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Determination of clindamycin in animal plasma by high-performance liquid chromatography combined with electrospray ionization mass spectrometry

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A method for the quantification of clindamycin in animal plasma using high-performance liquid chromatography combined with electrospray ionization mass spectrometry (LC/ESI-MS/MS) is presented. Lincomycin is used as the internal standard. The sample preparation includes a simple deproteinization step with trichloroacetic acid. Chromatographic separation is achieved on an RP-18 Hypersil column using gradient elution with 0.01 M ammonium acetate and acetonitrile as mobile phase. Good linearity was observed in the range 0–10 μg ml^{-1}. The limit of quantification of the method is 50 ng ml^{-1} and the limit of detection is 1.3 ng ml^{-1}. The method was shown out to be of use for pharmacokinetic studies of clindamycin formulations in dogs. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: clindamycin; pharmacokinetics; liquid chromatography/electrospray ionization tandem mass spectrometry; quantification; validation; plasma

INTRODUCTION

Clindamycin is a lincosamide antibiotic. The other member of this antibiotic family is lincomycin. Clindamycin is active against most Gram-positive bacteria, anaerobes and mycoplasma. Most Gram-negative bacteria are resistant towards this antibiotic. It is therefore used for the treatment of serious interior infections of Gram-positive bacteria and anaerobes, such as abscesses, prostatitis, arthritis, osteomyelitis and pyodermatitis.

Procedures for the determination of clindamycin in human plasma using high-performance liquid chromatography (HPLC) with UV detection have been described in the literature.[1–3] These methods are either not very sensitive or use time-consuming and labor-intensive sample preparation protocols. Only recently, two methods using HPLC coupled with mass spectrometric (MS) detection have been published for the quantification of clindamycin in human plasma.[4,5] Yu *et al*.[4] used an LC/ESI-MS/MS method, having acceptable linearity in the 0.05–20 μg ml^{-1} range and a limit of quantification of 0.050 μg ml^{-1}. Martens-Lobenhoffer and Banditt[5] used an LC/atmospheric pressure chemical ionization (APCI)-MS (not MS/MS) method for both human plasma and bone. The method showed good linearity in the 0.1–4 μg ml^{-1} range for plasma with a limit of quantification of 0.1 μg ml^{-1}.

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In this paper, we describe the development and validation of an LC/ESI-MS/MS method for the quantification of clindamycin in animal (dog) plasma. It has three principal advantageous modifications compared with the previously mentioned methods.[4,5]

1. It involves a minimal sample preparation, consisting of a deproteinization step with trichloroacetic acid. Most of the previously reported methods use acetonitrile for this purpose, which is not always beneficial for the LC part of the method: changing retention times, peak broadening, etc., especially when larger injection volumes are used.
2. The gradient elution used allows for a compromise between good retention of the components (low organic phase at the start of the run) and sufficient rinsing of the column between runs (high organic phase during the run). The previous isocratic LC/MS methods[4,5] are very fast (2 and 5 min, respectively), owing to the high initial organic phase content, but there is poor retention of the components of interest on the LC column, which can influence ionization (in various ways), since the components enter the mass spectrometer simultaneously with all non-retained matrix components.
3. The use of an MS-compatible buffered mobile phase (0.01 M ammonium acetate) avoids the use of an ion-pairing reagent such as TFA, often associated with non-reproducible retention times, and used in both the LC/MS methods published earlier for clindamycin detection.[4,5]

EXPERIMENTAL

Chemicals and standards

Clindamycin hydrochloride was a CRS standard from the European Pharmacopoeia (Strasbourg, France). A stock solution of 1000 μg ml^{-1} was prepared in methanol and stored at −20 °C. Working solutions at clindamycin concentrations of 50, 37.5, 25, 12.5 and 5 μg ml^{-1} were prepared by appropriate dilution of the stock solution with water. By spiking 200 μl plasma with 50 μl of these working solutions or 50 μl of water, clindamycin concentrations of 10, 7.5, 5, 2.5, 1, and 0 μg ml^{-1} plasma respectively (= calibration curve A: 0–10 ppm) were obtained. In the same way, working solutions at clindamycin concentrations of 2.5, 1.25, 0.5 and 0.25 μg ml^{-1} were prepared, yielding calibrators of 0.5, 0.25, 0.1 and 0.05 μg ml^{-1} plasma, respectively, which were used in combination with a 1 and a 0 μg ml^{-1} plasma sample (for preparation, see above) to construct a calibration curve B (0–1 ppm). All the working solutions were stored in a refrigerator (2–8 °C). Clindamycin stock and working solutions were stable for only 2 weeks, if stored as mentioned above.

