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Почитувани,

Ве известуваме дека ***Македонски фармацевтски билтен***, списанието на Македонското фармацевтско друштво, печати трудови на англиски и на македонски јазик, и тоа оригинални научни трудови, куси соопштенија и прегледи на англиски јазик, а стручни трудови на македонски или на англиски јазик. Трудовите се подложени на двојна рецензија од врвни стручњаци од соодветните области од земјата и од странство. Во тек се активностите за регулирање на статусот на Списанието во базата Chemical Abstracts и за перманентно цитирање во неа.

Ве поканuваме да придонесете во подигањето на научното и на стручното ниво на единственото списание од областа на фармацијата од нашата земја со приложување на Ваши трудови, подготвени во согласност со пропозициите на Списанието кои ќе ги најдете на последните страници од овој број.

Уредувачки одбор

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Original scientific paper

Quantitative determination of glycyrrhizinic acid by square-wave voltammetry and high-pressure liquid chromatography

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Abstract

Novel adsorptive stripping square-wave voltammetric method as well as a new high-pressure liquid chromatographic method for direct determination of glycyrrhizinic acid in dosage pharmaceutical preparation, used against virus infections, have been developed. Glycyrrhizinic acid is an electrochemically active compound, which undergoes irreversible reduction on a mercury electrode surface in an aqueous medium. Its redox properties were studied thoroughly by means of square-wave voltammetry, as one of the most advanced electroanalytical technique. The voltammetric response depends mainly on the pH of the medium, composition of the supporting electrolyte, as well as the parameters of the excitement signal. It was also observed that the voltammetric properties strongly depend on the accumulation time and potential, revealing significant adsorption of glycyrrhizinic acid onto the mercury electrode surface. Upon this feature, an adsorptive stripping voltammetric method for quantitative determination of glycyrrhizinic acid was developed. A simple, sensitive and precise reversed phase HPLC method with photodiode array UV detection has also been developed, mainly for comparison and conformation of the results obtained with the voltammetric method.

Key words: Glycyrrhizinic acid, square-wave voltammetry, high-pressure liquid chromatography

Introduction

Glycyrrhizinic acid (GA) is a saponin extracted from Glycyrrhiza glabra root. According to its chemical structure, it belongs to penthacyclical triterpenoid saponins, products of β-amyrin (1). GA and its aglycon 18β glycyrrhetinic acid are widely used against bacterial and virus infections, also as antiflogistics, expectorants, corrigens and as components with mineralocorticoid effect (2).

Many analytical methods have been employed for the determination of GA, such as: chromatographic techniques (high-pressure liquid chromatography, thin-layer chromatography, micellar electrokinetic chromatography, gas chromatography) (3-10); electrochemical techniques (oscillopolarography, single-sweep polarography) (11, 12); spectroscopic methods (13, 14). Immunoassays (ELISA and RIA techniques) were also used for determination of the glycyrrhizinic acid (15).

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The general purpose of the work was to develop simple, precise and accurate methods for quantification of glycyrrhizinic acid in pharmaceutical dosage forms.

Adsorptive stripping voltammetry is a valuable and well-known analytical method for trace analysis of electroactive compounds capable to adsorb onto the working electrode surface (16).

In the family of pulse techniques square-wave voltammetry appears to be one of the most advanced electroanalytical techniques (17). This technique is attributed with unique advantages, such as: high frequency and amplitude, solitary approach in the current-sampling procedure, as well as the effective ability to discriminate the capacity current. Thus, for developing a particular absorptive stripping voltammetric method for determination of miscellaneous species, square-wave voltammetry is most frequently a technique of choice.

In addition to the current voltammetric method, the paper also describes a new chromatographic method for determination of GA serving mainly for comparison and conformation of the results obtained with the voltammetric method.

Experimental

The analyzed sample was a pharmaceutical dosage in a form of spray. As declared by the manufacturer, the sample contains 0.1 % (w/w) glycyrrhizinic acid, while other components are maleic acid, fumaric acid, ascorbic acid, folic acid, polysorbate 80, propylen glycol and purified water. Glycyrrhizinic acid, standard substance, was a product of "Chemonova International, S. A." from Spain.

Voltammetric method

All used chemicals were of analytical reagent grade. KI, Na_2SO_4, $NaNO_3$, $KClO_4$, KBr, citric acid, and Na_2HPO_4 were all Merck products. Double distilled water was used. The buffer solutions were prepared by mixing of appropriate quantities of citric acid and Na_3PO_4. The stock solution of glycyrrhizinic acid was prepared by dissolving in redistilled water.

The oxygen removal from the electrolyzed solutions was performed by purging with highly purified nitrogen for ten minutes prior each measurement, and a nitrogen blanket over the solution was maintained thereafter. The measurements were carried out at room temperature.

All voltammograms were recorded with multi-mode polarograph Princeton Applied Research Model 384 B equipped with static mercury drop electrode Model PAR 303A. The reference was Ag/AgCl (sat. KCl) and a platinum wire was used as a third electrode. A small mercury drop with a surface of 0.0147 cm^2 was used as a working electrode.

The other experimental conditions are indicated in the caption of the figures.

HPLC method

All used chemicals were HPLC grade. The chromatograms were recorded on Waters HPLC system, equipped with Pump Waters 600 E, sample injector Rheodyne 7725i with 20 µl loop, Photodiode Array Detector 996 and column oven. Glycyrrhizinic acid was separated on a 5 mm Symmetry C18 column (25 cm x 4.6 mm i.d.) operated at 35 ^{0}C, using 25 mM potassium dihydrogen phosphate (pH 2.2) / acetonitril (60: 40 v/v) as a mobile phase (and solvent) at a flow rate of 1.6 ml/min and detection at 251 nm. The chromatographic system was controlled by software program Waters Millennium 32.

Results and discussion

Determination of glycyrrhizinic acid by voltammetric method

Glycyrrhizinic acid is both electroactive and surface-active compound. Its undergoes totally irreversible reduction onto the mercury electrode surface. Under conditions of square-wave voltammetry, the response consists of a single well-defined peak at potential of about -1.50 V as represented in Fig. 1. The SW voltammograms were analyzed for the peak position, height, and half-peak width. All these properties of the voltammetric responses are strong function of pH and chemical composition of the medium, accumulation time and potential, as well as the parameters of the excitement signal.

The redox reaction of GA could be measured only within the pH interval from 2.0 to 8.0. Beyond these boundaries, no voltammetric response could be detected. In strong acidic medium pH ≤ 2, the hydrolysis of GA occurs at appreciable rate (18) preventing its electrochemical detection. As pH increases from 2.0 to 4.9, the SW peak shifts slightly towards potential that is more negative (see data in Table 1). At the same time, the peak current depends non-linearly on pH, crossing through maximum value at about pH = 3.8. Within the interval 5.4 ≤ pH ≤ 7.5, the SWV response of GA splits in two peaks indicating existence of two different redox reactions. For analytical purposes, this phenomenon is strongly unfavorable.

The complexity of the pH effect upon the voltammetric response arises from the complexity of the redox reaction of GA as well as the complexity of its adsorption process onto the working electrode surface. Nevertheless, the electrolyte solution with 3.8 ≤ pH ≤ 5.0 provides a save medium for analytical application, as the SWV response in those solutions consists of a well-defined single peak. Moreover, the ratio $I_p/\Delta E_{p/2}$ (where I_p and $\Delta E_{p/2}$ is a peak current and half-peak width, respectively) should serve as a main criterion for selecting an optimal response for analytical purposes. This quantity reaches a maximum value for pH = 4.7 revealing that this medium is the most suitable for quantitative determination of GA.

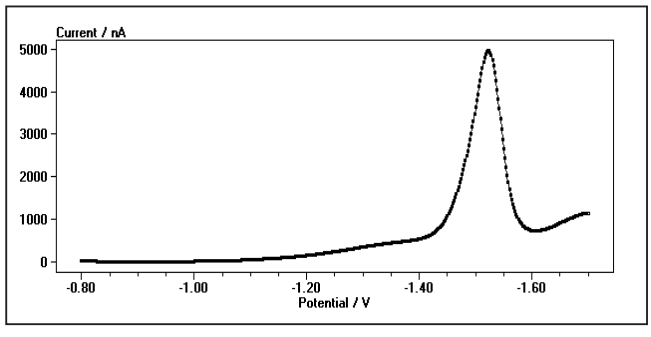

Fig. 1. Square-wave voltammogram of 1×10^{-5} mol/L glycyrrhizinic acid solution recorded in 0.1 mol/L KI electrolyte solution buffered to pH 4.7. Other experimental conditions are: initial potential E_s = -0.3 V, final potential E_f = -1.7 V, accumulation time $t_{acc.}$ = 100 s, accumulation potential $E_{acc.}$ = -0.3 V, SW frequency f = 100 Hz, SW amplitude E_{sw} = 25 mV and increment dE = 4 mV

Table 1. Dependence of peak potential and peak current of the voltammetric response of 5×10^{-6} mol/L glycyrrhizinic acid solution on pH recorded in citrate buffers. The other experimental conditions were: f = 100 Hz, E_{SW} = 25 mV, dE = 2 mV, E_S = -0,8 V and Ef = -1.7.

pH	$E_{p,1}$/V	$E_{p,2}$/V	$I_{p,1}$/nA	$I_{p,2}$/nA
2.0	No voltammetric response			
3.6	-1.476		603.7	
3.8	-1.478		1183	
4.1	-1.486		343.4	
4.3	-1.476		443.6	
4.5	-1.502		255.5	
4.7	-1.496		309.5	
4.9	-1.506		372.4	
5.4	1.414	-1.494	556.7	773.7
6.1	-1.37	-1.45	159	172
6.8	-1.358	-1.464	273.7	306.4
7.5	-1.382	-1.456	144.6	153.9
8.0	No voltammetric response		No voltammetric response	

Generally speaking, the adsorption of an electroactive compound onto the electrode surface is affected primarily by the chemical composition of the medium and the accumulation potential (16). The accumulation of GA on the mercury electrode surface was studied in four diverse supporting electrolytes. The results for the peak currents are listed in Table 2.

As it can be seen, the highest currents were obtained in 0.1 mol/L solution of KI indicating that in this medium the adsorption occurs at highest rate. Keeping in mind that the iodide ions are themselves adsorbed on the electrode surface, one can conclude that GA exhibits substantial tendency for adsorption. The data presented in Fig. 2 support this assumption. The Figure 2 illustrates the effect of the accumulation time upon the peak currents of GA investigated at various concentrations of the analyte. By increasing the accumulation time, the peak current becomes higher and the entire I_p-t_{acc} relationship obeys a low of an isotherm. The appear plateaus

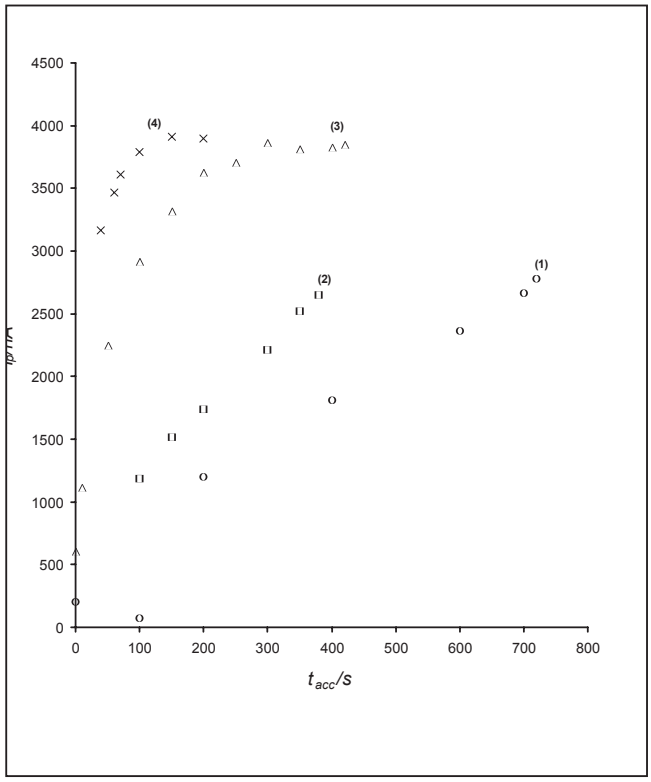

Fig. 2. Dependence of the peak currents on the accumulation time for different glycyrrhizinic acid concentration recorded in 0.1 mol/L KI buffered to pH = 4.7. Experimental conditions were: E_{acc} = -0.8 V, f = 100 Hz, E_{SW} = 25 mV, dE = 2 mV and c (glyc.) = 5×10^{-7} (1), 1×10^{-6} (2), 5×10^{-6} (3) 1×10^{-5} mol/L (4).

of the curves 1 and 2 corresponds to the saturation of the electrode with electroactive material. For analytical purposes, the electrode should be covered by a monomolecular layer of deposited GA molecules, which is fulfilled within the linear portions of the isotherms.

The influence of the accumulation potential on the peak current is presented in Fig. 3. The accumulation potential affects considerably the entire accumulation process through its influence upon the structure of the electric double layer adjacent to the electrode surface (16). The peak current depends sigmoidally on the accumulation potential in a form of a pseudopolarogram.

Table 2. Dependence of peak current of 5×10^{-6}mol/L glycyrrhizinic acid solution on the accumulation time recorded in diverse supporting electrolytes. Experimental conditions were: f = 100 Hz, E_{sw} = 25 mV, dE = 2 mV, E_{acc} = -0.8 V. All supporting electrolytes were buffered with citrate buffer to pH = 4.7.

	I_p/nA	I_p/nA	I_p/nA	I_p/nA	I_p/nA	I_p/nA
t_{acc}./s	Na_2SO_4	$NaNO_3$	$KClO_4$	KI	KBr	Citrate buffer
0	431.9	395.5	436.4	562.9	425.9	446
50						2323
60	2329	2099	2272	2839	2485	
100						3298
120	3396	3190	3264	3862	3272	

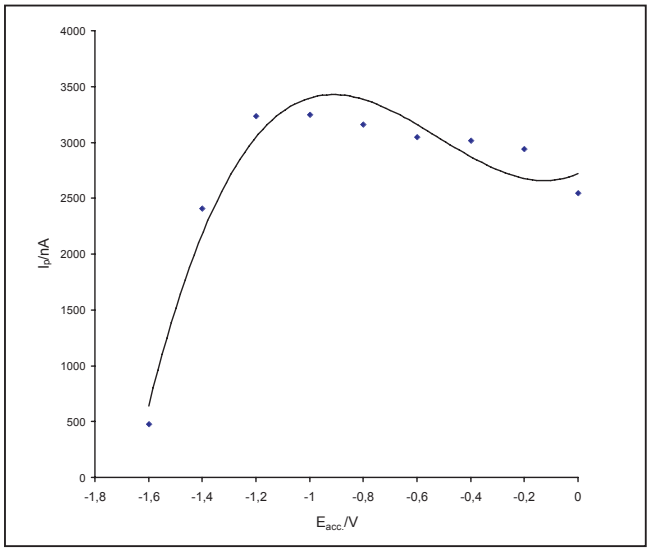

Fig. 3. Dependence of the peak current of glycyrrhizinic acid on the deposition potential. Experimental conditions were: f = 100 Hz, E_s = -0.8 V, E_{sw} = 25 mV, t_{acc} = 15 s, dE = 2 mV, c (glyc.) = 5 × 10^{-6} mol/L, supporting electrolyte 0.1 mol/L KI buffered to pH 4.7.

The peak current decreases considerably as the accumulation potential becomes more negative than -1.0 V. The most effective accumulation was observed under accumulation potential of about E_{acc} = - 0.80 V and hence, this value was used in the further measurements.

As mentioned previously, the sensitivity of the method depends markedly on the properties of the potential waveform applied in the course of the stripping step. Accordingly, an attempt was made to optimize the properties of the SW excitement signal such as SW amplitude E_{sw} (the height of the potential pulses), SW frequency f (the duration of single potential pulse), and scan increment dE (the step of the staircase potential ramp at which potential pulses of square shape are superimposed).

The peak current increases strongly by increasing the SW amplitude from 2 to 50mV. This phenomenon has already been predicted by the theory of SWV regarding a totally irreversible redox process, which proceeds from an immobilized state of the reactant.

As the amplitude becomes higher than 50mV, the peak current reaches a maximum value forming a plateau on the I_p-E_{sw} function (see curve 1 in Fig. 4). The peak potential shifts in positive direction within the interval 2mV ≤ E_{sw} ≤ 150mV. However, the amplitude also influences the shape of the voltammetric response, causing the SW peak to widen by the increase of the amplitude. The ratio $I_p/\Delta E_{p/2}$ gains a maximum value for Esw = 25mV, consequently this value was selected as an optimal for analytical measurements.

The signal frequency was varied over the interval from 10 to 120 Hz. It was observed that the frequency affects both the position and height of the SW response. A linear relationship between E_p (peak potential) and logarithm of the frequency was observed with a constant slope of $\Delta E_p/\Delta\log(f)$ = - 30.1 mV. At the same time, the peak current increases linearly with the SW frequency, which is typical for redox reaction occurring on the electrode surface via adsorbed reactant. The half-peak width is insensitive on the signal frequency. Thus, SW frequency of f = 120Hz was considered as an optimal value for further quantitative analysis.

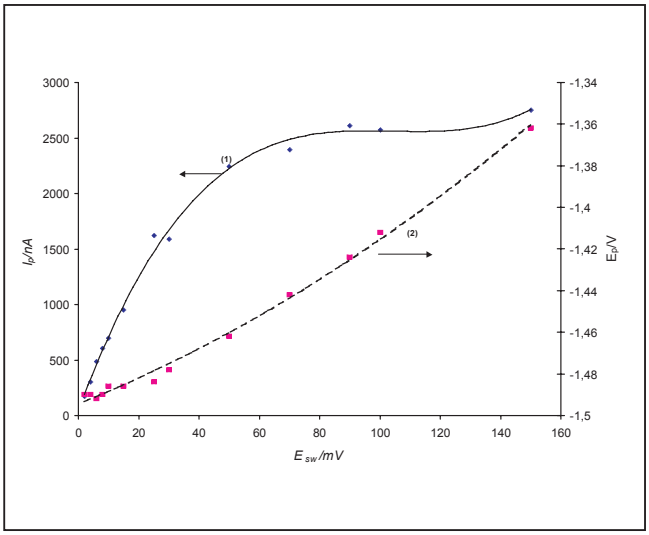

Fig. 4. Dependence of the peak currents (curve 1) and peak potentials (curve 2) on the signal amplitude. Experimental conditions were: f = 100 Hz, dE = 2 mV, E_{acc} = -0.8 V, t_{acc} = 15 s, c (glyc.) = 5 × 10^{-6} mol/L, supporting electrolyte 0.1 mol/L KI buffered to pH 4.7.

The scan increment exhibits rather similar effect to the voltammetric response as that of SW amplitude and consequently those results will be not repeated hereafter. The value of dE = 8 mV was selected as most relevant for quantitative measurements.

Therefore, the following set of experimental conditions are proposed for quantitative determination of GA: supporting electrolyte 0.1 mol/L KI buffered with citrate buffer to pH = 4.7, deposition potential E_{acc} = -0.8 V, SW frequency f = 120 Hz, SW amplitude E_{sw} = 25 mV, and scan increment dE = 8 mV. The duration of the deposition step depends on the analyte concentration range.

A calibration curve of GA was recorded in 0.1 KI buffered to pH = 4.7, over the concentration range from 5 × 10^{-7} mol/L to 7 × 10^{-6} mol/L. The other experimental conditions were: f = 100Hz, dE = 8mV, E_{sw} = 25 mV, E_{acc} = -0.8V and t_{acc} = 15s. The line is associated with a linear regression correlation coefficient of R = 0.9992.

To estimate the accuracy of the proposed adsorptive voltammetric method, a recovery analysis was undertaken and the corresponding results are listed in Table 3.

Table 3. Results of recovery analysis obtained by standard addition method. Experimental conditions were: f = 100 Hz, E_{sw} = 25 mV, dE = 2 mV, supporting electrolyte 0.1 mol/L KI buffered to pH 4.7.

Sample	Added (mol/L)	Found (mol/L)	Recovery (%)
Glycyrrhizinic acid - spray	4.97×10^{-6}	4.80×10^{-6}	96.58
	5.9×10^{-6}	6.06×10^{-6}	102.71
	9.95×10^{-6}	10.40×10^{-6}	104.50

For recovery analysis, the standard addition method and the Hon equation were utilized. The Hon formula for determination of the sample concentration reads:

$$c_s = \frac{c_{st} \cdot V_{st} \cdot I_{p,s}}{I_{p,st} \cdot (V_{cell} + V_{st}) - I_{p,s} \cdot V_{cell}}$$

where c_s is sample concentration, c_{st} is concentration of the standard addition, V_{st} is volume of the standard addition, $I_{p,s}$ is current associated with the sample, $I_{p,st}$ is current of the standard addition, and V_{cell} is volume of the electrolyzing solution.

Determination of glycyrrhizinic acid by HPLC method

A reversed phase HPLC procedure was developed as a comparative method for determination of GA in pharmaceutical preparation. GA was well resolved from the other formulation components using the previous established selective chromatographic conditions. The retention time of glycyrrhizinic acid was found to be approximately 4.8 minutes (Fig. 5).

Peak purity of glycyrrhizinic acid in the sample has been established with software program Waters Millennium 32 by comparing the purity angle (0.119) with purity threshold (0.269).

The dependence of the GA peak area on the concentration of the injected solution was linear over the investigated range of 10 to 500 μg/ml with a linear regression correlation coefficient of R = 0.9994.

The accuracy of the chromatographic procedure was confirmed by the method of standard addition (Table 4).

The recovery values of GA determinations range from 99.1 to 101.77 % confirming that HPLC method is both quantitative and accurate.

The results of GA determination in pharmaceutical preparation obtained by square-wave voltammetry and high-pressure liquid chromatography are given in Table 5.

As it can be seen, the results obtained by voltammetric and HPLC method are precise, accurate and reproducible.

