Srpsko hemijsko društvo

Serbian Chemical Society

Klub mladih hemičara Srbije

Serbian Young Chemists' Club

51. savetovanje Srpskog hemijskog društva

i

2. konferencija mladih hemičara Srbije

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51st Meeting of the Serbian Chemical Society

&

2nd Conference of the Young Chemists of Serbia

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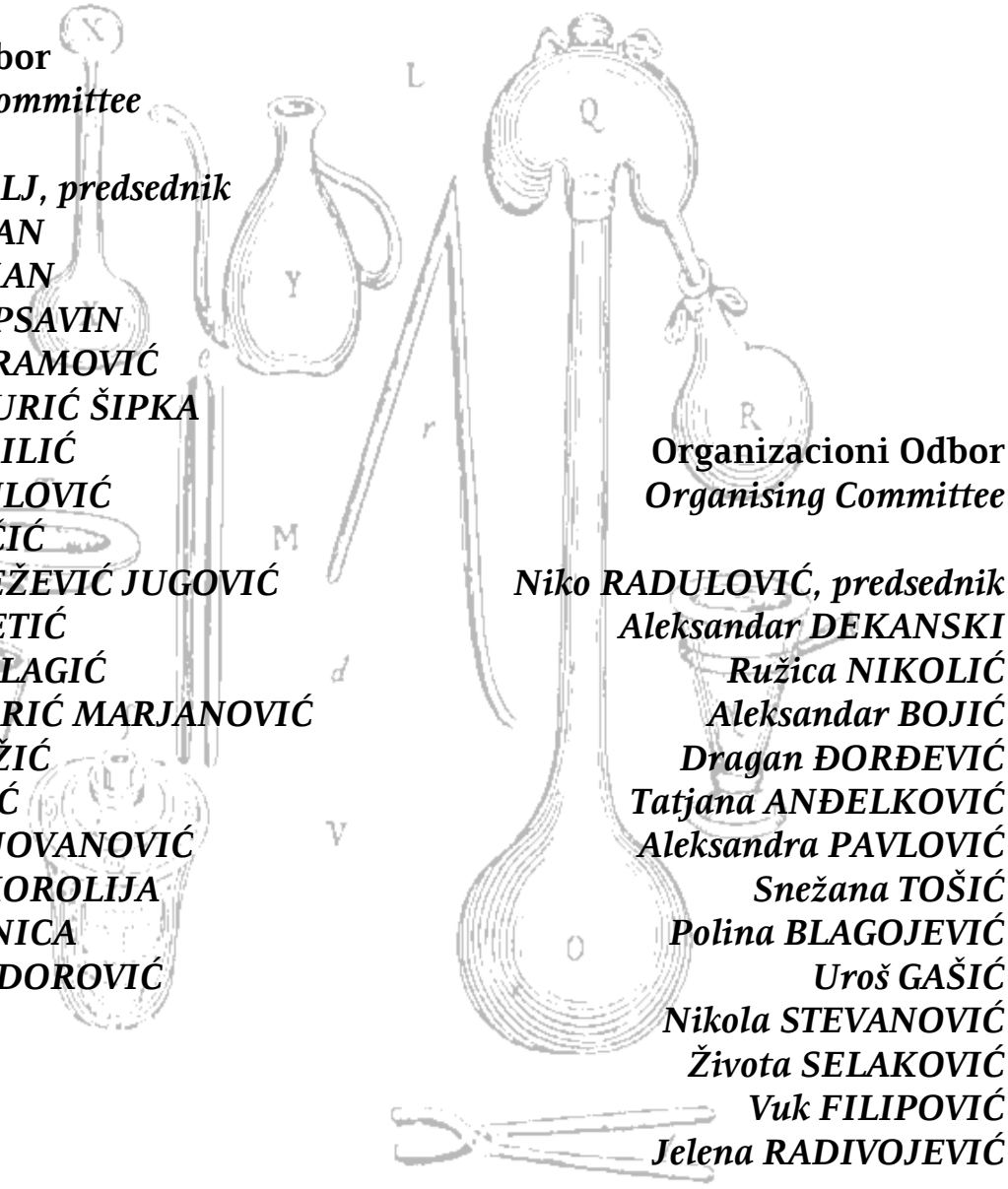