Lincomycin, used as internal standard (IS), was purchased from Sigma (Bornem, Belgium). A stock solution of 1000 μg ml^{-1} was prepared in methanol. The storage conditions were the same as for the clindamycin stock solution. A working solution at a concentration of 12.5 μg ml^{-1} was prepared by appropriate dilution of the stock solution with water. By spiking 250 μl of plasma with 50 μl of this working solution, a lincomycin concentration of 2.5 μg ml^{-1} was obtained. The internal standard working solution was also stored in a refrigerator (2–8 °C). Lincomycin stock and working solutions were stable for at least 1 month, if stored as mentioned above.

The solvents used for the mobile phase (water and acetonitrile) were of HPLC grade and were purchased from Acros (Geel, Belgium). Ammonium acetate, used as an additive to the mobile phase, was of analytical grade and purchased from Sigma. Trichloroacetic acid (TCA) and trifluoroacetic acid (TFA) were of analytical-reagent grade and were purchased from Merck (Darmstadt, Germany).

Biological samples

Known clindamycin-free plasma samples were obtained from Beagle dogs, which had not received clindamycin or lincomycin. Incurred plasma samples were obtained from dogs that had been treated orally with clindamycin tablets at a therapeutic dose. Blood was taken until 48 h after treatment. Plasma was removed by centrifugation and stored at −20 °C until analysis.

Plasma sample preparation

A 250 μl plasma sample was transferred into a capped 1.5 ml Eppendorff vial and spiked with 50 μl of the working solution of 12.5 μg ml^{-1} of the IS lincomycin. After vortex mixing for 15 s, 50 μl of a 20% (w/v) TCA solution in water were added. The sample was again vortex mixed for 15 s. After centrifugation at 6800 *g* for 10 min, the supernatant was transferred into an autosampler vial and a 20 μl aliquot was injected on to the HPLC column.

Chromatography

The HPLC system consisted of a P4000 quaternary gradient pump, an AS3000 autosampler with cooling device and a degassing kit using helium to sparge the eluents (all from Thermo Separation Products, ThermoQuest, San Jose, CA, USA). Chromatographic separation was achieved using an RP-18 Hypersil column (5 μm, 100 mm × 3 mm i.d.), in combination with a guard column of the same type (25 mm × 3 mm i.d.), from Chrompack (Middelburg, The Netherlands). Mobile phase A was a solution of 0.01 M ammonium acetate in water and the mobile phase B was acetonitrile. Mobile phase was delivered to the HPLC column at a constant flow-rate of 0.2 ml min^{-1}. The following gradient elution was used: 0 min, 70% A–30% B; 0–0.5 min, linear gradient to 30% A–70% B; 0.5–8 min, 30% A–70% B; 8–8.5 min, linear gradient to 70% A–30% B; 8.5–18 min, 70% A–30% B.