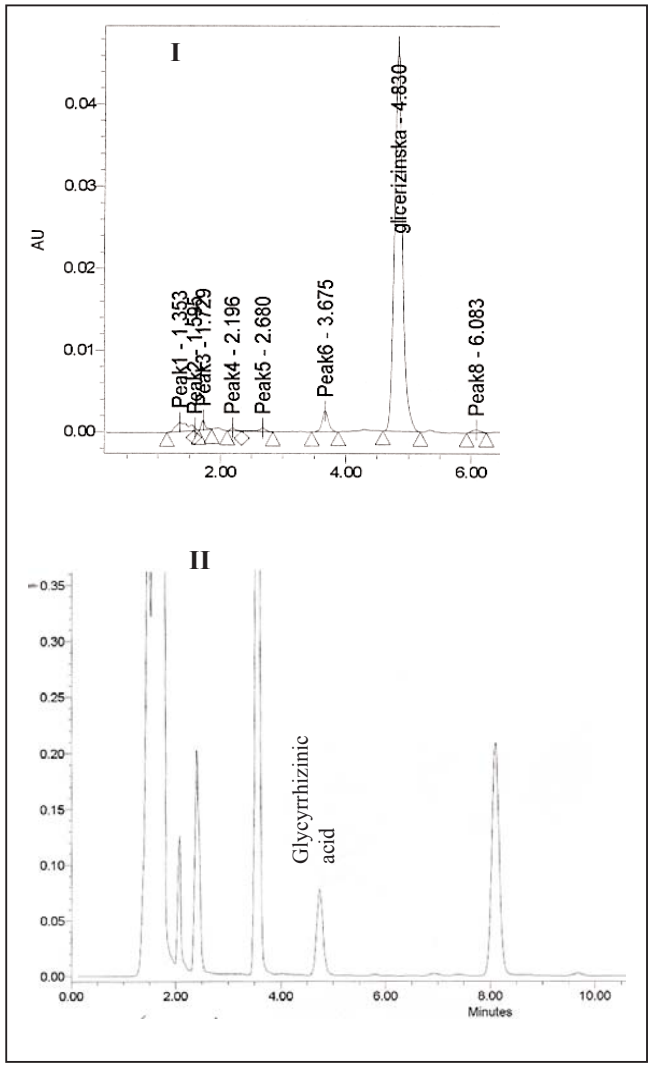

Fig. 5. Chromatograms of: I - glycyrrhizinic acid, standard substance (100 μg/ml), II – sample (glycyrrhizinic acid ~ 100 μg/ml).

Table 4. Results of glycyrrhizinic acid determination by method of standard addition, for estimating the accuracy of the HPLC method

Sample	Added (mg/ml)	Found(mg/ml)	Recovery (%)
Glycyrrhizinic acid - spray	-	0.9953	-
	0.25	1.2341	99.10
	0.50	1.4733	98.53
	1.00	2.0306	101.77

Table 5. Results of glycyrrhizinic acid determination in sample (spray) by square-wave voltammetry and high-pressure liquid chromatography

Method	Declared (mg/ml)	Determined (mg/ml)	RSD (%)	Recovery (%)
SWV	1.0	1.037	4.84	103.7
HPLC		0.993	1.54	99.3

Conclusion

A new adsorptive stripping square-wave voltammetric method for quantitative determination of glycyrrhizinic acid in pharmaceutical preparations is proposed. The method is simple, fast and precise. The voltammetric measurement itself takes a few seconds. Moreover, it is important to emphasize that the proposed method could be applied without previous treatment of the original preparation. The proposed method is also attractive due to the low cost of the electrochemical instrumentation. The conditions for quantification of GA applying high-pressure liquid chromatography are also established. The precision, accuracy and reproducibility of the voltammetric method are comparable to those of HPLC method and both may be applied for routine analysis and determination of glycyrrhizinic acid in pharmaceutical formulations.

References

1. M. Wichtl, *Herbal Drugs*, Medpharm Scientific Publishers, Stuttgart, 1994.
2. A.N. Carol, A.A. Linda and J.P. David, *Herbal Medicines*, The Pharmaceutical Press, London,1996.
3. M. Itoh, N. Asakawa, Y. Hashimoto, M. Ishibashi and H. Miyazaki, *J. Pharm. Soc. Jpn*., **12**, 1150-1154 (1985).
4. S.S. Nina, *Pharmaceutical Biomedical Analysis*, **5**, 289-293 (1987).
5. G. Lu and J. Liu, *Yaowu-Fenxi-Zazhi*, **8**, 137-139 (1988).
6. D.H. Yan, *Yaowu-Fenxi-Zazhi*, **13**, 381-383 (1993).
7. E. Hahn-Deinstrop, Thin-Layer chromatography in German Pharmacopeia, *Dtsch. Apoth. Ztg.*, **29**, 26-39, (1995).
8. X.J. Cui, Y.C. Chi, Y.H. Jin, W.L Dong and J. Zhang, *Yaowu-Fenxi-Zazhi*, **16**, 323-324 (1996).
9. R. Matissek and P. Spoeer, *Dtsch-Lebensm-Rundsch*., **92**, 381-387 (1996).
10. P. Wang, S.F.Y. Li and H.K. Lee, *J. Chromatogr A*., **811**, 219-224 (1998).
11. H. Song and W. Wang, *Yaowu-Fenxi-Zazhi*, **7**, 108-110 (1987).
12. W. Guo, J.F. Song, G. Chen and Y. Wang, *Fenxi-Shiyanshi*, **15**, 51-54 (1996).
13. J. Mau, *Fenxi-Huaxue*., **16**, 188-189 (1988).
14. M. Mizugaki, K. Itoh, M. Hayasaka, S. Ishiwata, S. Nozaki, N. Nagata, K. Hanadate and N. Ishida, *J. Immunoassay*, **15**, 21-34 (1994).
15. T. Kitagawa, Z. Yang, G. Bai, K. Fujiwara, A. Akahori, / S. Sonoi and S.Kondo, *Biol-Pharm-Bull*., **20**, 211-216 (1997).
16. W. Joseph, In *Electroanalytical Chemistry*, A.J.Bard (Ed.), Vol. 16, Dekker, New York, 1989.
17. J. Osteryoung, J.J. O'Dea, In *Electroanalytical Chemistry*, A.J.Bard (Ed.), Vol. 14, Dekker, New York, 1986.
18. L.A. Baltina, O.B. Flechter, Z.M. Putieva, R.M. Kondratenko, L.V. Krasnova and G.A. Tolstikov, *Khim. Farm. Zh.*, **30**, 47-49 (1996).

Резиме

Квантитативно определување на глициризинска киселина со квадратно-бранова волтаметрија и течна хроматографија под висок притисок

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Клучни зборови: глицеризинска киселина, квадратно-бранова волтаметрија, течна хроматографија под висок притисок

Развиен е метод на квадратно-бранова волтметрија и течна хроматографија под висок притисок за директно определување на глициризинската киселина во дозиран фармацветски препарат, што се користи против вирусни инфекции. Глициризинската киселина е електрохемиски активна суспстанција, што подлежи на иреверзибилна редукција на површината на живината електрода, во воден медиум. Редокс својствата на глициризинската киселина беа целосно проучени со примена на квадратно-брановата волтаметрија, како една од најефикасните електроаналитички техники. Волтаметрискиот одговор главно зависи од pH на средината, составот на основниот електролит, како и од параметрите на ексцитирачкиот сигнал. Исто така, утврдено е дека волтаметрискиот одговор е силно зависен од времето на акумулација и од потенцијалот, кои укажуваат на значителна адсорпција на глициризинската киселина на површината на живината електрода.

Методот на реверзно-фазна течна хроматографија под висок притисок беше развиен, главно за споредба и потврдување на резултатите добиени со волтаметрискиот метод.

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Original scientific paper

BSA - loaded gelatin microspheres: Comparative studies on biodegradation and drug release in presence of collagenase and trypsin

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Abstract

Certain variations in the process parameters (emulsification time, surfactant concentration) were performed in order to prepare BSA-loaded gelatin microspheres with particle size ranging from 1 to 10 μm and high loading efficiency using a procedure originally employed by Tabata and Ikada. In vitro degradation and drug release studies in the presence of trypsin and collagenase, respectively, were performed in order to evaluate the potential of gelatin microspheres as regulated and sustained release systems for oral vaccination. Degradation data showed that the preparation procedure had provided prolonged degradation in the presence of both enzymes, suggesting complete *in vivo* degradation. Exponential dependence of the amount of drug released on time was evidenced. The diffusion coefficients were superior to 0.5 indicating the Case II anomalous Fickian diffusion, except for the particles smaller than 5 μm where in the presence of *collagenase* the transition to Super Case II transport was observed due to the higher rate of polymer degradation and BSA diffusion through the matrix. The mathematical modeling of drug release showed a biphasic release pattern in the presence of both enzymes, where the rate constants for the initial time release confirmed the influence of the particle size and/or enzymatic degradation rate on the drug release rate.

Key words: Gelatin microparticles, BSA, controlled release, trypsin, collagenase

Introduction

There is a body evidence which suggests that the absorption of orally administered antigen in the intestines stimulate particular cells in the gut-associated lymphoid tissue (GALT), especially in the Peyer's patches (PP) leading to dissemination of B and T cells to mucosal effector tissues for subsequent antigen-specific secretory IgA responses. A number of potential delivery systems, including sustained-antigen releasing polymer based microparticulated carriers have been used for targeting the PP and protecting the antigen of interest from the harsh environment of the GIT (1-2). Among the natural polymers forming the particles, gelatin is known for its excellent biocompatibility due to low toxicity and poor immunogenic reaction (3). Furthermore, gelatin microspheres proved to evoke the immune response at the genito-urinary mucosa, suggesting the possibility to develop an oral vaccine (4). The strong adjuvant effect is considered to be derived from, at first, their function as carriers in the GIT to deliver antigens to the immuno-inductive site (PP), protecting them from enzymatic degradation. Gelatin microspheres taken up by PP gradually release the antigens, which are then recognized as infectious substances by B cells in the GALT. It is also possible to consider that the function of gelatin itself as an accelerator of macrophagocytosis might relate to the strong adjuvant effect of gelatin microspheres.

Variations in particle size can control particle uptake into mucosa subepithelial regions, while the control of mechanical characteristics as well as degradation kinetics

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via gelatin cross-linking could be achieved (5-6). The treatment of gelatin by glutarldehyde (GTA) gives rise to the formation of scarcely or non-soluble products which can slow down the release of the encapsulated drug. Cross-linking is optimal at neutral pH and requires free non-protonated ε-amino groups of lysine and hydroxylysine to form imines. GTA reacts not only with amino groups, but with carboxylic, amide and other groups of proteins, as well. Additional polymerization by aldol condensation results in covalent intramolecular cross-links connecting distant gelatin molecules.

Gelatin degradation by all cell types can occur either after phagocytosis or by extracellular protease acting at either neutral or acid pH. At neutral pH only specific collagenases simply attacks the Gly-X-Y sequence and are capable of cleaving the triple helix at multiple sites. Collagenase binds tightly to triple-helices at or near surface, whereas molecules in the interior become accessible to enzymes in the course of the progressive degradation from the outside (6). After the triple-helix is cracked, further degradation is facilitated by enzymes such as gelatinases and non-specific proteinases that cleave the primary fragments into small peptides and amino acids. Comparing to other neutral proteinases, trypsin appeared the most specific enzyme for gelatin microparticle degradation (7).

BSA-loaded gelatin microspheres were prepared by emulsification of aqueous solution of gelatin and BSA, into the particles in an oil (8-9). FTIR-ATR spectroscopic studies have confirmed that, during the preparation process and freeze-drying procedure, only minor conformational changes of protein (α-helix and β-sheet content) were induced, owing to the effective preservation provided by the presence of sorbitol (10-11). Variations in the formulation and process parameters were performed in order to prepare microspheres with high loading efficiency and an optimal size for uptake into subepithelial mucosa 1-10 μm. It was previously shown that microspheres with diameter from 5 to 10 μm remained in the PP, where they induced mucosal immunity, whereas the microspheres smaller than 5 μm in diameter passed through the PP within macrophages to stimulate systemic immunity (2, 12-13). "Model" protein, BSA, facilitated the understanding of the release kinetics. A key parameter appeared to be the particle size, so the correlation between the biodegradation and drug release rates in the presence of *collagenase* and *trypsin*, respectively and drug and polymer particle size was established as an object of this research.

Materials and Methods

Materials

Gelatin was purchased from Merck (Germany), bovine serum albumin, glutaraldehyde and sorbitan monooleate (SPAN 80®) from Sigma Chemical Co. (Germany), while sorbitol from Merck (Germany). Radiolabelling of BSA was performed by ^{131}I obtained from Biointernational (France). *Collagenase* (EC 3.4.24.3.) from *Clostridium histoliticium* lyophilised and *Trypsin*, EC 3.4.21.4. were kindly donated by Varus (Representative office of Merck, Germany for Macedonia).

Preparation of microspheres

The microspheres were prepared by emulsification of aqueous solution of gelatin and radiolabelled ^{131}I-BSA into the particles in an organic phase (9). In short, 0.2 ml gelatin aqueous solution (10 *w/v %*) with 5 mg BSA (2.5 *w/v %* in feed) was emulsified in a mixture of toluolum and chloroform (1:1) containing sorbitan monooleate (SPAN 80) in adequate concentration. The resulting emulsion was quickly poured into precooled mixture of toluolum and chloroform (1:3) containing 5% *m/V* SPAN 80. The gelatin was cross-linked with GTA-saturated toluene (0.05 mg mg^{-1} gelatin) in the emulsion at 0^0C for 4 hours. The resulting microspheres were successively washed with isopropanol, purified water, and then freeze-dried at 200 Pa and - 40 ^{0}C (Christ α 2-4, Bioblock, Scientific, France) in the presence of sorbitol as a lyoprotectant of protein structure. Variations in the formulation and process parameters, surfactant concentration and time of emulsification (Table 1) were performed in order to prepare microspheres with high microencapsulation efficacy and size ranging from 1 to 10 μm.

Table 1. Formulation and process variables of gelatin microsheres

Type of microspheres	SPAN 80 concentration in emulsion, w/v%	Time of emulsification, minutes
A	5	
A-1		15
A-2		30
A-3		45
B	8	
B-1		15
B-2		30
B-3		45
C	10	
C-1		15
C-2		30
C-3		45
D	12	
D-1		15
D-2		30
D-3		45

Microspheres size measurement

The mean diameter of the population of gelatin microspheres in aqueous dispersions was determined using laser diffractometry (Particle size analysette D LAB/22, Fritsh, Germany). The spherical form was based on optical micrographs of gelatin microspheres (camera Nikon E-800, Japan) using technique of phase contrast.

Determination of drug loading

The content of radiolabelled ^{131}I-BSA entrapped in gelatin matrix was calculated as a percent of total radioactivity present in the supernatant and in the filtrate after microsphere isolation ("well" counter Scaler Type N 529 D, EKCO Electronics, UK).

In vitro degradation of gelatin microspheres

In vitro degradation in the presence of collagenase. Gelatin microspheres (0.5 mg ml^{-1}) were suspended in *calcium* (0.05 mg ml^{-1} $CaCl_2$) containing PBS with *collagenase* (0.5 mg ml^{-1}). *In vivo* conditions (37 ^{0}C, 75 Str. min^{-1}) were simulated in a horizontal shaker (Haake SWB 20, Fisons, Germany). At regular time intervals, the dispersion was centrifuged at 8000 rpm for 15 min (Jouan MR 22i Centrifuge, France) and the amount of gelatin in the sediment was determined according to a ninhydrin method (14).

In vitro degradation in a presence of trypsin. Aqueous dispersion of microspheres (1.5 mg ml^{-1}) was used to determine *in vitro* enzymatic degradation rate in the presence of *trypsin* (0.4 mg ml^{-1}) according to the method proposed by Roser and Kissel (7). Turbidity was measured at the beginning and during regular time intervals by the absorbance at 546 nm (UV/VIS Lambda 16, Perkin Elmer Spectrophotometer, USA).

In vitro release of BSA from gelatin microspheres

The amount of BSA released from gelatin microspheres in the presence of *collagenase* and *trypsin*, respectively, was determined by measuring the radioactivity present in the supernatants in regular time intervals. To simplify the analysis of controlled release data, the empirical, exponential equation was used (11):

$$Mt/M_o = kt^n$$

where M_t/M_o is the fraction of drug released; t is the release time, n is a diffusional exponent characteristic of the release mechanism, and k denotes properties of the polymer and the drug. This equation applies until 60% of the total amount of drug is released. It predicts that the fractional release of drug is exponentially related to the release time and it adequately describes the release of drug from swellable and non-swellable polymer devices of varying geometry. For swellable controlled release systems, the slope (n) of the log (drug released) vs. log (time) plot is 0.45 for pure Fickian diffusion. An anomalous non-Fickian diffusion pattern (0.45-0.89) is observed when the rates of the solvent penetration and drug release are in the same transport (n > 1) is observed when drug diffusion is rapid compared to the constant rate of solvent induced relaxation and swelling in the polymer (15-17). In order to determin the initial and late time drug release rate, the *in vitro* release profiles from microspheres were described using biexponential function: $M_\infty - M_t/M_\infty = Ae^{-\alpha t} + Be^{-\beta t}$, where α and β are the rate constants for the initial and late time releases (18).

Results and Discussion

Effect of formulation and procedure parameters on microspheres size and loading efficacy

The mean diameter of microspheres prepared in this study was in the range from 1.20 ($\pm$1.96) to 7.03 ($\pm$1.23) μm, which is in the frame of optimal size for uptake into GALT and systemic immunoresponsive organs. By extending the time of emulsification and increasing the surfactant concentration, particle size decreased in all series of the gelatin microspheres. Two-factorial analysis of variance (α=0.05) confirmed that the time of emulsification dominantly influenced the microspheres size at all surfactant concentrations.

During the preparation procedure, due to the many functional groups BSA could be entrapped in the gelatin matrix not through the cross-linking reaction, only, but also by non-covalent bonding forces. High percentage of encapsulation is an important parameter to be considered in order to reduce the amount of administered microspheres to obtain *in vivo* activity. Considering the drug loading data, a very high percentage of encapsulation was achieved; from 80 to 95 % (Fig. 1).

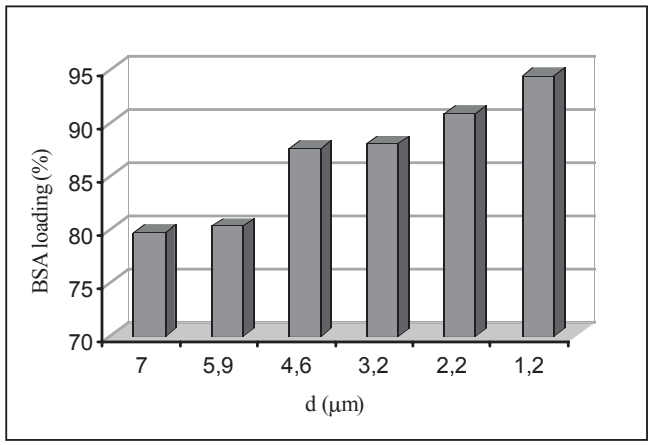

Fig. 1. BSA loading of gelatin microspheres (%) as a function of mean particle diameter;7.0 μm (A-1); 5.9 μm (B-1); 4.6 μm (D-1); 3.2 μm (A-2); 2.2 μm (C-2); 1.2 μm (D-3)

In vitro biodegradation studies of gelatin microspheres

Degradation data of gelatin microspheres in the presence of both enzymes showed that the GTA cross-linking degree, as well as additional cross-linking due to the freeze-drying procedure provided prolonged enzymatic degradation in all series of gelatin microspheres, suggesting complete *in vivo* degradation. For e.g., during 72 h at 37^{0}C between 84 and 90% of gelatin microspheres were degraded in the presence of *collagenase* (Fig. 2a). Similarly, between 80 and 91 % of microspheres were degraded during 96 h in the presence of *trypsin* (Fig. 2b). The difference in the degradation rate between the two enzymes suggests a degree of enzyme specificity. These results are in agreement with the data reported in the literature for collagen nanoparticles (19) and for gelatin nanoparticles where comparing to *protease* and *α-chymotrypsin, trypsin* appears to be the most suitable enzyme for gelatin nanoparticle degradation (7). The statistical analysis confirmed statistically significant difference at $\alpha = .05$ in degradation rate among the series with different particle size in the presence of both enzymes.

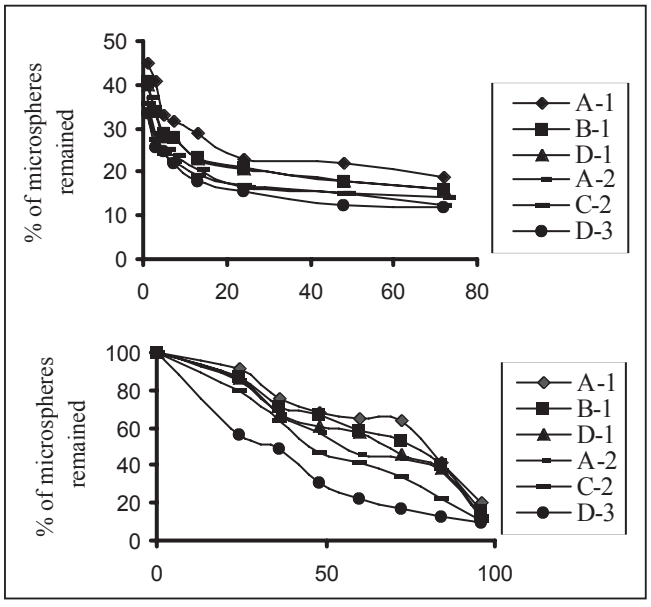

Fig. 2. Degradation profiles of gelatin microspheres in the presence of *collagenase* (a) and *trypsin* (b); particle size between 7.03 μm (A-1) and 1.20 μm (D-3)

In vitro release studies

In all series of gelatin microspheres, regardless the enzyme present, the BSA release profiles showed a biphasic modulation characterized by an initial relatively rapid release, followed by a continuous much slower release that decreased progressively until complete release of BSA. If such a release profile is reproduced *in vivo*, the drug effect will be obtained in a relatively short period to obtain therapeutic effect and the slow release period will serve to maintain this effect.

The BSA release followed the swelling behavior and enzymatic erosion of gelatin microspheres. Thus, the release kinetics was determined not only by the rate of enzymatic degradation, but also by the rate of water uptake and the rate of drug dissolution, associated with the movements of the swelling front, erosion front and diffusion front. Concerning the last one, termed also as undissolved drug front it should be emphasized that it is a function of drug solubility and loading. In addition, C-C bonds of cross-linked natural polymers, such as gelatin, are characterized with relatively high capability to overcome the potential energetic barrier for rotating when the polymer is in a rubbery state. Such freedom results in a series of changes in flexibility and conformation of polymer chains that influence on the drug liberation. Whereas interaction between water, polymer and drug are the primary factors for release control, various formulation parameters, such as polymer grade, drug/polymer ratio and drug and polymer particle size can influence on the drug release rate to greater or lesser degree, also. Concerning all these parameters strictly linked to the drug release kinetics, the differences in BSA release rate were observed in the series of gelatin microspheres with different particle size, and also, between the series of gelatin microspheres treated with both enzymes, separately.