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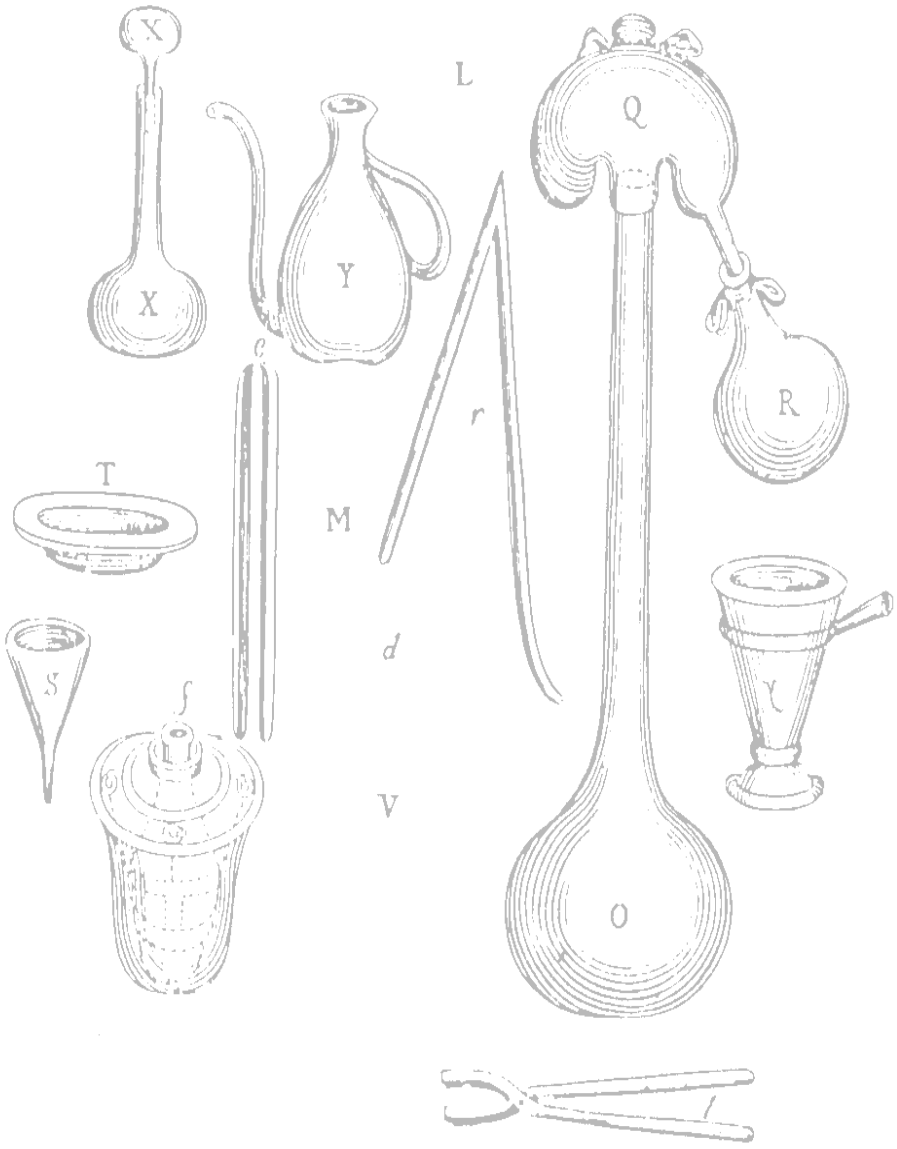

Determination of antibiotic anisomycin in tissue samples by liquid chromatography–tandem mass spectrometry

