl HCl 1 M (see Section 2.2), which was used for the major part of the validation work; blank 2 = left-over plasma of the Department of Medicine and Clinical Biology of Small Animals, Faculty of Veterinary Medicine, Ghent University, containing citrate-phosphate-dextrose solution with adenine, looking very clear, confirmed by the fact that little or no pellet was observed after the above mentioned ultracentrifugation step, which was used for a few determinations only; blank 3 = blank from the Department of Medicine and Clinical Biology of Small Animals, Faculty of Veterinary Medicine, Ghent University, that looks "very dirty" and "highly lipidic", confirmed by the consistent pellet after the ultracentrifugation step, as well as the lipidic layer above the supernatant, which was also used for a few determinations only.

The parameters evaluated during validation experiments, according to the guidelines of the EC where possible, were: linearity, accuracy and precision ("within-" and "between-day"), limit of quantification, limit of detection, carry over, specificity, stability in extract, extraction recovery, matrix effect:

- linearity: evaluated for a calibration curve in the lower range with the levels 0, 1, 2.5, 5, 10, 25, 50 and 100 ng ml^{-1}, and for a calibration curve in the higher range with the levels 0, 10, 25, 50, 100, 250, 500, 1000 and 2500 ng ml^{-1}, on 3 different analysis days. The curve was fitted with a linear curve with equation $y = ax + b$ with weighing $1/x$ (where y = instrument response and x = analyte

concentration); for good linearity the correlation coefficient r must be ≥0.99 and the goodness-of-fit coefficient g must be ≤10% for levels ≥10 ng ml^{-1}, and ≤20% for levels < 10 ng ml^{-1} [14,15].
- accuracy and precision (RSD): evaluated "within-day" at 3 levels 1, 10 and 100 ng ml^{-1} (each n = 6), the limits for accuracy are −20 to +10% for levels ≥10 ng ml^{-1}, −30 to +10% for levels >1 ng ml^{-1} and <10 ng ml^{-1}, and −50 to +20% for levels ≤1 ng ml^{-1}, while the tolerances for precision are given by the Horwitz equation: RSD$_{max}$ = 30.2, 21.3 and 15.1 for the 1, 10 and 100 ng ml^{-1} levels respectively. The evaluation of "between-day" accuracy and precision was not only performed at the same levels 1, 10 and 100 ng ml^{-1} levels (lower range), but also at 3 supplementary levels 25, 250 and 2500 ng ml^{-1} (higher range) (each n = 6, on 3 different analysis days), using the same tolerances for accuracy as above, while those for precision where 45.3, 32,0, 27.9, 22.6, 19.7 and 13.9 at the 1, 10, 25, 100, 250 and 2500 ng ml^{-1} levels respectively [14,16,17].
- limit of quantification (LOQ): the lowest level for which the criteria of accuracy and precision can be fulfilled [16].
- limit of detection (LOD): determined based on the average S/N value found for LOQ samples (n = 6), and using the S/N = 3 criterion [17].
- specificity: evaluated by controlling the presence or absence of peaks at the retention times of both ML-7 and ML-9 in a blank plasma sample extract [16].
- carry over: evaluated by injecting a solvent after the highest calibrator and controlling the presence or absence of peaks at the retention times of both ML-7 and ML-9.
- stability in extract: expressed as the ratio between the average area of ML-7 and ML-9 respectively, measured for 100 ng ml^{-1} plasma samples (n = 3) stored at 15 °C for 24 h in the autosampler after extraction, compared to the one for 100 ng ml^{-1} plasma samples (n = 3) analyzed directly after extraction.
- extraction recovery: expressed as the ratio between the average area of ML-7 and ML-9 respectively, measured for 10 ng ml^{-1} plasma samples (n = 3), compared to the one for blank plasma samples extracted and spiked after extraction with the same amount (n = 3).
- matrix effect: expressed as the ratio between the average area of blank samples spiked after extraction with the equivalent amount of ML-7 and ML-9 of a 10 ng ml^{-1} sample (n = 3), and the area of a reference solution containing the same amount of both components [18].

