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Determination of cucumarioside A_2-2 in mouse spleen by radiospectroscopy, MALDI-MS and MALDI-IMS

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ABSTRACT

The distribution of triterpene glycoside cucumarioside A_2-2, the main compound of medical lead Cumaside in immunodeficiency diseases, in mouse spleen was determined. For this purpose the stability and dynamics of glycoside content changes over time in Balb/c mouse spleen tissue homogenate as well as the study of the cucumarioside A_2-2 spatial distribution in tissue sections were investigated using radiospectroscopy, MALDI-MS and MALDI Imaging Mass Spectrometry (IMS), correspondingly.

Cucumarioside A_2-2 is reliably detected by MALDI-MS in the mouse spleen tissue after single intraperitoneal (*i.p.*) injection at a dosage of 5 mg/kg. The glycoside is stable in the spleen and does not undergo metabolic transformation in either tissue homogenates or in the intact organ within 24 h after *i.p.* injection. The cucumarioside A_2-2 was absorbed fairly rapidly: the glycoside maximum concentration (C_{max}) in tissue homogenate was observed in the first 30 min after injection; the minimum values were registered in 3 h. These results are in agreement with those obtained in the pharmacokinetic study of ^{3}H-cucumarioside A_2-2. It was established by MALDI-IMS that glycoside was mainly located in the tunica serosa part of the spleen and only a small amount was detected within the red and white pulp of the organ. MALDI MS images obtained 15–30 min post dosage clearly reflect high drug concentrations in the regions surrounding the organ followed by its decline in the surface part and a very slight redistribution to the internal part of the spleen.

1. Introduction

On the basis of triterpene glycosides isolated from Far Eastern sea cucumber *Cucumaria japonica*, a new immunomodulatory lead Cumaside has been created that consists of a complex of monosulfated glycosides (mainly cucumarioside A_2-2) with cholesterol in an approximate molar ratio of 1:2. This complex has been utilized for the prevention and treatment of human immunodeficiency states (Stonik et al., 2004). Cumaside, cucumarioside A_2-2 and some other sea cucumber glycosides clearly showed immunostimulatory effects. Thus, incubation of immune cells with the glycosides induces their activation that involves a sharp and reversible Ca^{2+} influx into cell cytoplasm, an increase in macrophage lysosomal and phagocytic activity and ROS-formation in macrophages. Injection of subtoxic doses causes an increase in the number of antibody producing plaque-forming cells in mouse spleen, increase in the number, size and acidity of lysosomes of macrophages, phagocytic index of the cells in intraperitoneal exudates, splenocyte proliferation (blast-transformation), an increase in the number of leukocyte and neutrophils of the blood and bone marrow, an increase in the weight and cell numbers in lymphoid organs including spleen and the percentage of survivability and average life span in the irradiated mice, and a significant enhancement in the resistibility in animals towards various bacterial infections (Agafonova et al., 2003; Aminin et al., 2001, 2008, 2011a, 2011b; Sedov et al., 1984, 1990).

The basis of immunomodulatory action of cucumarioside A_2-2 on mouse splenocytes is a result of the influence on expression of some proteins that participate in formation of the cell immune response. The glycoside regulates expression of proteins involved in the processes of maturation, activation and merging of lysosomes, phagocytosis, cytoskeleton rearrangement, cell adhesion, motility and proliferation of immune cells (Aminin et al., 2009). However, the pharmacokinetic behavior of the drug in target organs of the immune system is still unclear.

This article reports on an investigation of triterpene glycoside cucumarioside A_2-2, the main glycoside isolated from the Far-Eastern edible holothurian *C. japonica*. It describes a study of pharmacokinetic behavior of cucumarioside A_2-2 and presents the

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experimental results of the stability and dynamics of glycoside content changes over time in Balb/c mouse spleen tissue homogenate as well as the study of the cucumarioside A_2-2 spatial distribution on the surface of tissue sections investigated using radiospectroscopy, MALDI-MS and MALDI-IMS.

2. Materials and methods

2.1. Triterpene glycoside isolation

Triterpene glycoside cucumarioside A_2-2 or 3β-O-{[3-O-methyl-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 4)]-[β-D-xylopyranosyl-(1 → 2)]-β-D-quinovopyranosyl-(1 → 2)-4-O-sodium sulfate-β-D-xylopyranosyl}-holosta-7,25-diene-16-one was isolated from an ethanol extract of Far-Eastern holothurian *C. japonica* using hydrophobic chromatography on polytetrafluoroethylene powder Polychrom-1 (Biolar, Latvia) followed by chromatography on a Si gel column and HPLC as described previously (Avilov et al., 1990). Purity of the compound was checked by ^{13}C NMR and compared with published data. The chemical structure of cucumarioside A_2-2 is presented in Fig. 2A.