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Introduction

Anisomycin is a substance isolated from two bacterial species of *Streptomyces* (*S. griseolus* and *S. roseochromogenes*).[1] The primary effect of anisomycin is antibiotical, although it is not commercially available. Its application is limited to studies that investigate anisomycins' potential as suppressant of malignant tumor cell growth[2] or psychiatric drug,[3,4] etc. As a part of on-going research on anisomycins' potential as a radioprotector, the aim of this work was to develop sensitive method for its determination in various tissues (heart, brain, spleen, kidney, liver, and fat). To the best of our knowledge, there are no analytical methods for identification and quantification of anisomycin in tissue samples. Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) is nowadays the key technique for analysis of pharmaceuticals in tissues. Owing to sensitivity and selectivity, LC–MS/MS can be used for determination of traces of analytes in very complex matrices.

Experimental

Optimization of the sample preparation

In the optimization of anisomycin extraction from tissue samples, different extraction solvents and clean up cartridges were tested. For these experiments, spiked heart tissue samples were used. The heart tissue (200 g) was grinded and homogenized using a kitchen blender (Robert Bosch GmbH, Stuttgart, Germany). Spiked samples were prepared by adding 1 ml of standard anisomycin solution (in methanol, at concentration of 100 ng ml^{-1}) to 1.0 g of homogenized tissue and sonication in ultrasonic bath for one hour. Tested extraction solvents were methanol, 5% solution of trichloroacetic acid (TCA) and acetonitrile. The procedure was as follows: 1.0 g of the spiked tissue sample was extracted with 5 ml of the selected solvent in the ultrasonic bath for 30 min. Sample was then centrifuged for 10 min at 5000 rpm and extract was separated. Extraction was repeated one more time. Supernatants were combined, centrifuged again and transferred into separatory funnel. Then, hexane (5 ml) was added to the obtained extract in order to remove fat extracted from tissue sample.[5] By vigorous hand-shaking, liquid-liquid extraction of fat was performed, and lower methanol layer was separated and evaporated to dryness under a nitrogen stream at 30 ºC in a water bath. The residue was reconstituted in 10 ml of 5% solution of TCA. The solution of TCA is added for the reason of increasing the extraction efficiency by deproteinisation,[6,7] since it was shown that antibiotics structurally similar to anisomycin show affinity to bind to proteins. The resulting extract was transferred onto clean up cartridge (Oasis HLB, 200 mg/6 ml, Waters, Milford, MA, USA). It was preconditioned with 3 ml of methanol, followed by 3 ml of deionized water. The cartridge was then rinsed with 5% solution of TCA (3 ml) and dried by vacuum suction for 10 min. The analyte was eluted with 10 ml of methanol, evaporated to the volume of 1 ml, filtered through 0.45 µm polyvinylidene difluoride (PVDF) filter, acquired from Roth (Karlsruhe, Germany), and analyzed.

In the next experiment, additional clean up cartridge was tested. Beside Oasis HLB (hydrophilic-lipophilic balance), Strata X (200 mg/6 ml, Phenomenex, Torrance, CA, USA) was considered for the clean up procedure. The packing of two cartridges is polymeric and regarded as similar.

In the method optimization, external calibration was used with appropriate matrix-matched standards prepared by adding 1 ml of anisomycin standard solution (in methanol, at concentration of 100 ng ml^{-1}) to the blank extracts obtained by sample preparation procedure.

LC-MS/MS analysis

Liquid chromatography was performed using Surveyor LC system (Thermo Fisher Scientific, Waltham, MA, USA) with 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, pre-column was installed, 12.5 mm × 4.6 mm i.d. and 5 µm particle size (Agilent Technologies, USA). Mobile phase consisted of deionized water (A), methanol (B) and 10% acetic acid (C). Gradient was changing as follows: 0 min, A 69%, B 30%, C 1%; 5 min, B 100%; 11 min, B 100%. The initial conditions were then re-established and held for 3 min. The flow rate of the mobile phase was 0.5 ml min^{-1}. An aliquot of 10 µl of the final extract was injected into LC system.