3. Results and discussion

3.1. Liquid chromatography and mass spectrometry

ML-7, and a related compound ML-9, used as internal standard, could be easily separated on an ACQUITY UPLC™ BEH C$_{18}$ reversed-phase column (1.7 μm, 50 mm × 2.1 mm I.D.), using 0.1% formic acid and acetonitrile as the mobile phase, which is probably the most commonly used solvent system for LC–MS analysis. The only difference in the chemical structure of the components, an I-atom for ML-7 and a Cl-atom for ML-9 in the naphthalene part (see Fig. 1), gives however such a difference in polarity and in molecular mass (91.45 Da), making their isocratic separation not really a challenge. ML-9 is then the earlier eluting component with a retention time of 2.19 min, under the chromatographic conditions as described in Section 2.3, while ML-7 elutes about 1 min later at 3.28 min (see Fig. 2 for a chromatogram of a standard solution).

Fig. 1 shows besides the chemical structures of both ML-7 and ML-9, also the mass spectra obtained after direct infusion of a standard solution of 5 μg ml^{-1} of ML-7 and ML-9 in the ESI source, in combination with the LC mobile phase using a T-union. The best
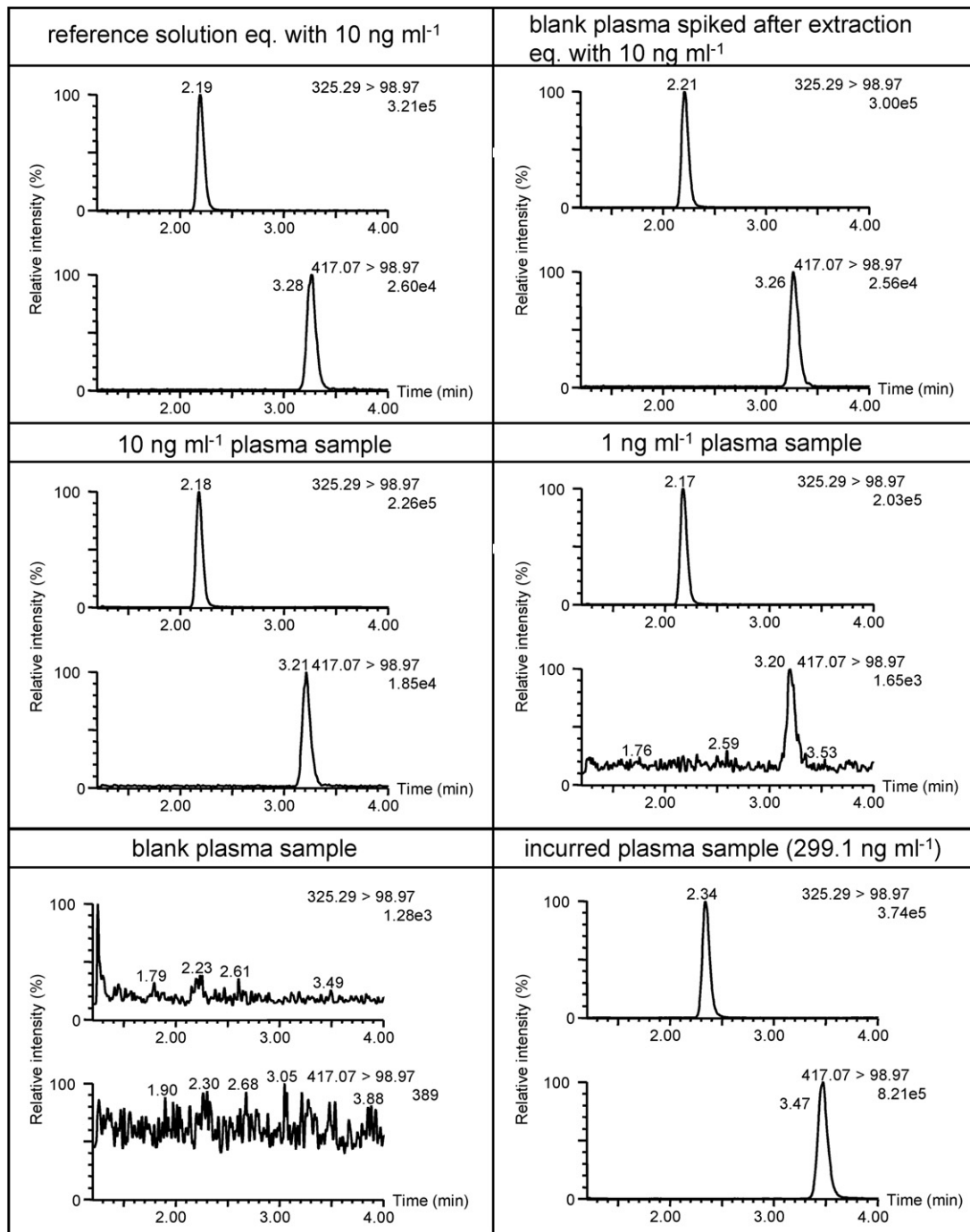
Fig. 2. MS/MS mass chromatograms of a standard solution of ML-7 and ML-9 (equivalent of 10 ng ml^{-1} sample), a blank plasma spiked with ML-7 and ML-9 (equivalent of 10 ng ml^{-1} sample) after extraction, a blank plasma spiked at 10 ng ml^{-1} of ML-7, a blank plasma sample spiked at 1 ng ml^{-1} of ML-7 (=LOQ), a blank plasma sample, and an incurred plasma sample (ML-7 concentration measured = 299.1 ng ml^{-1}, of a cat treated intravenously with 4 mg/kg BW, 2 h after administration). All chromatograms of spiked samples shown concern blank 1. ML-9 concentration in all plasma samples is 100 ng ml^{-1}. For each chromatogram, the upper trace represents ML-9, the lower trace is ML-7.