Mass spectrometry

The HPLC column effluent was pumped to an LCQ ion-trap mass spectrometer instrument (Finnigan MAT, ThermoQuest), equipped with an ESI ion source, which was used in the positive ion MS/MS mode. A divert valve was used to divert the HPLC effluent to the waste during the first 3 min and the last 8 min of the run. The instrument was calibrated with a solution of caffeine, MRFA (L-methionylarginylphenylalanylalanine acetate·H_2O) and Ultramark 1621, according to manufacturer's instructions. Thereafter, the instrument was tuned by direct infusion of a solution of 20 μg ml^{-1} clindamycin in the ESI source at 3 μl min^{-1}, followed by an infusion of the same solution in combination with the LC flow using a T-union. The following tune parameters were retained for optimal clindamycin detection: spray voltage, 3.5 kV; sheath gas flow-rate, 80 (arbitrary units); auxiliary gas flow-rate, 0 (arbitrary units); capillary voltage, 5 V; capillary temperature, 200 °C; tube lens offset, 5 V; octapole 1 offset, −2 V; lens voltage, −16 V; octapole 2 offset, −5 V; and octapole r.f. amplitude, 400 V_{p-p}. These tune parameters were also suitable for lincomycin detection, given the structural similarity between both components. The optimal collision energy in the MS/MS mode, corresponding to (nearly) 100% fragmentation of the protonated molecular ions of both clindamycin and lincomycin (m/z 425.3 and 407.3 respectively, for M_r of 424.99 and 406.54, respectively), was found to be 1.2 V. Under these conditions, typical product ions at m/z 126.3, 377.2 and 389.2 were obtained for clindamycin and at m/z 126.3, 359.2 and 389.2 for lincomycin. Quantification was effected with the LCQuan software (ThermoQuest), using the above-mentioned product ions. The first 6 min of the run the detector was scanning for lincomycin alone, then for the rest of the run it was scanning for clindamycin alone.

Validation criteria

The proposed method for the quantitative determination of clindamycin was validated by a set of parameters which are in compliance with the recommendations as defined by the EC:[6–8]

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1. Linearity: determined on calibration curves using spiked blank plasma samples (for levels, see the Chemicals and standards section). Peak area ratios between clindamycin and lincomycin were plotted against the concentration of clindamycin and a linear regression was performed. The acceptance criterion was then that the correlation coefficient r must be ≥ 0.99[8] and that the goodness of fit coefficient must be below 10%.[9]
2. Trueness: determined by analyzing six independent spiked blank plasma samples at the same spike level (two levels evaluated: 0.5 and 5 ppm). The trueness, expressed as the difference between the mean found concentration and the spiked concentration (in %), must be in the range −20% to +10%.[8]
3. Precision: expressed as the relative standard deviation (RSD, %), being the ratio between the standard deviation (SD) and the mean found concentration (%). It must fall within the values calculated according to the Horwitz equation: $RSD_{max} = RSD \times 2/3$, with $RSD = 2^{(1-0.5 \log C)}$.[8] It is determined on the same samples as for the trueness for the within-day precision, while the between-day precision is evaluated on samples with the same spike levels prepared and analyzed on different days.
4. Limit of quantification (LOQ): determined as the lowest concentration for which the method is validated with a trueness and precision that fall within the ranges recommended by the EU.[8]
5. Limit of detection (LOD): determined as the lowest measured content from which it is possible to deduce the presence of the analyte with reasonable statistical certainty, using the criterion of a signal-to-noise (S/N) ratio of 3.[8]

RESULTS AND DISCUSSION

Sample preparation and chromatography

Three different sample preparation protocols were evaluated, both with an original plasma sample volume of 250 µl, one using a concentrated 20% TCA solution, the second using concentrated TFA and the third using acetonitrile, to deproteinize the plasma samples. The added volumes of these reagents were 50, 20 and 250 µl, respectively. The recoveries were comparable in the three cases, which is not surprising since there is no real extraction. However, in the case of TFA and acetonitrile addition, there were problems with the chromatography of lincomycin especially, leading to the choice of the TCA procedure as the most practical one. On the TFA plasma extracts, there was a shift in retention time of lincomycin, when compared with a standard solution. Lincomycin then eluted shortly after the front and presented a distorted peak shape. With acetonitrile plasma extracts, an earlier eluting minor peak was observed on the lincomycin mass chromatogram, which in some cases was not completely separated from the major lincomycin peak, rendering reproducible integration of the lincomycin signal difficult. Clindamycin and lincomycin were both stable in the TCA plasma extracts, despite the low pH, for at least 48 h.