2.2. Animals

Female Balb/c mice weighing 18–20 g were purchased from the nursery RAMS «Stolbovaya» (Russia), and kept at the animal facility under standard conditions. All experiments were conducted in compliance with all rules and international recommendations of the European Convention for the Protection of Vertebrate Animals used for experimental studies.

2.3. Detection of ^3H-cucumarioside A_2-2

^3H-cucumarioside A_2-2 was obtained as described in Stonik et al. (2004). The water suspension of cucumarioside A_2-2 complex with cholesterol was administered intraperitoneally (*i.p.*) once to Balb/c mice at a dose of 5 mg/kg. Each experimental group contained five animals. The animals were euthanized at set time intervals, and the content of glycoside was determined in animal spleen. For this purpose an aliquot of wet spleen tissue was dissolved in hydrochloric acid and hydrogen peroxide under heating (Ozrina et al., 1979). After dissolving, a transparent yellowish liquid was formed in the vials. The vials were cooled to room temperature and neutralized with 1.5 M Tris. The prepared samples were placed into a Tri-Carb 2800 TR liquid scintillation counter (PerkinElmer/Packard, US). After incubation in the dark for 4 h, the radioactivity of the samples was determined.

The parameters of the cucumarioside A_2-2 pharmacokinetics in mouse spleen were calculated using an equation of nonlinear regression and the two-compartment model. The following calculated parameters and constants were taken into account: maximum concentration (C_{max}), the time to reach maximum concentration (T_{max}), half-elimination and half-absorption times ($T½$), elimination rate constant (K_e (Lz)), area under the concentration–time curve (AUC), total clearance (Cl), and mean residence time of the preparation (MRT).

2.4. MALDI-IMS

2.4.1. Tissue preparation

The water solution of cucumarioside A_2-2 was administered *i.p.* once to Balb/c mice at a dose of 5 or 15 mg/kg. Non-dosed mice were used as controls. At set time intervals mice were sacrificed by cervical dislocation and spleens were then surgically removed within 10 min. Tissues were slowly frozen at −80 °C in the freezer. Before analysis tissues were equilibrated to −20 °C followed by sectioning at −20 °C. Spleen tissue was sectioned at a thickness of 12 μm with Feather C35 80 mm blades (Japan) in a Microm HM 560 Criostat (Thermo Scientific, UK). The tissue sections were then mounted either onto microscopic glasses covered with poly-L-lysine for histological staining or onto pre-chilled Indium Tin Oxide (ITO) slides (Bruker Daltonics, Germany) for drug MALDI imaging. Mounted on ITO slides sections were desiccated for 45–60 min in vacuum of a desiccator at room temperature prior to analysis. A pure standard of cucumarioside A_2-2 was deposited onto MALDI slides and used to determine optimum MS parameters. To correlate the MALDI images with histological features, the sections mounted onto microscopic slides were stained with hematoxilin and eosin (H&E staining) and images were captured using AxioImager A1 microscope (Carl Zeiss, Germany) connected to a digital camera.

2.4.2. Matrix deposition

The ImagePrep station (Bruker Daltonics, Germany) was used and operated as per manufacturer's instructions to deposit homogeneous matrix layers onto tissue sections. α-Cyano-4-hydroxycinnamic acid (CHCA), trifluoroacetic acid (TFA), acetonitrile (ACN) were purchased from Sigma–Aldrich (France). CHCA at concentrations of 7 mg/ml in 1:1(v/v) ACN: H_2O/0.2% TFA were deposited using the default ImagePrep method. The tissue was analyzed immediately after the matrix deposition.

2.4.3. MALDI-IMS data acquisition and analysis

Slides for automated MALDI-IMS analysis were first scanned at 600 dpi using an HP scanjet 2400 digital flatbed scanner (Hewlett Packard, USA) to generate the optic images of spleen sections used in IMS analysis. Slides were then fitted into a Slide Adapter II MALDI target (Bruker Daltonics, Germany). An Ultraflex III MALDI TOF/TOF mass spectrometer equipped with a solid-state Smartbeam laser (Bruker Daltonics, Germany) with a frequency of 200 Hz operating in the reflection mode was used for IMS acquisition. The MALDI-IMS experiments were performed at a spatial resolution of 200 μm except for the detailed experiments that were performed with a spatial resolution of 100 μm. Data were collected between m/z 300–2500 Da in the negative ion mode, unless stated otherwise. Auto execute parameters were set by FlexControl software (version 3.0, Bruker Daltonics, Germany) and a fixed laser power was selected by the operator. Results from IMS acquisition were visualized and processed using FlexAnalysis 3.0, FlexImaging 3.0 (Bruker Daltonics, Germany) and BioMAP 3.8 (Novartis, Switzerland) software. For each spleen section, the related intensities were processed by discarding peaks with background in pulp and tunica serosa areas. The remaining intensities constituted the set of variables that were used for statistical analyses.

