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Determination of amiodarone and desethylamiodarone in horse plasma and urine by high-performance liquid chromatography combined with UV detection and electrospray ionization mass spectrometry

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Abstract

A rapid method for the quantification of amiodarone and desethylamiodarone in animal plasma using high-performance liquid chromatography combined with UV detection (HPLC–UV) is presented. The sample preparation includes a simple deproteinisation step with acetonitrile. In addition, a sensitive method for the quantification of amiodarone and desethylamiodarone in horse plasma and urine using high-performance liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) is described. The sample preparation includes a solid-phase extraction (SPE) with a SCX column. Tamoxifen is used as an internal standard for both chromatographic methods. Chromatographic separation is achieved on an ODS Hypersil column using isocratic elution with 0.01% diethylamine and acetonitrile as mobile phase for the HPLC–UV method and with 0.1% formic acid and acetonitrile as mobile phase for the LC–MS/MS method. For the HPLC–UV method, good linearity was observed in the range 0–5 μg ml^{-1}, and in the range 0–1 μg ml^{-1} for the LC–MS/MS method. The limit of quantification (LOQ) was set at 50 and 5 ng ml^{-1} for the HPLC–UV method and the LC–MS/MS method, respectively. For the UV method, the limit of detection (LOD) was 15 and 10 ng ml^{-1} for amiodarone and desethylamiodarone, respectively. The LODs of the LC–MS/MS method in plasma were much lower, i.e. 0.10 and 0.04 ng ml^{-1} for amiodarone and desethylamiodarone, respectively. The LODs obtained for the urine samples were 0.16 and 0.09 ng ml^{-1} for amiodarone and desethylamiodarone, respectively. The methods were shown to be of use in horses. The rapid HPLC–UV method was used for therapeutic drug monitoring after amiodarone treatment, while the LC–MS/MS method showed its applicability for single dose pharmacokinetic studies.

Keywords: Amiodarone; Desethylamiodarone; High-performance liquid chromatography; Liquid chromatography/electrospray ionization tandem mass spectrometry; Quantification; Validation; Plasma; Urine

1. Introduction

Amiodarone is a Class III antiarrhythmic agent. It is used for treatment of many ventricular and supraventricular arrhythmias, including atrial fibrillation. Desethylamiodarone is the main and *N*-dealkylated metabolite of amiodarone, which has similar pharmacodynamic activity.

Many procedures for the determination of amiodarone and desethylamiodarone in plasma using high-performance liquid chromatography with UV detection (HPLC–UV) have been described in the literature [1–11]. In most of the publications the limits of quantification (LOQs) ranged from 50 to 250 ng ml^{-1}. Sample preparation involved deproteinisation and liquid-liquid extraction. Recently, one method using HPLC coupled with mass spectrometric (MS) detection has been published for the quantification of amiodarone and desethylamiodarone in human plasma by Kollroser and Schober [12]. In this method, a very low limit of detection (LOD) (1 ng ml^{-1} for amiodarone and 0.5 ng ml^{-1} for desethylamiodarone) was found, but the LOQ

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was set at 50 ng ml^{-1}. It seems that this methodology has potential for achieving a much lower LOQ, which is of interest when studying elimination characteristics of the drug in pharmacokinetic studies. None of these manuscripts report the analysis of amiodarone and desethylamiodarone in urine.

The purpose of our study was to develop a rapid method for therapeutic drug monitoring of high concentrations (μg ml^{-1} range) of amiodarone and desethylamiodarone in horse plasma with HPLC–UV and to develop a sensitive method for the determination of low concentrations (ng ml^{-1} range) of amiodarone and desethylamiodarone in horse plasma and urine with liquid chromatography combined with tandem mass spectrometry (LC–MS/MS).

2. Experimental

2.1. Biological samples

The plasma samples for the HPLC–UV method originated from horses that were treated with an amiodarone infusion for atrial fibrillation [13].

The plasma and urine samples for the LC–MS/MS method originated from a single dose pharmacokinetic study of amiodarone in horses [14].

2.2. Chemicals and standards

Both amiodarone hydrochloride and desethylamiodarone were a gift from Sanofi (Brussels, Belgium). Tamoxifen was purchased from Sigma (Bornem, Belgium). HPLC grade water and methanol, isopropanol and methylene chloride were obtained from Acros (Geel, Belgium). Ammonia 25% and fuming hydrochloric acid 37% were purchased from Merck (Darmstadt, Germany). Acetic acid was delivered by Aldrich (Bornem, Belgium) and diethylamine by VWR (Leuven, Belgium). Isolute® SCX solid-phase extraction (SPE) cartridges (3 ml/100 mg) were supplied by Sopachem (Brussels, Belgium).

2.3. Instruments and methods

2.3.1. HPLC–UV

The HPLC–UV system consisted of a quaternary gradient pump P4000, an autosampler AS3000 with cooling device, an UV–DAD detector type UV 6000LP (all from Thermo Finnigan, San Jose, USA), run by PC 1000 software.

An ODS Hypersil column (5 μm, 100 mm × 3.0 mm i.d.) from Thermo Finnigan with a guard column of the same type (5 μm, 10 mm × 3.0 mm i.d.) was used for chromatographic separation. An isocratic run of 15 min was performed with a mobile phase of acetonitrile–0.01% diethylamine in water (80/20, v/v) and a flow-rate of 1.0 ml min^{-1}. The UV-detector was set at a wavelength of 245 nm.

2.3.2. LC–ESI–MS/MS

The LC–MS/MS analyses were performed using a Surveyor LC system consisting of a vacuum degasser, a quaternary pump, an autosampler with cooling device and a LCQ Advantage ion trap mass spectrometer equipped with an ESI source (all from Thermo Finnigan), run by XCALIBUR software (version 1.3).

For chromatographic separation, an ODS Hypersil Gold column (5 μm, 100 mm × 2.1 mm i.d.) from Thermo Finnigan with a guard column of the same type (5 μm, 10 mm × 2.1 mm i.d.) was used. An isocratic run of 5 min was performed with a mobile phase of acetonitrile–0.1% formic acid in water (80/20, v/v) at a flow-rate of 0.2 ml min^{-1}.