The aqueous part of the mobile phase consisted of 0.01 M ammonium acetate without pH adjustment. This mobile phase is fully compatible with LC/MS/MS, in contrast with the phosphate buffers used for some LC methods with UV detection.[2,3] It does not include the use of an ion-pairing reagent, which represents a further advantage over the mobile phase containing TFA used for MS detection in earlier papers,[4,5] or other ion-pairing reagents such as tetramethylammonium chloride.[1] Gradient elution was performed to obtain a good quality chromatogram for both components. Lincomycin has a poor retention and therefore the initial organic phase content of the mobile phase was set at only 30%. Lincomycin then elutes at 4.0 min (see Fig. 2). Immediately after the start of the run, the organic phase was increased to 70%, to elute also clindamycin as a sharp peak within a reasonable time interval (7.4 min) (see Fig. 2). This has the simultaneous advantage that the column is well rinsed between sample injections. Note that with the LC conditions used in the previously described LC/MS methods,[4,5] only poor retention of the components was observed, owing to the isocratic elution with high organic phase content, which might result in a simultaneous load of the source of the mass spectrometer with the components of interest and all non-retained matrix components, which can influence the ionization process. It is worth mentioning that we also evaluated initially UV detection at 201 nm as a means of clindamycin quantification, but two factors made this impossible. First, clindamycin produces only a weak UV signal owing to the lack of real chromophores and the detection sensitivity was therefore too low. Second, several important matrix interferences were observed owing to the low wavelength at which clindamycin absorbs.

Mass spectrometry

The structures of clindamycin and lincomycin are shown in Fig. 1, together with their MS and MS/MS traces, obtained after direct infusion of a standard solution of 20 µg ml^{-1} in the ESI source. In the MS mode, the most prominent ion for both compounds is the protonated molecular ion $[M - H]^+$: at m/z 425.3 for clindamycin and m/z 407.3 for lincomycin. In the MS/MS mode, three prominent product ions are formed for both compounds as can be seen in Fig. 1: at m/z 389.2, 377.2 and 126.3 for clindamycin and at m/z 389.1, 359.2 and 126.3 for lincomycin. For both components, these three product ions were used for quantification. The product ions at m/z 389 correspond to the loss of two and one H_2O, for clindamycin and lincomycin, respectively, those at m/z 377.2 and 359.2 correspond to the loss of CH_3SH and those at m/z 126.3 correspond to the 3-propyl-*N*-methylpyrrolidine ion after loss of the rest of the molecule.[10] For clindamycin, two minor product ions at m/z 335.2 and 407.1 are present in the tandem mass spectrum. The second one corresponds to the loss of one H_2O, and the first one corresponds to some unknown loss of 90 amu. In the negative ion mode, no signal was observed. On the APCI source, the signal was always lower compared with ESI (~75%).

Figure 2 shows different MS/MS mass chromatograms of clindamycin and lincomycin for a blank dog plasma sample, a blank dog plasma sample spiked at the LOQ