signal was obtained in the positive ion mode, probably because the components incorporate e.g. 2 N-atoms that can be easily protonated. In MS, a major ion at m/z 417.07 and 325.29 was observed for ML-7 and ML-9 respectively, corresponding to the protonated molecules [M+H]$^+$ of both components (molecular mass of respectively 416.28 and 324.83 as base). For ML-9, clearly also an ion lower in intensity at m/z 327.30 was observed due to the ^{37}Cl-isotope. The CID product ion spectra of both components show a predominant ion at m/z 98.97, which was therefore used in the MRM transition for quantification. According to the Mass Frontier

6.0 software (HighChem, Ltd., Bratislava, Slovakia) this corresponds to the 1,4-diazepine moiety of the components, after protonation and charge site rearrangement (rHc (γ)). This software has a module "Fragments and Mechanisms" which is an expert system providing information about basic fragmentation and rearrangement processes based on literature, starting from a user-supplied chemical structure. A major product ion with higher m/z was also observed, at m/z 253.10 and 160.93 for ML-7 and ML-9 respectively, according to Mass Frontier corresponding to the iodonaphthalene and chloronaphthalene moieties of the components, after

protonation and inductive cleavage (i). Also several common fragments at lower m/z were present in the product ion spectra: m/z 43.94, 55.88, 57.03, 58.10, 68.01, 68.96, 69.98, 82.03 and 85.02. These correspond all – according to Mass Frontier – to smaller fragments of the 1,4-diazepine moiety of the components, as the result of more complex fragmentation mechanisms.

The predominant fragment ions could be confirmed with reasonable certainty by infusion of a $10\,\mu g\,ml^{-1}$ solution of ML-7 and ML-9 in the hESI source of an Exactive® orbitrap mass spectrometer. This instrument allows high mass accuracy measurements of the fragment ions, which can then be related to the theoretical masses of the fragments generated by the Mass Frontier software. Mass errors (=$\Delta m/m \times 10^6$, with Δm the mass difference between the measured mass and the theoretical mass, and m the theoretical mass) of 0.50, 0.87 and 2.25 ppm were obtained for the fragment ions at m/z 98.97, 160.93 and 253.10 respectively, of ML-7 and ML-9. These low mass errors confirm the fragmentation mechanism proposed by the Mass Frontier software (see above).

3.2. Optimization of sample extraction and clean-up procedure

The aim was to develop an extraction procedure as simple and as rapid as possible, since in pharmacokinetic studies the number of samples can be substantial, but still robust enough to allow a reliable quantification of ML-7. Below several extraction and clean-up methods evaluated are briefly discussed.

A point of concern – independent of the preceding extraction and clean-up procedure – seemed to be the filter device used at the end of the extraction procedure to allow the extracts to be injected onto an UPLC system. It was shown that ML-7, as well as ML-9 (results not shown), were retained by several filter membrane types (see Table 1). The best result was obtained with the nylon filter device (13 mm, 0.20 μm) of Millipore. Remarkably, for some unknown reason, recovery was then even significantly above 100%, as confirmed by an independent second assay.