2.5. MALDI-MS

The water solution of cucumarioside A_2-2 was administered *i.p.* once to Balb/c mice at a dose of 5 mg/kg. At set time intervals mice were sacrificed by cervical dislocation and spleens were then surgically removed within 10 min. Each isolated spleen was then immediately homogenized with a glass homogenizer. Each tissue homogenate was mixed with water solution of frondoside A, triterpene glycoside isolated from sea cucumber *Cucumaria frondosa* (Avilov et al., 2007), as an internal standard (1 μM final concentration). Five microliters of each tissue homogenate was then spotted on a steel MALDI target, and the target was put into a desiccator to dry for a minimum of 60 min. After drying, the plates were spotted with CHCA matrix (7 mg/ml in 1:1 ACN: H_2O/0.2% TFA). Pure standards of cucumarioside A_2-2 and frondoside A mixed with matrix were deposited separately onto a MALDI target to determine optimum MS parameters and verify the results obtained with tissue

homogenates. Data were collected on the Ultraflex III MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Germany) in the negative ion mode to verify the presence of the peak of interest. The mass profiles were recorded by MALDI-MS using the same acquisition parameters as for tissue imaging. Peaks were labeled using FlexAnalysis 3.0 software. The cucumarioside A_2-2 amount in mouse spleen were expressed as a ratio of cucumarioside A_2-2/frondoside A signal intensity (ratio Cuc A_2-2/Frondo A) and parameters of pharmacokinetics were calculated as described above in Section 2.3.

2.6. Statistical analysis

Average value, standard error, standard deviation and *p*-values in all experiments were calculated and plotted on the chart using SigmaPlot 3.02 (Jandel Scientific, San Rafeal, CA, USA).

3. Results

3.1. Dynamics of tritium-labeled cucumarioside A_2-2 content in mouse spleen

A two-compartment first order pharmacokinetic model was used to describe the pharmacokinetics of ^{3}H-cucumarioside A_2-2 following *i.p.* administration. Fairly rapid ^{3}H-cucumarioside A_2-2 absorption was observed after a single application at a dosage of 5 mg/kg. The maximum concentration (C_{max}) in mouse spleen tissue homogenate was reached rapidly, i.e. within 10–30 min to a level of about 100 ng per mg of tissue, wet weight. This was a result of an immediate and rapid contact of the preparation with the organ (Fig. 1A). ^{3}H-cucumarioside A_2-2 was slowly eliminated with a systemic clearance rate of 21.5 ml/min from the mice. The half-elimination time ($T½$) in spleen was around 90 min, and the mean residence time of the preparation (MRT) was calculated as approximately 135 min. (Fig. 1A and Table 1).

3.2. Cucumarioside A_2-2 identification and quantification in mouse spleen homogenate by MALDI-MS

The full mass spectral data of cucumarioside A_2-2 was obtained by MALDI-MS on dry droplet samples of 1 μl of drug solution on mouse spleen tissue homogenate and 7 mg/ml CHCA as matrix. Example of typical full mass spectra of this drug is presented in Fig. 2B.

The MALDI-MS (negative ion mode) spectrum of cucumarioside A_2-2 exhibited signals of decationized molecule at *m/z* 1295.537 $[M_{Na} - Na]^-$, that allowed for the determination of molecular formulae of cucumarioside A_2-2 as $C_{59}H_{91}O_{29}SNa$. The MALDI-MS (positive ion mode) spectrum of cucumarioside A_2-2 showed the signal of the cationized molecule at *m/z* 1341.513 $[M_{Na} + Na]^+$ along with the peaks at *m/z* 1357.491 $[M_{Na} + K]^+$ and 1239.580 $[M_{Na} + Na - NaSO_3 + H]^+$. These results indicated that the sulfate group was detached from the carbohydrate moiety of the glycoside. The obtained data are consistent with the molecular formula (delta = 3.5 ppm).

Cucumarioside A_2-2 was diluted in spleen homogenate and its MS signal intensities were plotted within the concentration range of 1–1000 ng/ml using dry droplet sample preparation on a stainless steel target plate. In this series of experiments, we investigated the ionization properties of the compound and the linearity of the instrument response, which was expected to be similar, but with lower intensity from tissue sections. The average signal responses from five individual spectra were calculated. These data are presented in the diagram of Fig. 2D. In our observations a linear relationship within the glycoside concentration range of 1–1000 ng/ml with detection limit of 1 ng/ml was obtained.

