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Determination of anisomycin in tissues and serum by LC-MS/MS: application to pharmacokinetic and distribution studies in rats

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A selective, sensitive and fast method for extraction, identification and quantification of multifunctional drug anisomycin in various tissues and serum, based on liquid chromatography-tandem mass spectrometry, was developed, optimized and validated. The method was validated according to the FDA guidelines and generally demonstrated good selectivity, accuracy, precision, and stability of the analyte. In the optimized method, high extraction efficiency was achieved for tested tissues (heart, brain, spleen, kidney, liver and femoral muscle) and serum. The obtained values of lower limits of quantification (LLOQ, 1.0–11.0 ng g^{-1}) and limits of detection (0.3–3.3 ng g^{-1}) indicated that the method was suitable for determination of trace levels of anisomycin in the complex matrices. The method was linear ($R^2 \geq 0.990$) in the tested concentration range (LLOQ–2500 ng g^{-1}). The developed method was successfully applied in the first study on *in vivo* pharmacokinetics and distribution of anisomycin in tissues and serum of Wistar albino rats following subcutaneous injection (150 mg kg^{-1}). The peak concentration in most tissues was achieved within 3 h after injection. The highest anisomycin concentration was found in the brain and the lowest concentration was found in the serum.

Introduction

Anisomycin is an antibiotic isolated from two bacterial species of *Streptomyces* (*S. griseolus* and *S. roseochromogenes*).[1] It is still not used in therapy, but many studies investigate its potential applications. Anisomycin is a well-known inhibitor of protein synthesis that acts by binding to the 60S ribosomal subunit and blocks peptide bond formation and DNA synthesis.[2–4] Also, anisomycin binding to ribosome activates stress kinases, such as the c-Jun N-terminal kinase (JNK), a response that has been termed ribotoxic stress.[5] Death in cancer cells can be induced by triggering ribotoxic stress.[6] Therefore, there is a potential for anisomycin to be used as an antitumor agent since it suppresses malignant tumor cell growth.[7] The fact that anisomycin is also an immunosuppressant implies its possible application in treatment of some autoimmune diseases and in inhibiting transplantation rejection.[8,9] Furthermore, anisomycin affects memory by inhibiting the consolidation of new memories and can even cause amnesia. Its effects on memory and influence on behavior has been widely reported.[10–12] Studies that are focused on the mechanism of action of anisomycin have shown that it binds to the brain in such a way that inhibits protein synthesis which is necessary in forming fear memories.[13,14] Knowing its multifunctional properties, anisomycin represents the main subject of many studies, but some of the mechanisms of action and additional areas of its potential usage still remain unknown.[15] For example, our preliminary studies indicate that anisomycin can even be a very potent radioprotector. The knowledge of the pharmacokinetic properties (tissue distribution, concentration, clearance, *etc.*) of any substance is a necessary step in understanding its action. However, there are no such studies on anisomycin.

Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) is nowadays the key technique for analysis of pharmaceuticals in tissues. Owing to its sensitivity and selectivity, LC-MS/MS can be used for determination of traces of analytes in very complex matrices. The reported studies regarding distribution of various antibiotics in different animal tissues have used LC[16–18] or LC-MS/MS methods.[19–22] Anisomycin has been identified and analyzed using liquid chromatography, ultraviolet and infrared spectroscopy, mass spectrometry and nuclear magnetic resonance spectroscopy.[23–26] Yet, to the best of our knowledge there are no LC-MS/MS based methods (or any other) used for identification and quantification of anisomycin in tissue samples or serum. In our previous study, LC-MS/MS method was used as a comparative method for investigation of anisomycin electrochemical degradation in standard

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solution and in urine.[27] The aim of this work was to develop the sample preparation method for anisomycin determination in various tissues (brain, spleen, heart, femoral muscle, kidney, liver) and serum as well as to determine its *in vivo* pharmacokinetics and tissue distribution in rats.

Materials and methods

Chemicals and reagents

The anisomycin standard (purity ≥ 98%) was obtained from Fermentek (Jerusalem, Israel). All solvents used were HPLC grade from J. T. Baker (Avantor, Center Valley, PA, USA) or Sigma-Aldrich (St Louis, MO, USA). Analytical grade trichloroacetic acid (TCA) was supplied by Sigma-Aldrich. Deionized water was obtained by passing the distilled water through a GenPure ultrapure water system (TKA, Niederelbert, Germany). For the pH value adjustment of the samples, ammonia was used.

Preparation of stock and working solutions, calibration and quality control samples and matrix-matched standards

The stock solution of anisomycin was prepared in methanol at a concentration of 100 μg mL^{-1} and stored at 4 °C. Working standard solutions in the concentration range 1.0–2500 ng mL^{-1} were prepared by appropriate dilutions of the stock solution with methanol. The calibration samples used for the construction of the calibration curve were prepared by adding 1.0 mL of the appropriate working standard solution to homogenized blank tissues (1.0 g) or serum (1.0 mL) in order to obtain seven concentration levels. Although calibration using the internal standard is common in LC-MS analysis, isotope-labeled standard of anisomycin is not commercially available. Also, there are only a few rarely used compounds that can be regarded as structural analogues of anisomycin. The quality control (QC) samples, used for determination of validation parameters such as selectivity, accuracy, precision, recovery and anisomycin stability, were prepared by the same procedure in order to obtain required concentrations. Both calibration and QC samples were then sonicated for one hour and extracted using optimized sample preparation procedure.

Matrix-matched standards used for determination of recovery and matrix effect[28,29] were prepared by addition of the anisomycin standard solution at appropriate concentration to the blank extracts obtained at the very end of the sample preparation procedure. Blank tissue and serum samples were obtained from animals that were not exposed to anisomycin.