"Lag" phase of very low release was observed during the first hours of the study (1-3 h) depending on particle size and enzyme present. Namely, during the 1st hour of the study between 5 and 10%, of BSA were released in a presence of *collagenase*, while the same amount of BSA was released in a period of 3 h when *trypsin* was present. The time necessary for the dissolution medium to penetrate into the gelatin matrix and enzymatic complex with the polymeric particles to be formed can explain the delayed initial release of BSA, while the low release in a "lag" phase can be explained by the release of poorly entrapped and surface associated BSA.

The initial release of BSA was between 80 and 93% in a period of 24 h in the presence of *collagenase*, while in the same initial period nearly 67 to 85% of the entrapped BSA was released from gelatin microspheres treated with *trypsin*. At the same, *in vitro* conditions the faster water penetration into the spheres supported by the faster enzymatic degradation and thus, more free drug liberation resulted in a higher percent initially released in the series of gelatin microspheres with lower mean diameter. On contrary, increase in particle size decreased the biodegradation rate and increased the polymer relaxation time resulting in an extended duration of drug release. So, the high correlation between the particle size and the drug release rate in presence of both enzymes was observed.

Namely, in the series of gelatin microspheres with mean diameter smaller than 4 μm, between 40 and 55 % of total drug was released during 3 h in the presence of *collagenase*. The same percent of total drug (~ 37 %) in the series of gelatin microspheres with mean diameter higher than 4 μm was ~ 6 h (Fig. 3a). Similarly, in the presence of *trypsin* during 9 h between 40 and 80% of BSA were released from particles with mean diameter between 7.03 μm and 1.20 μm (Fig. 3b). These data are in correlation with degradation data in which *trypsin* and *collagenase* showed different degree of specificity. Namely, it is well known that the action of *trypsin* is restricted to peptide links involving the carboxylic groups of lysine and arginine. The hydrolytic reaction is slowed down when ε-amino groups of lysine residues are substituted; the splitting action being restricted to arginyl bonds (7).

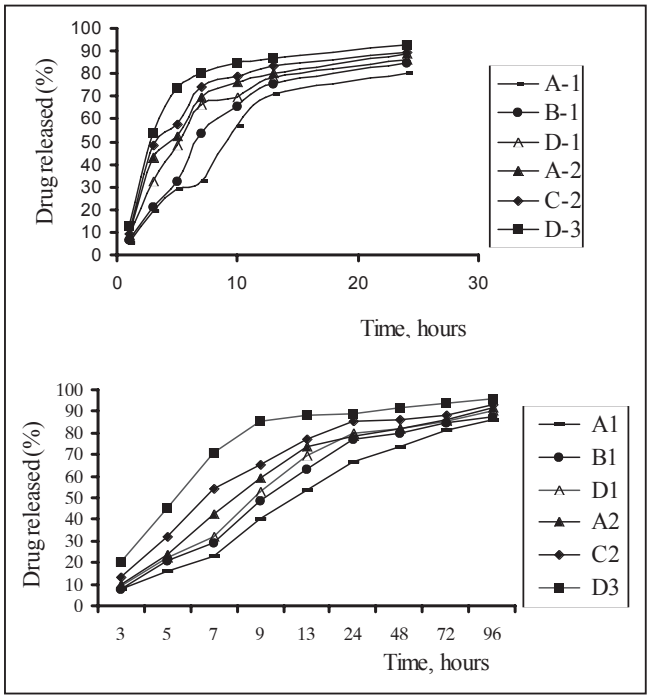

Fig. 3. BSA released (%) from gelatin microspheres in the presence of *collagenase* (a) and *trypsin* (b); particle size between 7.03 μm (A-1) and 1.20 μm (D-3)

Table 2. Exponential dependence of the amount of BSA released on time

Series of gelatin microspheres d, μm	Microspheres treated with *collagenase*		Microspheres treated with *trypsin*	
	n	r	n	r
A-1 (7.03)	0.99	0.991	1.02	0.987
B-1 (5.89)	1.05	0.997	1.04	0.975
D-1 (4.63)	1.17	0.990	1.03	0.962
A-2 (3.20)	1.15	0.994	1.04	0.962
C-2 (2.19)	1.18	0.980	0.79	0.978
D-3 (1.20)	1.33	1.000	0.58	1.000

Exponential dependence of the amount of BSA release on time was evidenced; the diffusion exponents in the presence of *trypsin* were superior to 0.45 (0.58-1.04) indicating the influence of polymer relaxation and diffusion through the matrix in BSA release. Considering the exponential dependence of BSA released on time in the presence of *collagenas* was observed indicating complete water penetration into the spheres supported by the faster enzymatic degradation and thus, more free drug liberation (Table 2).

Literature data (14, 17) confirmed that such a biphasic modulation of drug release is typical for swellable polymer matrices regardless their shape, particle size and drug loading. Considering this, the constants for the initial (α) and late time release (β) were determined using biexponential function that includes degradation, swelling and diffusion rate (Table 3).

Conclusion

With the aim of obtaining cross-linked gelatin-based microparticles as potential adjuvant in oral immunization the resistance to enzymatic degradation and control of drug release rate in the presence of *collagenase* and *trypsin* was evaluated. The diffusional exponents (*n*) according to the general exponential release equation indicate anomalous (non-Fickian) and Case-II transport mechanism in BSA release from swellable gelatin ma-

Table 3. Rate constants for the initial (α) and late time releases (β) according to the biexponential function

Series of gelatin microspheresd, μm	Kinetic parameters according to biexponential function							
	Microspheres treated with *collagenase*				Microspheres treated with *trypsin*			
	r_1	α ,h^{-1}	r_2	β, h^{-1}	r_1	α, h^{-1}	r_2	β, h^{-1}
A-1 (7.03)	0.977	0.058	0.930	0.066	0.967	0.050	0.997	0.012
B-1 (5.89)	0.999	0.081	0.957	0.071	0.986	0.094	0.948	0.012
D-1 (4.63)	1.000	0.151	0.998	0.072	0.991	0.114	0.966	0.012
A-2 (3.20)	1.000	0.155	0.948	0.072	0.997	0.129	0.981	0.012
C-2 (2.19)	1.000	0.248	0.963	0.074	0.988	0.136	0.937	0.012
D-3 (1.20)	1.000	0.321	0.938	0.078	0.999	0.289	0.989	0.013

trices. At the same cross-linking degree, the particle size has an impact on the drug release rate through the polymer relaxation time, diffusive processes and degradation rate. The difference in enzymatic specificity in degradation rate reflects the BSA release, so the higher release rates in the presence of *collagenase* were observed. The faster water uptake into the spheres supported by the faster enzymatic degradation in the presence of *collagenase* resulted in more free drug liberation in the series of gelatin microspheres with lower mean diameter where a transition toward Super-Case II transport in BSA release was observed.

References

1. H.O. Alpar, K.R. Ward and E.D. Williamson, *S. T. P. Pharma. Sci.,* **10** (4) 269-78 (2000).
2. S. Trolle, A. Andremont and E. Fattal, *S.T.P. Pharma. Sci.,* **8** (1), 19-30 (1998).
3. C. Weber, C. Coester, J. Kreuter and K. Langer, *Int. J. Pharm.,* **194** (1), 91-102 (2000).
4. M. Nakamura, S. Yamashita, Y. Tsume, T. Nadai, H. Sezaki, T. Kohno, Y. Tabata and Y. Ikada, *S.T.P. Pharma. Sci* **8.,** (1), 67-73 (1998).
5. R. Cortesi, E. Esposito, M. Osti, G. Squarzoni, E. Menegatti, S. S. Davis and C. Nastruzzi, *Eur. J. Pharm. Biopharm.,* **47** (2), 153-60 (1999).
6. F. Friess, *Eur. J. Pharm. Biopharm.,* **45** (2), 113-36 (1998).
7. E. Leo, M. A. Vandelli, R. Cameroni and F. Forni, *Int. J. Pharm.,* **155** (2), 75-82 (1997).
8. J. Kreuter, *Colloidal drug delivery systems*, Marcel Dekker, Inc., 1994, pp 219-342.
9. Y. Tabata and Y. Ikada, *Pharm Res* **6** (2), 422-27 (1994).
10. K. Griebenow, A. M. Santos and K. G. Carrasquillo, *The Internet J Vibrational Spectroscopy*, Vol. 3, 1st Ed. (1999).
11. K. Mladenovska, Lj. Klisarova, E. I. Janevik and K. Goracinova, *Acta Pharm* **52** (2), 91-100 (2002).
12. H. O. Alpar, J. E. Eyles and E. D. Wiliamson, *S.T.P. Pharma. Sci.,* **8** (1), 31-9 (1998).
13. K. Mladenovska, E. I. Janevik, M. D. Glavas, E. F. Kumbaradzi and K. Goracinova, *Int. J. Pharm.,* **242** (1-2), 247-49 (2002).
14. E. Sawicki, T. R. Hanser, T. W. Stanley and W. Elbert, *Anal. Chem.,* **33** (3), 93-6 (1961).
15. F. A. Radu, M. Bause, P. Knabner, L. W. Geoffrey and W. C. Friess, *J. Pharm. Sci.,* **91** (4), 964-72 (2002).
16. C. S. Brazel and N. A. Peppas, *Eur. J. Pharm., Biopharm.,* **49** (2), 47-58 (2000).
17. Y. Tabata and Y. Ikada, *Adv. Drug. Deliv. Rew.,* **31** (2), 287-301 (1998).
18. M. I. Ugwoke, N. Verbeke and R. Kinget, *Int. J. Pharm.,* **148** (2), 23-32 (1997).
19. M. El Samaligy and P. Rohdewald, *J. Pharm. Pharmacol.,* **35** (3), 537-39 (1983).

Резиме

Желатинските микросфери како потенцијални носачи на антигени: компаративни испитувања на брзината на деградација и ослободување во присуство на колагеназа и трипсин

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Клучни зборови: желатински микросфери, албумин, контролирано ослободување, колагеназа, трипсин

Подготвени се желатински микросфери во кои е инкорпориран албумин со користење на методот на Табата и Икада. Со варијации во производните и во формулациските параметри подготвени се честички со висока ефикасност на инкорпорирање и големина помеѓу 1 и 10 μm. Со цел да се оцени потенцијалот на желатинските микросфери како носачи за контролирано и насочено ослободување антигени по орална примена следени се брзината на деградација и брзината на ослободување на албуминот во присуство на ензимите трипсин и колагеназа. Експоненцијалната зависност на количината на ослободен албумин во време упатува на Case II видоизменета Fick-ова дифузија, освен кај честичките со дијаметар помал од 5 μm во кои во присуство на колагеназа се забележува премин кон Super Case II транспорт поради поголемата брзина на деградација и дифузија на албуминот низ матриксот. Овие резултати се во согласност со биодеградацијата на честичките, при што се забележува одредена разлика во специфичноста на дејствувањето на соодветните ензими. Математичката обработка на резултатите на ослободување на албуминот покажува бифазна модулација, при што константите за иницијалното, односно одложеното ослободување го потврдуваат влијанието на големината и/или брзината на деградација на честичките врз брзината на ослободување на албуминот.

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Design, synthesis and determination of physical and chemical characteristics of glycoconjugates as model for oligosaccharide vaccines against vibrio cholerae

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Abstract

Cholera is toxin-mediated enteroinfection, with epidemic character and there are approximately 120000 death cases per year worldwide. Protection against cholera has not been accomplished due to deficiencies in the licensed vaccines. Serum vibriocidal activity mediated by LPS antibodies is the only immune segment correlated with the resistance of cholera.

On the basis of literature data (Robbins JB, 1990; Ogawa Y, 1996) we synthesized glucoconjugates, composed of detoxified LPS from *Vibrio cholerae* and protein carriers. Conjugate vaccines were prepared by binding acetic acid and hydrazine-treated lipopolysaccharide (LPS) from *Vibrio cholerae* O1, serotype Inaba, to cholera toxin B-subunit (CT-B) and bovine serum albumin (cBSA). Adipic acid dihydrazide was used for derivatization of oligosaccharides and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as conjugating agent. SDS-PAGE, glycoprotein detection and TLC dot-blot were used for physical and chemical analysis of the prepared four types of conjugates. Safe level of endotoxins, measured by LAL assay was detected in all conjugates. The synthesized conjugates can be used for monitoring immunization schemes on experimental animals. It is to be expected that conjugated vaccines are safe and efficient and that will have high immunogenic and T-dependant characteristics with long immune protection against cholera.

Key words: vaccines, glycoconjugates, Vibrio cholerae, LPS, cholera toxin

Introduction

Cholera is caused by *Vibrio cholerae* strains belonging to two serogroups, O1 and O139, based on their LPS structure. Serogroup O1, which is responsible for the seventh cholera pandemic, is divided into three serotypes, Inaba, Ogawa and Hikojima (1). In the year 2002, World Health Organization (WHO) estimated that over 220,000 people were stricken with cholera each year. Natural infection and currently available vaccines offer incomplete protection of relatively short duration. The long-term control of cholera depends on good personal hygiene, uncontaminated water supply and appropriate sewage disposal. However, the improvement of hygiene is a distant goal for many countries. Thus, the availability of an effective cholera vaccine is important for the prevention of cholera in these countries. The only vaccines recommended from WHO for massive immunization of population in cholera epidemic regions is WC/rBS per oral cell vaccines. The vaccines CVD 103-HgR is used mainly for immunization of people who travel in cholera epidemic regions.

A conjugate vaccine composed of the detoxified LPS of *V. cholerae* O1 serotype Inaba bound to cholera toxin elicits vibriocidal antibodies against both Ogawa and Inaba serotypes (2,3). The Ogawa and Inaba serotypes of *V. cholerae* O1 differ only by a 2-O-methyl group that is present in the non-reducing terminal sugar of the Ogawa O-specific polysaccharide (O-SP) of the LPS, but is absent from the Inaba O-SP (4,5,6). It is thus most likely that the protection induced in vaccine trials by Ogawa whole-cell vaccines is due to the induction of antibodies recognizing only this serotype-specific antigenic determinant.

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The new method in design of vaccines for Gram-negative bacteria is trough synthesis of glycoconjugates structures. The concept of the conjugates is linking the polysaccharides from bacteria with protein carrier. Immune response to conjugates with sugar ligand can be MHC-I/ T-dependent or T-independent (7). It is to be expected that T-dependent carbohydrate antigens with peptide carriers to induce T-dependant humoral immunity towards carbohydrate determinants. Development of glycoconjugate vaccines is expected to provide protection from other human bacterial pathogens with the utilization of capsular polysaccharides, LPS oligosaccharides and other carbohydrate determinants (8).

The new method in the synthesis, linkage and use of new protein carriers and better characterization of the conjugate vaccines will allow understanding of the mechanism of induction of immunity and will help in the rational design of high immunogenic and efficient vaccines.

Study design

The chosen model of design for synthesis of glycoconjugate immunogens is trough conjugation of carbohydrate component of bacterial lipopolysaccharide from *Vibrio cholerae* 01 serotype Inaba with two different protein carriers. This design should provide production of immunogenic molecule with carbohydrate antigen determinant and peptide hapten carrier. LPS as main bacterial antigen determinant with immunogenic characteristics will be used for synthesis of glycoconjugates. Because of the high toxicity of lipopolysaccharide molecule, detoxification for elimination of the toxic Lipid A is done with acetic and hydrazine hydrolysis of the LPS. The obtained oligosaccharides will still have immunogenic characteristics of the LPS (9,10). For multiple points linkage between carbohydrate and protein molecule adipic acid derivatization is used (11). Bacterial B-subunit of cholera toxin (CT-B) and bovine serum albumin (BSA) are chosen as protein carriers. Cholera toxin is strong virulent determinant with antigenic characteristic, and with adjuvant properties in the conjugate. The use of B-subunit is because of its nontoxicity and adherent character. BSA is neutral antigen protein in the design concept of the glycoconjugates, and a hapten carrier (12).

The linker used for conjugation should provide separation of the two main glycoconjugate components, making them available for recognition and interaction with the elements of the immune system. With carboimide conjugation and use 1-etil-3, 3-dimetilaminopropil carboimid (EDC) this need is accomplished. EDC conjugation is very effective one step method for production of a large number of peptide-carrier immunogens (13).

Experimental

Materials. - Anhydrous hydrazine, adipic acid dihydrazide (ADH); cianogenbromide (CNBr); Cholera toxin B-subunit (CT-B) from *V. cholerae* 569B, biotype classical, serotype Inaba; LPS was of *V. cholerae* O1 569B, biotype classic, serotype Inaba, lot VC12-19, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and agarose were from Sigma Chemical Co., St. Louis, Mo.; Sepharose CL-4B, Sephadex G-25 and G-50 were from Pharmacia AB, Uppsala, Sweden; bovine serum albumin (BSA) standard solution, Coomassie blue protein assay reagent, and triethylamine (TEA) were from Pierce, Rockford, Ill.; *Limulus* amebocyte lysate pyrogen was from BioWhittaker, Inc., Walkersville, Md.; Cellular cholera vaccine was obtained from Frank McCarthy, Wyeth-Ayerst Laboratories, Marietta, Pa.

Derivatisation of the protein carrier BSA. - Native BSA is with acid character (pI 4,5-5,1). For decreasing the acidity cationisation is done, which makes the protein molecule of BSA suitable for linkage with carbohydrate ligand (14). Cationisation is done with 1,8 g EDC on 5 g native BSA with addition of 350 ml a.d. and mixed for 2 hours. The reaction is stopped with addition of 30 ml acetic buffer. The product is dialyzed for 70 hours, lyophilised and named cationic BSA (cBSA). The protein carrier CT-B is used in its native form.

Detoxification of oligosaccharides. - LPS was detoxified by two methods. For acid hydrolysis 10 mg of LPS was suspended in 10 ml of 1% acetic acid, and was heated at 100 °C for 90 min. The reaction mixture was ultracentrifuged (60,000 rpm, 10°C, 5 h), dialyzed against water and passed through a sterile 0.25-µm pore-size membrane and freeze dried. The product was designated as O-SP and stored at -20°C. For hydrazine hydrolysis, LPS 800 mg was suspended in 80 ml of anhydrous hydrazine and placed in a 37°C water bath for 2 h with stirring. The resultant precipitate was washed with cold 90% acetone, centrifuged (35,000 rpm, 10°C, 5 h), and passed through a 2.5- by 50-cm column of G-50 Sephadex in pyrogen-free water; the void volume fractions were sterile filtered through a 0.25-µm pore-size membrane and freeze dried. The product was designated as deacylated LPS (DeA-LPS**)** and stored at -20°C.

Derivatisation of oligosaccharides. - O-SP and DeA-LPS (10 mg/ml) activated at pH 10.5 with CNBr and bound to adipic acid dihydrazide (ADH) at pH 8.5 were designated as O-SP-ADH and DeA-LPS-ADH, respectively. The extent of derivatization was determined by 2,4,6-trinitrobenzene sulfonic acid assay (Pierce, Rockford, Ill.) using ADH as a standard.

Conjugation of oligosaccharides with protein carriers. - cBSA or CT-B were mixed with OS-P-LPS-ADH and DeA-LPS-ADH in pyrogen-free saline in equal weight, and 450 mg of 1-ethyl-3-3-dimethylaminopropyl carbodiimide was added. The reaction mixture was maintained at pH 5.5 to 5.7 for 2 h and passed through a 2.5- by 90-cm column of G-50 Sephadex in 0.2 M NaCl-0.005 M sodium phosphate (pH 7.3). Fractions containing protein and polysaccharide were pooled, thimerosal was added to 0.01%, and the preparations were sterile filtered and designated as O-SP-cBSA and DeA-$_{LPS}$-cBSA conjugates with cBSA as carrier, and O-SP-CT-B and DeA-$_{LPS}$-CT-B conjugates with CT-B as carrier, respectively.

Determination of physical and chemical characteristics of conjugates

Using electrophoretic separation with PAGE-SDS, glycoprotein detection of the carbohydrate parts of the conjugate and determination of the free unbound oligosaccharides with thin layer chromatography, characterization of the chemical and physical characteristics of the glycoconjugates was done.

SDS-PAGE analysis: LPS, oligosaccharides and synthesized conjugates (in concentration 50 mg proteins per well) were analysed using polyacrylamide gel-electrophoresis in sodium-dodecyl sulfate (PAGE-SDS) on a 10-15% running gel and a 4% stacking gel (Bio-Rad). Conditions for PAGE-SDS were standard (start:100-200V 4 ^{o}C, final:200-300V 12-14 ^{o}C). Synthesized glycoconjugates after PAGE-SDS were visualized with two staining methods with different sensitivity and affinity to protein and carbohydrate components of the conjugates. Coomassie brilliant blue has high affinity and microgram detection to proteins but highly glucolised proteins are hard to be identified with this dye. Because of its sensitivity to carbohydrate fractions (0,1ng/ml), a silver staining is done after the Coommassie brilliant blue (15) using Silver stain kit (Bio-Rad Labaratories).

Glycoprotein detection of the carbohydrate component in the synthesized glycoconjugates. - We have used biotin enzymatic reaction for detection of glycoproteins and visualization was done with streptavidin-alcaline phosphatase. Using dot-blot method, detection of the carbohydrate part of the glycoproteins is done (Immun-Blot Kit for glycoprotein detection, Bio-Rad Laboratories). The samples are immobilized an nitrocellulose membrane (20 mg/ml per sample), the membrane is incubated with biothynilated solution, washed with PBS twice and treated with 5 ml streptavidin alkaline phosphatase.

Determination of present impurity in synthesized glycoconjugates using thin layer chromatography. - TLC is used to detect the impurity from eventually present LPS or free unbound oligosaccharides of O-SP$_{LPS}$ and DeA$_{LPS}$ in the synthesized glycoconjugates through specific coloring with sulfuric acid. The samples in concentration of 50 mg/ml are immobilized on chromatographic plate (TLC silica gel plate 60 F_{254}, 20x20 cm), in mobile phase (ethanol/methanol). The plate is sprayed with "CHAR" (sulfuric acid solution) reagent and determination of the carbohydrate is in form of dark spots on the plate.