3.2.1. Deproteinization with TCA

The simplest extraction procedure that was tried consisted of a deproteinization step with TCA: briefly, to a 250 μl plasma sample, 25 μl of a 20% (w/v) TCA solution were added. After vortex mixing for 15 s, the sample was centrifuged (13,000 rpm, 10 min). The supernatant was transferred over a nylon filter device and a 5 μl aliquot was injected onto the LC column.

The mean signal of ML-7 of spiked plasma samples ($n = 3$) was only 20.7% compared to a reference solution with the same amount and treated in the same way, either due to a low recovery or an important matrix effect. No further investigation was performed to elucidate this.

3.2.2. Deproteinization with acetonitrile and liquid–liquid extraction with dichloromethane

A second extraction procedure tested was a deproteinization step with acetonitrile, followed by a liquid–liquid extraction with dichloromethane, adapted from [19]: briefly, to a 250 μl plasma sample, 800 μl acetonitrile were added. After vortex mixing for 15 s, the sample was centrifuged (13,000 rpm, 10 min). 1.5 ml of dichloromethane were added to the supernatant. After vortex mixing for 15 s, the sample was centrifuged (13,000 rpm, 10 min), the upper aqueous layer was transferred over a nylon filter device, and a 5 μl aliquot was injected onto the LC column.

This procedure showed to be of no use, since no peaks were measured, probably due to the fact that ML-7 and ML-9 do not remain in the aqueous phase.

3.2.3. Liquid–liquid extraction with ethyl acetate

A third extraction procedure evaluated was a liquid–liquid extraction with ethyl acetate: briefly, to a 250 μl plasma sample – conditioned or not with either 100 μl HCl 1 M solution or 100 μl 1 M NaOH solution – 3 ml of ethyl acetate were added. The sample was then rolled for 20 min on a roll bank. After centrifugation (4000 rpm, 10 min), the upper organic phase was transferred into a glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted into 250 μl of a methanol/water (1/1, v/v) mixture, transferred over a nylon filter device, and a 5 μl aliquot was injected onto the LC column.

As expected, the best result was obtained here when the plasma sample was conditioned with 100 μl NaOH 1 M solution. The N-groups of ML-7, and ML-9, are then deprotonated, favourizing the transfer to the organic phase. The mean signal of ML-7 of spiked plasma samples ($n = 3$) was then 61.1% compared to a reference solution with the same amount (32.6% for acidified plasma, and 26.0% for non-conditioned plasma). In further assays – using blank plasma of a different origin – a different result was observed. The mean signal of ML-7 of the spiked plasma samples ($n = 3$) was then much lower: 37.2% compared to 61.1% before. However, it was shown that there was a significant matrix effect, since extracted blank plasma samples spiked with ML-7 showed a decrease in signal to only 51.6% ($n = 3$) compared to that of a reference solution of the same level. Actual recovery is therefore still yet 72.2% instead of the apparent 37.2%. For a blank plasma of yet another origin, recovery was very low, 22.1% for ML-7, while again a significant matrix effect of even 21.3% in this case was observed, compromising the establishment of an LOQ as low as $1\,ng\,ml^{-1}$. It is evident that an extraction method with such a variable matrix effect as observed above, does not allow for reliable quantification.

No improvement was observed when ethyl acetate was replaced by dichloromethane as the extraction solvent: apparent recoveries of 9.0, 17.5, and 29.6% were obtained, respectively for non-conditioned, alkalinized and acidified plasma. The fact that apparent recovery in acidic conditions is higher that in alkaline conditions might indicate that matrix effect is even more pronounced when using dichloromethane as extraction solvent, compared to ethyl acetate.

3.2.4. Solid-phase extraction on HLB extraction cartridge

A fourth extraction procedure involved the use of a solid-phase extraction clean-up step. A procedure using an Oasis® HLB (30 mg, 1 ml) extraction cartridge was tested where a plasma sample was first treated with 750 μl 0.1% (v/v) ammonia solution, and thereafter loaded onto the cartridge conditioned successively with 1 ml MeOH, 1 ml H_2O and 1 ml 0.1% (v/v) ammonia solution. The cartridge was then washed with 1 ml H_2O. After drying under vacuum, the components were eluted with 1 ml of acetonitrile. The eluate was evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted into 250 μl of a methanol/water (1/1, v/v) mixture, transferred over a nylon filter device, and a 5 μl aliquot was injected onto the LC column.