Optimization of the sample preparation

Reported methods for antibiotic determination in complex matrices, such as animal tissues, mainly require sample preparation procedures which involve protein precipitation, fat removal, and solid-phase extraction as the method for extract clean-up.[29–31] Parameters for optimization of the sample preparation were selected based on the literature dealing with analysis of different antibiotic classes in biological samples (Table 1). Extraction parameters optimized in the study were the extraction solvent and the pH-value of the extract prior to clean-up. Tested extraction solvents were methanol,[18,22,33,38,41] 5% solution of TCA[28,30,34,37] and acetonitrile.[19,32,35–40,42] Also, the packing type of the clean-up cartridge was optimized, and Oasis HLB (hydrophilic–lipophilic balance, 200 mg/6 mL, Waters, Milford, MA, USA)[28] and Strata X cartridges[43] (200 mg/6 mL, Phenomenex, Torrance, CA, USA) were tested.

For these experiments, spiked pig heart tissue samples were used. Spiked samples were prepared by adding 1.0 mL of a standard anisomycin solution at a concentration of 100 ng mL^{-1} to 1.0 g of tissue and sonicating in an ultrasonic bath for one hour. The optimized extraction procedure (Fig. 1) was as follows: 5.0 mL of methanol was added to 1.0 g of the spiked tissue sample. The mixture was homogenized using an Ultra-Turrax T-25 homogenizer (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) and subsequently sonicated in the ultrasonic bath for 30 min. The sample was then centrifuged for 10 min at 5000 rpm and the extract was separated. Extraction was repeated one more time. Supernatants were combined, centrifuged again and transferred into the separatory funnel. Hexane (5.0 mL) was added to the obtained extract in order to minimize the lipid content extracted from the tissue sample and the potential interferences during the analysis.[29] By vigorous hand-shaking, liquid–liquid extraction (LLE) of fat was performed, and the lower methanol layer was separated and evaporated to dryness under a nitrogen stream at 30 °C in a water bath. The residue was reconstituted in 10.0 mL of 5% solution of TCA. The TCA solution was used to increase the extraction efficiency by deproteinisation,[28,34] since anisomycin shows affinity for binding with proteins. The resulting extract was transferred onto a clean-up cartridge (Oasis HLB), preconditioned with 3.0 mL of methanol and 3.0 mL of deionized water. The cartridge was then rinsed with 5% solution of TCA (3.0 mL) and dried by vacuum suction for 10 min. The analyte was eluted with 10.0 mL of methanol, evaporated to the volume of 1.0 mL, filtered through 0.45 μm polyvinylidene difluoride filter (PVDF, Roth, Karlsruhe, Germany) and analyzed. The performance of the finally developed and optimized method was demonstrated by analysis of different rat tissues obtained in the *in vivo* distribution study of anisomycin.

LC-MS/MS analysis

Liquid chromatography was performed using a Surveyor LC system (Thermo Fisher Scientific, Waltham, MA, USA) with a reverse-phase Zorbax Eclipse® XDB-C18 column, 75 mm × 4.6 mm i.d. and 3.5 μm particle size (Agilent Technologies, Santa Clara, CA, USA). In front of the separation column, the pre-column was installed, 12.5 mm × 4.6 mm i.d. and 5 μm particle size (Agilent Technologies). The mobile phase consisted of deionized water, methanol and 10% acetic acid (69 : 30 : 1, v/v/v). Acetic acid was used for improving the analyte ionization. After 5 min, the column was rinsed with methanol for 4 min. The initial conditions were then re-established and held for 5 min. The flow rate of the mobile phase was 0.5 mL min^{-1}. An aliquot of 10.0 μL of the final extract was injected into the LC system.

Mass spectra were obtained by the LTQ XL (Thermo Fisher Scientific) linear ion trap mass spectrometer. Electrospray was used as the ionization technique in the positive mode.

Table 1 Selected analytical methods for determination of different antibiotic classes in biological samples[c]

Analyte	Matrix	Extraction solvent	Analytical technique	Recovery, %	Linear range, ng g^{-1}	Sensitivity, ng g^{-1}	Ref.
Doxorubicin	Plasma	35% perchloric acid	LC-FLD	95–101	5–1000[a]	5[a]	16
Ethambutol	Plasma	Methanol	LC-UV	94–101	250–30 000[a]	250[a]	18
Doxorubicin	Plasma, heart, liver, spleen, brain	5 mM ammonium acetate and acetonitrile	LC-ESI-MS	84–112	0.1–10 000[b]	0.3–2[b]	19
Thiosemicarbazones	Plasma	Methanol	LC-ESI-MS	69–101	180–2800[b]	20[b]	22
Aminoglycosides	Muscle, kidney, liver	5% TCA	LC-ESI-MS	61–116	0–10 × MRL	11–5539	28
Tetracyclines	Muscle, kidney, liver	0.1 M sodium succinate and 20% TCA	LC-ESI-MS	7–60	100–1200	50–300	30
Penicillins	Muscle	Water and acetonitrile	LC-TIS-MS	50–101	LLOQ–2 × MRL	0.2	32
Aminoglycosides, macrolides	Muscle	Methanol	LC-ESI-MS	70–96	5–200	5–20	33
Aminoglycosides	Muscle, kidney, liver	5% TCA	LC-ESI-MS	27–93	10–500	1–60	34
Macrolides	Kidney	Acetonitrile and 0.3 M phosphate buffer	LC-ESI-MS	68–76	5–50	0.5–2	35
Sulfonamides	Muscle, kidney, liver	Acetonitrile and hexane	LC-ESI-MS	52–120	1–200	0.1–1	36
Aminoglycosides, macrolides, lincosamides, sulfonamides, tetracyclines, quinolones	Muscle	Acetonitrile and 2% TCA	LC-ESI-MS	71–119	0.5–1.5 × MRL	1–120	37
Veterinary antibiotics	Muscle	Acetonitrile, methanol and hexane	LC-ESI-MS	46–118	0.5–30	0.1–10	38
Sulfanilamide, nitroimidazoles, quinolones, macrolides, lincosamides, praziquantel	Muscle	Acetonitrile	LC-TIS-MS	21–121	0.5–45	0.3–3	39
Tulathromycin	Plasma	Acetonitrile	LC-ESI-MS	95–110	2–500[a]	4[a]	40
Marbofloxacin	Plasma	Methanol	LC-API-MS	93–96	5–2500[a]	5[a]	41
Levofloxacin, moxifloxacin	Serum	Acetonitrile	LC-ESI-MS	96–109	0.1–1000[a]	0.1–0.2[a]	42

[a] ng mL^{-1}. [b] nM. [c] FLD: fluorescence detector; UV: ultraviolet; ESI: electrospray ionization; MRL: maximum residue level; TIS: turbo ionspray; API: atmospheric pressure ionization.