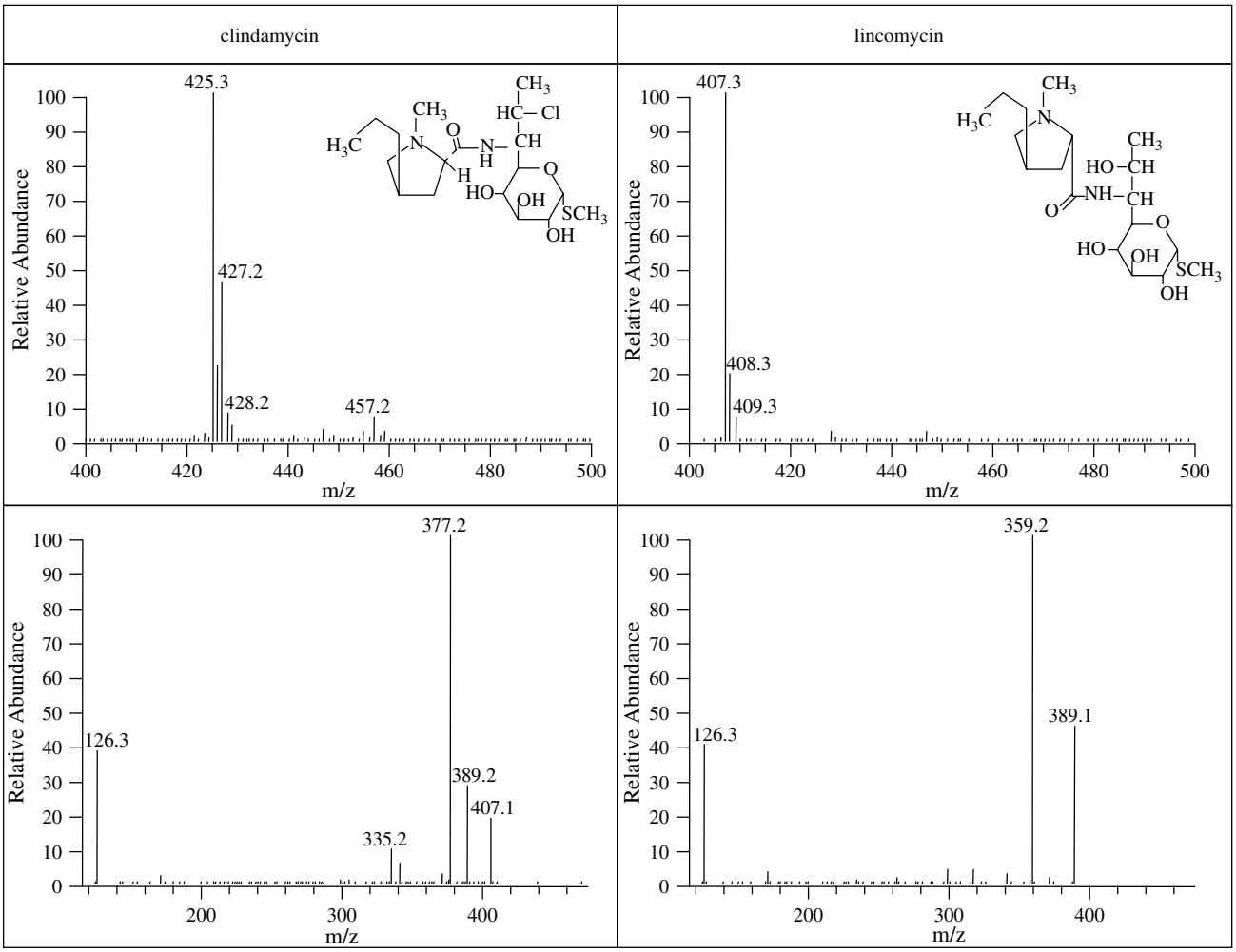

Figure 1. Structure, MS and MS/MS spectra of clindamycin and lincomycin, obtained after direct infusion of standard solutions of 20 μg ml^{-1} of clindamycin and lincomycin (ESI, positive ion mode, collision energy in MS/MS = 1.2 V).