Operating conditions for the ESI source used in the positive ionization mode were optimized by constantly adding a mixture of amiodarone, desethylamiodarone and tamoxifen each at a concentration of 1 μg ml^{-1} in methanol to the HPLC flow by a syringe pump via a T connector in the infusion mode. The following tune parameters were obtained for optimal amiodarone detection: spray voltage, 4.5 kV; sheath gas flow-rate, 80 (arbitrary units); auxiliary gas flow-rate, 20 (arbitrary units); capillary voltage, 3 V; capillary temperature, 200 °C; tube lens offset, −16 V; octapole 1 offset, −2.75 V; lens voltage, 55 V; octapole 2 offset, −5 V and octapole r.f. amplitude, 400 $V_{p\text{-}p}$. These tune parameters were also suitable for detection of desethylamiodarone, given the structural similarity between these components, and were also useful for the detection of the internal standard tamoxifen. The optimal collision energy in the MS–MS mode, corresponding to nearly 100% fragmentation of the protonated molecular ions of amiodarone (*m/z* 646 for M_r of 645.31), was found to be 1.8 V. For desethylamiodarone (*m/z* 618 for M_r of 617.25) the optimal collision energy was determined at 2.3 V and at 2.0 V for tamoxifen (*m/z* 372 for M_r of 371.51). Under these conditions, most abundant product ions at *m/z* 573, 547 and 327 were obtained for amiodarone, desethylamiodarone and tamoxifen, respectively. Quantification was effected with the LCQuan software, using the above-mentioned product ions.

2.4. HPLC–UV extraction method

2.4.1. Calibration standards and quality control samples

Stock solutions of amiodarone, desethylamiodarone and tamoxifen (internal standard), of 1000 μg ml^{-1} were prepared in methanol and stored at −20 °C. The stock solutions of amiodarone and desethylamiodarone were combined and diluted with acetonitrile to obtain working solutions containing 0.05, 0.1, 0.2, 1, 2, 5 and 10 μg ml^{-1} amiodarone and desethylamiodarone. By adding 50 μl of these working solutions or 50 μl acetonitrile to 100 μl of plasma, amiodarone and desethylamiodarone concentrations of 25, 50, 100, 500, 1000, 2500, 5000 and 0 ng ml^{-1} in plasma, respectively, were obtained. All the working solutions were stored in a refrigerator (2–8 °C). The stock solution of the internal standard was also diluted in acetonitrile to a final concentration of 10 μg ml^{-1}. Quality control samples were prepared in a similar way using the working solution of 1 μg ml^{-1} yielding a concentration of 500 ng ml^{-1} of each analyte.

2.4.2. Sample preparation

A 100 μl plasma sample was transferred into a capped 1.5 ml Eppendorff vial. All plasma samples, including calibration standards and quality control samples, were spiked with 100 μl of the

diluted internal standard (10 μg ml^{-1}), except for the blanks. To all samples acetonitrile was added to a final volume of 300 μl. After vortex mixing for 30 s, the samples were centrifuged at 10,000 × *g* for 10 min. The supernatant was transferred into an autosampler vial and a 50 μl aliquot was injected onto the HPLC column.

2.5. LC–MS/MS extraction method

2.5.1. Calibration standards and quality control samples

Working solutions of amiodarone and desethylamiodarone were prepared by diluting the stock solution (for preparation, see above) with methanol. Combined working solutions of amiodarone and desethylamiodarone concentrations of 0.05, 0.1, 0.25, 0.5, 1, 5, and 10 μg ml^{-1} were obtained. By spiking 900 μl blank plasma or urine with 100 μl of these working solutions or 100 μl methanol, amiodarone and desethylamiodarone concentrations of 5, 10, 25, 50, 100, 500, 1000 and 0 ng ml^{-1} in plasma, respectively, were obtained. These working solutions were also stored in a refrigerator (2–8 °C). The stock solution of the internal standard was diluted with methanol to a final concentration of 10 μg ml^{-1}. In a similar way quality control samples were prepared using the working solution of 0.1 μg ml^{-1} yielding a concentration of 10 ng ml^{-1} plasma or urine of each analyte.

2.5.2. Sample preparation

An aliquot of 1000 μl plasma or urine sample was transferred into a capped 1.5 ml Eppendorf vial and spiked with 100 μl of the working solution of 10 μg ml^{-1} of the IS tamoxifen. To all samples, excluding the calibration standards and the quality control samples, an additional 100 μl of methanol was added to obtain the same grade of deproteinisation. After vortex mixing for 15 s, 25 μl glacial acetic acid was added. The sample was again vortex mixed for 30 s and centrifugated at 10,000 × *g* for 10 min.

Solid phase extraction was performed using Isolute® SCX cartridges, a vacuum manifold device and a vacuum source (Alltech, Lokeren, Belgium). SPE cartridges were conditioned and equilibrated with 1 ml of methanol and 1 ml of water. The supernatant of the acidified sample was applied to the cartridge and passed slowly through the bed. Cartridges were sequentially washed with 1 ml of 0.1 M hydrochloric acid and 1 ml of methanol. Analytes were eluted with 2 ml of methylene chloride/isopropanol/ammonia (78/20/2, v/v/v). The elution liquid was evaporated at 40 °C under a gentle nitrogen stream. The