Bioassays The LPS concentration, assayed by the Limulus amebocyte lysate (LAL) assay done under standard procedure recommended by the kit manual (Bio-Whittaker), is expressed in endotoxin units relative to the US standard (16).

Results and discussion

Chemical characterization of conjugates

The extents of derivatization of O-SP and DeA-LPS with ADH are shown in Table 1.

The extent of derivatization of DeA-LPS and O-SP with ADH is very similar (1,14% and 1,33%). The carbohydrate/protein ratio (wt/wt) ranges from 0,67 for O-SP-cBSA to 1,30 for DeA-$_{LPS}$-CT-B. The yield for all of the conjugates was approximately 80%, as calculated by the recovery of saccharides in the conjugates compared with the derivative polysaccharide. The results obtained from derivatization of oligosaccharides and determination of protein composition of the conjugates, suggested that satisfactory level of conjugation between oligosaccharides and proteins was accomplished.

Table 1. Characterization of V.cholerae O-SP and DeA-LPS protein conjugates

Conjugate	ADH / OS* (wt/wt)	ADH/ protein (wt/wt)	OS/ protein (wt/wt)	Yield (%)	Composition of the conjugates (mg/ml)	
					protein	OS
O-SP-cBSA	1,21	8,6	0,67	88	1,8	1,22
O-SP- CT-B	1,14	7,8	0,60	75	1,0	0,55
DeA-$_{LPS}$- cBSA	1,30	8,1	0,75	79	0,35	0,25
DeA-$_{LPS}$-CT-B	1,33	8,7	1,30	88	0,9	1,45

* oligosaccharides (O-SP and DeA-LPS)

Determination of the electrophoretical mobility of the synthesized glycoconjugates with polyacrilamide gel electrophoresis

Synthesized conjugates analyzed with reductive PAGE-SDS are stained with two different colors with different sensitivity and affinity towards protein and carbohydrate part of the glycoconjugates. Electrophoretic mobility of the conjugates are determinate corresponding to protein standard (SDS-PAGE Standard Broad range 6-200 kDa) and to CT-B, cBSA, O-SP_{LPS}, DeA-$_{LPS}$ and LPS.

Coomassie Brilliant blue staining

After Coomassie staining, the electrophoretic mobility was determinate for the following proteins and glycoproteins: protein carrier CT-B showed molecular weight of approximately 12 kDa, protein molecule of cBSA had 60 kDa, conjugates with protein carrier of cBSA had molecular weight more than 60 kDa, while conjugates with protein CT-B had molecular weight of 12 kDa.

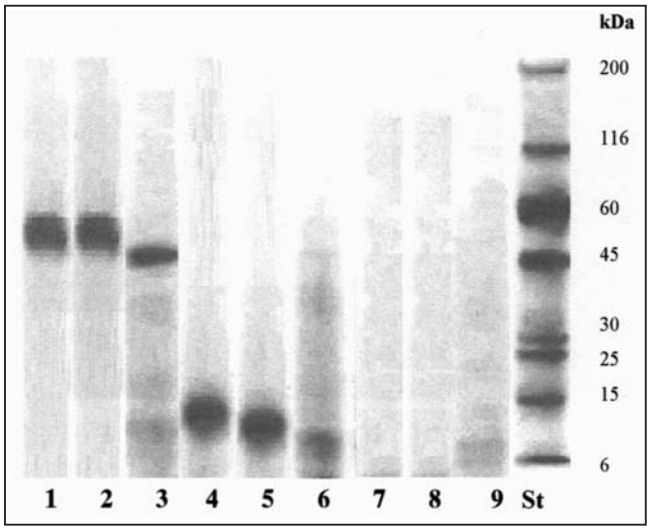

Fig.1 Comassie brilliant blue staining of SDS-PAGE gel
1. DeA-LPS-cBSA; 2. O-SP- LPS -cBSA; 3. cBSA;
4. DeA-LPS-CT-B; 5. O-SP-LPS-CT-B; 6. CT-B; 7. O-SP;
8. DeA-LPS 9. LPS

Samples from O-SP, DeA_{LPS} and LPS did not show signal, because of selective staining only of the proteins with Coomassie brilliant blue. The carbohydrates present in the glycoconjugates are decreasing the mobility of the conjugates due to the increasing of molecular weight of 1,5 -2 kDa, which is difficult to detect in respect to unbound cBSA and CT-B.

Silver staining

Using this staining method after the Coomassie stain, except for the bands of ~12 kDa (DeA-$_{LPS}$-CT-B and O-SP-$_{LPS}$-CT-B) and ~60 kDa (DeA-$_{LPS}$-cBSA and O-SP-$_{LPS}$–cBSA) of the synthesized conjugates, the bands from oligosaccharides and LPS are also detected. O-SP had molecular weight of 6 kDa, DeA_{LPS} of ~8 kDa and LPS of ~13 kDa.

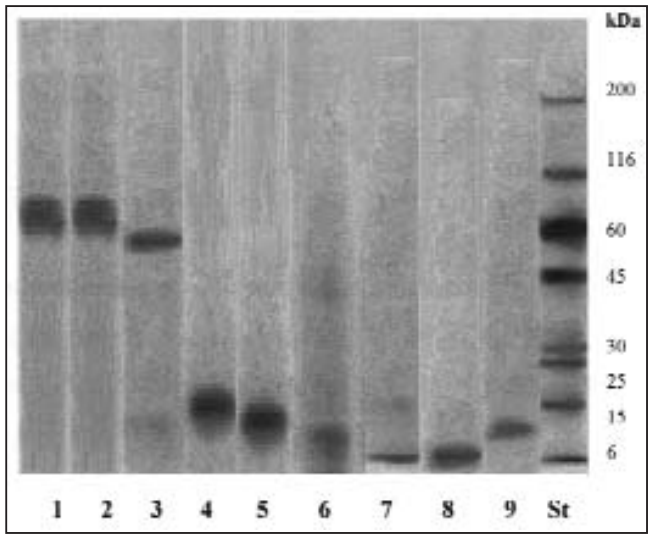

Fig. 2 Silver staining of SDS-PAGE gel 1. DeA-LPS-cBSA;
2. O-SP-LPS-cBSA; 3. cBSA; 4. DeA-LPS-CT-B;
5. O-SP-LPS-CT-B; 6. CT-B; 7. OS-P; 8. DeALPS ; 9.LPS

Glycoprotein detection of the carbohydrate component in the synthesized glycoconjugates

Glycoprotein detection of the carbohydrate component in the synthesized glycoconjugates is done using dot-blot immobilization of the samples on nitrocellulose membrane. The samples are applied in duplicates in concentration of 20 mg/ml. Protein carriers CT-B and cBSA are used as negative control, and LPS, O-SP and DeA-$_{LPS}$ as positive control.

Positive signal is detected in all conjugates and in carbohydrate components LPS, O-SP and DeA-$_{LPS}$,while protein controls from cBSA and CT-B did not show signal. The positive signal in synthesized glycoconjugates is confirmation of the conjugation between carbohydrate and protein components in the conjugates.

Determination of present impurity in synthesized glycoconjugates

Using TLC and specific "CHAR" reagent, the eventually present impurity from present LPS or free unbound oligosaccharides of O-SP_{LPS} and DeA_{LPS} in the synthesized glycoconjugates is detected. LPS, O-SP-$_{LPS}$ and DeA-$_{LPS}$ are identified on the starting place of application and are not mobile in the used mobile phase. In all conjugates the carbohydrate component is detected, but single bands from LPS, O-SP-$_{LPS}$ and DeA_{LPS} are not present which points to high purity of the synthesized glycoproteins.

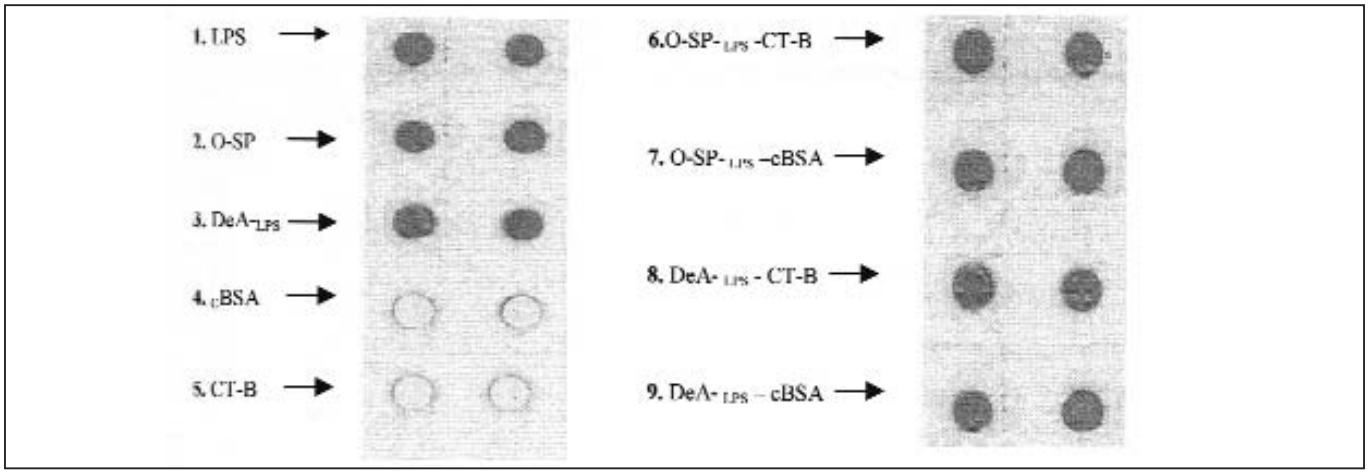

Fig. 3 Dot-blot analysis in duplicates of the synthesized glucoconjugates and their protein and carbohydrate components 1. LPS; 2. O-SP ; 3. DeA- LPS; 4. CBSA; 5. CT-B; 6. O-SP- LPS -CT-B; 7. O-SP- LPS -cBSA; 8. DeA- LPS - CT-B ; 9. DeA- LPS - cBSA

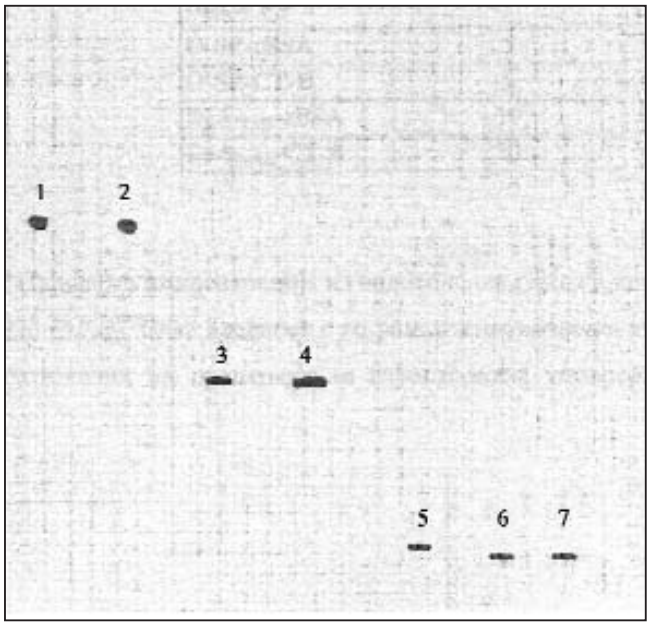

Fig. 4. TLC profile of the synthesized glycoconjugates
1. DeA-LPS-CT-B; 2. O-SP- LPS -CT-B;
3. DeA-LPS- cBSA; 4. O-SP-LPS- cBSA; 5. LPS;
6. OS-P; 7. DeA-LPS

Table 2. Concentration of endotoxins (EU/mg) in the synthesized conjugates

Immunogen	Concentration of Endotoxins (EU/μg)
O-SP	2
DeA-LPS	3
O-SP-cBSA	5
O-SP-CT-B	5
DeA-$_{LPS}$-cBSA	10
DeA-$_{LPS}$-CT-B	20

Determination of the concentration of the endotoxins present in the synthesized glycoconjugates

Concentration of bacterial endotoxins is determined in all synthesized conjugates and oligosaccharides. The conjugate DeA-$_{LPS}$-CT-B had a higher endotoxin level (20 EU/mg). This value is in the rang of the approved concentration for parenteral preparations for human use (16).

Vaccines are the most cost-effective way of preventing and controlling infectious diseases caused by microorganisms. The success of the Hib conjugate vaccine and promising results obtained from clinical trials of meningococcal and pneumococcal vaccines have assured an exciting future for glycoconjugate vaccines. Glycoconjugates can be prepared, using a variety of coupling strategies, from surface carbohydrates such as capsular polysaccharides, lipopolysaccharides from bacteria or synthetic saccharides. Progress will be made in the development of novel vaccines to protect against a wide variety of other human and animal bacterial pathogens based on surface carbohydrates such as capsular polysaccharides, LPS saccharides and less common saccharides.

Conclusions

We have synthesized glycoconjugates composed of carbohydrate component from oligosaccharide specific polysaccharide and deacetyled lipopolysaccharide of *Vibrio cholerae* 01 serotype Inaba and protein carrier cationic bovine serum albumin and B-subunit of cholera toxin. The synthesized glycoconjugates DeA$_{LPS}$-CT-B, O-SP-CT-B, DeA$_{LPS}$-cBSA and O-SP-cBSA have been analyzed with glycoprotein detection method, and conjugation between the carbohydrate and protein part in the glycoconjugates is conferred. The presents of impurity from the free unbound oligosaccharides and lipopolysaccharides in the synthe-

sized glycoconjugates were not detected using TLC analysis. Electrophoretic separation and determination of the molecular weight of glycoconjugates was done using SDS-PAGE and two different staining. Concentration of bacterial endotoxins in all synthesized conjugates and oligosaccharides is determined using LAL test. Low level of endotoxin concentration found in all conjugates is proof that synthesized glycoconjugates are safe and can be used for future immunization of experimental animals. The advances in synthetic methodology, coupling technology, introduction of novel carrier proteins and better characterization of conjugate vaccines will permit greater understanding of the mechanisms by which these vaccines induce protective responses and facilitate the rational design of highly immunogenic and protective vaccines.

References

1. U.H. Stroeher, L. E. Karageorgos, R. Morona and P. A. Manning, *Proc. Natl. Acad. Sci. USA,* **89**, 2566-2570 (1992).
2. R. K. Gupta, S. C. Szu, R. A. Finkelstein and J. B. Robbins. *Infect. Immun.,* **60**, 3201-3208 (1992).
3. R. K. Gupta, D. N. Taylor, D. A. Bryla, J. B. Robbins and S. S. C. Szu. *Infect. Immun*., **66**, 3095-3099 (1998).
4. K. Hisatsune, S. Kondo, Y. Isshiki, T. Iguchi and Y. Haishima , *Biochem. Biophys. Res. Commun.,* **190**, 302-307 (1993).
5. T. Ito, T. Higuchi, M. Hirobe, K. Hiramatsu and T. Yokota, *Carbohydr. Res.,* **256**, 113-128 (1994).
6. D. Wang and E. Kabat. Carbohydrate antigens (polysaccharides), In *Structure of antigens*, vol. 3. M. H. V. Van Regenmortel (ed.), CRC Press, New York, N.Y., 1996 pp. 247-276
7. M. L. Levine, N. F. Pierce, *Immunity and vaccine development in Cholera*, D. Barua and W. B. Greenough, ' ed. III. New York: Plenum, 1992, pp. 285–327
8. Z. Kossaczka, J. Shiloach, V. Johnson, D. N. Taylor, R. A. Finkelstein, J. B. Robbins and S. C. Szu. *Infect. Immun*., **68**, 5037-5043 (2000).
9. I. J. Goldstein, G. W. Hay, B. A. Lewis and F. Smith, *Methods Carbohydr. Chem.* **5**, 361-370 (1970).
10. O. Westphal and K. Jann, *Methods Carbohydr. Chem.,* **5**, 83-87 (1965).
11. C. Y. Chu, B. K. Liu, D. Watson, S. S. Szu, D. Bryla, J. Shiloach, R. Schneerson, and J. B. Robbins. *Infect. Immun.,* **59**, 4450-4458 (1991).
12. S. C. Szu, R. Gupta and J. B. Robbins. In *Vibrio cholerae and cholera*, I. K. Wachsmuth, P. A. Blake, and O. Olsvik (ed.), American Society for Microbiology, Washington, D.C. 1994, pp. 381-394.
13. L. Moroder, *Biol. Chem.,* **369**, 381-385 (1988).
14. D. L. McDonalds, *J. Lipid. Res.,* **21**, 642-645 (1980)
15. C. M. Tsai and C. E. Frasch. *Anal. Biochem.,* **119**, 115-119 (1982).
16. H. D. Hochstein, Role of the FDA in regulating the Limulus amoebocyte lysate test. In R. B. Prior (ed.), Clinical applications of the Limulus amoebocyte lysate test. CRC Press, Boca Raton, Fla, 1990, pp. 38-49

Резиме

Дизајнирање, синтеза и определување на физичко-хемиските карактеристики на гликоконјугатни структури како модел на олигосахаридни вакцини на Vibrio Cholerae

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Клучни зборови: вакцини, гликоконјугати, Vibrio cholerae,LPS, колера токсин

Колерата е токсин посредувана ентероинфекција, со епидемични размери и со приближно 120 000 смртни случаи годишно во светската популација. Заштита од колера не е постигната заради ограничувањата на достапните вакцини. Користејќи сознанија од литературата (Robbins JB. 1990; Ogawa Y, 1996) синтетизиравме гликоконјугатни имуногени со шеќерна компонента од детоксициран LPS од *Vibrio cholerae* и протеински носачи. Конјугатите беа припремени преку врзување на липополисахаридот од *Vibrio cholerae* O1, серотип Inaba третиран со кисела и хидразинска хидролиза (O-SP and DeALPS) и колера токсин Б-субединица (CT-B) и говедскиот серум албумин (cBSA). Дихразидот од адипинска киселина беше користен за дериватизација на олигосахаридите, а како конјугирачко средство го користевме 1-ethil-3-(3-dimethilaminopropil) carbodiimide (EDC). Со редуктивна SDSPAGE, гликопротеинска детекција и TLC дот-блот анализа извршено е физичко-хемиско профилирање на добиените конјугати. Безбедна концентрација на ендотоксини беше утврдено кај сите конјугати, користелќи LAL тест. Вака синтетизираните гликоконјугати може да се користат за следни имунизации на експериментални животни. Се очекува конјугатните вакцини да се сигурни и ефикасни и да покажат висока имуно геност и Т-клеточно зависни својства обезбедувајќи долготраен заштитен имунитет кон колерата и можност за разјаснување на механизмот за појава на болеста.

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Original scientific paper

Biopharmaceutical characterization of topical liposome formulations bearing 5-fluorouracil

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Abstract

Liposome dispersions and liposome gel formulations for topical administration were evaluated as modified release delivery systems for 5-fluorouracil. Drug substance has been entrapped in the internal aqueous compartment of liposomes during the preparation. The concentration of 5-fluorouracil in the hydration medium was varied and the effect on the liposome characteristics was considered. Liposome gel formulations were prepared by incorporation of liophylized liposomes into a structured vehicle of chitosan. The decrease of the amount of aqueous phase bearing total drug quantity (drug/aqueous phase ratio from 1:100, 1:60, 1:40) led to an increase of the percentage of liposome-entrapped drug, and decreased percentage of drug release, while particle size analysis showed no changes in vesicle size. Liposome gel formulations showed initially a higher drug release rate in comparision with liposome dispersions, which could be related to the release of "free" 5-fluorouracil, leaked from liposomes due to the process of liophylization. This was followed by slower release (after 1.5 hour) as a result of the influence of the viscosity of the gel matrix.

Key words: liposomes, hydration conditions, topical application, drug release.

Introduction

In recent years liposomes (phospholipid bilayer vesicles) have attracted increasing attention as a novel drug delivery system for a variety of drugs such as anticancer, antifungal, anaesthetic and antibiotic agents (1-3). With regard to the topical application, liposomes may serve as rate-limiting membrane barriers for modulation of systemic absorption, penetration enhancers or microreservoirs for sustained and controlled release of encapsulated drug (4-8). Topical liposome formulations (as dispersions or hydrogels) could be more effective and less toxic than the conventional topical formulations (9, 10). This potential advantage is especially of great interest in topical application of highly toxic drugs, such as antineoplastics.

5-fluorouracil is one of the most effective antineoplastic agent used for treatment of variety of tumours in many organs. Also, it is used topically in the treatment of different skin carcinomas (11, 12). Having in mind that liposomes as drug carriers may provide controlled drug release, site-directed and site-avoiding delivery (13), the encapsulation of 5-FU in topical liposome formulation is a reasonable approach for improvement of drug effectiveness and reduction of the side - effects.

Considering the above-mentioned, the aim of our study was to prepare and evaluate liposome formulations with 5-fluorouracil intended for topical application. The effect of the hydration conditions on the physical and biopharmaceutical properties of the prepared formulations has also been considered. In order to determine physical properties of the prepared liposomes as a function of different hydration conditions, microscopic study, particle size analysis and determination of the encapsulation efficiency were carried out. Also, drug release studies from liposome dispersions and corresponding liposome gel formulations were performed to follow the effect of hydration conditions and the influence of gel matrix over the release profile of 5-fluorouracil.

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Materials and methods

Materials

For the preparation of liposomes and topical gel formulations, the following materials were used: Phospholipon 90H - gel state (PL 90H, Natterman Phospholipid, Germany), cholesterol (CHOL, Galenika, Yugoslavia), 5-fluorouracil (5-FU, Ebewe Arzneimittel, Austria), chitosan (Katakura Chikkarin, Japan) and saccharose (Merck, Germany). All other used chemicals were of analytical grade.

Preparation and characterization of liposomes

Liposomes containing 5-FU were prepared by the modified lipid film hydration method (14). The lipid components, PL 90H and CHOL, were dissolved in chloroform and the organic solvent was removed by evaporation under vacuum (65 ^{0}C). Afterwards, the dried lipid film was hydrated with different quantities of aqueous phase bearing total drug quantity (5-FU in phosphate buffer pH 7.4), samples LD1, LD2 and LD3, respectively (Table 1).