For more precise cucumarioside A_2-2 quantitation an internal standard, frondoside A, was used when performing MS analysis of cucumarioside A_2-2 changes in spleen homogenates over time after drug *i.p.* administration (Fig. 2C). It was found that after single drug application at a dosage of 5 mg/kg the maximum concentration of cucumarioside A_2-2 in mouse spleen tissue homogenate was achieved rapidly, i.e. within 30 min (Fig. 1B). Cucumarioside A_2-2 was moderately slowly eliminated from the spleen. The half-elimination time ($T½$) in spleen was around 80 min, and the mean residence time of the preparation (MRT) was calculated as approximately 140 min. (Fig. 1B and Table 1).

3.3. Cucumarioside A_2-2 stability in mouse spleen

It was established that the cucumarioside A_2-2 is stable in the spleen homogenates during incubation at 37 °C within a 24 h period. The glycoside peaks were clearly detectable by MALDI-MS in the negative ion mode in each homogenate sample obtained after 0–240 min and 24 h of mouse exposure to the drug. Cucumarioside A_2-2 did not undergo metabolic transformation in either tissue homogenates while incubated *in vitro* at 37 °C or *in vivo* within a 24 h period in the intact organ of the mouse after *i.p.* injection (Fig. 3).

3.4. Cucumarioside A_2-2 spatial distribution in mouse spleen after drug single administration

MALDI-IMS was performed with the mouse spleens sampled after 15 min of cucumarioside A_2-2 *i.p.* administration at doses of 15 mg/kg. Successive sections were stained with H&E for identifying the regions where IMS was performed. Sections were prepared

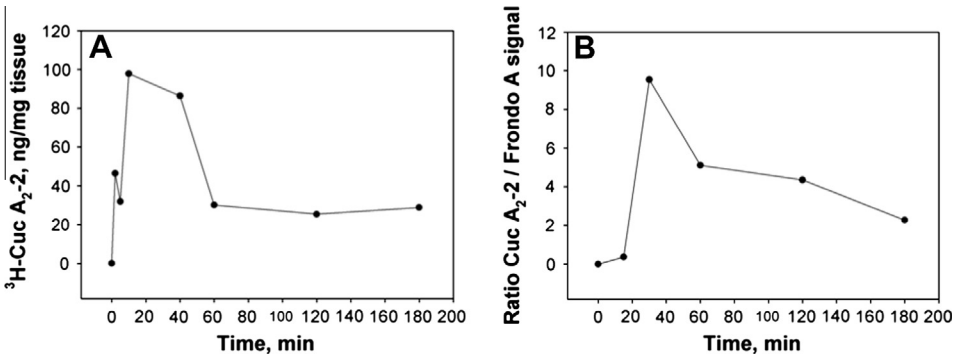

Fig. 1. Dependence of cucumarioside A_2-2 concentration in Balb/c mouse spleen at the time of exposure after a single *i.p.* administration at a dose of 5 mg/kg body weight. The content of cucumarioside A_2-2 was estimated by radiospectroscopy (A) and MALDI-MS (B) methods.