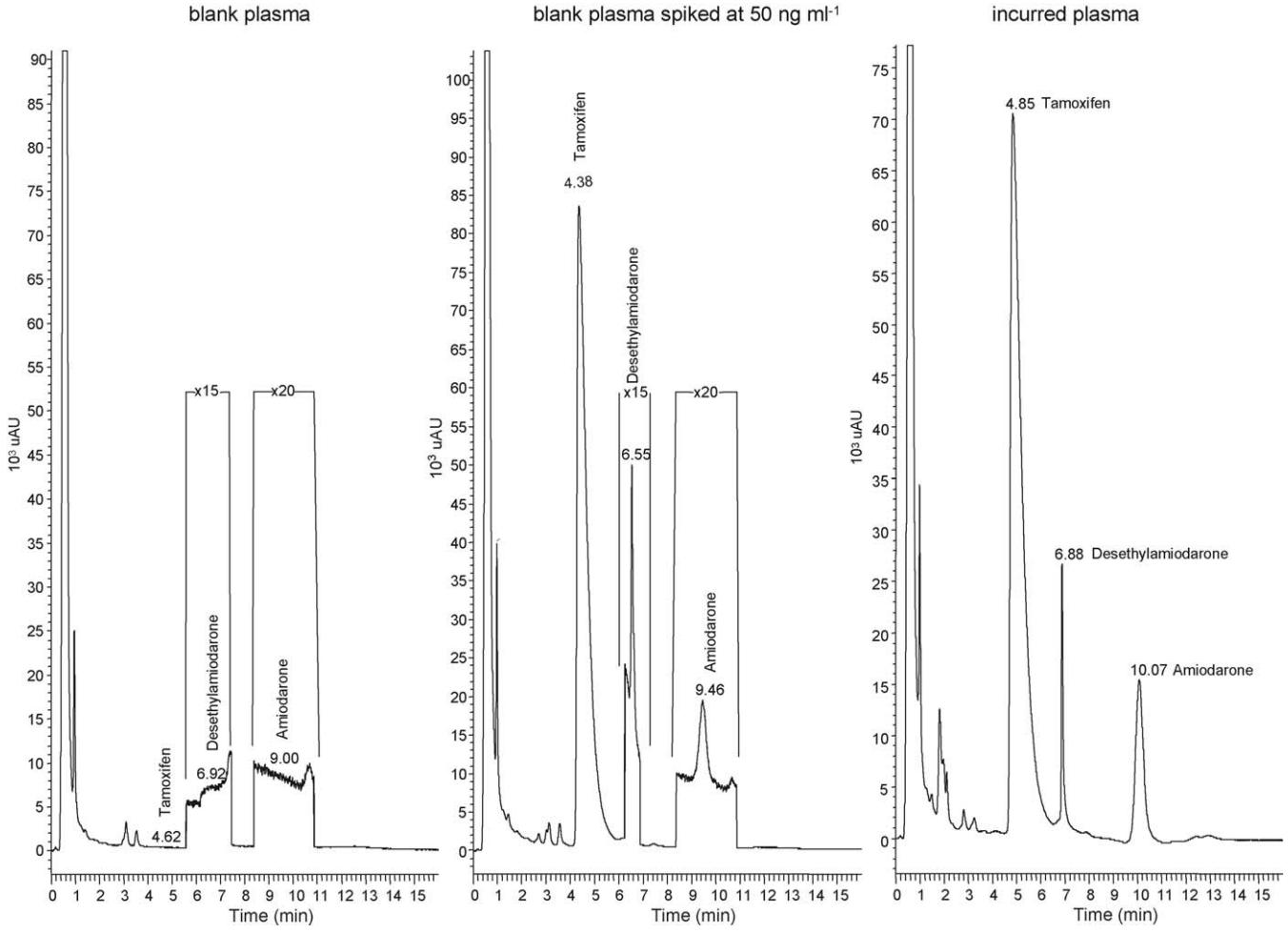

Fig. 1. UV chromatogram of amiodarone, desethylamiodarone and tamoxifen for a blank plasma sample (left), a blank plasma spiked with 50 ng ml^{-1} (=LOQ) (middle) and an incurred plasma sample containing 1682 ng ml^{-1} amiodarone and 542 ng ml^{-1} desethylamiodarone (right).

dry samples were dissolved in 250 μl of 0.1% formic acid in water/methanol (20/80, v/v). An aliquot of 100 μl was injected.

3. Validation procedure

The proposed methods for the quantitative determination of amiodarone and metabolite was validated by a set of parameters which are in compliance with the recommendations as defined by the EC [15–17]:

1. *Linearity*: Determined on calibration curves using spiked blank plasma or urine samples (for levels, see Sections 2.4.1 and 2.5.1). Peak area ratios between amiodarone or desethylamiodarone and tamoxifen were plotted against the concentration of amiodarone or desethylamiodarone and a linear regression was performed. The acceptance criterion was a correlation coefficient $r \geq 0.99$ and a goodness-of-fit coefficient $\leq 10\%$.
2. *Trueness*: Determined by analyzing six independently spiked blank plasma or urine samples at the same spike level (two levels evaluated for the HPLC–UV method: 100 and 1000 ng ml^{-1}, and two levels for the LC–MS/MS method: 10 and 50 ng ml^{-1}). The trueness (in %), expressed as the difference between the mean found concentration and the spiked concentration, must be in the range −30 to +10% for levels ≤ 10 ng ml^{-1} and −20 to +10% for levels >10 ng ml^{-1}.
3. *Precision*: Expressed as the relative standard deviation (R.S.D., %), being the ratio between the standard deviation (S.D.) and the mean found concentration. For within-day precision, the R.S.D. should be lower than the values calculated according to two thirds of the Horwitz equation: $\text{R.S.D.}_{\max} = 2/3 \times 2^{(1-0.5 \log C)}$, with C being the concentration at which the sample is fortified. It is determined using the same samples as for the trueness.The between-day precision is evaluated on samples with the same spike levels prepared and analyzed on different days. The R.S.D. must be lower than the $\text{R.S.D.}_{\max} = 2^{(1-0.5 \log C)}$.
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.
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.
6. *Ion suppression and matrix effect*: For the ion suppression a post-column infusion technique was used. A blank plasma sample was injected onto the LC–MS instrument.