Recovery was only very poor: 27.6% for a standard mixture, and 8.8% for a spiked plasma sample (each time $n = 3$), indicating that ML-7 is not well retained on the HLB cartridge.

3.2.5. Solid-phase extraction on MCX extraction cartridge

A much more promising result was obtained when using an MCX instead of an HLB cartridge, as described in Section 2.2. The different steps of the extraction procedure were evaluated to obtain an extract combining a high recovery with a sufficient clean-up, allowing analysis by UPLC–MS/MS and minimizing possible matrix effects on the analytes response, as observed for the liquid–liquid extraction procedure.

Table 1
Behaviour of ML-7 (standard solution of 1 µg ml^{-1} in MeOH/H$_2$O (1/1, v/v) solution) on passage over different filter membrane types (each assay mean result of n = 3).

Membrane	Manufacturer	% passage through filter
Durapore® Membrane (PVDF) (13 mm, 0.22 µm)	Millipore	2.4
Millipore Express® Membrane (PES) (13 mm, 0.22 µm)	Millipore	59.3
Nylon Membrane (13 mm, 0.20 µm)	Millipore	128.0[a]/125.6[b]
Regenerated Cellulose Membrane (15 mm, 0.20 µm)	Macherey-Nagel	2.1

[a] First assay.
[b] Second (independent) assay.

Table 2
Evaluation of the influence of: (1) the wash steps, and (2) the ammonia 25% concentration in the elution solvent, on the recovery of ML-7 and ML-9 from an MCX cartridge (evaluated on a 100 ng ml^{-1} solution of ML-7 and ML-9 in water, each result n = 3).

	ML-7 recovery (%)	ML-9 recovery (%)
Wash procedure		
1 wash step 3 ml 0.1 M HCl	69.5	69.6
2 wash steps 3 ml 0.1 M HCl, 3 ml water	30.0	43.2
2 wash steps 3 ml water, 3 ml 0.1 M HCl	79.2	78.5
3 wash steps 3 ml water, 3 ml 0.1 M HCl, 3 ml methanol	42.6	52.6
Ammonia content (%)		
5	87.6	85.5
10	78.2	79.4
25	69.2	77.3

The replacement of the 100 µl 1 M HCl solution by 50 µl of a TCA 20% (w/v) solution, added to the plasma before the ultracentrifugation step preceding the loading of the sample onto the MCX cartridge, resulted in a cleaner extract, since the TCA solution is more powerful to remove plasma proteins. However, recovery was only marginal (±10%) for both components, despite the fact that a cleaner solution was applied onto the cartridge.

The results of the evaluation experiments of the rinsing procedure of the MCX cartridge after the loading of the sample, which allows washing off residual matrix components, are summarized in Table 2. As can be seen, the best recovery was obtained with 2 wash steps, successively with water and 0.1 M HCl solution. Surprisingly, for some unknown reason, the same washing procedure in the opposite order resulted in an important loss of both components. A supplementary third wash step with an organic solvent such as methanol, adding a supplementary selectivity for washing additional to the aqueous phases, also resulted in an important loss of analyte, and was therefore not included in the final procedure.

Ammonia was added to the elution solvent methanol to disrupt the binding of ML-7 and ML-9 by deprotonating their N-groups, facilitating the recovery of both components. Since recovery decreases with increasing ammonia concentration in the elution solvent (see Table 2), the lowest concentration of ammonia (5%) was retained in the final extraction method.

The equivalent of the MCX cartridge of Isolute (Biotage, Uppsala, Sweden), the SCX column (500 mg, 10 ml), was also evaluated as an alternative extraction cartridge. Although recoveries seemed to be quite comparable, a problem occurred when injecting the extracts onto the UPLC column, namely excessive peak broadening and shift in retention time.

3.3. Validation experiments and applicability of sample extraction and clean-up method involving the MCX cartridge

The extraction procedure based on solid-phase clean-up over an MCX cartridge, as described in Section 2.2, was subjected to validation experiments to evaluate the performance characteristics of the method. The results are summarized in Table 3.