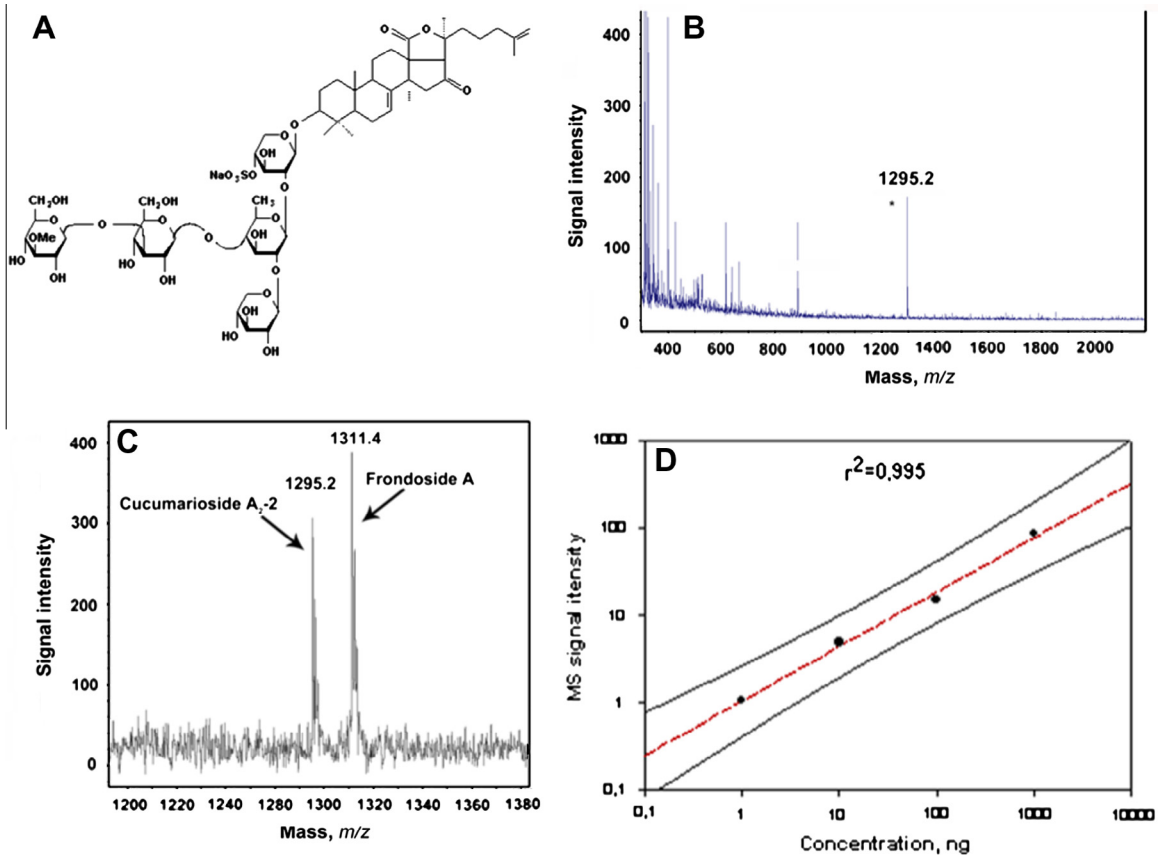

Fig. 2. Chemical structure of cucumarioside A_2-2 (A); full mass spectrum of cucumarioside A_2-2 (negative ion mode) as obtained in the dry droplet experiments (B); MALDI-MS spectrum of cucumarioside A_2-2 (left) and frondoside A (right) as an internal standard (C) in mouse spleen tissue homogenate and CHCA as matrix; diagrams of MS signal intensities of cucumarioside A_2-2 in a concentration range of 1–1000 ng/ml obtained from a stainless steel target plate (D).

Table 1
Pharmacokinetic parameters of cucumarioside A_2-2 in mice after *i.p.* administration at a dose of 5 mg/kg obtained by radiospectroscopic and MALDI-MS analysis.

Method of analysis	C_{max} ng/mg	T_{max} min	AUC min ng	K_e (Lz) 1/min	MRT min	Cl ml/min	$T½$ min
Radiospectroscopy, ^{3}H-cucumarioside A_2-2	97.77 a.u.	10 min	104,381 min a.u.	0.0076 1/min	134.5 min	21.475 ml/min	91.61 min
MALDI-MS, ratio Cuc A_2-2/Frondo A	95.37	30	104,222	0.0085	141.5	23.363	82.01

from the center part (midsection) of the spleens (Fig. 4A) and the tunica serosa, red and white pulp regions of the spleen were defined (Fig. 4B). In the reconstituted MS image using BioMAP software, distinct cucumarioside A_2-2 localizations were observed at *m/z* 1295.2 (Fig. 4C and E). These images revealed the concentration of the glycoside was mainly located in the tunica serosa part of the spleen and not within the red and white pulp of the organ as suggested by MALDI-IMS. On images obtained with a higher resolving power of the instrument, it is shown that at 100 μm resolution, the glycoside is almost uniformly distributed along the serous membrane of the spleen.

The next set of experiments was to evaluate the effects of a single cucumarioside A_2-2 dose over time. For this purpose mice were given a single dose of 15 mg/kg cucumarioside A_2-2 (*i.p.*) and then sacrificed in groups according to the time point they would represent. Groups were sacrificed 15 min, 30 min, 1 h, 2 h or 3 h after administration of the glycoside dose. The distribution of cucumarioside A_2-2 was determined in mouse spleen tissues to elucidate if the drug was penetrating through the serosa barrier. A semi-quantitative analysis was performed by correlating the glycoside MS intensities and spleen structures. For the acutely dosed mice it can be seen that the drug signal is strong at 15–30 min post dose in the tunica serosa regions surrounding the organ. From there the amount of detected cucumarioside A_2-2 decreases (Figs. 5 and 6). The images obtained 60–180 min post dosages clearly reflect drug concentration decline in the surface part (tunica serosa) and a very slight redistribution to the internal part of spleen corresponding to the red and white pulp regions.