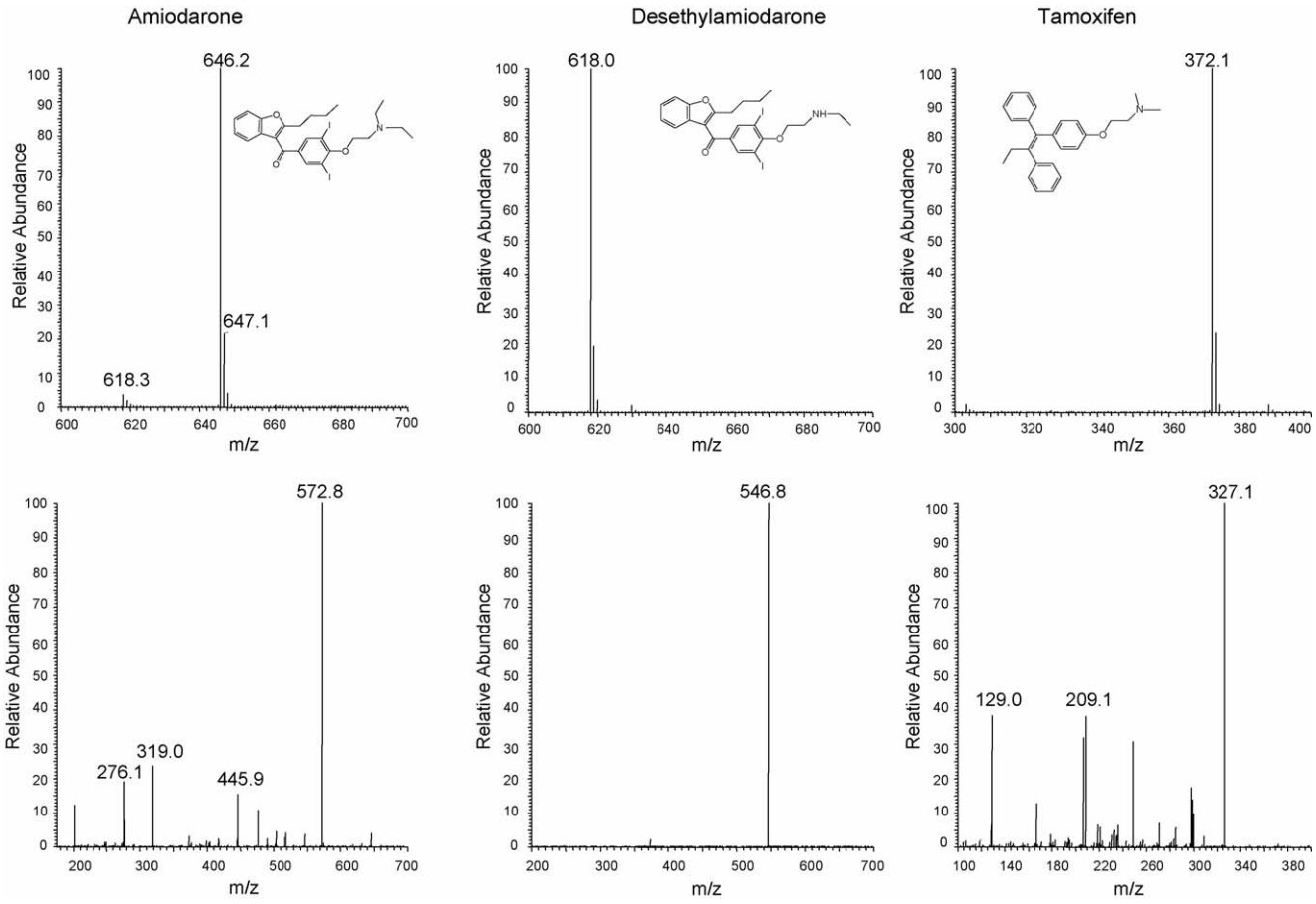

Fig. 2. Structure, MS (upper trace) and MS–MS spectra (lower trace) of amiodarone, desethylamiodarone and tamoxifen, obtained after direct infusion of standard solutions of 1 μg ml^{-1} of amiodarone, desethylamiodarone and tamoxifen (ESI positive mode, collision energy in MS–MS = 1.8, 2.3 and 2.0 V for amiodarone, desethylamiodarone and tamoxifen, respectively).

A standard solution containing amiodarone, desethylamiodarone and tamoxifen, was continuously infused through a T-coupling device into the LC eluate. This allows to visualize sections in the chromatogram where ion suppression is noticed. For the study of the matrix effect, three blank plasma samples were extracted and spiked afterwards with amiodarone, desethylamiodarone and tamoxifen. The peak area ratio (analyte/internal standard) of these samples was compared with the peak area ratio of a standard solution containing the same amount of the three analytes.

4. Results and discussion

4.1. Sample preparation and chromatography

4.1.1. HPLC–UV method

For the chromatographic method several columns were evaluated. A Nucleosil C18 column (Varian, 100 mm × 3.0 mm) gave too much retention so the analytes did not elute at an acceptable time. A polymeric reversed-phase column (Polymer laboratories, 150 mm × 2.1 mm) on the other hand gave too little retention so the peaks eluted with the front. On a Microsorb CN column (Varian, 100 mm × 4.6 mm) and an Inertsil C8 column (Varian, 100 mm × 3 mm), no peak separation was achieved. A Hypersil column (Thermo Finnigan, 100 mm × 3.0 mm) was chosen because a good peak shape and acceptable retention times were obtained. There was also sufficient separation between the components, as can be seen in Fig. 1. Tamoxifen was chosen as internal standard based on an article of Pollak [18] who evaluated different products that can be used as an internal standard.

Fig. 1 shows the chromatograms, obtained with UV detection, of blank horse plasma, blank horse plasma fortified at the LOQ level (50 ng ml^{-1}) and an incurred sample containing 1682 ng ml^{-1} amiodarone and 542 ng ml^{-1} desethylamiodarone. The retention time is 9.7, 6.5 and 4.5 min for amiodarone, desethylamiodarone and tamoxifen, respectively. The chromatogram of the blank sample shows that there is no interference of endogenous components at the elution time zones of amiodarone, desethylamiodarone and tamoxifen.

4.1.2. LC–MS/MS method

The HPLC–UV method was not sensitive enough for analysis of samples to study pharmacokinetic properties (e.g. terminal elimination phase), so there was a need to switch to another, more sensitive, detection system and to concentrate the sample. The concentration of the sample was achieved using SPE-extraction.