Table 1. Formulations of liposomes

Samples	LD1	LD2	LD3
Lipid phase composition PL 90H : CHOL	12:1	12:1	12:1
Hydration conditions			
Mass ratio lipid/aqueous phase PL 90H-CHOL/phosphate buffer pH 7.4	1:31.3	1:18.8	1:12.5
Mass ratio drug/aqueous phase	1:100	1:60	1:40
1 % Chitosan gel base	LG1	LG2	LG3

After 24 h, in order to remove an unentrapped portion of the drug substance, liposomes were washed with phosphate buffer pH 7.4 and ultracentrifuged (20000 rpm, 45 min., 3 times; Ultracentrifuge MLW K24D, Yugoslavia). Liposomes were lyophilized (temperature -40^{0}C, pressure 200 Pa; Crist alpha 2-4, Bioblock, Scientific, France) using saccharose as a cryoprotector, incorporated on both sides of the phospholipid lamellae (lipid phase:saccharose = 1:1.25).

Liposome gel formulations (LG1, LG2, LG3) intended for topical application, were prepared by incorporation of lyophilized liposomes (series LD1, LD2 and LD3) in structured vehicle of chitosan (1% m/m) in ratio 1:3.

Photomicrographs (standard microscope with camera Nikon, Japan; method by Nomarsky, magnification x600) were taken immediately after the preparation, after dilution with phosphate buffer pH 7.4 (1:1). Particle size analysis was carried out by use of laser difractometry (Fritsch particle size analysette D LAB/22, France).

The encapsulation efficiency of 5-FU in liposomes was quantified UV spectrophotometrically (266 nm; Perkin Elmer, Lambda 16, USA) after dissolving of liposomes in chloroform:methanol mixture.

In vitro dissolution studies

Release of the drug substance from freshly prepared liposome dispersions and liposome gel formulations was followed *in vitro* using dialysis through hydrophilic membrane of regenerated cellulose against phosphate buffer pH 7.4 at 37 ^{0}C. Quantity of the released 5-FU in dialyzing medium, within a period of 8 h, was analysed spectrophotometrically. All experiments were carried out in triplicate and average values are presented. To deduce the mechanism of the drug release from the prepared formulations, the release data were mathematically processed.

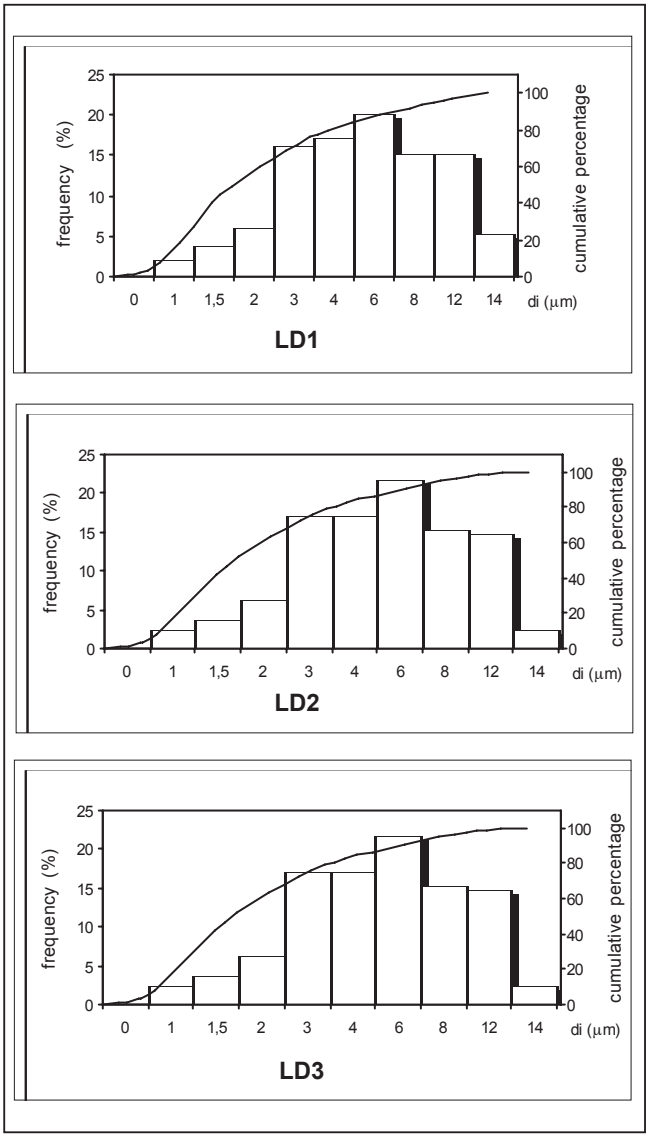

Fig. 1. Particle size-frequency distribution curves and cumulative distribution plots for samples LD1, LD2 and LD3

Results and discussion

Characterization of liposomes

Microscopic observations confirmed formation of spherical vesicles with average size of 5 μm. The size-frequency distribution curves and cumulative distribution plots for the prepared formulations are presented in Fig. 1.

By varying the drug/aqueous phase mass ratio during the preparation of liposomes, different efficiency of 5-FU encapsulation has been achieved. Lower concentration of the drug substance in the hydration medium resulted in lower incorporation efficiency. At a concentration of 10 mg/ml, 16.66 mg/ml and 25 mg/ml of 5-FU in hydration medium (samples LD1, LD2, LD3), the efficiency of 5- FU encapsulation was 5.2, 9.4 and 15.4 %, respectively (Fig. 2). The encapsulation of hydrophilic drug substance into

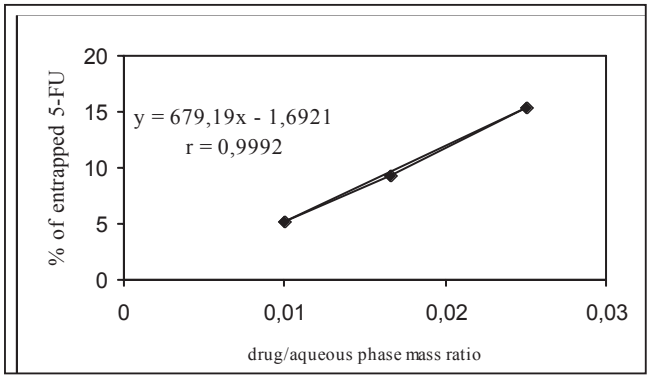

Fig. 2. Effect of hydration conditions on the percentage of incorporated 5-FU into liposomes

liposomes bears relationship to the overall volume of aqueous phase that is encapsulated during liposome formation (15). Having in mind that the prepared vesicles are of similar size (mean geometric diameters were 5.04 (± 2.01), 4.53 (± 2.02) and 4.75 μm (± 1.89) for samples LD1, LD2 and LD3 respectively), higher encapsulation efficiency of 5-FU into liposomes could be related to the higher drug concentration in the hydration medium.

Drug release from liposome dispersions and liposome gel formulations

Drug releases from liposome dispersions are presented in Fig. 3a. As it can be seen, by increasing the drug/aqueous phase mass ratio (from 1:100, 1:60, 1:40), the release rate of 5-FU decreased. Bearing in mind the percentage of liposome-encapsulated 5-FU, it is obvious that formulations with higher percentage of encapsulated drug showed slower release rate. This may suggest that liposome bilayer acts as a rate-limiting membrane barrier for the release of encapsulated drug substance.

The release of 5-FU from liposome gel formulations was found to be a function of liposome characteristics and prop-

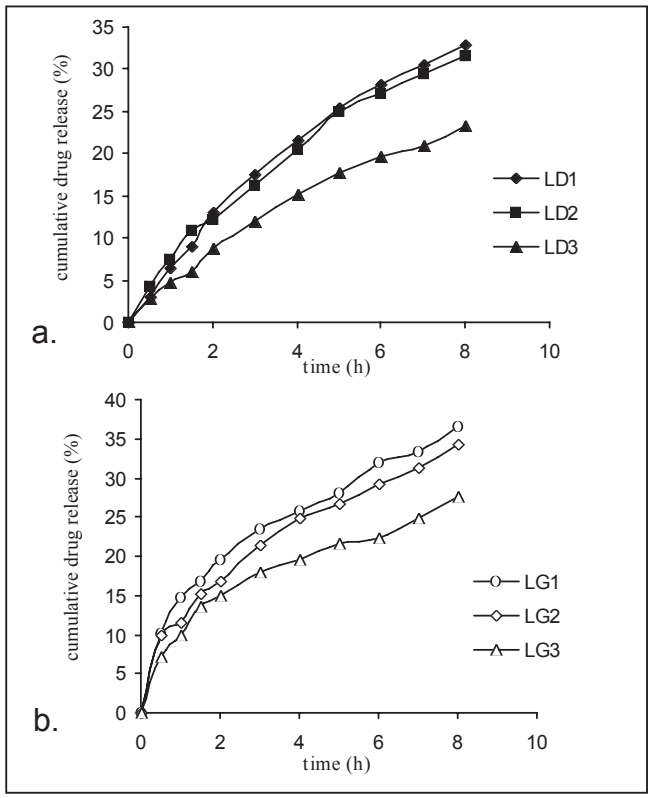

Fig. 3. Release of 5-FU from liposome dispersions and liposome gel formulations, n=3

erties of the gel matrix. Gel liposome formulations (Fig. 3b) showed initially faster release rate (first 1.5 hours) compared to corresponding liposome dispersions, followed by a continuous much slower release rate. The release pattern during the first 1.5 hour could be related to the portion of drug substance that leaked out of liposomes during the process of lyophilization (1, 16). Slower release rate after 1.5 hour correlates to the release of liposome entrapped 5-FU and the influence of the viscosity of the gel matrix.

To examine the mechanism of the release of 5-FU from liposome dispersions and liposome gel formulations, the Higuchi diffusion model and zero order kinetic were applied. Kinetic data indicated that the release of 5-FU followed the diffusion model of Higuchi (rate constant $k = 8.55 – 14.47$ %$h^{-1/2}$

Table 2. Release rate constants for the prepared formulations (r>0.985)

Samples	Diffusion model k (% $h^{-1/2}$)	Diffusion model * k (% $h^{-1/2}$)	Zero order kinetic ** k (% h^{-1})
LD1	14.47	-	3.29
LD2	13.17	-	3.22
LD3	8.55	-	2.00
LG1	11.90	13.10	2.89
LG2	11.68	9.98	2.87
LG3	11.61	11.39	2.85

* 0 – 1.5 hour
** after 1.5 hour

for liposome dispersions and $k = 11.61 – 11.90$ %$h^{-1/2}$ for liposome gel formulations), while the release rate after 1.5 hour obeyed the zero order kinetic (rate constant $k = 2 – 3.29$ %h^{-1} for liposome dispersions and $k = 2.85 - 2.89$ %h^{-1} for liposome gel formulations) (Table 2). This may suggest that liposome vesicles act as a reservoir system for controlled release of encapsulated drug substance 5-FU.

Conclusion

As a conclusion, incorporation of aqueous solution of 5-FU into a lipid mixture of phospholipid PL 90H and cholesterol under the proposed hydration conditions, enable formation of liposomes with a potential use as a sustained release depot. Incorporation of liposomes bearing 5-FU into a structured vehicle of chitosan provided formulations suitable for topical application.

References

1. D.J.A. Crommelin and K. Schreier, in *Colloidal drug delivery systems*, J. Kreuter, Ed., Marcel Dekker New York, 1994, pp 73-123.
2. R. Singh and S. P. Vyas, *J. Dermatol. Sci.,* **13**, 107-111 (1996).
3. G. Lopez–Berestein and E. J. Fidler, Liposomes in the therapy of infection diseases and cancer, *New York Liss*, 1989.
4. M. H. Schmid and H. C. Korting, *Adv. Drug Deliv. Rev*., **18**, 335-342 (1996).
5. K. Egabaria and N. Weiner, *Adv. Drug Deliv. Rev.*, **5,** 287-300 (1999).
6. N. Skalko, M. Cajkovac and I. Jalsenjak, *Int. J. Pharm.*, **85**, 97-101 (1992).
7. M. K. Kim, S. J. Chung, M. H. Lee, A. R. Choy and C. K. Shim, *J. Control. Release*, **46**, 243-251 (1997).
8. V. B. Patel, A. Mirsa and Y. S. Marfatia, *AAPS Pharm. Sci. Tech.*, **3**, 56-62 (2001).
9. V. Gabrielcic and M. Sentjuri, *Int. J. Pharm.*, **118,** 207-212 (1995).
10. E. Touitou, H. E. Junginger, N. D. Weiner, T. Nagai and M. Mezei, *J. Pharm. Sci.*, **83,** 1189-1203 (1994).
11. H. Boussen, J. Zwik, I Mili-Boussen, N. Rammeh, N. Bouaouina, S. E. Gritli and A. May, *Therapie*, **56,** 751-754 (2001).
12. M. E. Unis, *Dermatology and Surgery*, **21,** 162-163 (1995).
13. S. B. Kulkarni and G. V. Betageri, in *Pharmaceutical particulated carriers, Therapeutical application*, A. Rolland, Ed., Marcel Dekker, New York 1993, pp 82-135.
14. A. D. Bangham, *J. Mol. Biol.*, **13,** 238-252 (1965).
15. B. Elorza, M. A. Elorza, G. Frutos and J. R. Chantel, *Biochim. Biophys. Acta*, **1153**, 135-142 (1993).
16. Y. Ozer, H. Talsma, D. J. A. Crommelin and A. A. Hincal, *Acta Pharm. Technol.*, **34,** 133-139 (1989).

Резиме

Биофармацевтска карактеризација на липозоми со енкапсулиран 5-флуороурацил за дермална примена

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Клучни зборови: липозоми, услови на хидрирање, дермална примена, ослободување на лековитата супстанција.

Со користење на модифицирана техника на хидрирање на липиден филм подготвени беа различни формулации на липозомски дисперзии со вградена хидрофилна лековита супстанција 5-флуороурацил. Со цел да се согледа влијанието на различните пристапи во подготовката на липозомите, како и да се определи нивната потенцијална примена како депоа за продолжено ослободување на вградената лековита супстанција, формулациите беа подготвени под различни услови на хидрирање на липидниот филм (однос лек/водена фаза) и беа инкорпорирани во цитозански гел матрикс.

Со варирање на количината на водена фаза во која е растворена лековитата супстанција беше постигната различна ефикасност на енкапсулација на 5-флуороурацилот, при што намалувањето на количеството на водена фаза во која е растворен 5-флуороурацилот (масен однос лек/водена фаза), резултираше со повисока ефикасност на енкапсулација.

In vitro следењето на брзината на ослободување на лековитата супстанција од липозомските дисперзии и гел липозомските формулации покажа дека гел липозомските формулации во споредба со соодветните липозомски дисперзии даваат поголем процент на иницијално ослободена лековита супстанција. Ова може да се припише на процесот на лиофилизација на липозомите, која неминовно доведува до оштетување на дел од везикулите, проследено со излегување на дел од лековитата супстанција надвор од везикулите. Во понатамошниот тек на ослободувањето, брзината на ослободување на 5-флуороурацил од гел липозомските формулации не покажа поголеми разлики во однос на липозомските дисперзии.

Математичката обработка на резулататите од ослободувањето на вградената лековита супстанција од липозомските дисперзии и гел липозомските формулации покажаа дека ослободувањето го следи моделот на дифузија. Истовремено по 1.5 часа, ослободувањето ја следи кинетиката од нулти ред што ја потврдува можноста на липозомските везикули да функционираат како депоа на лековитата супстанција од каде таа може контролирано и успорено да биде ослободена.

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Original scientific paper

HPLC identification and determination of myricetin, quercetin, kaempferol and total flavonoids in herbal drugs

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Abstract

A new and rapid HPLC method for identification and determination of myricetin, quercetin, kaempferol and total flavonoids in ten herbal drugs of Macedonian origin is presented. Preparation of samples (*Uvae ursi folim*, *Pruni spinosae flos, Sambuci flos*, *Betulae folim*, *Primulae flos*, *Herniariae herba*, *Centaurii herba*, *Tiliae flos, Robiniae pseudoacaciae flos*, *Bursae pastoris herba)* included hydrolysis of glycosides and extraction of total aglycones with ethyl acetate. HPLC analysis with UV-diode array detection was carried out on RP C18 column, using 5% acetic acid and acetonitrile in agradient elution mode and column temperature of 30 °C. The monitoring of the elution is performed in the whole UV-range and the acquisition of data for quantitative analysis at 367 nm. Screening of the extracts showed presence of quercetin in nine, kaempferol in seven and myricetin in only one sample. The quantitative analysis showed that the content of quercetin ranged from 0.026-0.506 % (m/m), while for kaempferol it was from traces to 1.246 %. *Uvae-ursi folium* and *Pruni spinosae flos* were rich in content of quercetin (0.482 % and 0.506 %, respectively), while *Pruni spinosae flos* and *Robiniae pseudoaccaciae flos* contained the highest amounts of kaempferol (1.246 % and 0.892 %, respectively). Myricetin was identified and determined only in *Betulae folium* (0.102 %). The content of total flavonoids in the investigated samples expressed in terms of quercetin ranged from 0.040 to 1.680 %. The proposed HPLC method is convenient for use in routine analysis of myricetin, quercetin and kaempferol, as well as for estimation of total flavonoids content in herbal drugs.

Key words: flavonols, quercetin, kaempferol, HPLC, determination

Introduction

A lot of commonly used herbal drugs are appreciated in phytotherapy because of their flavonoid pattern. Flavonoids have showed wide range of pharmacological activities, including antioxidant, antimicrobial, spazmolytic, diuretic, capilary protection, citostatic, *etc* (1-3). Quercetin is the most important flavonol and many of the above mentioned activities are due to its presence (1-6). Besides quercetin, kaempferol and myricetin are also widely spread in the plant kingdom (7).

The content of total flavonoids in plant materials has been usually determined by spectrophotometric method with $AlCl_3$ (8). The reaction between flavonoids with OH-group at C-3 and/or C-5 positions and Al^{3+} ions was used for establishing a spectrophotometric method for determination of total flavonoids in *Betulae folium* (9). The same method can be used for analysis of total flavonoids in *Sambuci flos* or *Tiliae flos* (5). For the other herbal drugs, it is recommended to check the accuracy and reproducibility of the method.

Nowadays, high performance liquid chromatography (HPLC) with spectrophotometric or electrochemical detection is the method of choice for identification and quantification of flavonoids. Besides other advantages, the method gives the opportunity of simultaneous identification and determination of flavonoids in plant extracts. Some of recently published data considering HPLC measurements of flavonols in fruits, vegetables, beverages (10-13) and berry wines (14, 15) confirm the wide range of possibilities of HPLC in flavonoid analysis in different kinds of natural materials.

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Examination of flavonoids in Macedonian medicinal plants was recently made for *Helychrisum plicatum*, *Hypericum perforatum*, a large number of wild *Thymus* species, *etc.* (16-18). Continuing our work on flavonoids in medicinal plants, several herbal drugs used in Macedonian folk medicine as spazmolytic, diuretic, antimicrobial and diaphoretic agents (19) were chosen for further examination. According to literature data, determination of total flavonoids with the above mentioned spectrophotometric method is recommended for some of them (5, 8, 9). For the other specimens no method for determination of flavonoids has been suggested until now. The present work is a contribution to the research of developing methods for identification and quantification of flavonoids in different herbal drugs using HPLC method.

Experimental

Materials

Plant material: parts of plants (leaves, herbs, flowers) were collected in the blooming season during summer 1999 and 2000 at different locations throughout the whole territory of Macedonia. The materials were air-dried, milled, packed in paper bags and kept in a dark and cool place until analysis. The following specimens were included in the investigation: *Uvae ursi folim*, leaves of *Arctostaphylos uva-ursi* (L.) Spreng; *Pruni spinosae flos*, flowers of *Prunus spinosa* L.; *Sambuci flos*, flowers of *Sambucus nigra* L.; *Betulae folim*, leaves of *Betula pendula* Roth.; *Primulae flos*, flowers of *Primula veris* L., *Herniariae herba*, herbs of *Herniaria glabra* L.; *Centaurii herba*, herbs of *Erythraea centaurium* (L.) Pers.; *Tiliae flos,* flowers of *Tilia platyphyllos* Scop.; *Bursae pastoris herba*, herbs of *Capsela bursa pastoris* (L.) Med., and *Robiniae pseudoacaciae flos*, flowers of *Robinia pseudoacacia* L.

Reagents and authentic samples: The applied reagents were of the highest purity (>99.95), acetonitrile HPLC grade and glacial acetic acid (Merck, Darmstadt, Germany), authentic samples of myricetin, quercetin, and kaempferol (Extrasynthese, Lyon, France).

Extraction procedure

Milled plant material (2 g) was extracted twice with 50 ml of acetone, 2 ml of concentrated HCl and 1 ml of 1 % solution of urotropine in water, each time. The extraction was performed in an Erlenmeyer flask with reflux on a boiling water bath for 30 min. The extract was then cooled, filtered and filled to volume with acetone (100 ml). Then 25 ml of this extract were transferred to a separating funnel, 50 ml of water was added and extraction with ethyl acetate was repeated 3 times with 15 ml each. The ethylacetate fractions were collected and washed three times with 50 ml of water each, then dried with anhydrous Na_2SO_4, filtered and evaporated to dryness under low pressure. The residue was dissolved and filled to 10 ml with methanol and this solution was used for identification and quantification of flavonols by HPLC.

HPLC analysis

A Varian HPLC system equipped with a ternary pump Model 9012 and UV-Diode Array detector Model 9065 and a reverse phase column C18 (250 x 4.6 mm, 5 mm particle diameter) were used. The mobile phase consisted of two solvents: 5 % CH_3COOH (A) and CH_3CN (B) and the elution program was the following: 0-10 min 70% Asocratic, 10-20 min gradient to 40 % A and then 20-30 min 40 % A isocratic again. The flow rate was 1.0 ml/min and the temperature was set to 30 ϵC. The elution was monitored in the UV range and the acquisition of data for quantitative analysis was performed at 367 nm.

Calibration was made in the concentration range of 0.049-0.490 mg/ml myricetin; 0.086-0.860 mg/ml quercetin and 0.094-0.940 mg/ml kaempferol. The limit of detection (LOD) and limit of quantification (LOQ) were established by construction of a calibration curve in the low concentration region (0.005-0.05 mg/ml) for quercetin.