As can be seen, the ML-7 signal measured in MRM mode on the MS instrument was linear over a wide range from 1 to 100 ng ml^{-1}, with $r \geq 0.99$ and $g \leq 20\%$, as required for good linearity in this range. The same was true in the higher range with ML-7 levels ranging from 10 to 2500 ng ml^{-1}. The accuracy of the analysis method, evaluated on 3 different levels (1, 10 and 100 ng ml^{-1}, each n = 6) was very good, compared to the allowed tolerances (see Table 3). The "within-day" precision (RSD) of the analysis method, evaluated on the same 3 levels, was even excellent, taking into account the RSD$_{max}$ values given by the Horwitz equation (see Table 3). The same behaviour was also observed for samples spiked at 10 ng ml^{-1} (n = 3), but using blanks of different origin and with a different behaviour during extraction (blanks 2 and 3, see Section 2.5), indicating the robustness of the method (see Table 3). Also "between-day" results for accuracy and precision, evaluated at 1, 10, and 100 ng ml^{-1} levels in the lower range, and also at 25, 250 and 2500 ng ml^{-1} levels in the higher range, for 3 different analysis days, were very satisfactory (see Table 3).

LOQ was established at 1 ng ml^{-1}, as the lowest level at which the criteria for accuracy and precision could be fulfilled. LOD was calculated to be 0.38 ng ml^{-1}, by determining the average S/N of the six 1 ng ml^{-1} samples used for accuracy and precision evaluation, and using the S/N = 3 criterion. The specificity of the analysis method was guaranteed since no interfering peaks were observed at the retention times of ML-7 and ML-9, for blanks 1 (see Fig. 2), 2 and 3. Carry-over was of no concern since no peaks for both ML-7 and ML-9 were measured when a solvent methanol/water (1/1, v/v) mixture was injected immediately after a 2500 ng ml^{-1} spiked plasma sample. The stability of ML-7 and ML-9 in extracts stored at 15 °C during 24 h was verified, and no decrease in signal of both components was observed during this time period.

Recovery of the analysis method was acceptable for both components: 72.1% and 70.4% for ML-7 and ML-9 respectively. More important however is that the method is free of any significant matrix effect, as well for ML-7 as for ML-9, as shown by comparing the area of blank samples spiked after extraction and the area of a standard solution containing the same amount (see Fig. 2 for chromatograms typically obtained for such samples), and this for all 3 blanks tested, despite their different composition and behaviour during extraction (see Section 2.5). The biggest decrease in signal – of about 8% – is observed for ML-7 in combination with blank 1.

The method was shown to be of practical use for the determination of ML-7 in plasma samples of cats treated with an ML-7 formulation, either by intravenous or subcutaneous injection or oral administration, to determine concentration versus time profiles necessary for pharmacokinetic evaluation. Such a typical profile obtained for one cat after intravenous administration of ML-7 is shown in Fig. 3. As can be seen, the compound shows a typical profile for a two-compartmental pharmacokinetic model, indicating a good distribution of ML-7 in the body.

The applicability of the method was further evaluated towards the determination of ML-7 in plasma of other animal species and in human plasma. Therefore, extraction recovery and matrix

Table 3
Results of validation experiments for the analysis method with sample extraction and clean-up on MCX cartridge, performed on blank 1, unless specified otherwise on blank 2 and blank 3.

Linearity		
Lower range: $1 - 100$ ng ml^{-1}		
day 1: a= 1.175; b= -0.2797	r= 0.9993	g= 5.7 %
day 2: a=1.321; b= -0.5131	r= 0.9989	g= 6.9 %
day 3: a= 1.159; b= 0.02160	r= 0.9997	g= 3.5 %
Higher range: $10 - 2500$ ng ml^{-1}		
day 1: a= 0.7683; b= -1.360	r= 0.9998	g= 4.5 %
day 2: a= 0.7159; b= -0.003445	r= 0.9997	g= 3.3 %
day 3: a= 0.6737; b= -0.6513	r= 0.9997	g= 4.1 %

Accuracy and precision ("within-day")		
level	mean conc. found ± SD	accuracy / precision (RSD)
1 ng ml^{-1}	1.0 ± 0.029	+ 4.8 % / 2.8 %
10 ng ml^{-1}	9.1 ± 0.36	- 8.7 % / 3.9 %
	blank 2: 9.0 ± 0.03 (n= 3)[*]	- 10.3 % / 0.3 %
	blank 3: 9.1 ± 0.23 (n= 3)[*]	- 8.6 % / 2.5 %
100 ng ml^{-1}	100.5 ± 2.0	+ 0.5 % / 2.0 %