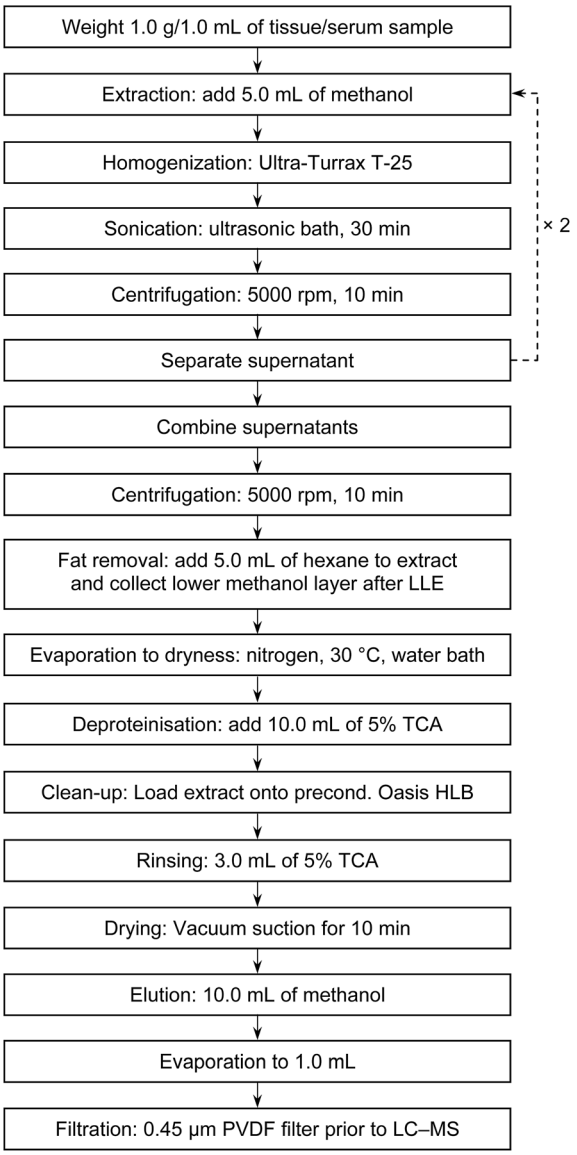

Fig. 1 Schematic diagram of optimized sample preparation procedure for extraction of anisomycin from different tissues and serum.

Fragmentation reaction of the most abundant ion in the MS spectrum to the most intensive fragment ion was selected for quantification of anisomycin in the selected reaction monitoring (SRM) mode. Other transitions were used for confirmation purposes. The optimized source working parameters were: source voltage (5.0 kV), capillary temperature (300 °C) and sheath gas (47 au, *i.e.* 47 arbitrary units, on the scale in the 0–100 range defined by the LTQ XL system).

Method validation

A previously developed and optimized method for determining the presence of anisomycin in pig heart tissue was tested using other pig tissues, such as brain, spleen, kidney, liver, femoral muscle as well as serum. Validation was performed by evaluating selectivity, accuracy, precision, recovery, calibration curve, matrix effect, and stability of the drug according to the guidelines for Bioanalytical Method Validation published by US Food and Drug Administration.[44]

The selectivity of the method was investigated by analyses of blank samples for each tissue type and serum from six different sources. Chromatograms of blank samples were compared to chromatograms of blank samples spiked at the concentration corresponding to the lower limit of quantification (LLOQ). In order to differentiate and quantify the analyte in the presence of other components in the sample, the presence or the absence of undesirable peaks at the retention time of anisomycin was tested.

The accuracy and precision of the method were evaluated by analysis of five replicates of QC samples at LLOQ, low (50 ng g^{-1}), medium (500 ng g^{-1}) and high (2500 ng g^{-1}) concentrations. Accuracy was calculated as the percent deviation of the mean determined concentration from the true concentration of the analyte. Precision describes the closeness of individual determinations of the analyte. It was calculated as the relative standard deviation (RSD). For determination of intra- and interday accuracy and precision experiments were performed on the same day and in three successive days.

The recovery was evaluated at six concentration levels (50, 100, 250, 500, 1000 and 2500 ng g^{-1}) in triplicate by comparing the peak area of anisomycin extracted from QC sample to the peak area of analyte obtained for the blank extract spiked at the appropriate concentration, *i.e.* matrix-matched standard.

Calibration curve was obtained by plotting the anisomycin peak area obtained for the calibration samples *vs.* corresponding concentrations of the analyte. The experiments were carried out at seven concentration levels, in duplicate, in the concentration range LLOQ–2500 ng g^{-1}, for each tissue type and serum. The linear regression analysis was performed in order to determine correlation coefficient (R^2), intercept and slope, and to establish method linearity.