4. Discussion

For more than 40 years triterpene glycosides from sea cucumbers (holothurians) have attracted the attention of chemists, biochemists, pharmacologists, and biologists-taxonomists. These compounds demonstrate a wide spectrum of biological effects: antifungal, antitumor, hemolytic, cytostatic, pro-apoptotic and immunomodulatory activities. The application of many preparations from sea cucumbers in traditional oriental medicine is known. The medicinal properties of these preparations are attrib-

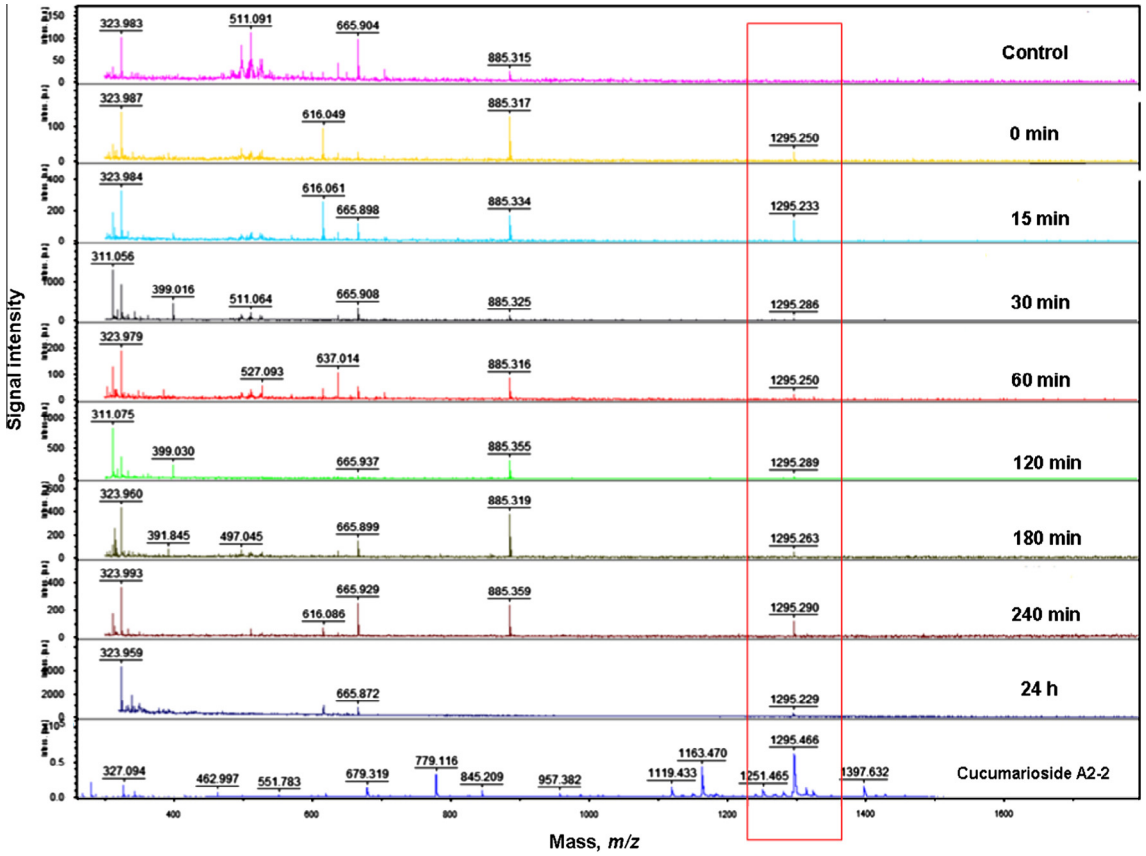

Fig. 3. Cucumarioside A_2-2 MALDI-MS spectral profiles obtained from mouse spleen homogenates incubated *in vitro* with drug over time at 37 °C. Control – homogenate incubated with physiological saline solution; Cucumarioside A_2-2 – standard of glycoside deposited separately onto a MALDI target. Peaks corresponding to cucumarioside A_2-2 on MS spectra are selected in box.

uted to triterpene glycosides (Kalinin et al., 2008). Despite the rather long intensive and detailed study of the biological activity of triterpene glycosides of sea cucumbers, the pharmacokinetics of these compounds is practically unexplored. There are several studies related to the assessment of pharmacokinetic parameters of some glycosides of plant origin. These works are mostly associated with the development of new approaches and methods for the quantitative estimation of glycosides in the blood plasma of animals.

Thus, the highest concentration of the tritium-labeled glycoside of *Eleutherococcus*, eleuteroside B, after *i.p.* administration was observed in rat blood 15 min after administration, which was taken as evidence of its intensive absorption into the blood after injection. Such a high concentration is maintained in the blood for up to 30 min before dropping sharply in the interval from 30 min to 4 h, which evidently is associated with its incipient elimination in the excrement (Bezdetko et al., 1981). The plant glycoside, astragaloside IV, with cardioprotective and immunomodulatory properties was moderately eliminated in plasma, following intravenous administration in rats which was estimated with a highly sensitive and accurate analytical LC/MS/MS method for quantitative glycoside detection (Zhang et al., 2005). In another study the decline of ginseng saponins, ginsenoside Rb1 and Rg1, in serum has been described by a two-compartment model, where the rapid reduction of the compound in blood was registered using an HPLC method (Xu et al., 2003).

