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It is our great pleasure to present this Supplement Issue on "*Macedonian Pharmaceutical Bulletin*" to the scientific and professional community. This supplement includes the short communications from the *Sixth Congress of Pharmacy in Macedonia with International participation*, as the largest gathering for the pharmacy profession held in the Republic of Macedonia. The main theme of the Congress was "Modern pharmacist - bridging science with practice".

A broad spectrum of topics within the pharmaceutical sciences and practice carefully selected for this special occasion in order to build up a highly interesting and comprehensive program were covered. The contributions submitted to the Congress included 6 plenary lectures, 84 section lectures, and more that 240 posters. This Congress, followed the excellent international tradition, was attended by close to 1000 domestic and foreign participants. We received 326 short paper submissions from more than 25 countries. These numbers show that our Congress is aiming for the highest scientific standards, and that it can be considered a well-established venue for researchers in the broad fields of Pharmaceutical sciences and practice.

We would like to thank all internationally prominent researchers for their contribution to reinforcing the overall quality of the Congress. They give the state of the art of the recent advances in the field of pharmacy research.

Sincere thanks to the hosts of the Sixth Congress of Pharmacy in Macedonia with International participation, Macedonian Pharmaceutical Association and Faculty of Pharmacy, Ss Cyril and Methodius University in Skopje for their vision and commitments.

We acknowledge the sponsoring companies: the platinium sponsor AD ALKALOID, Skopje, the golden sponsor PLIVA, the silver sponsor EUROFARM and the bronze sponsor SEPTIMA, for the permanent support to our efforts during the organization.

We would also like to thank our members of the Scientific Committee for their volunteer time and dedication to the critical peer review process and in the organization of the program. We also wish to thank all the members of the Organizing Committee, whose work and commitment was invaluable.

On behalf of the Advisory and Scientific Committees, we would like to especially thank the authors, whose work was the essential part of the congress and contributed to a very successful event. Besides the many academic staff and professionals who contributed to the success of the Congress, we are grateful to the students who participated with oral presentations and posters.

The pharmaceutical sciences continue to grow as dynamic scientific interdisciplinary fields. We believe that published short communications will be an excellent source of scientific material in the fast evolving fields in Pharmaceutical sciences and practice.

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The present issue *of Macedonian Pharmaceutical Bulletin* is a special issue of the 6th Congress of Pharmacy in Macedonia with international participation.

This issue of *Macedonian Pharmaceutical Bulletin* contains short papers accepted by the scientific committee for the presentation at the Congress.

The authors are fully responsible for the contents of their short papers.

All reviewers that were involved in the short papers revision process are sincerely acknowledged.

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Short communication

Approaches in evaluation of freeze-dried antibody conjugates

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Introduction

Antibodies, proteins and other biotechnological products are often challenging in terms of their in-solution stability. Various physical and chemical changes occur during their in-solution storage, leading to shorter shelf-life. Freeze drying is often proposed as a method of choice, since removal of water has been reported to greatly reduce the rate of degradation, both in physical and chemical terms. Still, during freeze-drying antibodies and other protein pharmaceuticals can experience in-process changes that may reduce their physicochemical biological and/or pharmacological properties. In this context, many attempts have been made to reduce these changes, both in optimization of the freeze-drying process and in optimization of solution formulation, adding various buffers, cryoprotectants, etc.

The presented experience was in freeze-drying of monoclonal antibody – rituximab, conjugated with three types of bifunctional chelating agents, p-SCN-Bn-DOTA, p-SCN-Bn-DTPA, and 1B4M-DTPA, and evaluation of possible changes in post-freeze-drying phase. In order to assess possible defragmentation of the antibody, protein integrity test was performed, using SDS-PAGE electrophoresis and the analysis of several structural elements of FT-IR and Raman spectra pre- and post- freeze-drying process, provided an insight in possible changes in the structure.