The sensitivity of the method was evaluated by determination of lower limit of quantification (LLOQ) and limit of detection (LOD) for six tissue types and serum. LLOQ and LOD were determined as minimum detectable concentrations of the analyte producing signal to noise ratios of 10 and 3, respectively.[45]

The matrix effect *i.e.* suppression or enhancement of the analyte signal in the matrix solution was estimated for each tissue type and serum at three concentrations (50, 500 and 2500 ng g^{-1}) using the following equation (eqn (1)):

$$\text{Matrix effect } (\%) = \frac{A_{\text{matrix}}}{A_{\text{solvent}}} \times 100 - 100 \quad (1)$$

The anisomycin peak area of the matrix-matched standard (A_{matrix}) was divided by the analyte peak area of the appropriate working standard solution *i.e.* solution of the analyte in methanol (A_{solvent}). From the obtained number (in %), the value of 100 was subtracted in order to determine the percentage of signal suppression (negative values) or enhancement (positive values) by the matrix components.

The stability study reflected conditions encountered during the sample handling, storage, preparation and analysis. The long-term stability of anisomycin was tested over 15 days at −80 °C. The post-preparative stability of anisomycin in

processed sample was evaluated after 12 h in the autosampler maintained at 20 °C. The experiments were performed at low, medium and high concentrations using three replicates of QC samples. The stability of stock solution and working standard solutions stored at 4 °C for 15 days was also tested.

Animal studies

The experiments were performed on male Wistar albino rats weighting 200 g (±5%). The animals were obtained from the parent colony which has been grown in the nursery. The animals were housed in cages at ambient temperature (20–23 °C) and a relative humidity of 50 ± 20%, 2 per cage, with a light and dark cycle (12/12 h) for 2 months before the beginning of the experiment. Food and water were supplied *ad libitum*. All animal experiments were performed in compliance with the Animal Welfare Law, Republic of Serbia, and procedures of vivarium of Faculty of Biology, University of Belgrade, Serbia. Experiments were approved by the Ethics Committee, Faculty of Biology (reg. no. 04/2013). The rats had no previous exposure to any antibiotic, and no other drugs were given to the animals during the study period. A dose of 150 mg kg^{-1} of anisomycin was subcutaneously injected. The solution for injection was prepared by dissolving anisomycin in saline and an adjustment of pH to 7.3. A volume of 1.0 mL of solution was used per injection. The rats were sacrificed at the predetermined sampling time (0.25, 0.5, 1, 3, 7, 12 and 24 h) after the antibiotic was injected. Samples were collected from 3 animals at each time point. Tissue samples (heart, brain, spleen, kidney, liver and femoral muscle) were collected, quickly dissected and kept frozen at −80 °C until the analysis. Serum samples were obtained by centrifugation of the blood samples, deprived of coagulum, at approximately 3000 rpm for 10 min and stored frozen until the analysis. Samples of tissues and serum were prepared using the optimized sample preparation procedure.

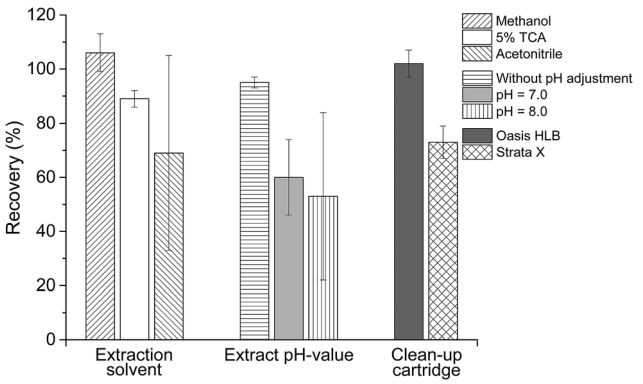

Fig. 2 Anisomycin recoveries from heart tissue using different sample preparation parameters.

Pharmacokinetic study

To characterize the distribution kinetics of anisomycin, the concentration data in the serum and tissues were estimated by a non-compartmental analysis using the Matlab software (MathWorks, Natick, MA, USA). The maximum serum and tissue concentration (C_{max}) and the time to reach maximum concentration (t_{max}) were read directly from the fitted anisomycin concentration–time profile. Also, the model-independent parameters include: the area under the concentration–time curve during the observation period (AUC_{0-t}), the mean residence time (MRT), the elimination half-life ($t_{1/2}$) and the elimination rate constant (λ_z, obtained from the slope of the terminal phase). The model is quite common and has been frequently used in similar studies.[16,46,47]

Results and discussion

Optimization of the sample preparation

In the optimization of anisomycin extraction from tissue samples and serum, different extraction solvents, the sample