In our study, we compared the two approaches for pharmacokinetic studies of cucumarioside A_2-2 in mouse spleen: an evaluation using radioactively-labeled drug and a MALDI-MS method. The main disadvantage of the radiospectroscopic method is the lack of confidence in the fact that we watch for pharmacokinetic behavior of the original material, but not fragments or metabolites after conversion in the body, because in these experiments only tritium is registered. The MALDI-MS approach allows for recording the entire fate of the analyte molecules and from its beginning through its degradation and metabolism. The cucumarioside A_2-2 pharmacokinetic behavior and its rapid elimination in the mouse spleen is not related to its metabolism or to the degradation of the molecule. The glycoside is stabile as shown by incubation in tissue homogenates, and in the whole organ for 24 h. Both methods, radiospectroscopy and MALDI-MS analysis, yielded similar results in cucumarioside A_2-2 pharmacokinetic properties. Correlations were found between parameters such as T_{max}, $T½$, AUC, total clearance, K_e and MRT as calculated from using both applied approaches. Some of them are similar to the results obtained with glycosides from other plants (Bezdetko et al., 1981; Zhang et al., 2005; Xu et al., 2003).

MALDI Imaging Mass Spectrometry is an emerging tool for the analysis of biological and clinical tissue samples. It has been shown to be amenable for the analysis of proteins, peptides, lipids, and small molecules (drugs and endogenous metabolites). Spatial relationships of molecules within a specimen are preserved since intact tissue is directly analyzed without homogenization. In this way, molecules can be interrogated in their native environments providing new insights into the biological processes involved (Seeley and Caprioli, 2008).

In our study MALDI images were acquired for each of the spleens within their respective dosing groups. The cucumarioside A_2-2 shows no signal in the control tissues, but its signal was rather strong at 15 min post dosage in the spleen sections and in