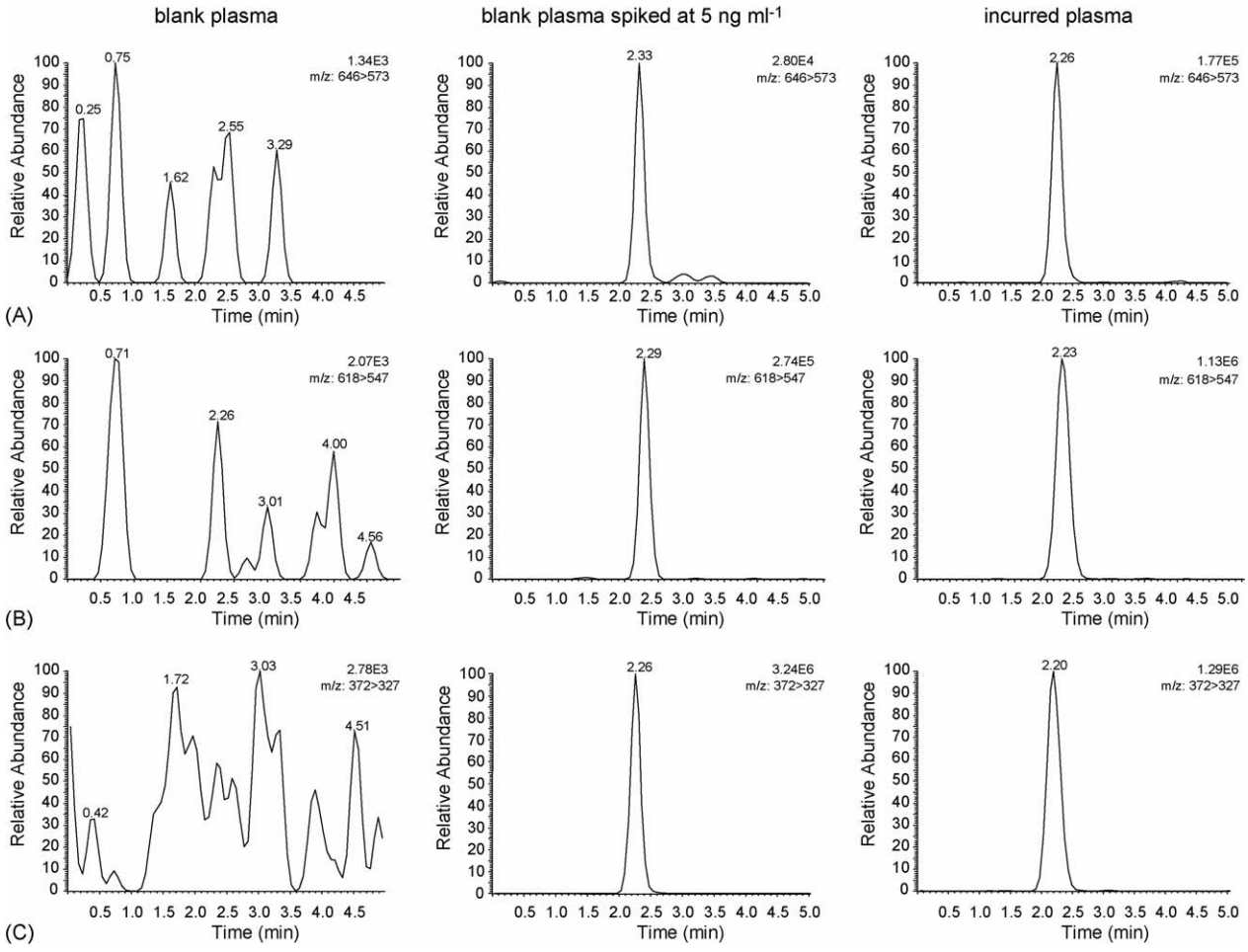

Fig. 3. Extracted-ion chromatogram of amiodarone (A), desethylamiodarone (B) and tamoxifen (C) for a blank plasma sample (left), a blank plasma spiked with 5 ng ml^{-1} (=LOQ) (middle) and an incurred plasma sample containing 113 ng ml^{-1} amiodarone and 75 ng ml^{-1} desethylamiodarone (right).

The organic compound in the solvent used to redissolve the dry residue after SPE was methanol, because a better peak shape was obtained with methanol than with acetonitrile.

The extraction and chromatographic method was based on the method of Kollroser and Schober [12]. However a Hypersil ODS column was used because this type of column was used in our HPLC–UV method. The Hypersil ODS Gold column was chosen because this column produced a better peak shape then the normal Hypersil ODS column. Also an internal diameter of 2.1 mm was optimal for a flow-rate of 0.2 ml/min, used in LC–MS/MS.

Diethylamine, used in the HPLC–UV method, gave ion suppression in LC–MS/MS for all components, so weak signals were obtained. Therefore, mobile phases containing 0.1% acetic acid and 0.1% formic acid were evaluated. With acetic acid in the mobile phase, amiodarone and desethylamiodarone did not elute at an acceptable time. The retention time was over 40 min. On the other hand, with 0.1% formic acid, the analytes eluted much faster and no ion suppression was detected. The retention time of all components was 2.3 min.

In comparison to the extraction method of Kollroser and Schober, orthophosphoric acid was replaced by glacial acetic acid and for solid phase extraction a SCX-column was used instead of a MCX-column, because of availability and price.

Care should be taken to avoid sample spilling after the addition of glacial acetic acid to the urine. This is probably due to the formation of carbon dioxide from bicarbonate after the addition of glacial acetic acid to horse urine.

4.2. Mass spectrometry

The structures of amiodarone, desethylamiodarone and tamoxifen are shown in Fig. 2, together with their MS and MS-MS traces, obtained after direct infusion of a standard solution of 1 $\mu g\,ml^{-1}$ in the ESI source. In the MS mode the most prominent product ion for all compounds is the protonated molecular ion $[M+H]^+$: at *m/z* 646 for amiodarone, *m/z* 618 for desethylamiodarone and *m/z* 372 for tamoxifen. In the MS–MS mode, the most intense product ion, *m/z* 573 for amiodarone, can be explained by the loss of the diethylamino group (loss of *m/z* 73). The loss of *m/z* of 71, the most abundant product ion for desethylamiodarone results in ion at *m/z* 547 and originates from the loss of ethylene-ethylamine. For tamoxifen the most intense