Mass spectra were obtained by LTQ XL (Thermo Fisher Scientific, USA) linear ion trap mass spectrometer. Electrospray was used as ionization techniques in the positive ionization mode. Fragmentation reaction of the most abundant ion in MS spectra to the most intensive fragment ion was selected for quantification of anisomycin in selected reaction monitoring (SRM) mode. The optimized source working parameters were: source voltage (5000 V), capillary temperature (300 ºC) and sheath gas (47 au, *i.e.* 47 arbitrary units, from the scale of arbitrary units in the 0–100 range defined by the LTQ XL system). Results were processed using Xcalibur software package 2.2 (Thermo Fisher Scientific, USA).

Method validation

Previously developed and optimized method for determination of anisomycin in heart tissue was tested and validated using other tissue samples, as brain, spleen, kidney, liver and fat tissue. Validation of the method was performed at six concentration levels in the range 50–2500 ng g^{-1}, for all six tissue samples. Spiked samples were prepared by adding 1 ml of standard anisomycin solution (in methanol, at different concentrations) to 1.0 g of homogenized tissue and sonication in ultrasonic bath for one hour. Recoveries at each concentration level, and repeatability of the method, expressed as the relative standard deviation (RSD), determined by analysis of three replicate samples, as well as limits of detection (LODs) and quantification (LOQs) were determined as criteria for validation of the analytical method. LODs and LOQs were determined as minimum detectable concentrations of analyte that would give signal to noise ratios of 3 and 10, respectively. The results were also used for determination of the method linearity by plotting anisomycin peak area of the matrix-matched standard *i.e.* spiked blank extract *vs*. analyte concentrations. Correlation coefficients (R^2) were calculated for each tissue sample.

For every tissue, the matrix effect, *i.e.* suppression or enhancement of the analyte signal in the matrix solution, was estimated at each concentration level. Anisomycin peak area of the matrix-matched standard was divided by anisomycin peak area of the standard solution *i.e.* solution in methanol producing the value of matrix effect.

Results and discussion

Optimization of the sample preparation

In the selection of extraction solvent, the highest recovery of anisomycin was obtained using methanol (106%), with good method repeatability (RSD 7%). Solution of TCA was less efficient for anisomycin extraction from heart tissue (89%, RSD 3%), whereas acetonitrile provided lowest recovery (69%) with poor method repeatability (RSD 36%). When clean up cartridges were tested, recoveries were significantly lower with Strata X (73%, RSD 6%) compared to HLB (106%, RSD 5%) cartridge. It was finally determined that the best anisomycin recovery was achieved using methanol as extraction solvent and Oasis HLB as clean up cartridge.

Anisomycin mass spectrum

The standard solution of anisomycin was prepared in methanol at 100 ng ml^{-1}. It was found to be stable up to one month. The recorded mass spectrum (Fig. 1) showed that protonated molecule ($[M+H]^+$) of anisomycin (*m/z* 266) was dominant and it was selected as the precursor ion. Fragmentation reaction of the precursor ion to the most intensive fragment ion (*m/z* 206) was selected for quantification in SRM mode (Fig. 1). Transition to the fragment ion *m/z* 188 was used for confirmation purposes.

Method validation

Results of method validation are presented in Fig. 2 and Table 1. Anisomycin recoveries from different tissues at six concentration levels using optimized extraction procedure (Fig. 2) were good for majority of tested samples, as for heart (72–96%), brain (76–127%), spleen (72–99%), kidney (62–95%) and

liver (59–108%). Lower recoveries were obtained when fat was extracted (38–63%). The RSDs of the optimized method were good (≤ 12%) regardless of the sample matrix or the spiking level.