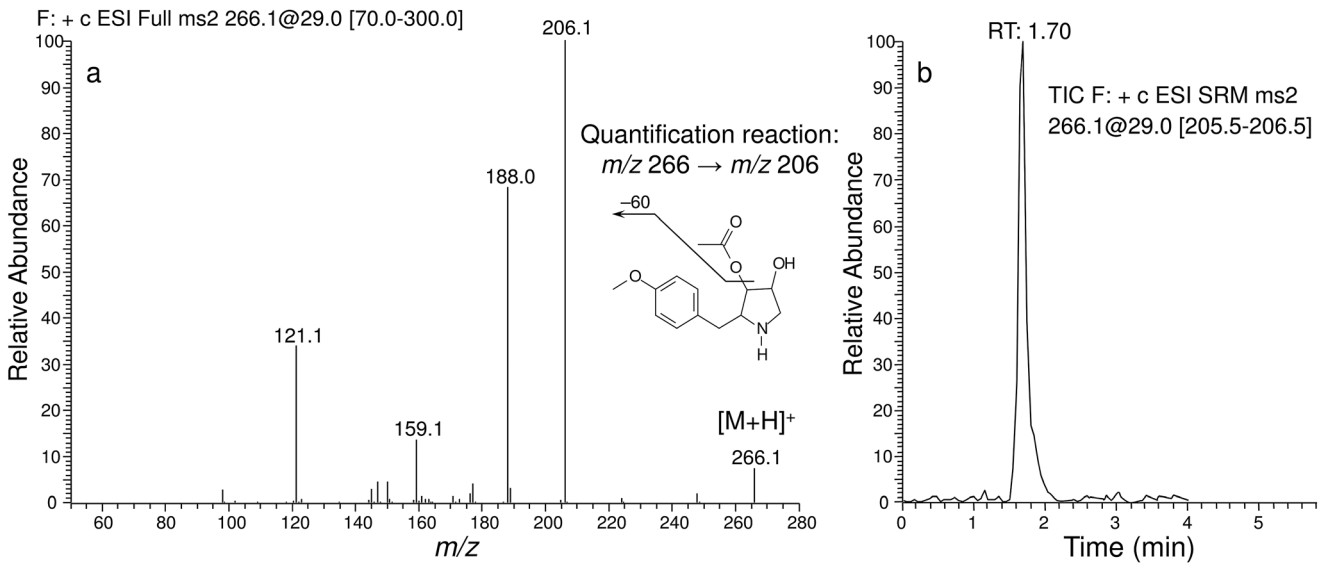

Fig. 3 (a) MS/MS spectrum of anisomycin with the fragmentation reaction selected for quantification; (b) SRM chromatogram of anisomycin.

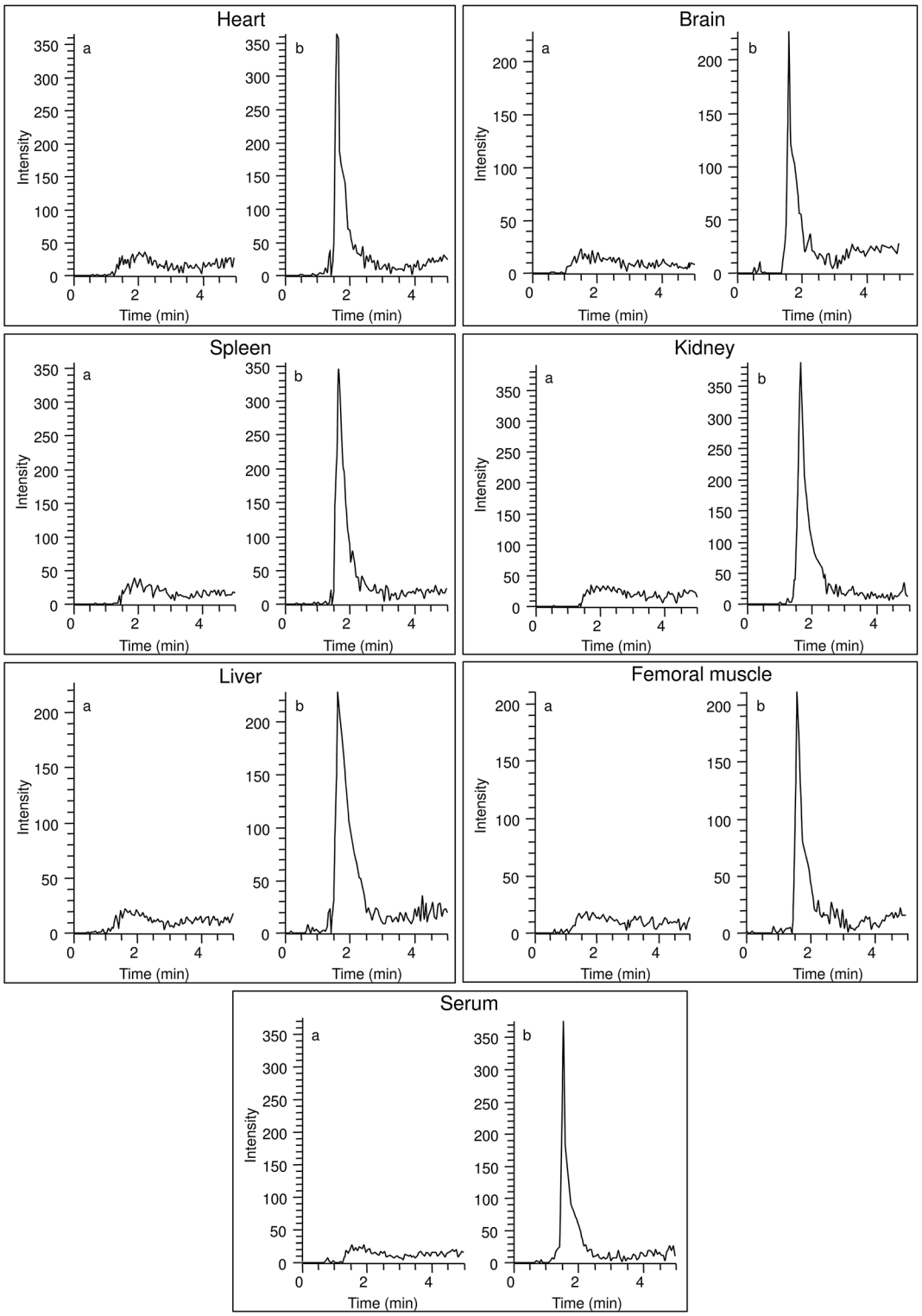

Fig. 4 Representative SRM chromatograms of: (a) blank samples and (b) QC samples at the LLOQ concentration for six studied tissues and serum.

pH and clean-up cartridges were tested. The results are presented in Fig. 2. In the selection of the extraction solvent, the highest recovery of anisomycin was obtained using methanol (106%), with good method repeatability (RSD 7%). The solution of TCA was less efficient for anisomycin extraction from heart tissue (89%, RSD 3%), whereas acetonitrile provided the lowest recovery (69%) with poor method repeatability (RSD 36%). Based on previous reported results summarized in Table 1, methanol generally exhibits good extraction capability,[18,22,33,41] while TCA[28,30,34] and acetonitrile[32,35,36,39] are slightly less efficient

Table 2 Intra- and interday accuracy and precision and matrix effect obtained for six different tissues and serum