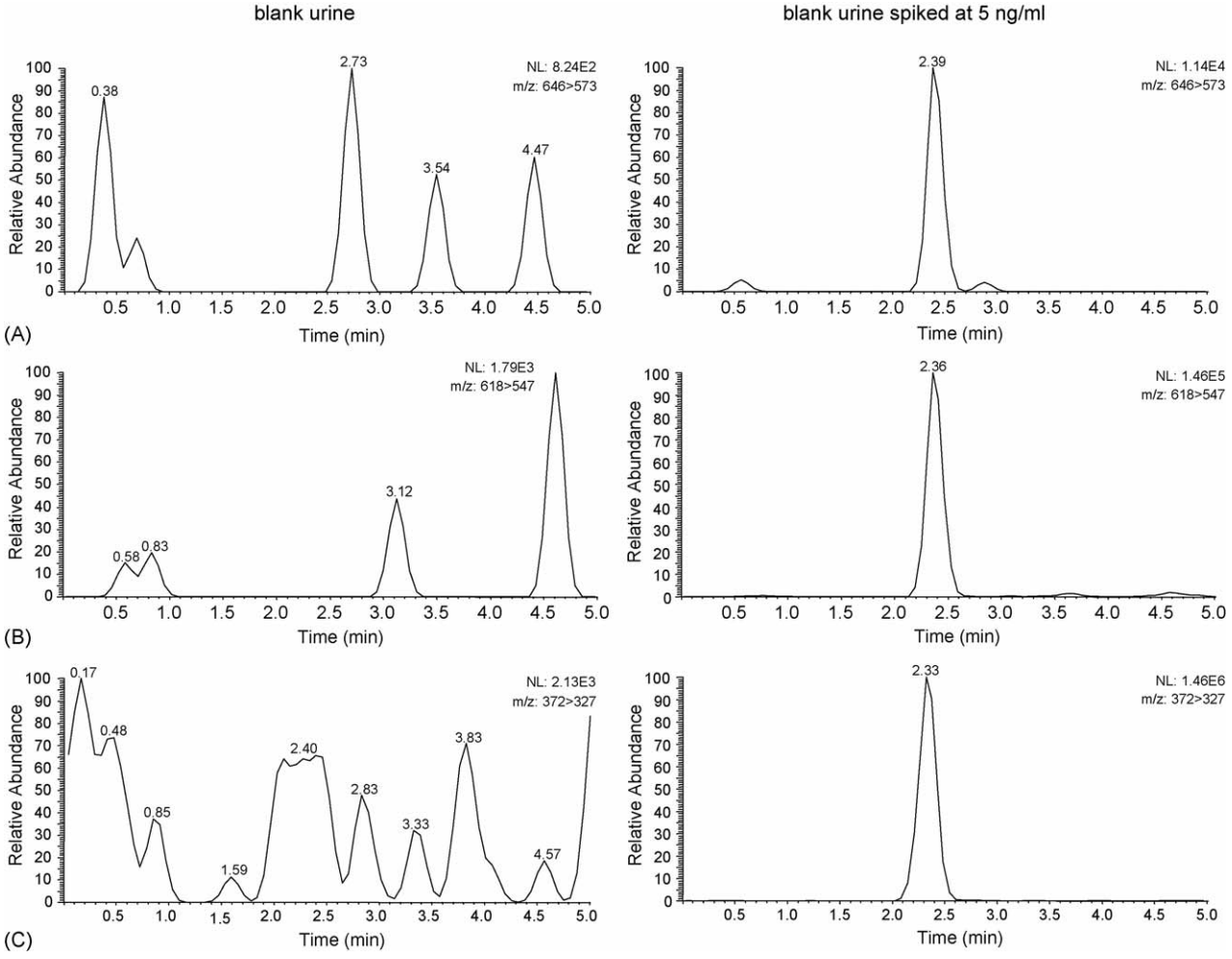

Fig. 4. Extracted-ion chromatogram of amiodarone (A), desethylamiodarone (B) and tamoxifen (C) for a blank urine sample (left) and a blank urine sample spiked with 5 $ng\,ml^{-1}$ (=LOQ) (right).

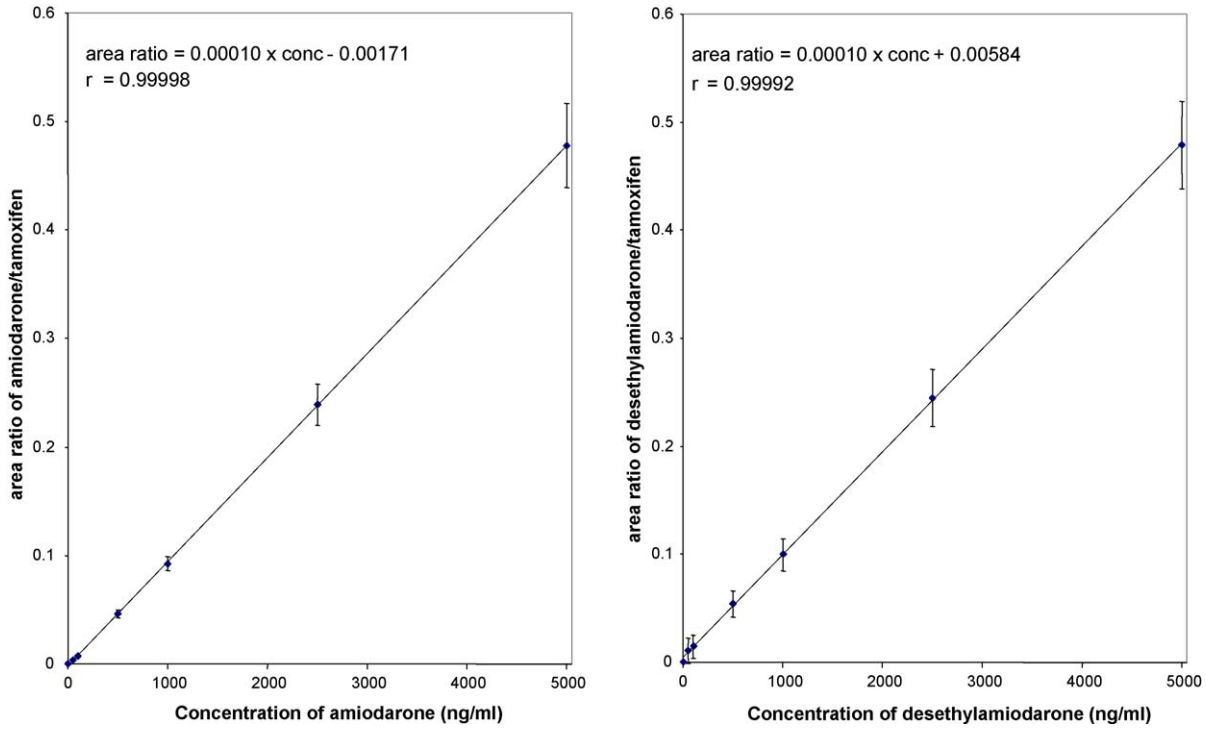

Fig. 5. Calibration curves of amiodarone (left) and desethylamiodarone (right), obtained by the HPLC–UV method, represented by the mean ± S.D. of 5 calibration curves constructed over a period of 24 days, each individual calibration curve resulting from a new set of extractions.

product ion is at *m*/*z* 327. The loss of the dimethylamino group is also proposed.