Accuracy and precision ("between-day")		
level	mean conc. found ± SD	accuracy / precision (RSD)
1 ng ml^{-1}	1.1 ± 0.08	+ 12.8 % / 7.2 %
10 ng ml^{-1}	8.6 ± 0.5	- 14.5 % / 5.4 %
25 ng ml^{-1}	25.2 ± 1.2	+ 0.6 % / 4.9 %
100 ng ml^{-1}	102.9 ± 2.7	+ 2.9 % / 2.6 %
250 ng ml^{-1}	244.7 ± 7.8	- 2.1 % / 3.2 %
2500 ng ml^{-1}	2352.8 ± 81.1	- 5.9 % / 3.4 %

LOQ= 1 ng ml^{-1}, based on results of accuracy and precision evaluation

LOD= 0.38 ng ml^{-1}, based on average S/N value found for 1 ng ml^{-1} LOQ samples

Specificity: no peaks at retention times of ML-7 and ML-9 in blank sample[**]

Carry over: no peaks in solvent injected after highest calibrator

Stability in extract (15 °C, 24 h): 100.2 % for ML-7, 109.1 % for ML-9

Recovery= 72.1 % for ML-7, 70.4 % for ML-9

Matrix effect		
blank	ML-7	ML-9
blank 1	91.8 %	96.9 %
blank 2	99.3 %	95.6 %
blank 3	98.5 %	94.0 %

[a] Results for the 10 ng ml^{-1} samples ($n=3$) of blank 2 and blank 3 were obtained using the calibration curve obtained with spiked blank 1 samples.
[b] Evaluated for blank 1, 2, and 3.

effect were evaluated on canine, rabbit, chicken, porcine, bovine, and human plasma. The results are summarized in Table 4. As can be seen, the method gives extraction recoveries ranging from 67.6 to 81.3% and from 70.3% to 82.2% for ML-7 and ML-9 respectively, for canine, rabbit, chicken, porcine and human plasma, the lowest values corresponding to canine plasma, while the highest recoveries were obtained for human plasma. These recoveries are comparable to the ones found for feline plasma (see Table 3). Only for bovine plasma, extraction recovery was significantly lower, close to 40%. This is due to the fact that the pellet after the ultracentrifugation step of the plasma sample after the addition of 100 μl 1 N HCl solution was very substantial and with a gelatinous aspect, resulting in a more important loss of analyte during this step, compared to the plasma of other species. However, this lower extraction recovery
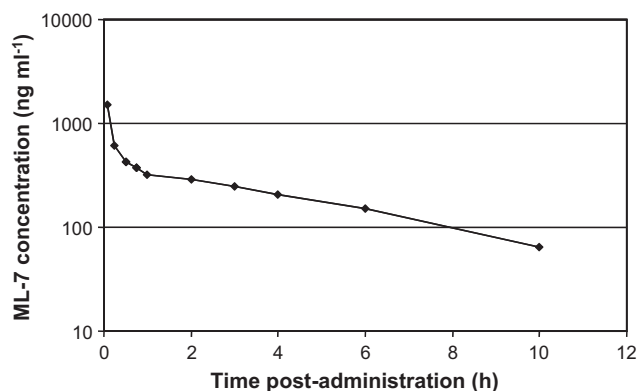
Fig. 3. Typical concentration versus time profile of ML-7 after intravenous administration to a cat at 4 mg/kg BW.

Table 4
Evaluation of extraction recovery and matrix effect of the analysis method on plasma of other species (canine, rabbit, chicken, porcine and bovine plasma) and human plasma, each determination at $n = 3$, with ML-7 and ML-9 levels both at 100 ng ml^{-1}.

Plasma origin	Extraction recovery (%) ML7/ML-9	Matrix effect (%) ML-7/ML-9
Canine	67.6/70.3	107.7/102.0
Rabbit	79.9/79.5	96.7/90.1
Chicken	71.7/75.9	115.3/105.3
Porcine	77.5/78.5	105.5/96.1
Bovine	37.6/41.7	100.4/95.4
Human	81.3/82.2	90.9/94.0

remains reproducible (RSD = 3.5%, $n = 3$). A minor modification of the pre-SPE step might be considered for bovine plasma to increase this lower extraction recovery. The matrix effect is very acceptable for the plasma of all different animal species tested, as well as for human plasma, with the least-positive results being a 15.3% increase of the ML-7 signal in the case of chicken plasma, and a 9.9% decrease of the ML-9 signal in the case of rabbit plasma. The overall ratio of the ML-7 to the ML-9 signal, which is actually used for quantification, of all samples involved in the experiment summarized in Table 4, is 66.1 ± 3.4, giving an RSD = 5.1%, indicating that the quantification of ML-7 is relatively independent of the origin of the plasma, indicating that the method is more generally applicable, and is not designed to be limited for feline plasma only. This can be of interest, since at least also for dogs, systemic corona virus infection has been reported [20].