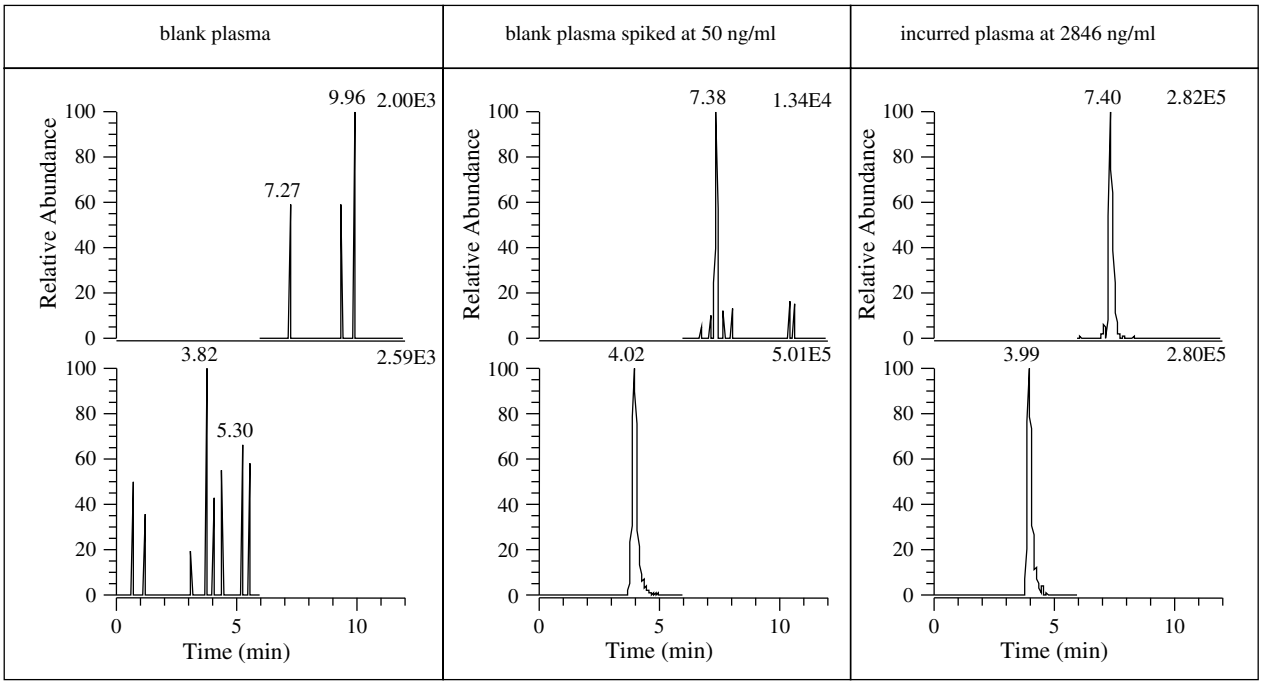

Figure 2. Mass chromatograms of clindamycin (top) and lincomycin (bottom) for a blank plasma sample (left), a blank plasma spiked with 50 ng ml^{-1} (= LOQ) (middle), and an incurred plasma sample at 2846 ng ml^{-1} (right).

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level (= 50 ng ml^{-1}, see below) and an incurred dog plasma sample (clindamycin concentration 2846 ng ml^{-1}). The chromatograms of the blank plasma sample are clean and free from endogenous interferences at the elution time zones of clindamycin and lincomycin, as a consequence of the high specificity of the LC/MS/MS technique. Lincomycin elutes at 4.0 min and clindamycin at 7.4 min, as sharp and symmetrical peaks, as well for the spiked as for the incurred samples.

Method validation

In Fig. 3, both the high ((A) 0–10 ppm) and the low ((B) 0–1 ppm) calibration curves are presented as the means of 14 calibration curves made over a period of more than 30 days, each individual calibration curve being for a new set of extractions. Good linearity was observed: the goodness-of-fit coefficients (g) of the individual calibration curves were all <10% and the correlation coefficients all >0.99.

The trueness and within-day precision of the method were determined using six independently spiked blank plasma samples at 5000 ng ml^{-1} (calibration curve A) and at 500 ng ml^{-1} (calibration curve B). The results are summarized in Table 1. The trueness fell within the range −20 to +10%, testifying to the good trueness of the method. The precision for all matrices also fell within the maximum RSD values. The between-day precision was determined using blank plasma samples independently spiked at the same levels as those mentioned above, and which were used as quality control (QC) samples during a period of sample analysis of more than 30 days. The results are summarized in Table 2. Also here, the trueness and precision fell within the specified ranges. The LOQ was established by analyzing six blank plasma samples, which were spiked with clindamycin at a level of 50 ng ml^{-1}. The results are summarized in Table 1. Since the 50 ng ml^{-1} level could be quantified fulfilling the criteria for trueness and precision, it was set as the LOQ of the method. The LOD was determined using