Results and discussion

The results from the screening HPLC analysis of the extracts obtained after hydrolysis showed that the main flavonol component in the majority of samples was quercetin, followed by kaempferol. Identification of flavonols

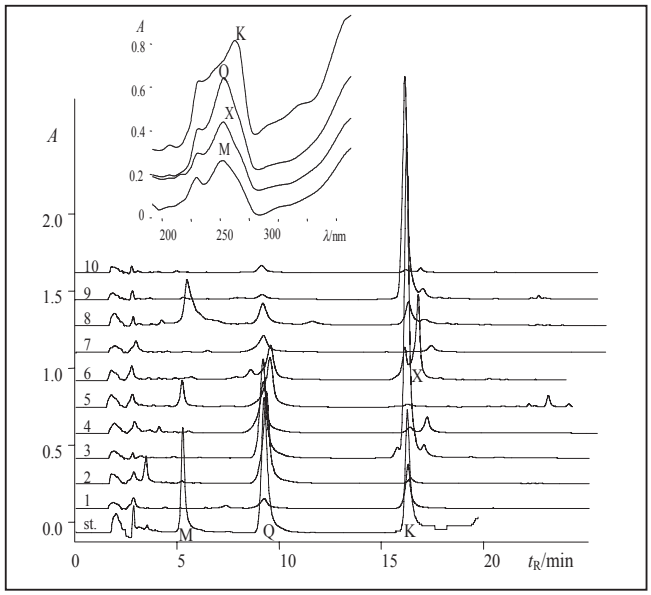

Fig. 1. Chromatograms obtained for a mixture of authentic samples of myricetin (M), quercetin (Q) and kaempferol (K) and from the extracts numbered from 1-10 as in Table 2, with detection at 367 nm (X is the unknown flavonol component) together with the UV-spectra of the flavonoid components

Table 1. Results of the qualitative analysis of flavonols in plant extracts after hydrolysis

	Plant material	Myricetin (M) t_R-5.4	Quercetin (Q) t_R-9.4	Kaempferol (K) t_R-16.3	Unknown (X) t_R-16.9
1	*Tiliae flos*	-	+	+++	-
2	*Uvae ursi folium*	-	+++++	+	-
3	*Pruni spinosae flos*	-	+++++	+++++	+
4	*Sambuci flos*	-	++++	+	++
5	*Betulae flos*	+	++++	-	-
6	*Primulae flos*	-	+++	++	++++
7	*Herniariae herba*	-	++	traces	++
8	*Centaurii herba*	-	++	++	-
9	*Robiniae pseudoacaciae flos*	-	traces	+++++	-
10	*Bursae pastoris herba*	-	+	+	-

* not found in the extract; ** relative quantity approximated in relation to peak area at 367 nm.;
t_R - retention time in min

was performed by comparing the chromatograms obtained from the samples to the one obtained for the mixture of the authentic samples of myricetin, quercetin and kaempferol (Fig. 1, Table 1), using the retention times together with the UV-spectra of the studied flavonols. The retention times obtained for myricetin, quercetin and kaempferol were 5.4; 9.4 and 16.3 min, respectively. This elution sequence is directed by their structural characteristics. All three flavonols have the same substitution pattern in the A flavone ring. Consequently, their elution pattern depends only on the substitution in the B ring, which is as follows: kaempferol has one OH-group in position 4', quercetin two OH-groups at 3', 4' and myricetin three OH-groups at 3', 4', 5' in the B-ring. As expected, the retention is affected by the number of OH-groups and the order of elution from the reverse phase column follows the rule: more polar (OH) groups » less retention on the reversed phase (non-polar) column » shorter retention time.

Screening of the extracts showed that quercetin is the most abundant flavonol in *Uvae ursi folium*, *Pruni spinosae folium*, *Sambuci flos* and *Betulae folium*, lesser amount was found in *Primulae flos*, *Herniariae herba*, *Centaurii herba*, *Tiliae flos* and *Bursae pastoris herba*, and only traces in *Robiniae pseudoacaciae flos.* Kaempferol was found to be most abundant in *Pruni spinosae flos, Robiniae pseudoacaciae flos* and *Tiliae flos*, whereas myricetin was identified only in *Betulae folium*. An unknown flavonoid component was found in the extracts of *Primulae flos, Herniariae herba, Sambuci flos* and *Pruni spinose flos*. Its UV-spectrum, which is almost identical to the one of quercetin, and retention time close to kaempferol suggests that this is most probably quercetin with one methylated OH-group (20).

The HPLC method used for screening analysis was then employed for quantitative analysis of myricetin, quercetin and kaempferol. 367 nm was selected as optimal wavelength for taking the areas of the peaks for quantification because of the better selectivity for all three components and the better sensitivity for myricetin and kaempferol compared to 254 nm (for quercetin no significant difference in sensitivity was observed). The linear dependence of the mass concentration of myricetin, quercetin and kaempferol injected in the column was established in the examined ranges and the linear regression equations, obtained at 254 and 367 nm, with the corresponding coefficients of correlation are the following:

myricetin

254 nm: area = $6{,}4198 \cdot 10^6 \gamma$

$R^2 = 0{,}9995$

367 nm: area = $7{,}2800 \cdot 10^6 \gamma$

$R^2 = 0{,}9989$

quercetin

254 nm: area = $8{,}8234 \cdot 10^6 \gamma$

$R^2 = 0{,}9995$

367 nm: area = $8{,}7838 \cdot 10^6 \gamma$

$R^2 = 0{,}9992$

kaempferol

254 nm: area = $6{,}7326 \cdot 10^6 \gamma$

$R^2 = 0{,}9989$

367 nm: area = $8{,}0530 \cdot 10^6 \gamma$

$R^2 = 0{,}9999$

The sensitivity of the method was determined for quercetin by construction of a calibration curve in the concentration region approximated as a detection and quan-

tification limit (550 mg/ml). The regression equation or the low concentration curve for quercetin is: area = 8.7913·106 g (quercetin), *SD* = 11278, R2 = 0.9980.

The limit of detection was calculated as three times the ratio between the *SD* and the slope of the low concentration curve (LD=3·*SD/slope*) and the limit of quantification as ten times this ratio (LQ=10·*SD/slope*) (21). For quercetin the LD and LQ were found to be 0.004 mg/ml and 0.013 mg/ml, respectively. Having in mind the comparable characteristics of these three compounds, the values for LD and LQ for myricetin and kaempferol were estimated according to the ratio of the slopes of the calibration curves for quercetin and the other two flavonols. In this way the LD for myricetin and kaempferol was estimated as 0.005 and 0.004 mg/ml, and the LQ as 0.016 and 0.014 mg/ml, respectively.

The content of myricetin, quercetin and kaempferol in investigated herbal drugs were determined in the extracts obtained after acid hydrolysis of the plant material and extraction of aglycones with ethylacetate. The results are presented in Table 2. The content of total quercetin ranged from 0.026 % in *Bursae pastoris herba* to 0.506 % in *Pruni spinosae flos,* while the content of total kaempferol ranged from 0.026% in *Uvae ursi folium* to 1.246 % in *Pruni spinosae flos*. 0.102 % myricetin was determined in *Betulae folium*.

According to the obtained chromatograms, only the investigated three flavonols together with the one marked as X were identified as flavonoid compounds. They all have similar UV-spectra (especially myricetin, quercetin and X) which justifies the introduction of a method for estimating the quantity of total flavonols in terms of quercetin as the most readily available of all flavonoids. This method involves summation of the areas of the peaks attributed to flavonols (according to UV-spectra) obtained at 367 nm and calculating the content of total flavonols expressed in terms of quercetin. The results from application of this approach are presented in the last column of Table 2, where according to this criterion the flavonol content ranges from 0.040 to 1.680 % for *Pruni spinosae flos* which is the richest sample in content of flavonols.

It is well known that *Betulae folium* and *Sambuci flos* are appreciated in phytotherapy because of their flavonoid content. Among other measurements, quality control of these herbal drugs includes spectrophotometric determination of total flavonoids with $AlCl_3$ (5). Thus the content of total flavonoids in *Betulae folium* should not be less than 1.5 % expressed as hyperozid, as well as 0.8 % for *Sambuci flos*, calculated as isoquercitrin (5). In quality control of *Tiliae flos* determination of the swelling index is accepted as a quantitative standard although it is well-known that flavonoids are probably the most important active components in this drug. Other specimens, such as *Primulae flos, Pruni spinosae flos* and *Robiniae pseudoacaciae flos,* contain predominantly quercetin and kaempferol as flavonol components (1-5). No method for quantitative determination of these components was suggested until now. On the other hand, in quality control of *Uvae ursi folim* determination of arbutin was accepted as quantitative standard. Among other investigations, bitterness index for *Centaurii herba* and hemolytic index for *Herniariae herba* were accepted as tests for evaluation of their quality, as well.

The present work offers a new and rapid HPLC method for assay of flavonols and determination of myricetin, quercetin, kaempferol and total flavonoids in herbal drugs, which can be used in routine analysis of these plant materials. The method is particularly recommended for the herbal drugs for which no method for quantitative standardization has been proposed yet.

Table 2. Results from determination of myricetin, quercetin, kaempferol and total flavonoids in air dried plant material (in %)

	Plant material	Myricetin ω / %	Quercetin ω / %	Kaempferol ω / %	Total flavonoids (in quercetin)* ω / %
1.	*Tiliae flos*	–	0.046±0.002**	0.194±0.004	0.238
2.	*Uvae ursi folium*	–	0.482±0.004	0.026±0.001	0.520
3.	*Pruni spinosae flos*	–	0.506±0.010	1.246±0.020	1.680*
4.	*Sambuci flos*	–	0.281±0.008	0.028±0.002	0.388*
5.	*Betulae flos*	0.102±0.002	0.312±0.014	–	0.410
6.	*Primulae flos*	–	0.197±0.008	0.122±0.002	0.606*
7.	*Herniariae herba*	–	0.102±0.002	traces	0.226*
8.	*Centaurii herba*	–	0.092±0.008	0.072±0.002	0.182
9.	*Robiniae pseudoacaciae flos*	–	0.047±0.002	0.892±0.016	0.842
10.	*Bursae pastoris herba*	–	0.026±0.002	traces	0.040

* the flavonol X is also considered;
** mean average content ±SD (n=3).

Conclusion

HPLC analysis of flavonols in 10 herbal drugs of Macedonian origin, showed that quercetin is the most abundant flavonol in *Uvae ursi folium* and *Pruni spinosae flos*. Kaempferol was most abundant in Pruni spinosae flos and *Robiniae pseudoacaciae flos*, whereas myricetin was identified only in Betulae folium. The content of quercetin ranged from 0.026-0.506 %, while for kaempferol it was from traces to 1.246 %. The proposed HPLC method for identification and determination of myricetin, quercetin, kaempferol and total flavonoids is rapid and suitable for use in routine analysis of flavonols in herbal drugs.

References

1. I. Asenov and S. Nikolov. *Farmakognozia*, Medicina i fiskultura, Sofia, 1988.
2. J. Bruneton, *Pharmacognosy, Phytochemistry, Medicinal Plants*, Lavoisier Publishing, Paris, 1995.
3. N. Kovacevic, *Osnovi farmakognozije*, MJM, Beograd, 2000.
4. N. Newall, L.A. Anderson and J.D. Phillipson, *Herbal Medicines*, *A Guide for Helthcare Proffesionals*, The Pharmaceutical press, London, 1996.
5. M. Wichtl, *Herbal Drugs and Phytopharmaceuticals*, Medpharm Scientific Publishers, Stuttgart, 1994.
6. P.C.H. Hollman, M. G. L. Hertog and M. B. Katan, *Food Chem.*, **57** (1) 43-6 (1996).
7. J. B.Harborne and T. J. Mabry, In *The Flavonoids: Advances in Research*, Ed. J. B. Harborne, T. J. Mabry, Chapman and Hall, London-New York, 1982.
8. T. J. Mabry, k. R. Markham and M. B. Thomas, *The Systematic Identification of Flavonoids*, Springer-Verlag, New York-Heidelberg-Berlin, 1970.
9. *Pharmacopoeia Helvetica editio septima*, Eidgenossiche drucksachen – und Materializentrale, 3000 Bern, 1987.
10. S. Kawaii, Y. Tomono, E. Katase, K. Ogawa and M. Yano, *J. Agric. Food Chem.* **47,** 3565-71 (1999).
11. S. Karakaya and S. N. El, *Food Chem.,* **66**, 289-92 (1999)
12. S.H. Hakkinen, S. O. Karenlampi, I. M. Heinonen, H. M. Mykkanen and A. R. Torronen, *J. Agric.Food Chem.*, **47**, 2398-2403 (1999).
13 A. Crozier, M. E. J. Lean, M. S. McDonald and C. Black, *J. Agric. Food Chem.*, **45**, 590-595 (1997).
14. H. Vuorinen, K. Maatta and R. Torronen, *J. Agric. Food Chem.* **48**, 2802-2806 (2000).
15. M. Ollanketo and M. L. Riekkola, *J. Liq. Chromatogr. Relat. Technol.*, **23**, 1339-1351 (2000).
16. S. Kulevanova, M. Stefova and T. Stafilov, *Pharmazie* **55**, 391-392 (2000).
17. S. Kulevanova, M. Stefova and T. Stafilov, *Acta Pharm.*, **50**, 29-37 (2000).
18. S. Kulevanova, T. Stafilov, F. Anastasova, M. Ristic and D. Brkic, *Pharmazie* **52**, 886-888 (1997).
19. V. Dervenxi, Sovremeno lekuvawe so lekoviti bilki, Tabernakul, Skopje, 1994.
20. V. Bankova, S.S. Popov and N. L. Marekov, *J. Chromatography*, **242**, 135-143 (1982)
21. Reviewer Guidance, *Validation of Chromatographic Methods*, Center for Drug Evaluation and Research (CDER) FDA, 1994.

Резиме

HPLC идентификација и определување мирицетин, кверцетин, кемферол и вкупни флавоноиди во растителни дроги

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Клучни зборови: флавоноли, кверцетин, кемпферол, HPLC

Во трудот е претставен нов и брз HPLC метод со спектрофотометриска детекција за идентификација и определување кверцетин, кемферол, мирицетин и вкупни флавоноиди во десет растителни дроги од македонско потекло. Подготовката на растителниот материјал (*Uvae ursi folim*, *Pruni spinosae flos, Sambuci flos*, *Betulae folim*, *Primulae flos*, *Herniariae herba*, *Centaurii herba*, *Tiliae flos, Bursae pastoris herba)* вклучува хидролиза на хетерозидните облици на флавоноидите и екстракција на вкупните флавонски агликони со етил ацетат. Во HPLC анализата е користена реверзно фазна колона C18, градиентно елуирање со смеша од 5% оцетна киселина и ацетонирил и температура на колона 30 ^{o}C. Квалитативната анализа на флавоноидите покажа присуство на кверцетин во девет испитувани примероци, додека кемферолот беше најден во седум. Квантитативната анализа покажа дека кверцетинот е застапен во количество од 0,03-0,51 %, а кемферолот од траги до 1,25 %. *Uvae ursi folium* (0,49 %) и *Pruni spinosae flos* (0,51 %) се суровини најбогати со кверцетин. Суровини богати со кемферол се *Pruni spinosae flos* (1,25 %) и *Robiniae pseudoacacie flos* (0,89 %). Предложениот HPLC метод е погоден за рутинско определување кверцетин, кемпферол, мирицетин и вкупни флавоноиди во наведените растителни дроги.

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Original scientific paper

HPLC determination of encapsulated 5-fluorouracil in lyoiphilized liposomes

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Abstract

5-fluorouracil is used as an antineoplastic agent in the treatment of several malignant neoplasm, alone or in a combination with other antineoplastic drugs. Due to the difficulties in delivery of antineoplastic drugs on the target place, serious adverse events and hazardous effects, recent advanced researches are focused on the topical colloidal systems as drug carriers for the chemical substance. Colloidal drug carriers, as liposomes, can improve the therapeutic effects of the chemical substances by modifying their distribution and increasing their efficacy.

The aim of this study was to develop HPLC method for quantitative determination of encapsulated 5-fluorouracil in lyophilized liposomes.

HPLC method was developed using the following chromatographic conditions: column- LiChrospher® 60, RP Selected B, 125 x 4 mm, 5 μm; mobile phase - 0.02 M phosphate buffer pH 4.7; flow rate of 1.0 ml/min; column temperature 20 єC; UV - detection at 266 nm; injection volume - 20 μl. Linear relationship was obtained in the concentration range of 10-100 μg/ml, r=0.9999. Accuracy of the method was confirmed by standard addition method. Determination of encapsulated 5-fluorouracil in lyophilized liposomes was carried out with RSD of 0.71 %.

The proposed method could be used for rapid and precise quality control of lyophilized liposomes with 5-fluorouracil, used as drug delivery system, prepared for further incorporation in pharmaceutical dosage form-gel.

***Key words*:** 5-fluorouracil, liposomes, quantitative determination, HPLC

Introduction

Existing scientific data show that from approximately 1000000 analysed chemical substances that are in a preclinical trial phase, performed in a scientific research and development institutions in the world, only one or two are with physic-chemical, pharmacological and safety characteristics, suitable for further development and modification with the final target of producing active substance with desired therapeutic effects (1). Having these facts as a background, most of the pharmaceutical companies are oriented to design a new pharmaceutical form, instead of research and design of new drug products. Targeting drugs today's through carrier system is a central theme of pharmaceutical development and accent is paid on the use of the existing potential resource of marketed and patented drug substances with known therapeutic effect and modification of their pharmaco-therapeutic characteristics (2). Improvement in their pharmaco-therapeutic characteristics is achieved with decreasing/minimizing their side effects through chemical modification of drug substance and further incorporation in suitable drug system-carriers. Colloidal drug carriers as liposomes and nanoparticles can be used to improve the therapeutic characteristics of marketed and new drugs, by modifying their distribution and increasing their efficacy/safety and by reducing their system toxicity (3). Design of the liposome carrier concept in order to achieve a pharmaceutical level and development of liposomes as potential drug carrier for therapeutic agents took more than 2 decades (4). In recent years, liposomes are widely used as carrier systems for many drugs, including small molecular weight drugs, therapeutic proteins, chemotherapeutic agents, antigens, immunomodulators, chelating compounds, haemoglobin, diagnostic agents etc.

5-fluorouracil is inactive, pyrimidine "prodrug", widely used as an antineoplastic agent in the treatment of common malignacies, in the form of injection, cream, topical solution, tablets etc. Due to the various side effects of 5-fuorouracil, use of liposomes as a drug delivery system gives a site-spe-

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cific delivery of the active substance and increased duration of action with increased therapeutic efficacy. Furthermore, incorporation of liposomes with 5-fluorouracil in pharmaceutical dosage form, for example gel, gives more comfortable way of administration to the patients (5).

The official monographs of 5-fluorouracil in USP XXIV and Ph. Eur. 2000, prescribe quantitative determination of 5-fluorouracil with titrimetry technique using 0.1 N tetrabutylammonium hydroxide in a presence of thymol blue as indicator solution (6,7).

USP XXIV prescribed UV-spectrophotometric determination of 5-fluorouracil in injection, cream and topical solution at 266 nm, using phosphate buffer pH 4.7 as a solvent (6). Previous treatment of the samples for cream and topical solution included multiple extraction procedure using ethyl ether and chloroform.

Several methods based on high-performance liquid chromatography (HPLC) have been developed for assay of 5-fluorouracil (8-10). Reported reversed-phase HPLC methods are used for quantitative determination of 5-fluorouracil in various material as serum, plasma and solid pharmaceutical dosage forms. Mobile phases used in these methods are 0.02 - 0.05 M phosphate buffers pH 4.7 and UV detection at 266 nm. Ion pair HPLC method has been reported for the determination of 5-fluorouracil in human serum (11) using mobile phase, which was a combination of tetrabuthylammonium bromide, tetrabutylammonium hydrogen sulfate, methanol, sodium acetate and phosphate buffer and UV detection at 266 nm. Another ion pair HPLC method was used for separation of 5-fluorouracil from its related substances uridin and uracil in plasma (12). The method employs the use of internal standard and organic extraction procedure. A sensitive reversed-phase HPLC method was developed for quantitative determination of 5-fluorouracil traces on vials (13). Mobile phase used in this method is 0.05% phosphoric acid pH 2.5 and UV detection at 266 nm.

The aim of our study was to develop HPLC method for quantitative determination of encapsulated 5-fluorouracil in lyophilised liposomes used as drug delivery system, formulated for further incorporation in pharmaceutical dosage form - gel.

Experimental

I. Preparation of liposomes with 5-fluorouracil

Liposome formulation containing 5-fluorouracil were prepared at the Institute of Pharmaceutical Technology at the Faculty of Pharmacy, Skopje, using film hydration technique (14). Optimized formula for the preparation of liposomes, which gives the highest % of encapsulation of 5-fluorouracil of approximately 25% was used for the preparation of the sample (Phospholipon 90H/cholesterol =15:1 and drug/aqueos phase = 1:40) (15).

Lipid components were dissolved in chloroform and the solvent was evaporated under reduced pressure at 65°C until no chloroform odour could be detected in the vessel. An aqueous phase was prepared separately by dissolving 5-fluorouracil in phosphate buffer pH 7.4. Lipid film was hydrated with aqueous phase and unentrapted drug was removed by washing the liposomes with phosphate buffer pH 7.4 and ultracentrifugation (25 000 rpm, 20 min, three times). Phosphate buffer was used for reconstitution of pellets and for lyophilization.

Empty liposomes (without active substance 5-fluorouracil) were prepared in the same manner and same time as liposomes with 5-fluorouracil.

II Quantitative determination of encapsulated 5-fluorouracil in lyophilized liposomes

Standard solution

The stock standard solution with 5-fluorouracil (1 mg/ml) was prepared in phosphate buffer pH 4.7. Standard solutions for the construction of the calibration curve (in the range of 10-100 μg/ml) and working standard solution (40 μg/ml) were prepared with further appropriate dilutions with phosphate buffer pH 4.7.

Sample solution

Quantity of lyophylized liposomes of approximately 0.8 g (accurately weighed) was transferred to the volumetric flask of 50 ml, 20 ml chloroform was added and the flask was placed in the ultrasonic bath for 15 min, with slightly heating. After cooling at the room temperature, the flask was filled with phosphate buffer pH 4.7 to the volume. The sample was centrifugated on 3000 rpm for 25 min. 1.0 ml of the clear centrifugate was transferred to the volumetric flask of 10.0 ml and filled with phosphate buffer pH 4.7 to the volume.

Sample solution of empty lipsomes was prepared according to the same procedure.

All solutions were filtered through 0.45 μm membrane filter, before injection.