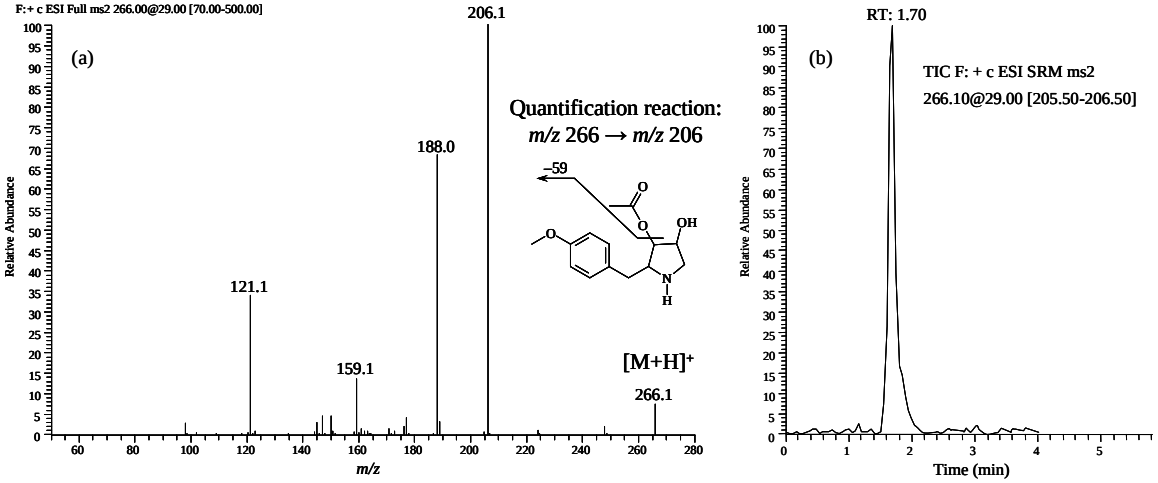

Fig. 1. *(a) MS/MS spectrum of anisomycin with the fragmentation reaction selected for quantification; (b) SRM chromatogram of anisomycin.*

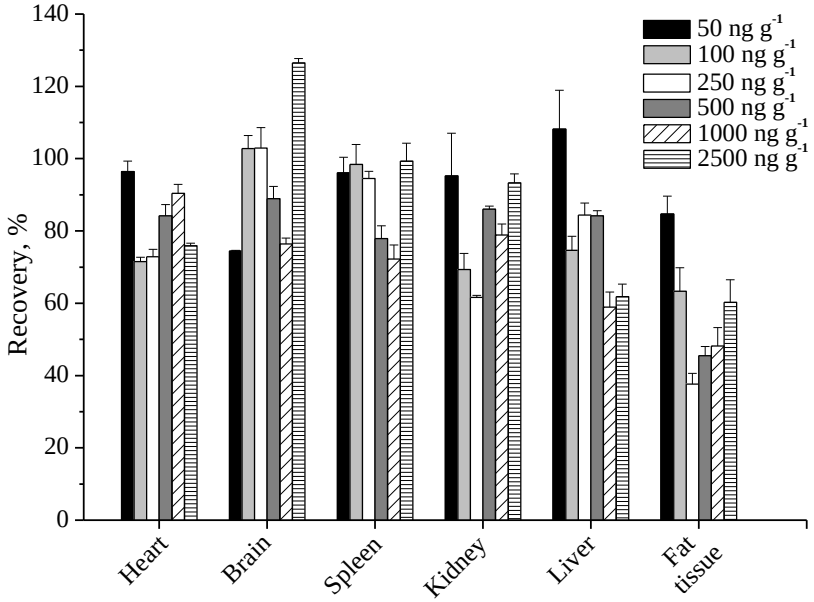

Fig. 2. *Anisomycin recoveries from six different tissues at spiking levels in the range 50–2500 ng g^{-1}.*

For all tested tissues, the developed method provided low limits of detection (0.6–3.8 ng g^{-1}, Table 1) and quantification (2.4–11.8 ng g^{-1}) indicating that it is sensitive and acceptable for determination of trace levels of anisomycin.