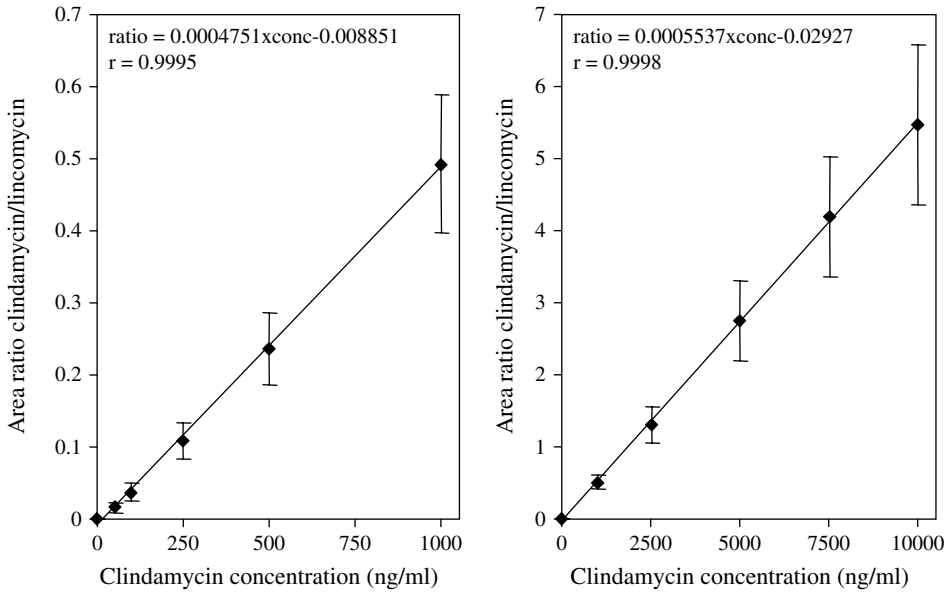

Figure 3. Calibration curves in the high ((A) 0–10 ppm; right) and the low ((B) 0–1 ppm; left) range, represented as the means of 14 calibration curves constructed over a period of more than 30 days, each individual calibration curve being for a new set of extractions.

Table 1. Results of the trueness and within-day precision evaluation experiments

Concentration level (ng ml^{-1})	Measured concentration (average) (ng ml^{-1})	RSD (%)	RSD_{max} (%)	Trueness (%)	n
50	40.6	6.7	16.7	−18.7	6
500	535.9	5.1	11.8	+7.2	6
5000	5079.0	2.7	8.4	+1.6	6

Table 2. Results of the trueness and between-day precision evaluation experiments

Concentration level (ng ml^{-1})	Measured concentration (average) (ng ml^{-1})	RSD (%)	RSD_{max} (%)	Trueness (%)	n
500	490.0	8.9	11.8	−2.0	31
5000	4768.6	5.2	8.4	−4.6	31

the criterion of S/N ratio = 3:1. The mean S/N ratio for the six samples at 50 ng ml^{-1} (see LOQ determination) was 118. This corresponds by calculation to an LOD of 1.3 ng ml^{-1}. The stability of clindamycin in plasma samples between sampling and analysis was verified by analyzing six samples spiked at 1000 ng ml^{-1} and stored thereafter for 40 days at −20 °C. The mean measured value was 1079.7 ng ml^{-1} (trueness = +8.0%, precision = 4.9%).

The above method for the quantitation of clindamycin in dog plasma was used in a pharmacokinetic study, after oral administration of a tablet (results not shown). To demonstrate the practicability and applicability of the method, the following data can be mentioned: total number of unknown incurred samples analyzed, 420; number of calibrators analyzed, 140, for a total of 14 calibration curves; number of QC samples analyzed, 31 at 500 ng ml^{-1}, 31 at 5000 ng ml^{-1} and 15 unknown blind ones. All samples were analyzed on the same HPLC column, while the guard column was replaced twice during the study. All QC samples were within the −20 to +10% limits for trueness, so no batches of samples had to be rejected (Table 2). Only one out of 15 unknown blind QC samples was found to be outside the limits for trueness.

CONCLUSION

The procedure described in this paper for the quantitation of clindamycin in animal plasma is fast, selective and sensitive. The use of an MS detector working in the MS/MS mode allows a minimal sample preparation, namely a simple deproteinization step with TCA. The chromatographic separation of lincomycin and clindamycin is advantageous compared with previously published methods for MS detection:[4,5] the gradient elution, although it prolongs the duration of the LC run, allows for better retention of the components and the use of an MS-compatible buffered mobile phase avoids the use of ion-pairing reagents often associated with poorly reproducible retention times. The good linearity in the 0–10 μg ml^{-1} range and the low limit of quantification (50 ng ml^{-1}) make this LC/ESI-MS/MS method of real practical use for pharmacokinetic studies of clindamycin formulations in e.g., dogs.

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