III. Apparatus

A Waters HPLC equipped with Waters 600 E pump, Rheodyne 7725i manual injector and Photodiode Array Detector 996 was used. Column used was LiChrospher® 60, RP Selected B, 125 x 4 mm, 5 μm. The mobile phase was 100 % 0.02 M phosphate buffer pH 4.7. Chromatographic condition set for this method was: flow rate 1.0 ml/min; column temperature 20°C; UV detection at 266 nm; injection volume 20 μl.

Results and Discussion

Determination of the content of encapsulated 5-fluorouracil in lyophilized liposome formulation involves usage of organic solvent capable to destroy lipid structure,

to release encapsulated 5-fluorouracil and to extract the released active substance in suitable solvent. 5-fluorouracil is sparingly soluble in water, slightly soluble in alcohol and practically insoluble in ethyl ether and chloroform. In the manufacturing procedure, chloroform was used as a solvent for the preparation of the lipid film. In the analytical procedure, chloroform was also used as a solvent for lyophilized liposomes and the extraction of the released 5-fluorouracil was carried out with phosphate buffer pH 4.7. Phosphate buffer pH 4.7 was suitable solvent because it was used as mobile phase as well.

Under the proposed chromatographic conditions, the retention time of approximately 2.4 min for 5-fluorouracil was obtained. The chromatograms of standard solution (a), empty liposome sample solution (b) and liposome sample solution (c) are presented in Fig. 1.

Linear relationship over the concentration range of 10 – 100 μg/ml of 5-fluorouracil was obtained. The calibration curve was constructed by the results obtained for the peak areas *versus* concentration. The linear regression equation obtained by a least-squares method was $y = 1.0517 + 0.3392\ c$; $r = 0.9999$.

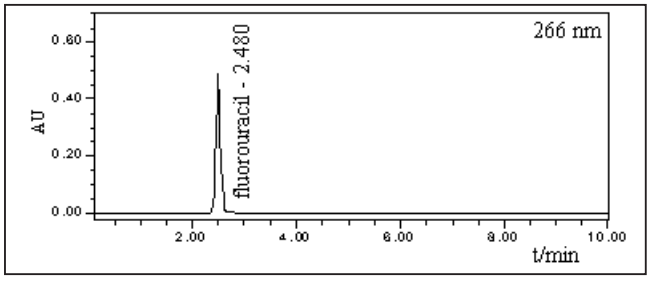

Fig. 1a. Chromatogram of standard solution of 5-fluorouracil (40μg/ml)

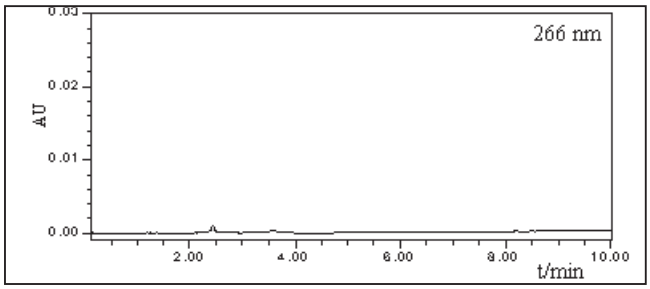

Fig.1b. Chromatogram of the empty liposomes solution

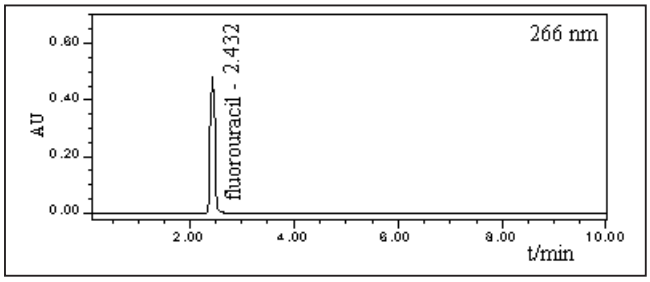

Fig. 1c. Chromatogram of the sample solution of 5-fluorouracil (40 mg/ml)

Precision of the method was confirmed by determination of:

- Repeatability of the system with 6 successive injection of standard solutions at working concentration with RSD value 0.22 % and

- Repeatability of the method with 6 successive injections of sample solutions at working concentration with RSD value 0.71 %.

The results of the determination of encapsulated 5-fluorouracil in a lyophilized liposomes are presented in Table 1.

Table 1. Results of the determination of encapsulated 5-fluorouracil in lyophilized liposomes*

Measured mg/g*	Encapsulated mg/g*	SD mg/g*	RSD %
100	25.47	0.18	0.71

*prepared according to the optimized formula (15) using film hydration method: drug/lipid phase = 1:3.2; drug/aqueous phase = 1:40

The results obtained have shown that the encapsulated amount of 5-fluorouracil in a lyophilized liposomes prepared with the lipid film hydration technique is 25.47 %. These results are in accordance with the latest literature reported data indicating that the highest % of encapsulation of active substance in liposomes prepared according to this technique is up to 30 % (14).

Accuracy of the method was confirmed by the standard addition method performed by adding 5-fluorouracil standard solution in amount of 50, 75 and 100% over the determined encapsulated content in lyophilized liposomes and results are shown in Table 2.

Table 2. Results of 5-fluorouracil determination in lyophlized liposomes by standard addition method*

Encapsulated mg/g*	Added mg/g*	Calculated mg/g*	Determined mg/g*	Recovery %
25.47	12.00	37.47	37.17	99.22
	18.00	43.47	42.05	96.74
	24.00	49.47	48.18	97.40

drug/lipid phase = 1:3.2; drug/aqueous phase = 1:40

The obtained recovery values of 96.74 – 98.22 % indicated that the proposed method is quantitative and accurate.

Detection limit and quantification limit were determined with the method based on the calibration curve studied using samples containing an analyte in the range of approximate DL and QL. The obtained values for DL and QL were 0.24 μg/ml and 0.78 μg/ml, respectively.

Conclusion

A simple and rapid reversed-phase HPLC method for quantitative determination of 5-fluorouracil in lyophilized liposomes was developed. The proposed method is precise, accurate and reproducible. The content of encapsulated 5-fluorouracil in lyophilized liposomes determined by the proposed HPLC method was found to be 25.47 % which is in accordance with the reports in the literature indicating maximum 30% encapsulation of active component in liposomes using lipid film hydration technique for their preparation (14).

The method could be also used for determination of the active substance in the pharmaceutical dosage forms (gel) prepared with incorporation of 5-fluorouracil loaded liposomes, as well as in-process quality control of a previous prepared intermediate product (liposome dispersion).

References

1. J.R. Andersen., *Guidelines for Management of Discovery Projects*, NN Project Manual, Copenhagen, July 2000
2. S.Vemuri and C.T. Rhodes, a review, *Pharm. Acta Helv*. **70**, 95-111, (1995).
3. G.M.Barrat , *Pharmaceutical Science and Technology Today* **3,** 163-171, (2000).
4. D.J.A. Crommelin, G. Storm, *Pharmaceutical Science and Technology Today* **1**, 19-31, (1998).
5. R.H.Williams, J.D.Wilson and D.W. Foster (Editors) *Willaim's Textbook of Endocrinology*, 7-th Ed., W.B. Saunders, Philadelphia, 1995
6. *United States Pharmacopoeia XXIV*, United Pharmacopeial Convection, Inc., Twinbrook Parkway, Rockville, 2000
7. *European Pharmacopoeia*, 4th, Council of Europe, Strasbourg, Supplement 2000
8. E.J. Quebbeman, *J. Liquid Chro.* **7**, 1489-1494 (1984).
9. C.L. Hornbeck, R.A. Floyd, J.C. Griffith and J.E. Byfield, *J. Pharm.Sci.*, **70**, 1163-1166, (1981).
10. CromCircle, *Merck Applications*, Merck KgaA Darmstadt Germany, (1998).
11. M.W. De Gregorio and W.M. Holleran, *Anal. letters* **18(B1),** 51-55, (1985).
12. G.J. Peters and I.Kraal, *J. Chromatography* **307**, 464-468, (1984).
13. J. Delporte, P. Chenoix and Ph. Hubert, *Science* **5 (3),** 119-121, (1999).
14. A. Chonn and P.R. Cullis, *Curr. Opin. Biotechnol.* 6, 698-708, (1995).
15. M.G. Dodov, Liposomes as biodegradabile carriers of 5-FU-formulation and characterization, MSc thesis. University of "Ss Cyril and Methodius", Faculty of Pharmacy, Skopje 2002.
16. E.G.C.Clarke, *Clark's Isolation and Identification of drugs*, 2nd Ed., The Pharmaceutical Press, London, 1986

Резиме

HPLC определување на енкапсулиран 5-флуороурацил во лиофилизирани раствори

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Клучни зборови: 5-флуороурацил, липозоми, квантитативно определување, HPLC

5-флуороурацил се употребува како антинеопластичен агенс при лекување на повеќе малигни неоплазми, сам или во комбинација со други антинеопластични лекови. Поради потешкотиите во испорачувањето до целното место, како и поради многуте несакани дејства на антинеопластичните лекови, најновите истражувања се однесуваат на употребата на колоидни системи како носители за активната супстанција. Колоидни носители, како липозомите, можат да го подобрат терапевтскиот ефект на хемиските супстанции преку модификување на нивната дистрибуција и зголемување на терапевтската ефикасност.

Целта на овој труд беше да се разработи HPLC метод за квантитативно определување на енкапсулиран 5-флуороурацил во лиофилизирани липозоми. Разработен е HPLC метод со употреба на реверзно фазна колона LiChrospher® 60 RP B selected, 125 x 4 mm, 5μm; како мобилна фаза беше користен 0,02M фосфатен пуфер pH 4,7; проток од 1,0 ml/min; температура на колона 20 °C; UV-детекција на 266 nm и волумен на инјектирање 20 μl. Линеарноста на методот беше испитана и утврдена во опсегот на концентрации од 10-100 μg/ml, r=0,9990. Точноста на методот беше потврдена со методот на стандардни додатоци. Содржината на енкапсулиран 5-флуороурацил во лиофолизираните липозоми беше определена со вредност на RSD од 0,71%.

Предложениот HPLC метод може да биде употребен за брза и прецизна контрола на квалитет на лиофилизирани липозоми со 5-флуороурацил, како систем за испорака на лековита супстанција, подготвени за понатамошно вградување во дозиран фармацевтски облик-гел.

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Short communication

Effect of cyclosporine therapy in transplanted patients-diagnostic values of tubular markers

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Abstract

The introduction of cyclosporine A (CsA) into the clinical practice has resulted in a major improvement in the short-term outcomes of solid organ transplantation and treatment of autoimune diseases. Chronic ScA nephrotoxicity has been described in kidneys of recepients of renal and other organ allografts. However, the exact mechanism underlying the development of fibrosis in chronic CsA nephrotoxicity has remained poorly understood. Evaluation with the validation data set showed that noninvasive urine protein differentiation might be a useful diagnostic strategy in nephrology. Over the past decade numerous studies in patients after transplantation have demonstrated that renal tubular cell injury after a toxic insult, results in sloughing of tubular debris and cell into the tubular lumen with eventual obstruction of tubular flow, increased intratubular pressure and backleak of glomerular filtrate out of the tubule. Urinary enzymes and low molecular proteins have been recommended as useful markers for the detection of changes in the kidney tissue in cases after renal transplantation.

The aim of our study was to monitor the concentration and eventual nephrotoxic effect of Cyclosporine A using the concentration of low molecular proteins α-1-microglobulin and β–2-microglobulin, serum Cystatin C, as well as the concentration of isoform of GST-α and π.

Key words: Cyclosporine A, α-1-microglobulin, β–2-microglobulin, Cystatin C, α/π GST

Introduction

The renal tubular system consists of several segments with special functions. It is known that various pathological processes and toxins influence on specific parts of the system, initially very fast, leaving the others undamaged (1). Therefore, diagnosis of processes affecting specific segments of the tubular system requires complementary methods specific for different portions of the nephron. Urinary enzymes and specific proteins in urine have been recommended as useful markers for the detection of changes in the kidney tissue in cases with acute renal failure, acute tubular necrosis, acute rejection episode and nephrotoxicity in cases after transplantation (1,2).

The most common and potential limiting consequence of Cyclosporine therapy is renal dysfunction (3). Coupled with renal pathological changes and systemic hypertension, this adverse effect can lead to chronic and even end-stage kidney failure (4).

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Cyclosporine is a neutral, cyclic polipeptide with 11 amino acids, which is extremely hydrophobic. This is related partialy to its wide volume of distribution and high concentration in fat, liver and pancreas.The kidney does not preferentially accumulate extentsive tissue stores of cyclopsporine. Cyclosporines is highly bound to red cells and lipoproteins in blood, and in fact, only 5-10% of the drug remains “free”. Therefore, variation in lipoprotein and hematocrit values may affect therapeutic efficacy and toxicity. Due to limited excretion of unchanged cyclosporine or its metabolites by the kidney, no dosage adjusments are required for patients with pre-existing renal dysfunction (3).

Throughout the extensive metabolism, cyclosporine is metabolized to multiple metabolites by hepatic cytochrome P-450, mixed-function-oxidase enzymes. This leads to clinical relevant drug interactions. Drugs that inhibit cytochrome P-450 enzymes increase concentration of parent cyclosporine and may cause nephrotoxicity, hypertension and other side-effects. Conversely, drugs that induce these enzymes reduce parent cyclosporine concentration and lead to reductions in the drugs immunosuppressive to the unchanged drug.

Cyclosporine (CsA) has improved one-year allograft survial in renal, liver, heart and pancreas transplantation, and has lately also been advocated as a promising treatment for some autoimmune diseases (3). However, the use of CsA is associated with major nephrotoxicity, very likely being a consequence of its toxic effect on vascular endothelium, consistently documented in experimental animals and humans. Evidence is now available that in kidney transplant recipients, each oral dose of CsA if followed by a transient reduction in renal plasma flow and glomerular filtration rate that results from a form of acute reversible renal hypoperfusion (5).

The aim of our study was:

- To select the urinary enzymes and low molecular urinary proteins which have diagnostic and predictive value for rejection after renal transplantation

- To point out the diagnostic and clinical importance of follow-up study of glomerular and tubular proteins after transplantation

The present investigation is focused especially on pointing out the differences between acute rejection episode (ARE) and CsA nephrotoxicity, using specific urinary enzymes and proteins as markers. It also aims at creating diagnostic laboratory background for the clinicians, and to providing helpful information for identifying the differences between the relevant different complications prior to clinical symptoms, without expansive and dangerous invasive investigation.

Exprimental

The control group comprised 30 healthy individuals, with no cardiac, liver or renal diseases or hypertension and with normal urine analysis and normal serum urea, creatinine and Cystatin C concentration.

Urine samples were collected and evaluated from 48 patients after renal transplantation during a period of 24h at room temperature, and their volume was measured sometimes second morning urine was also used.

Patients with transplants were classified in two groups due to their clinical diagnosis:

Patients with normal graft function (NGF), N=28, with spontaneous decrease in serum creatinine or Cystatin C level immediately after transplantation

Patients with acute rejection episode (ARE), N=20, characterized with increasing serum creatinine, Cystatin C concentration, diminished daily urine volume, graft tenderness and enlargement, and nephrotoxic effects of immunosuppressive therapy (Cyclosporine A)

The concentration of α-1-microglobulin, β-2-microglobulin, IgG, albumin in urune, Cystatin C and β-2-microglobulin in serum were determined using immunoturbidimetric method according to DAKO-Instruction cataloque (mg/l) (6). Urinary enzyme activities of N-acetyl-β-d-glucosaminidase (β-NAG) (7), alanin aminopeptidase (AAP), (U/mmol creatinine) α/π GST(ng/l) were determined with standard validated methods (8).

Results and disscusion

The activity of urinary enzymes (β, NAG, AAP, α/pi GST) and concentration of low molecular proteins are presented in Table 1. The results showed that the obtained values (SD) were in close correlation with the already published ones (4,9,10,11).

Table 1. Reference interval of investigated parameters

	N	X	SD	Min-Max
α-1- Microglobulin urine,mg/l	50	10.0	2.0	8.0-14.0
Albuminuria Mg/l	50	12.0	6.0	6.0-20.0
β-NAG- U/mmol creatinine	50	0.72	0.5	0.2-1.20
β-2 Microglobulin serum mg/l	50	1.65	0.66	0.65-2.75
б -GST ng/l	50	3.7	2.3	1.4-6.0
π-GST ng/l	50	13.4	5.3	8.1-18.7

The excretion of urinary enzymes β-NAG and AAP has controversial diagnostic value 2-7 postoperative days, although these enzymes can indicate the ability of the graft to regenerate. In significant enzymuria in the early postoperative period, the values decrease to the normal range for 3-5 days. It is important to note that mean values of the investigated enzymes were lower that those of the control group (Fig.1), and they reached the reference values after 6-7 days (AAP 1.38 + 0.18, β-NAG 0.68 + 0.35 U /mmol creatinine).

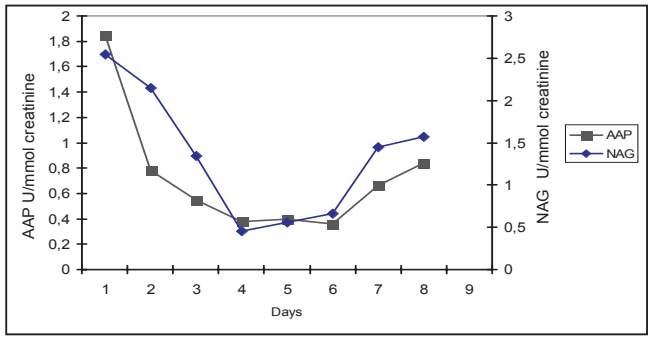

Fig.1. Activity of AAP and β-NAG in patients with stable graft function

At the same time, monitoring the concentration of Cystatin C in serum, the new biomarker of glomerular filtration rate, our results have shown that it decreased more rapidly than creatinine (Fig.2).

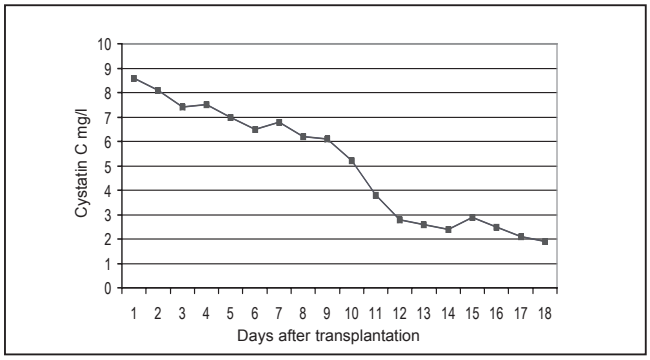

Fig. 2. Concentration of Cystatin C in serum in patients with stable graft function

The ability (capacity) to detect a reduction in GFR was also shown to be better for serum Cystatin C, than for serum creatinine. Similar results were obtained by Bricon group (12).

In cases of acute rejection episode induced by Cyclosporine A, toxicity changes of Cystatin C concentration is higher compared with creatinine concentration (Fig.3).

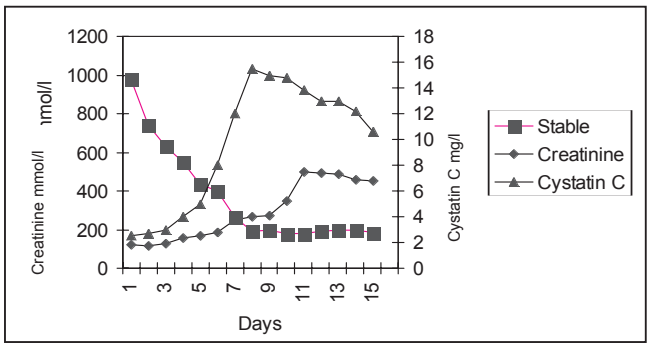

Fig.3. Concentration of creatinine and Cystatin C in transplanted patients with normal graft function and acute rejection episode induced by high doses of Cyclosporine A.

The changes of α-1 microglobulin concentration have confirmed that this low molecular protein is a very sensitive marker of Cyclosporine A toxicity (Fig.4).

Results of the correlation between ARE and CysA toxicity showed high significante elevation in the first two days after transplantation. These results revealed early proximal

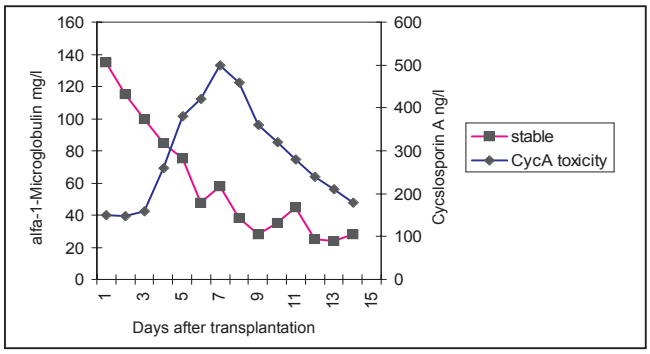

Fig.4. Concentration of α-1 microglobulin in urine of patients with stable graft function and patients with acute rejection episode (CsA toxicity)

tubular dysfunction. Similar results were shown by Hoffman et al. (13) with application of Urinary Protein expert system (UPES) in the differential diagnosis of kidney diseases.

In case of tubular dysfunction, the measurement of plasma protein β-2 microglobulin have to provide the most sensitive induction of tubular damage (Fig.5).

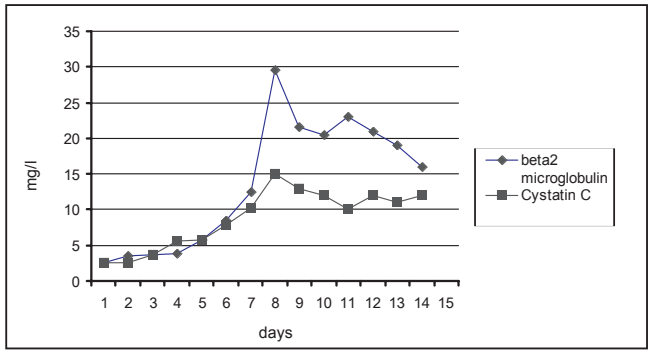

Fig. 5. Concentration of β-2- microglobulin in serum of pateints with stable graft function and patients with acute rejection episode

Gluthatione S transferases (α/pi isoforms) are presented in many different tissues and play a central role in the biotransformation of drugs and toxic chemicals. High concentration of these isoforms can indicate toxic damages (cyclosporine, aminoglycosides), tubular necrosis and rejection of the transplant.

In case of acute rejection, in the early stages of CsA-induced nephrotoxicity, a significant amount of GST-α is excreted into the urine (Fig.6).