Calibration curves using matrix-matched standards were generated using linear regression analysis over concentration range 50–2500 ng g^{-1}. It was determined that method was linear in the tested concentration range (R^2 ranging from 0.975 for liver to 0.998 for heart tissue, Table 1). Generally, matrix constituents induced suppression of anisomycin signal, the most pronounced for spleen tissue (up to 90%, Table 1). Also, reduced analyte ionization was determined for brain (up to 79%) and kidney tissue (77%). In the case of heart sample, signal suppression was less pronounced (up to 23%). For liver and fat tissue, signal suppression was observed for lower spiking levels (up to 78% for liver and 35% for fat), whereas enhancement of the analyte signal was noted for higher spiking levels (up to 17% for liver and fat). Significant matrix effect was successfully eliminated using matrix-matched standards, proving that this type of calibration must be used when dealing with complex tissue matrices.

Table 1. *Method validation parameters and matrix effects obtained for six tissues at spiking levels in the range 50–2500 ng g^{-1}.*

Tissue	Method validation parameters			Matrix effect, %					
	LOD, ng g^{-1}	LOQ, ng g^{-1}	R^2	Spiking level, ng g^{-1}					
				50	100	250	500	1000	2500
Heart	2.0	4.8	0.998	83	79	83	77	87	92
Brain	2.0	2.4	0.991	51	39	35	46	21	40
Spleen	3.8	11.8	0.993	16	14	10	13	13	10
Kidney	1.8	7.1	0.987	23	29	28	25	28	30
Liver	1.3	3.6	0.975	28	46	22	93	117	80
Fat	0.6	3.0	0.985	93	65	80	104	117	109

Conclusions

With the aim of development of sensitive analytical method for determination of drug anisomycin in tissue samples, extraction procedure was optimized. The best recoveries were achieved using methanol as extraction solvent and Oasis HLB as clean up cartridge. For majority of tissue samples, anisomycin was efficiently extracted, as for heart (72–96%), brain (76–127%), spleen (72–99%), kidney (62–95%) and liver (59–108%) tissue. Lower recoveries were obtained when fat was extracted (38–63%). The developed method provided low limits of detection (0.6–3.8 ng g^{-1}) and quantification (2.4–11.8 ng g^{-1}) indicating that it is sensitive and suitable for determination of anisomycins' trace levels. The method was linear in the tested concentration range 50–2500 ng g^{-1}, with correlation coefficients ranging from 0.975 for liver to 0.998 for heart tissue. Significant matrix effect was successfully eliminated using matrix-matched standards, proving that this type of calibration must be used when dealing with complex tissue matrices.

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Određivanje antibiotika anizomicina u tkivima metodom tečne hromatografije sa tandem masenom spektrometrijom

Cilj ovog rada bio je razvoj osetljive analitičke metode za određivanje leka anizomicina u različitim tkivima. U optimizovanoj proceduri ekstrakcije, metanol je odabran kao rastvarač za ekstrakciju, a Oasis HLB kao kertridž za prečišćavanje ekstrakta. Prilikom validacije razvijene metode, anizomicin je efikasno ekstrahovan iz većine tkiva, kao što su srce, mozak, slezina, bubrezi i jetra, dok su za masno tkivo dobijeni niži prinosi. Niske granice detekcije (0,6–3,8 ng g^{-1}) i kvantifikacije (2,4–11,8 ng g^{-1}) ukazuju da je razvijena metoda pogodna za određivanje tragova anizomicina. U ispitivanom opsegu koncentracija (50–2500 ng g^{-1}) metoda je linearna (R^2 > 0,975). Značajan uticaj matrice eliminisan je upotrebom standarda koji odgovaraju matrici uzorka. Na osnovu rezultata validacije utvrđeno je da je razvijena metoda osetljiva i pouzdana za određivanje leka anizomicina u različitim tkivima korišćenjem tečne hromatografije sa tandem masenom spektrometrijom.

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