		Intraday		Interday		
Tissue	Concentration, ng g^{-1}	Accuracy, %	Precision, %	Accuracy, %	Precision, %	Matrix effect, %
Heart	LLOQ	1	2	6	8	
	50	4	6	3	9	−2
	500	−11	3	−12	2	−10
	2500	−15	2	−14	3	−5
Brain	LLOQ	−1	5	−3	6	
	50	−10	1	−9	5	−23
	500	−8	2	−11	3	−33
	2500	−14	3	−15	4	−25
Spleen	LLOQ	1	7	4	2	
	50	3	4	5	3	−36
	500	−5	2	−8	1	−40
	2500	3	2	4	2	−34
Kidney	LLOQ	2	4	−4	6	
	50	−4	9	−1	10	−19
	500	−13	2	−14	6	−27
	2500	−4	1	−7	2	−20
Liver	LLOQ	1	4	−3	8	
	50	10	3	13	1	−17
	500	−14	1	−15	2	−32
	2500	−7	3	−12	3	−21
Femoral muscle	LLOQ	3	6	−3	10	
	50	2	6	9	11	−49
	500	1	1	3	5	−40
	2500	−14	2	−15	1	−46
Serum[a]	LLOQ	−14	1	−12	5	
	50	−15	3	−10	3	−10
	500	−13	1	−12	5	−5
	2500	−13	2	−15	1	−9

[a] Concentration in ng mL^{-1}.

for extraction of antibiotics from different biological matrices, which is in accordance with the obtained results.

The pH-value of the extract prior to clean-up (*i.e.* loading onto the HLB cartridge) was adjusted using ammonia. In the sample preparation, after methanol extraction, evaporation and reconstitution in 5% solution of TCA, the pH-value of the obtained extract was 1.7. The pH adjustment was used in order to enhance hydrophobic retention of anisomycin on the cartridge since analyte ionization would be minimized at the pH-values close to its pK_a.[28,48] The tested pH-values were 7.0 and 8.0 (anisomycin pK_a = 7.9), and an additional experiment without pH adjustment was performed. The highest recovery of anisomycin was obtained without pH adjustment (95%, RSD 2%). When the pH-value was adjusted to 7.0 (59%, RSD 9%) and 8.0 (54%, RSD 22%) a significant decrease in recovery was observed. In the next experiment, testing of the additional Strata X clean-up cartridge showed that recoveries were significantly lower (73%, RSD 6%) compared to the HLB cartridge (106%, RSD 5%). It was finally determined that the optimal sample preparation should be performed with methanol as an extraction solvent, without pH adjustment of the TCA extract, using Oasis HLB as the clean-up cartridge.

LC-MS/MS analysis

The recorded mass spectrum of anisomycin showed that the protonated molecule ($[M + H]^+$) of anisomycin (*m*/*z* 266) was dominant and it was selected as the precursor ion. In Fig. 3a, fragmentation of the precursor ion (MS/MS spectrum) is presented. The fragmentation reaction of the precursor ion to the most intensive fragment ion (*m*/*z* 206) was selected for anisomycin quantification in the SRM mode (Fig. 3b). Transition to the fragment ion *m*/*z* 188 was used for confirmation purposes.

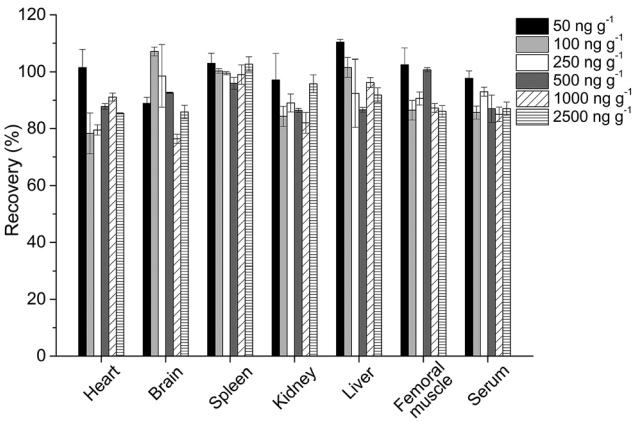

Fig. 5 Anisomycin recoveries from six different tissues and serum at six spiking levels in the range 50–2500 ng g^{-1} (n = 3).

Table 3 Method validation parameters obtained for six different tissues and serum

Tissue	Linear regression equation	R^2	LLOQ, ng g^{-1}	LOD, ng g^{-1}
Heart	$y = 45.1x + 68.6$	0.996	2.7	0.8
Brain	$y = 41.9x + 3086.0$	0.994	5.7	1.7
Spleen	$y = 13.7x + 996.6$	0.995	11.0	3.3
Kidney	$y = 43.5x + 1249.0$	0.993	4.7	1.4
Liver	$y = 98.1x + 2001.0$	0.992	3.7	1.1
Femoral muscle	$y = 30.6x + 73.6$	0.990	6.0	1.8
Serum[a]	$y = 57.7x + 1184.0$	0.993	1.0	0.3

[a] LLOQ and LOD in ng mL^{-1}.

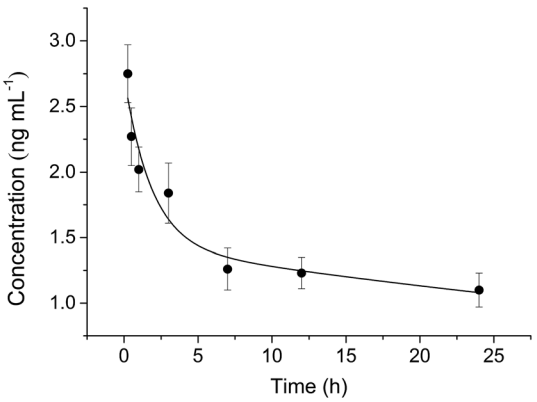

Fig. 6 Mean serum concentration–time curve of anisomycin in rats after a single dose of 150 mg kg^{-1} ($n = 3$).

Method validation

Typical SRM chromatograms of blank samples and QC samples at LLOQ for each tissues type and serum (Fig. 4) clearly demonstrate selectivity of the method. Significant interferences from matrix components were not detected at the retention time of anisomycin.