Materials and methods

Commercially available rituximab (Mabthera®) was conjugated with three bifunctional chelating agents, p-SCN-Bn-DOTA, p-SCN-Bn-DTPA, and 1B4M-DTPA (Macrocyclics Inc. USA). The conjugates were synthesized, purified, adjusted to concentration of 1 mg/mL and freeze dried, using Labconco Free Zone Stoppering Tray Dryer (USA), as previously described (Smilkov et al., 2014). The protein integrity was assessed using SDS-PAGE that was performed in about 5 μL of reconstituted samples and 1 mg/mL purified, commercial rituximab (Mabthera®). Samples were mixed with sample buffer and boiled 5 min at 95 °C. Approximately 5 μL of each preparation was applied in 12% bisacrylamide under reducing conditions. Visualization of the bands was enabled using Coomassie Brilliant Blue R-250 (Sigma, USA). For comparison, low molecular weight marker (Amersham GE Healthcare, UK) was used.

For determining protein structure, FT-IR spectroscopy was conducted on PARAGON 1000 (Perkin Elmer, USA) spectrophotometer in the spectral range 2000–500 cm^{-1}. Attenuated Total Reflectance (ATR) spectra were acquired at a resolution of 4 cm–1. The obtained data was processed with Grams_32 software (Thermo Scientific). Raman spectra (2000–400 cm^{-1}) were recorded on a micro-Raman multichannel spectrometer Horiba JobinYvon LabRam 300 Infinity, using He:Ne laser. The spectral resolution was set to 4 cm^{-1}. The acquisition time and the accumulation number were set to 10 s and 10 scans, respectively (Gjorgieva Ackova et al., 2015).

Results and discussion

Using SDS-PAGE electrophoresis it is possible to determine the purity and, therefore possible defragmentation. The electrophoresis in reducing conditions resulted in two distinct Mw species which migrated in two bands in all

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three rituximab conjugates. The upper band corresponded to ~50 kDa and the lower band to ~25 kDa. This migration pattern is characteristic of IgG antibodies that have two identical subunits, each composed of two polypeptide chains: two heavy and two light chains, linked via four disulfide bonds. Under the action of the reducing agent DTT, the antibody is separated to heavy and light chains, with molecular weight corresponding to the two formed bands (Maleki et al., 2013; Nebija et al., 2011). The lyophilization protocol used did not affect structure properties and caused no post-lyophilization modification, as shown in the reducing SDS-PAGE lane patterns, compared to commercially available rituximab sample.

Spectroscopy studies can reveal information to witness preserved secondary structure upon freeze-drying, a mandatory prerequisite for immunoconjugates. Protein denaturation upon lyophilization is usually monitored by IR spectroscopy (Murphy et al., 2012), although Raman spectroscopy can also be applied (Wen, 2007).

The IR spectra of all three rituximab conjugates revealed higher percentage of β-sheet conformation (antiparallel and parallel) in the structure (strong band in the region between 1612 and 1640 cm^{-1}, followed by a weaker band around 1685 cm^{-1}), followed by α-helices (bands at 1655 or 1656 cm^{-1}), as obtained in the band frequencies for amide I, II and III bands which are used as diagnostic bands. We observed that the freeze-dried rituximab conjugates regain their native conformation upon rehydration (reversible unfolding).

Thermally-induced aggregation processes of the majority of proteins can also be studied by FT-IR and Raman spectroscopy. Strong absorption bands below 1620 cm^{-1} can be correlated with aggregation, usually associated with the formation of new strong beta-sheet structures (Schüle et al., 2007). With the lowest frequency band detected at 1620 cm^{-1} (in all samples analyzed), we concluded no obvious aggregation in all three freeze-dried antibody conjugates.

Conclusion

Among the many techniques available for evaluation of the structure and stability of freeze-dried antibody conjugates, SDS-PAGE electrophoresis, FT-IR and Raman spectroscopy can be employed in assessing structural properties as well as in determination of stability of these potential drug candidates.

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