Fig. 3 shows different extracted-ion chromatograms of amiodarone, desethylamiodarone and tamoxifen for a blank horse plasma sample, a blank horse plasma sample spiked at the LOQ level (5 ng ml^{-1}) and an incurred horse plasma sample (amiodarone concentration: 113 ng ml^{-1}, desethylamiodarone concentration: 57 ng ml^{-1}). The chromatograms of the blank plasma

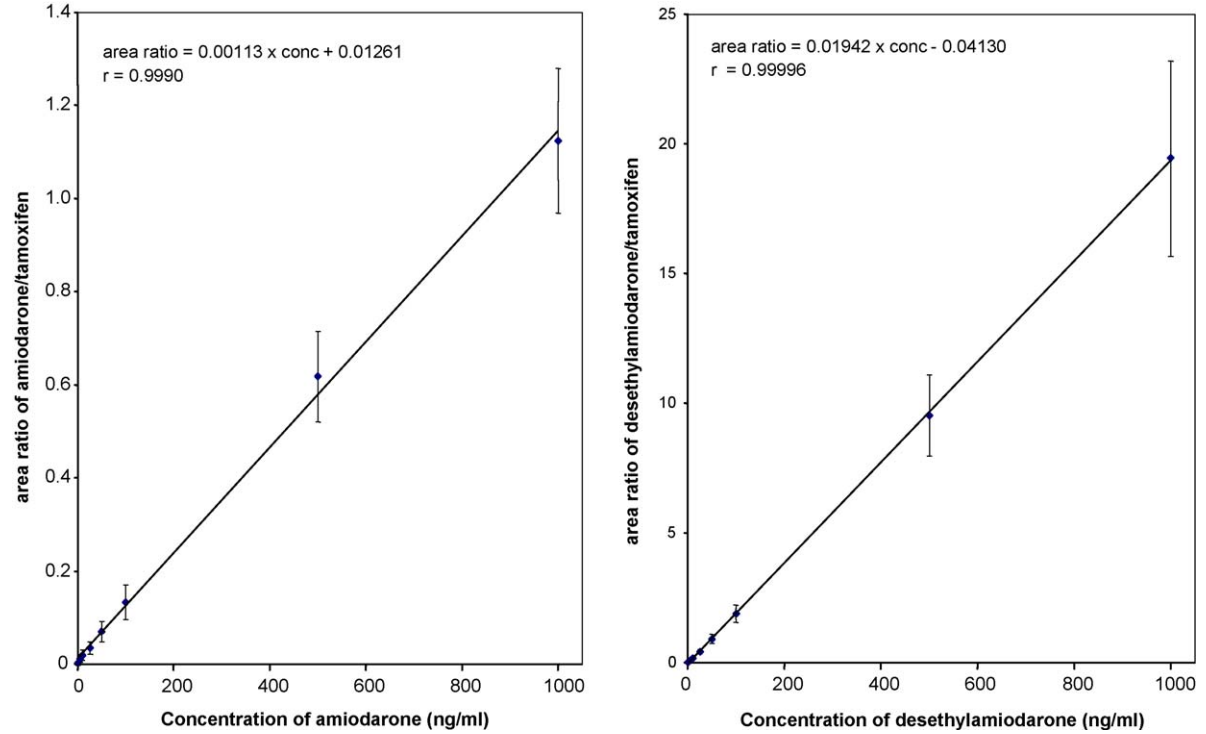

Fig. 6. Calibration curves of amiodarone (left) and desethylamiodarone (right), obtained by the LC–MS/MS method, represented by the mean ± S.D. of 12 calibration curves constructed over a period of 22 days, each individual calibration curve resulting from a new set of extractions.

sample are free from endogenous interferences at the elution time zones of amiodarone, desethylamiodarone and tamoxifen, as a consequence of the high specificity of the LC–MS/MS technique.

The same is shown in Fig. 4 for urine. A chromatogram of an incurred sample is not shown, because all the incurred samples were found to contain no amiodarone, nor desethylamiodarone. These findings indicate the lack of renal excretion of amiodarone and its metabolite in horses, which was not reported previously to our knowledge. Again the chromatogram of a blank horse urine sample is free from endogenous interferences at the elution time zones of the analytes.

4.3. Method validation

In Fig. 5 the calibration curve of the HPLC–UV method of amiodarone and desethylamiodarone is presented as the mean of 5 calibration curves made over a period of 24 days, each calibration curve originating from a new set of extractions. The same is shown in Fig. 6 for the LC–MS/MS method. The mean of 12 calibration curves, made over a period of 22 days, is presented. Good linearity was observed for all calibration curves: 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 100 and 1000 ng ml^{-1} for HPLC–UV and at 10 and 50 ng ml^{-1} for LC–MS/MS. The blank urine samples were independently spiked at 25 and 500 ng ml^{-1}. The results are summarized in Table 1. The trueness fell within the range of −30% to +10% for concentrations ≤10 ng ml^{-1} and −20% and +10% for concentrations >10 ng ml^{-1}, testifying the good trueness of the method. The precision also fell within the maximum R.S.D. values. The between-day precision was determined using blank plasma samples independently spiked at 100 ng ml^{-1} for HPLC–UV and 10 ng ml^{-1} for LC–MS/MS and were used as quality control (QC) samples during the analysis of the incurred samples. The results are also summarized in Table 1. The trueness and precision also fell within the specified ranges.

The LOQ was established by analyzing six blank plasma samples, which were spiked with amiodarone and desethylamiodarone at a level of 50 ng ml^{-1} for HPLC–UV and 5 ng ml^{-1} for LC–MS/MS, for both plasma and urine. The results are summarized in Table 1. Since the 50 and 5 ng ml^{-1} levels could be quantified fulfilling the criteria for trueness and precision, they

Table 1
Results of the trueness, the within-day and between-day precision evaluation experiments of plasma and urine samples obtained by the HPLC–UV method and LC–MS/MS method