Results obtained for intra- and interday accuracy and precision of the method are presented in Table 2. The intra- and interday accuracy of the method were within acceptable ranges. It was found that the percent deviations of the mean determined value from the true value were less than 15%, for both intra- and interday experiments. The determined precision of the method was in accordance with FDA guidelines,[44] for all tissue types and serum. Calculated values of intra- and interday precision were below 9% and 11%, respectively, indicating the closeness of individual determinations.

Anisomycin recoveries from different tissues and serum at six concentration levels using the optimized extraction procedure were high for tested samples, as in the case of the heart (78–102%), brain (76–107%), spleen (99–103%), kidney (82–96%), liver (87–110%), femoral muscle (86–103%) and serum (85–98%) (Fig. 5). The calculated RSD values of the method recovery were less than 12% regardless of the sample matrix or the spiking level, indicating that the method was consistent, precise and reproducible, as suggested by FDA guidelines.[44]

The calibration curves were linear over the tested concentration range (LLOQ–2500 ng g^{-1}) with the correlation coefficients (Table 3) ranging from 0.990 for femoral muscle to 0.996 for heart tissue, proving method linearity for all investigated matrices.

The developed method provided low LLOQs (1.0–11.0 ng g^{-1}, Table 3) and LODs (0.3–3.3 ng g^{-1}), indicating that method is sensitive and suitable for determination of trace levels of anisomycin in different tissues and serum.

Regarding the matrix effect, it was determined that the matrix constituents induced suppression of the anisomycin signal, the most pronounced for femoral muscle (up to 49%, Table 2). In the case of heart and serum samples, signal suppression was the least pronounced (up to 10%). Because of the existence of matrix effect, matrix-matched calibration was used in order to achieve correct quantification.

The results of stability study indicated that anisomycin was stable in the long-term for 15 days when samples were kept frozen at −80 °C. Also, anisomycin was stable post-preparatively, when processed samples were kept in autosampler for 12 h at 20 °C. Additionally, stock solution and working

Table 4 Anisomycin concentration in tissues of rats at different times after injection ($n = 3$)

	Concentration ± SD, ng g^{-1}						
Tissue	0.25 h	0.50 h	1 h	3 h	7 h	12 h	24 h
Brain	1005 ± 46	1267 ± 141	1652 ± 39	1799 ± 175	207 ± 40	29 ± 6	22 ± 0
Spleen	653 ± 30	838 ± 60	1162 ± 220	773 ± 88	111 ± 13	15 ± 8	11 ± 5
Femoral muscle	73 ± 52	150 ± 83	143 ± 54	531 ± 30	28 ± 1	10 ± 1	18 ± 1
Heart	332 ± 105	689 ± 185	283 ± 117	164 ± 14	60 ± 7	4 ± 0	3 ± 1
Liver	25 ± 6	40 ± 8	54 ± 6	49 ± 4	10 ± 5	9 ± 4	5 ± 1
Kidney	6 ± 1	6 ± 2	9 ± 1	40 ± 2	24 ± 2	9 ± 1	5 ± 2

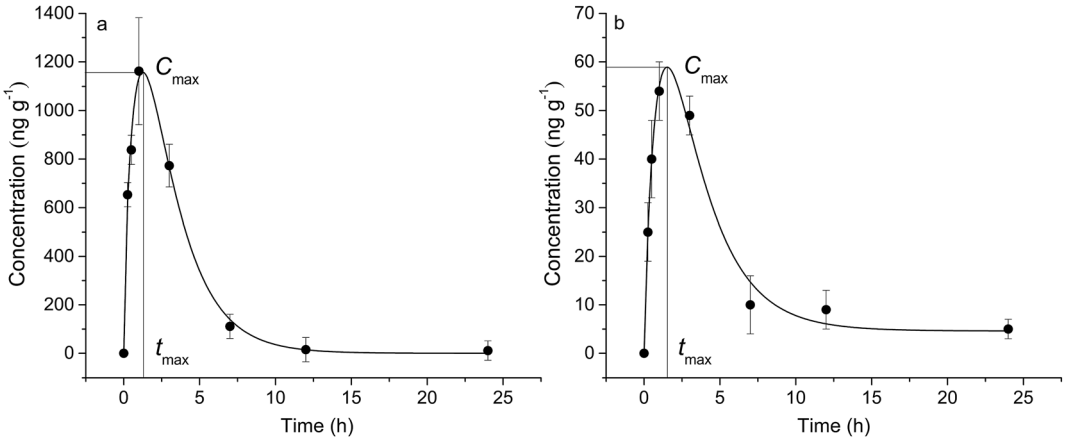

Fig. 7 Fitting of data with non-compartmental model for (a) spleen and (b) liver. Parameters that can be obtained from the curve (c_{max} and t_{max}) are labeled.

standard solutions did not exhibit significant loss of analyte when stored at 4 °C for 15 days. In all three experiments, the loss of anisomycin was less than 11%.

In vivo serum and tissue pharmacokinetic and distribution study

The developed and validated method was applied in pharmacokinetic and distribution study of anisomycin in tissues and serum of Wistar albino rats after a single subcutaneous dose of 150 mg kg^{-1}. Fig. 6 shows the concentration–time profile of anisomycin in serum. The absolute value of the concentration of anisomycin in the serum is rather low when compared to the majority of similar studies on other antibiotics, but roughly the same low values have been found in some other studies.[16,20,21] The mean residence time of anisomycin in serum was found to be 10.81 ± 2.45 h, whereas elimination half-life was 6.5 ± 1.1 h. Determined anisomycin half-life is at the lower end of the reported values, which, depending on the type of antibiotic and the route of administration, are in the range 2–66 hours.[16,40,47,49] Low λ_z value of 0.0035 ± 0.0004 h^{-1} indicate that anisomycin is eliminated slowly from serum in the terminal phase.