4. Conclusions

An UPLC–MS/MS method was developed for the quantitation of ML-7 in feline plasma. A solid-phase extraction on an MCX cartridge was found to be necessary as sample clean-up step, to avoid matrix effect on the detection of ML-7 on the MS instrument. However, the method remains rather simple and not very time consuming, allowing the analysis of a sufficient number of samples in a reasonable time period. Validation of the method showed its good performance characteristics, possibly also for plasma of other animal species and human plasma. Also the practical use of the method was shown to determine plasma concentration versus time profiles of ML-7 after its administration to cats, to determine the pharmacokinetics needed to design an adequate dosage regimen.

References

[1] N.C. Pedersen, J. Feline Med. Surg. 11 (2009) 225.
[2] H.L. Dewerchin, E. Cornelissen, H.J. Nauwynck, J. Arch. Virol. 150 (2005) 2483.
[3] H.L. Dewerchin, E. Cornelissen, H.J. Nauwynck, J. Gen. Virol. 87 (2006) 1685.
[4] E. Cornelissen, H.L. Dewerchin, E. Van Hamme, H.J. Nauwynck, J. Vet. Microbiol. 121 (2007) 131.
[5] E. Cornelissen, H.L. Dewerchin, E. Van Hamme, H.J. Nauwynck, J. Virus Res. 144 (2009) 285.
[6] H.L. Dewerchin, E. Cornelissen, E. Van Hamme, K. Smits, B. Verhasselt, H.J. Nauwynck, J. Gen. Virol. 89 (2008) 2731.
[7] M. Saitoh, T. Ishikawa, S. Matsushima, M. Naka, H. Hidaka, J. Biol. Chem. 262 (1987) 7796.
[8] L. Ferrier, L. Mazelin, N. Cenac, P. Desreumaux, A. Janin, D. Emilie, J.F. Colombel, R. Garcia-Villa, J. Fioramonti, L. Bueno, Gastroenterology 125 (2003) 795.
[9] H. Eutamene, V. Theodorou, F. Schmidlin, V. Tondereau, R. Garcia-Villar, C. Salvador-Cartier, M. Chovet, C. Bertrand, L. Bueno, Eur. Respir. J. 25 (2005) 789.
[10] A. Ait-Belgnaoui, S. Bradesi, J. Fioramenti, V. Theodorou, L. Bueno, Pain 113 (2005) 141.
[11] R. Moriez, C. Salvador-Cartier, V. Theodorou, J. Fioramenti, H. Eutamene, L. Bueno, Am. J. Pathol. 167 (2005) 1071.
[12] Z.M. Goeckeler, R.B. Wysolmerski, J. Cell Biol. 130 (1995) 613.
[13] J.R. Turner, B.K. Rill, S.L. Carlson, D. Carnes, R. Kerner, R.J. Mrsny, J.L. Madara, Am. J. Physiol. 273 (1997) C1378.
[14] Commission Decision (2002/657/EC) of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Off. J. EC (L221) (2002) 8.
[15] J. Knecht, G. Stork, Fresen. Z. Anal. Chem. 270 (1974) 97.
[16] Extract of volume 8, notice to applicants, veterinary medicinal products: establishment of maximum residue limits (MRLs) for residues of veterinary products in foodstuffs of animal origin: development and validation of a proposed regulatory method, EMEA/CVMP/573/00-FINAL.
[17] R.J. Heitzman (Ed.), Veterinary Drug Residues, Report Eur 15127-EN, Commission of the EC, Brussels–Luxemburg, 1994.
[18] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng., Anal. Chem. 72 (2003) 3019.
[19] T. Reyns, S. De Baere, S. Croubels, P. De Backer, J. Mass Spectrom. 41 (2006) 1414.
[20] V. Zappulli, D. Caliari, L. Cavicchioli, A. Tinelli, M. Castagnaro, Res. Vet. Sci. 84 (2008) 278.