HPLC–UV	Amiodarone (ng ml^{-1})			Desethylamiodarone (ng ml^{-1})		
	50.0	100.0	1000.0	50.0	100.0	1000.0
Within-day (n = 6)						
Average	41.9	90.1	1037.4	49.8	95.1	1003.7
R.S.D. (%)	2.0	11.3	2.5	2.8	6.7	4.4
R.S.D.$_{max}$ (%)	16.7	15.1	10.7	16.7	15.1	10.7
Trueness (%)	−16.2	−9.9	+3.7	−0.4	−4.9	+0.4
Between-day (n = 28)						
Average		97.0			92.4	
R.S.D. (%)		8.1			9.0	
R.S.D.$_{max}$ (%)		22.6			22.6	
Trueness (%)		−3.0			−7.6	
LC–MS/MS plasma samples	5.0	10.0	50.0	5.0	10.0	50.0
Within-day (n = 6)						
Average	5.2	7.6	44.7	5.2	10.8	49.2
R.S.D. (%)	8.0	18.8	8.8	6.6	3.3	6.6
R.S.D.$_{max}$ (%)	23.7	21.3	16.7	23.7	21.3	16.7
Trueness (%)	+3.5	−23.9	−10.7	+3.9	+7.5	−1.6
Between-day (n = 28)						
Average		9.7			10.1	
R.S.D. (%)		20.1			11.5	
R.S.D.$_{max}$ (%)		32.0			32.0	
Trueness (%)		−3.3			+0.7	
LC–MS/MS urine samples	5.0	25.0	500.0	5.0	25.0	500.0
Within-day (n = 6)						
Average	4.3	25.2	496.1	4.2	21.9	497.1
R.S.D. (%)	13.0	11.6	9.2	10.4	6.2	9.3
R.S.D.$_{max}$ (%)	23.7	18.6	11.8	23.7	18.6	11.8
Trueness (%)	−13.5	+1.0	−0.8	−15.4	−12.3	−0.6

were set as the LOQ of the HPLC–UV and LC–MS/MS method, respectively.

For the determination of the LODs of the HPLC–UV method, the signal-to-noise (S/N) ratio of the amiodarone and desethylamiodarone peak in the LOQ samples was calculated by measuring the height and the concentrations corresponding to a S/N ratio of 3 were determined. LODs of 15 ng ml^{-1} for amiodarone and 10 ng ml^{-1} for desethylamiodarone were obtained.

The LOD for the LC–MS/MS method was determined using the same criterion of a S/N ratio of 3. For the plasma samples the mean S/N ratio for the six LOQ samples at 5 ng ml^{-1} was 152 for amiodarone and 394 for desethylamiodarone. This corresponds by calculation to an LOD of 0.10 ng ml^{-1} for amiodarone and 0.04 ng ml^{-1} for desethylamiodarone. For the urine samples the mean S/N ratio for the six samples fortified with 5 ng ml^{-1} was 93.5 for amiodarone and 168.0 for desethylamiodarone resulting in an LOD of 0.16 ng ml^{-1} for amiodarone and 0.09 ng ml^{-1} for desethylamiodarone.

Ion suppression was detected with the post-column infusion method, since a decrease in the analyte response was seen. However, because of the coelution of the analytes and the internal standard, no matrix effect was noticed. According to a Student's *t*-test there was no significant difference for both components in the peak area ratio ($\alpha = 0.05$).

4.4. Analysis of biological samples

The above methods for the quantitation of amiodarone and desethylamiodarone in horse plasma were used in two pharmacokinetic studies [13,14]. One study used the fast UV method for quantification of 'high' level samples after intravenous (IV) infusion and the other study used the sensitive LC–MS method for quantification of 'low' level samples after IV bolus administration. The plasma concentration-time profiles of amiodarone in two different horses, administered according to these different protocols, are shown in Fig. 7. The protocols were either an IV infusion at 5 mg/kg/h during 1 h, followed by 0.83 mg/kg/h for 23 h, and then 1.9 mg/kg/h for 30 h or an IV bolus administration at 5 mg/kg. Moreover, to demonstrate further the practicability and applicability of the LC–MS/MS method, the following data can be mentioned. The total number of unknown incurred plasma and urine samples analyzed was 156 and 29, respectively, the number of calibrators analyzed was 96, for a total of 12 calibration curves. In addition, 28 QC samples were also run. All samples were analyzed on the same HPLC column, while the guard column was replaced once during the study.

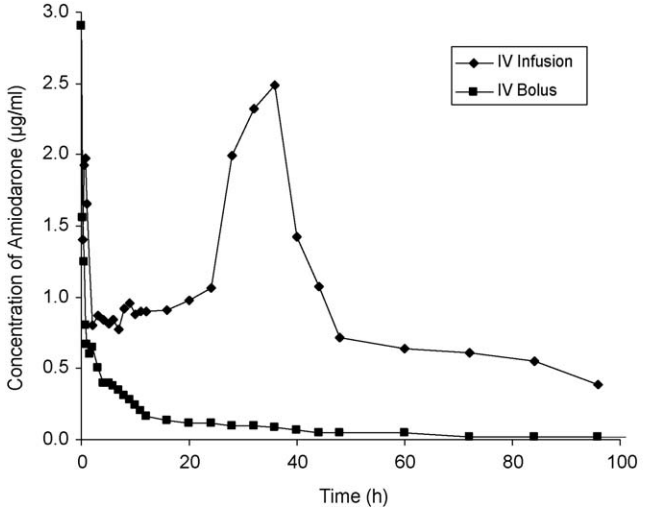

Fig. 7. Plasma concentrations of amiodarone in a horse that received an intravenous (IV) infusion administration (♦) and in a horse that received an intravenous bolus administration (■).

The same can be done for the HPLC–UV method. The total number of unknown incurred plasma samples was 253, in addition to 35 calibrators, for a total of 5 calibration curves. The number of QC samples analyzed was 28. All samples were analyzed on two HPLC columns, while the guard column was several times replaced during the study. These findings indicate that a sample clean-up based on deproteinisation only, resulted in a shorter life time of guard columns and/or analytical columns, in comparison to a clean-up based on SPE.

5. Conclusion

The HPLC–UV method described in this paper for the quantitation of amiodarone and desethylamiodarone is a fast procedure. The minimal sample preparation, namely a simple deproteinization, allows the extraction of many samples a day.

The LC–MS/MS method was developed because there was a need for a more sensitive method. Combined with the solid phase extraction, the LC–MS/MS method is ten times more sensitive. On the other hand, the LC–MS/MS method is more time consuming and more expensive than the HPLC–UV method.

In this paper a fast HPLC–UV method for the detection of amiodarone and desethylamiodarone in plasma is described. This method is suitable for therapeutic drug monitoring and was successfully applied. Additionally, a more sensitive LC–ESI–MS/MS method was described which was successfully applied for the analysis of plasma and urine samples during a pharmacokinetic study.

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