Table 4 shows the distribution of anisomycin at various time points following injection. Data for all tissues display the same general pattern of relatively fast absorption with concentration reaching maximum within 3 h and gradual elimination. Differences in concentrations among tissues are quite large when compared to other antibiotics.[16,19–21,46] Some conclusions can be reached from these data, but proper analysis can be performed only when data are fitted to the model which has been used in almost all similar studies.

Fig. 7 shows the fitting of pharmacokinetic data for two selected organs. Experimental points for the spleen lay nicely on the curve, which is not the case for the liver, demonstrating the need for using the model, rather than raw data, for extracting essential pharmacokinetic parameters.

Results obtained from the data fit are shown in Table 5. The fastest accumulation was observed in the heart (t_{max} = 0.46 h), whereas the longest time to reach maximum concentration was noted for kidney (2.88 h). Other tissues reached the peak concentration at nearly the same time (between 1.26 and 1.92 h). Similar results were obtained for fluoroquinolone achieving peak concentration in most of the tissues between 0.45–1.0 h.[46] It is difficult to compare anisomycin data to other studies since those experiments were performed at only three time points[20,21] or even just one.[16] However, it appears that the relative distribution of antibiotic is almost independent from the time point of sampling (see Table 4 as well). For this reason, distributions of antibiotics obtained in various studies at different time points can be qualitatively compared (Table 6).

Two facts can be learned from this literature survey: the liver and/or spleen are at the top of the accumulation order in all studies, and the brain accumulates insignificant amounts of these antibiotics. The results of this work have shown that distribution of anisomycin is quite the opposite from the

Table 5 Pharmacokinetic parameters of anisomycin for *in vivo* distribution study in rats (subcutaneous injection 150 mg kg^{-1})

Tissue	C_{max}, ng g^{-1}	t_{max}, h	$AUC_{0.25-24}$, h ng g^{-1}	MRT, h	$t_{1/2}$, h	λ_z, h^{-1}
Brain	1748 ± 183	1.37 ± 0.42	7482 ± 1197	3.94 ± 0.89	4.00 ± 1.28	0.010 ± 0.002
Spleen	1156 ± 123	1.26 ± 0.39	4565 ± 730	3.62 ± 0.67	3.74 ± 1.17	0.011 ± 0.002
Heart	652 ± 69	0.46 ± 0.15	1419 ± 227	3.27 ± 0.74	1.07 ± 0.31	0.010 ± 0.002
Femoral muscle	553 ± 59	1.92 ± 0.57	1257 ± 207	6.94 ± 1.57	5.69 ± 1.79	0.005 ± 0.001
Liver	58 ± 8	1.51 ± 0.45	355 ± 62	6.62 ± 1.57	4.58 ± 1.41	0.018 ± 0.003
Kidney	40 ± 4	2.88 ± 0.92	350 ± 58	8.31 ± 1.91	7.55 ± 2.38	0.021 ± 0.003

Table 6 Distribution of different antibiotics in animal tissues

Antibiotic	Sampling time, h	Distribution in tissues	Ref.
Doxorubicin	72	Spleen > liver > kidney > heart > muscle > brain	16
Doxorubicin	8	Spleen > liver > heart > brain	19
Gemifloxacin	3	Liver > kidney > heart > brain	21
Roxithromycin	3	Liver > spleen > kidney > heart > muscle > brain	20
Fluoroquinolone	0.5–1	Liver > kidney > spleen > heart > brain	46

expected antibiotic distribution according to literature. The highest C_{max} was reached in brain (1748 ng g^{-1}, Table 5) and the lowest in kidney (40 ng g^{-1}). In accordance with maximum tissue concentration, a measure of anisomycin total amount in a tissue over time ($AUC_{0.25-24}$) also exhibits the highest value for brain (7482 h ng g^{-1}), and the lowest for kidney (350 h ng g^{-1}). The highest anisomycin concentration found in brain is in agreement with the fact that the primary site of action of this drug is the brain where it inhibits protein synthesis.[13,14] Therefore, this is a good demonstration that the majority of anisomycin goes to its targeted organ. This level of accumulation in the brain can be found for some antipsychotics.[50] According to previous results the following distribution of anisomycin in tissues can be derived: brain > spleen > heart > femoral muscle > liver > kidney.

The obtained MRT values for anisomycin were in the range 3.27 h for heart to 8.31 h for kidney (Table 5). The values of MRT indicate that anisomycin stays the longest in kidney, whereas shorter residence times are observed for heart, spleen and brain. The results of previously reported study for fluoroquinolone show that the residence time of this drug was approximately 8 h for all tested tissues.[46] The values of λ_z for all tested tissues were generally low (Table 5) pointing to low terminal elimination rate, *i.e.* long terminal phase of anisomycin elimination from tissues. According to values of $t_{1/2}$, time necessary for concentration of anisomycin to fall to 50% during the elimination phase is the longest for kidney (7.55 h, Table 5) and the shortest for heart (1.07 h). The comparable results were obtained for fluoroquinolone, which also exhibited the highest elimination half-life value for kidney.[46] Although both residence and elimination times were the longest for anisomycin in kidney, the total amount of drug in kidney over time (exposure over time) was the lowest. Additionally, anisomycin exhibited both the fastest accumulation and elimination for heart.

Conclusions

In this paper, a fast, selective and reliable method for determination of anisomycin in various tissues and serum using LC-MS/MS analysis was developed, optimized and validated. The method displayed good accuracy and precision and high extraction efficiency with good repeatability. Low LLOQ and LOD values indicated that the method was suitable for determination of trace levels of anisomycin in different tissues and serum. The method was applied in the study of *in vivo* anisomycin distribution in the rat tissues and serum after subcutaneous injection. The anisomycin concentration in rat serum was much lower than that in rat tissues. The results showed that the highest anisomycin impact was observed for the brain, followed by the spleen, heart, femoral muscle, liver and kidney, which is an important finding related to the biological function of anisomycin.

Conflict of interest

The authors report no conflict of interest.

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