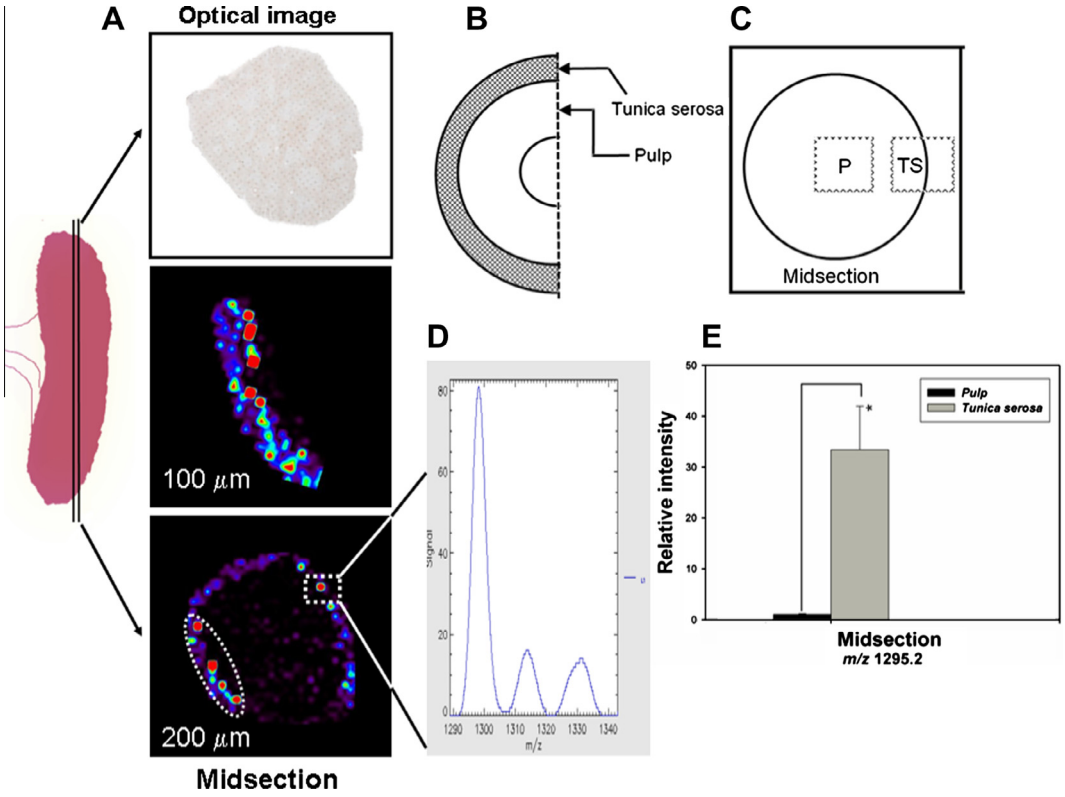

Fig. 4. Cucumarioside A_2-2 spatial distribution determined by MALDI-IMS in the spleen tissue after 15 min of single *i.p.* administration. (A) Optical images of spleen section covered by matrix (upper) and obtained by MALDI-IMS from middle sections at 100 μm and 200 μm resolution (middle and lower); (B) the regions of the investigated area on spleen sections; (C) areas (ROI) enclosed within the dashed-line rectangles were measured to quantify the MS intensity across the pulp (P) and tunica serosa (TS) regions; (D) cucumarioside A_2-2 spectrum obtained from ROI on spleen section using BioMap software; (E) total MS intensity across the different part of spleen sections. Obtained values were commuted to per unit area. The values are expressed in terms of mean ± se. $^*p < 0.05$ with student's *t*-test. $n = 5$.

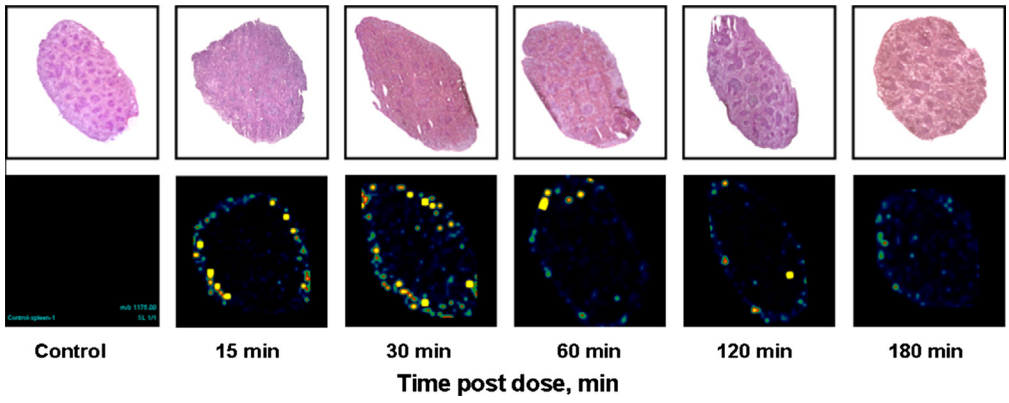

Fig. 5. Cucumarioside A_2-2 MALDI images for the acutely dosed mouse spleen samples. The top-most row displays the H&E stained section for the representative spleens. The bottom row shows the cucumarioside A_2-2 spatial distribution for a representative spleen at each acute time-point.

reasing further at 30 min. From the image data it can be determined that the cucumarioside A_2-2 was getting to the spleen and concentrating in the organ surface, but it did not significantly cross the tunica serosa upon acute glycoside *i.p.* dosing. Such a distribution of the drug after injection is obviously related to the method of administration. In this case the drug injected into the peritoneal cavity, at the earliest time point will contact the outer surface of the organs.

The subsequent decline in the glycoside concentration in the spleen tunica serosa displays its rapid elimination over time. This process is associated with its redistribution in the spleen and the very slow penetration into the deeper layers and pulp after about 2 h post injections that reflected in glycoside decrease in tunica serosa and its slight increase in pulp at 3 h. The MALDI-IMS results correspond to the radiospectroscopic studies of ^{3}H-cucumarioside A_2-2 dynamics and to MALDI-MS data.

In summary, the data obtained in this study allowed for a determination of cucumarioside A_2-2 dynamic in the spleens of acutely dosed mice using three different approaches. The MALDI-IMS techniques provided a rapid, reproducible method to find the drug accumulation in tissue while maintaining spatial integrity. Further work needs to directly correlate drug distribution with pharmacological response and provide insight into the concentration–effect relationship.

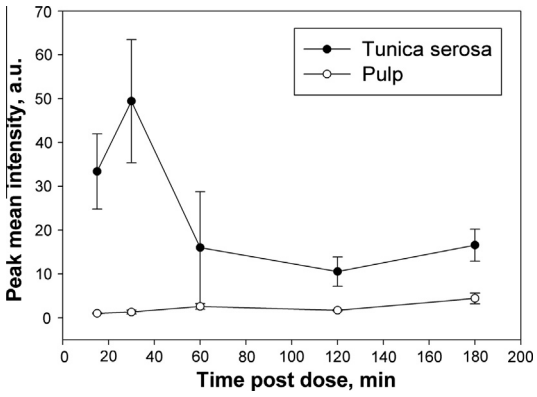

Fig. 6. Cucumarioside A_2-2 quantification results in peak intensity of spleen section ROI for the acutely dosed Balb/c mice. Obtained values were converted to per unit area. The values are expressed as mean ± se (n = 10).

Acknowledgments

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