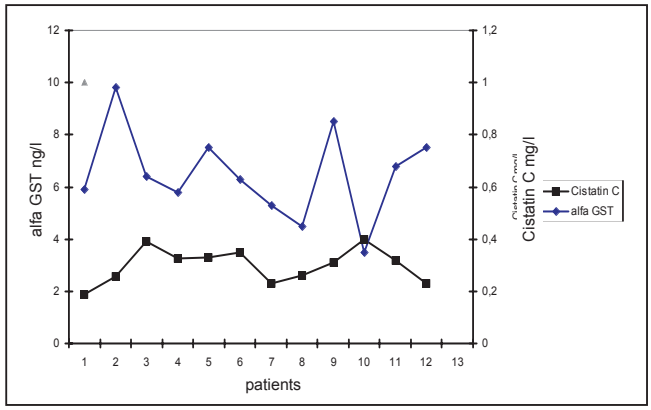

Fig. 6. Concentration of α-GST and Cystatin C in cases with CsA toxicity

In this case no significant elevation of urinary GST-π was noticed.These infindings are in agreement with the understanding that in the initial phase of such toxicity, only the proximal tubules are damaged, the distal portion being affected only at a later stage. There two explanations for the appearance of GST-transferases in the urine: excretion from plasma by glomerular filtation or release from damaged cells in the proximal tubular epithelium (14). CsA induced nephrotoxicity in human renal transplant was accompanied

by high recipients release of α–GST ($p<0.001$) and low significant values of π–GST ($p < 0.05$) in urine indicating toxic effect of the drug in the proximal tubular system.

It appears that the assay of different proteins and enzymes in urine and their determination can be used as powerful tool in renal diagnosis in the future, since direct methods like needle biopsy, are relatively complicated and very invasive. Further studies in order to determine to what extent these tests are useful in identifying differences between glomerular and tubular diseases are also needed.

References

1. L.A.H. Campbell, A.V. Corrigal, A. Guy, R.E. Kirsch, *Cancer*, **67**,1608-1613 (1991).
2. R. Corbert, G.J. Gardner, P.R.N. King, A.E. Thompson, R. Price, *Clin.Chim.Acta*, **128**,141-150 (1983).
3. W.M. Bennet, E.A. Burdmann,T.F. Andoh, D.C. Houghton, J. Lindsley and L.W. Elzinga, *Nephrol. Dial. Transpl*., **9**, Suplpl. 4, 141-145 (1994).
4. E. Coli, A. Botey and L. Alvares, *Am. J. Kidney Dis*., **36**, 29-34 (2000).
5. L. Backman, E.L. Appelkvist, O. Ringden and G. Dallner, *Kidney Inter*., **33**, 571-577 (1988).
6. Dako Cytomation: *Clinical Immunochemistry*, 239-246 (2003).
7. G. Klein and E. Geibel, *Prodr. Med. Lab.,* **4**/4, 450 (1990).
8. W.P. Raab, *Clin. Chem*., **18**,5-25 (1972).
9. E. Sarvary, P. Borka, B. Sulyok, A. Peter and Z. Vass, *Transpl. Inty*., **9** (Suppl), S68-S72, (1996).
10. S. Tian, E. Kisano, J. Ohara, K. Tabri, Y. Iton and T. Kava, *Clin. Nephr*., **48**, 104-108 (1997).
11. R.D. Perrowe and N.E. Madias, *Clin. Chem.,* **88**, 1933-53 (1992).
12. T. Bricon, E. Thervel and M. Froisaat, *Clin. Chem*., **46**, 1206-1207 (2000).
13. W. Hoffman and W. Guder, *Clin. Nephrology*, **38** Suppl.1, S3-S7 (1992).
14. A.Sundberg, E.L. Appelkvist and L. Backman, *Nephron*, **67**, 308-316 (1994).

Резиме

Ефект на циклоспоринската терапија кај трансплантирани пациенти - дијагностичко значење на тубуларните маркери

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Клучни зборови: циклоспорин А, нефротоксичност, α-1 микроглобулин, β-2 микроглобулин, Cistatin C, α/π GST, NAG

Примената на циклоспорин А во клиничката практика кај пациенти со извршена бубрежна трансплантација резултира со нарушувања во функцијата на бубрезите, доведувајќи до компликации, како акутна нефротоксичност, хронична тубулоинтестицијална фиброза и појава на хемолитички уремичен синдром. Со употребата на релевантни т.н. тубуларни и гломелуларни маркери постигнат е напредок во следењето на состојбата на бубрежната функција во првите денови по трансплантацијата. Следењето на концентрацијата на циклоспорин А во серум , како и тубуларните маркери во урина како : α-1 микроглобулин, β-2 микроглобулин, α/pi Глутатион С трансфераса, NAG, AAP како и Cystatin C (нов биомаркер за гломеруларната филтрациона брзина) во голема мера ја дополнуваат клиничката слика кај овие пациенти.

Целта на оваа студија е да се укаже на осетливоста и на раните промени на биохемиските маркери за навремено укажување на локализацијата и степенот на бубрежното оштетување под влијание на нефротоксични агенси. Врз основа на добиените резултати, обработени со помош на UPES (urinary protein expert system), се добива комплетна биохемиско-клиничка слика за моменталната состојба на трансплантантот (стабилна фаза, фаза на почетна реакција, акутна реакција на отфрлување, односно нефротокичност предизвикана од имуносупресивната терапија).

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Стручен труд

Валидација на метод за определување микробиолошка чистота на Кафетин® таблети

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Апстракт

Извршена е валидација на методот за определување микробиолошка чистота на Кафетин® таблети. За таа цел користени се тест-микроорганизми: *Escherichia coli, Pseudomonas aeruginosa ,Staphylococcus aureus, Candida albicans* и *Aspergillus niger,* од колекцијата ATCC. Употребен е неспецифичен хранлив медиум за аеробни микроорганизми и специфични хранливи медиуми за соодветни тест микроорганизми: ENDO, Cetrimid, Baird-Parker и Sabouraud хранлив агарен медиум. Користени се два начина на изведување на методот: директна инокулација во хранлив медиум и мембранска филтрација. Притоа е користен тестот на предизвик (Challenge test) и тестот на броење на пораснати колонии (CFU/ml). Вршена е пресметка на факторот што претставува однос помеѓу пораснати микроорганизми на инокулиран хранлив медиум, без и со додавање на испитуваниот препарат, како критериум за прифатливост на добиените аналитички резултати. Добиените вредности за факторот како критериум за прифатливост покажаа задоволителни вредности. Тоа укажува дека методот со кој се работи може да се употребува за определување на микробиолошка чистота кај Кафетин® таблети.

Клучни зборови: микробиолошка чистота, валидација на метод, Кафетин® таблети

Вовед

Микробиолошката чистота како параметар кај цврстите фармацевтски форми покажува присуство на дозволен број од некои непатогени микроорганизми што не го загрозуваат здравјето на корисниците, а исто така немаат негативно влијание на квалитетот на фармацевтскиот препарат и отсуство на патогени микроорганизми (1, 6).

Поголем број непатогени микроорганизми од дозволениот може да доведе до појава на несакани ефекти кај пациентите, додека присуството на патогени микроорганизми може да биде вистинска опасност по здравјето на корисниците. Микробиолошката чистота е значаен параметар не само во моментот на производство, туку и до рокот на употреба на препаратот. Ако почетните вредности за микробиолошка чистота не соодветствуваат може да се очекува дека до декларираниот рок на траење нивниот број ќе се зголеми. Микроорганизмите преку своите ензими и метаболити, можат да стимулираат и да предизвикаат различни процеси како оксидација, хидролиза, редукција и сл. Тоа може да доведе до појава на несакани физички и хемиски промени кај препаратите како промена на боја, појава на несакан мирис, опаѓање на тврдината, опаѓање на содржината на активната компонента, појава на разградни продукти.

Несоодветна контактна амбалажа може да доведе во услови на несоодветно чување на фармацевтските препарати до иницирање раст на микроорганизми и како последица на тоа можат да се случат процеси на интензивна деградација, иако препаратите во моментот на производство ги задоволувале критериумите за микробиолошка чистота.

Затоа е многу важно при испитување микробиолошка чистота методолошката постапка да е валидирана, да се користат хранливи медиуми на кои пред-

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ходно им е испитан фертилитетот и да бидат задоволени условите на ДПП/ДЛП во микробиолошката лабораторија во која што се изведува тестот (5).

Определување микробиолошка чистота на Кафетин® таблети се изведува според монографиите во современите фармакопеи. Притоа, како прифатливи се земаат критериумите:

– вкупен број аеробни бактерии - дозволен лимит 10^3 - максимално прифатлив лимит 5 x 10^3

– вкупен број фунги - дозволен лимит 10^2 - максимално прифатлив лимит 5 x 10^2

Escherihia coli - да не биде присутна (2).

За таа цел користени се лиофилизати од тест-микроорганизми со деклариран број колониформни единици (околу 100 CFU/ml). Употребени се директна инокулација на цврст хранлив медиум и паралелна употреба на мембранска филтрација како потврда на валидација на методот. Притоа е користен тестот на предизвик (Challenge test) и тестот на броење на плоча (3). Факторот како критериум на прифатливост е пропишан во фармакопеите и тој не смее да биде поголем од фактор пет (2). Кога некој препарат се работи прв пат за чии ексципиенти и активни компоненти нема потполни литературни податоци за нивното антимикробно дејство, како претходно испитување се прави тест на антимикробно дејство на препаратот (4).

Експериментален дел

Материјали

Стандардна апаратура и прибор во микробиолошка лабораторија ;

Пуферен раствор на пептон со натриум хлорид ;

Хранливи медиуми:

Trypcase soy agar (TSA),
Cetrimide агарен медиум,
Baird Parker агарен медиум,
ENDO агарен медиум,
Sabouraud агарен медиум.

Тест-микроорганизми со деклариран број на CFU/ml:

Escherichia coli ATCC 8739,
Pseudomonas aeruginosa ATCC 9027,
Staphylococcus aureus ATCC 6538,
Candida albicans ATCC 10231,
Aspergillus niger ATCC 16404 и
Bacillus subtilis ATCC 6633

Суспензиите на тест-микроорганизми од лиофилизати приготвени се според упатството на производителот на тест-културите.

Определување антимикробно дејство на Кафетин® таблети

За оваа цел се подготвени во дупликат петриеви садови во кои е аплициран подготвениот препарат што се испитува. Извршена е инокулација со *Bacillus suptilis,Candida albicans* и *Aspergillus niger* и прелиени се со соодветни хранливи медиуми. Инкубирани се 48-72 часа и отчитани се резултатите за вкупен број бактерии и фунги.

За определување на антимикробното дејство со специфични тест микроорганизми подготвена е суспензија од пуферен раствор на пептон со испитуваниот препарат и тест микроорганизми: *Staphylococcus aureus, Escherichia coli* и *Pseudomonas aeruginosa.* Од вака подготвените суспензии инокулиран е дел во претходно подготвени хранливи медиуми во петриеви садови во дупликат. Инкубирани се 48 часа и отчитани се резултатите за специфичните тест микроорганизми.

Фактор на прифатливост при определување микробиолошка чистота кај Кафетин® таблети

Користени се пет видови тест-микроорганизми: *Escherichia coli ATCC 8739,Pseudomonas aeruginosa ATCC 9027, Staphylococcus aureus ATCC 6538, Candida albicans 10231* и *Aspergillus niger ATCC 16404*.

Во 90,0 ml пуферен раствор од пептонска вода со pH 7,2 суспендирани се 10,0 g Кафетин® таблети.

Во пет стерилни епрувети (со капак за завртување), со стерилна пипета, префрлен е дел од суспензијата на Кафетин® таблети.

Во секоја епрувета со суспензија од Кафетин® таблети, со стерилна пипета, инокулиран е дел од суспензија на тест микроорганизам (во секоја епрувета по еден од тест микроорганизмите).

Содржината од секоја епрувета (добро затворена) е хомогенизирана со мешалка.

За потврда на добиените резултати користени се два начина за изведба на тестот.

Директна инокулација во хранлив медиум

Методот е изведен во согласност со наведените податоци:

Мембранска филтрација

Од епруветата со суспензија од Кафетин® таблети и соодветен микроорганизам по добра хомогенизација, со стерилна пипета, аплицирана е одре-

Препарат со микроорганизам	Хранлив медиум	Температура за инкубација (°C)	Период на инкубација
Кафетин® таблети/ *Echerichia coli*	TSA и ENDO	44– 2	Пет дена
Кафетин® таблети/ *Pseudomonas aerug.*	TSA и Cetrimide	32-35	Пет дена
Кафетин® таблети/ *Staphylococcus aureus*	TSA и Baird Parker	32-35	Пет дена
Кафетин® таблети/ *Candida albicans*	Sabouraud	22-25	Седум дена
Кафетин® таблети/ *Aspergillus niger*	Sabouraud	22-25	Седум дена

дена количина во еден држач за филтер од системот за филтрација, во кој веќе е поставен мембрански филтер со мрежа и големина на пори од 0,45μм. По извршена филтрација, филтерот е промиен со три порции од по 100ml пуферен раствор од пептон. Со стерилна пинцета филтерот е пренесен во петриев сад со хранлив медиум.

Хранливите медиуми, температурата и времетраењето на инкубацијата се исти како и при директна инокулација.

Резултати и дискусија

Според прикажните резултати во Табела 1, активните компоненти и ексципиентите во Кафетин® таблети не покажаа инхибирачко дејство врз испитуваните тест микроорганизми поради што нема потреба од инактивација.

Резултатите прикажани на Табелата 2 покажаа дека факторот на прифатливост се движи од 1,71 до 2,12 при директна инокулација врз хранлив медиум или 1,50 до 1,75 со мембранска филтрација, што е во рамките на критериумот на прифатливост.

Резултатите прикажани на Табелата 3 покажаа дека факторот на прифатливост се движи од 1,30 до 2,26 при директна инокулација врз хранлив медиум или 1,53 до 3,22 со мембранска филтрација, што е во рамките на критериумот на прифатливост.

Резултатите прикажани на Табелата 4 покажаа дека факторот на прифатливост се движи од 2,25 до 2,50 при директна инокулација врз хранлив медиум или 1,5 до 2,00 со мембранска филтрација, што е во рамките на критериумот на прифатливост.

Резултатите прикажани на Табелата 5 покажаа дека факторот на прифатливост се движи од 1,25 до 1,48 при директна инокулација врз хранлив медиум или 1,24 до 1,37 со мембранска филтрација, што е во рамките на критериумот на прифатливост.

Резултатите прикажани на Табелата 6 покажаа дека факторот на прифатливост се движи од 0,92 до 1,02 при директна инокулација врз хранлив медиум или 0,65 до 0,74 со мембранска филтрација, што е во рамките на критериумот на прифатливост.

Резултатите во Табела 7 покажуваат дека факторите на прифатливост за петте тест микроорганизми, при валидација на методот за микробиолошка чистота кај Кафетин® таблети со директна инокулација се движат од 0,92 до 2,26, а со мембранска филтрација од 0,65 до 3,22 и се во рамките на критериумот на прифатливост.

Табела 1. Определување антимикробно дејство на Кафетин® таблети

микроорганизам во суспензија од препарат	хранлив медиум	I петриев сад	II петриев сад	Контролен петриев сад (без препарат)
Escherichia coli	ENDO	+	+	+
Bacillus subtilis	TSA	+	+	+
Staphylococcus aureus	Chapman	+	+	+
Pseudomonas aeruginosa	Cetrimid	+	+	+
Candida albicans	Sabouraud	+	+	+
Aspergillus niger	Sabouraud	+	+	+

- нема пораст на колонии; ! слаб пораст на колонии; + добар пораст на колонии;

Табела 2. Фактор на прифатливост при определување микробиолошка чистота на Кафетин® таблети со суспензија од *Escherichia coli* ATCC 8739

тест - материјал		директно нанесување на хранлив медиум						
		вид на медиум	24h cfu	48h cfu	72h cfu	96h cfu	пет дена cfu	F
пептонска вода + суспензија од *Escherichia coli*	I	1,0ml на TSA	17	17	19	20	24	
	II	1,0ml на TSA	9	11	17	17	17	
	I	1,0ml на ENDO	7	9	11	11	12	
	II	1,0ml на ENDO	8	11	12	14	14	
суспензија од Кафетин® таблети + суспензија од *Escherichia coli*	I	1,0ml на TSA	13	13	14	14	14	1,71
	II	1,0ml на TSA	5	7	8	8	8	2,12
	I	1,0ml на ENDO	3	5	5	6	6	2,00
	II	1,0ml на ENDO	0	4	8	8	8	1,75
тест - материјал		мембранска филтрација						
		вид на медиум	24h cfu	48h cfu	72h cfu	96h cfu	пет дена cfu	F
пептонска вода + суспензија од *Escherichia coli*	I	1,0ml на TSA	3	7	13	13	14	
	II	1,0ml на TSA	0	3	6	7	11	
	I	1,0ml на ENDO	0	0	6	7	7	
	II	1,0ml на ENDO	0	2	5	6	6	
суспензија од Кафетин® таблети + суспензија од *Escherichia coli*	I	1,0ml на TSA	0	3	5	8	8	1,75
	II	1,0ml на TSA	0	3	3	5	7	1,57
	I	1,0ml на ENDO	0	1	4	4	4	1,75
	II	1,0ml на ENDO	0	0	3	4	4	1,50

F - фактор на прифатливост I - прв петриев сад II - втор петриев сад

Табела 3. Фактор на прифатливост при определување микробиолошка чистота на Кафетин® таблети со суспензија од *Pseudomonas aeruginosa* ATCC 9027

тест - материјал		директно нанесување на хранлив медиум						
		вид на медиум	24h cfu	48h cfu	72h cfu	96h cfu	пет дена cfu	F
пептонска вода + суспензија од *Pseudomonas aeruginosa*	I	1,0ml на TSA	161	169	170	170	173	
	II	1,0ml на TSA	170	171	175	175	175	
	I	1,0ml на Cetrimid	19	120	122	125	125	
	II	1,0ml на Cetrimid	43	153	160	162	165	
суспензија од Кафетин® таблети + суспензија од *Pseudomonas aeruginosa*	I	1,0ml на TSA	117	127	128	133	133	1,30
	II	1,0ml на TSA	113	128	130	132	132	1,32
	I	1,0ml на Cetrimid	0	78	80	88	88	1,42
	II	1,0ml на Cetrimid	0	64	73	73	73	2,26
тест - материјал		мембранска филтрација						
		вид на медиум	24h cfu	48h cfu	72h cfu	96h cfu	пет дена cfu	F
пептонска вода + суспензија од *Pseudomonas aeruginosa*	I	1,0ml на TSA	160	162	166	166	споени	
	II	1,0ml на TSA	154	154	155	155	споени	
	I	1,0ml на Cetrimid	0	63	63	63	63	
	II	1,0ml на Cetrimid	0	44	45	46	46	
суспензија од Кафетин® таблети + суспензија од Pseudomonas aeruginosa	I	1,0ml на TSA	70	70	72	72	72	2,30
	II	1,0ml на TSA	34	47	48	48	48	3,22
	I	1,0ml на Cetrimid	0	33	33	33	33	1,90
	II	1,0ml на Cetrimid	0	29	30	30	30	1,53

F - фактор на прифатливост I - прв петриев сад II - втор петриев сад

Табела 4. Фактор на прифатливост при определување микробиолошка чистота на Кафетин® таблети со суспензија од *Staphylococcus aureus* ATCC 6538

тест- материјал		директно нанесување на хранлив медиум						
		вид на медиум	24h cfu	48h cfu	72h cfu	96h cfu	пет дена cfu	F
пептонска вода + суспензија од *Staphylococcus aureus*	I	1,0ml на TSA	23	25	25	27	27	
	II	1,0ml на TSA	22	22	22	24	25	
	I	1,0ml на B-Parker	0	18	20	20	22	
	II	1,0ml на B-Parker	0	15	18	19	21	
суспензија од Кафетин® таблети + суспензија од *Staphylococcus aureus*	I	1,0ml на TSA	0	9	11	11	12	2,25
	II	1,0ml на TSA	0	7	9	10	10	2,50
	I	1,0ml на B-Parker	0	7	7	8	9	2,44
	II	1,0ml на B-Parker	0	5	7	7	9	2,33
тест- материјал		мембранска филтрација						
		вид на медиум	24h cfu	48h cfu	72h cfu	96h cfu	пет дена cfu	F
пептонска вода + суспензија од *Staphylicoccus aureus*	I	1,0ml на TSA	11	13	13	16	17	
	II	1,0ml на TSA	13	13	14	14	14	
	I	1,0ml на B-Parker	0	11	11	12	12	
	II	1,0ml на B-Parker	4	8	8	9	9	
суспензија од Кафетин® таблети + суспензија од *Staphylococcus aureus*	I	1,0ml на TSA	10	11	11	11	11	1,54
	II	1,0ml на TSA	0	5	7	7	7	2,00
	I	1,0ml на B-Parker	5	7	7	7	7	1,71
	II	1,0ml на B-Parker	3	4	5	5	5	1,80

F - фактор на прифатливост I - прв петриев сад II - се работи прв пат некој препарат втор петриев сад

Табела 5. Фактор на прифатливост при определување микробиолошка чистота на Кафетин® таблети со суспензија од *Candida albicans* ATCC 10231

тест – материјал		директно нанесување на хранлив медиум							
		вид на медиум	24h cfu	48h cfu	72h cfu	96h cfu	пет дена cfu	седум дена cfu	F
пептонска вода + суспензија од *Candida albicans*	I	1,0 ml на Sabouraud	0	48	120	160	споени	споени	
	II	1,0 ml на Sabouraud	0	70	86	190	190	споени	
суспензија од Кафетин® таблети + суспензија од *Candida albicans*	I	1,0 ml на Sabouraud	0	27	128	128	128	128	1,25
	II	1,0 ml на Sabouraud	0	26	44	128	128	128	1,48
тест - материјал		мембранска филтрација							
		вид на медиум	24h cfu	48h cfu	72h cfu	96h cfu	пет дена cfu	седум дена cfu	F
пептонска вода + суспензија од *Candida albicans*	I	1,0 ml на Sabouraud	0	15	37	37	37	37	
	II	1,0 ml на Sabouraud	0	20	31	31	31	споени	
суспензија од Кафетин® таблети + суспензија од *Candida albicans*	I	1,0 ml на Sabouraud	0	11	23	27	27	27	1,37
	II	1,0 ml на Sabouraud	0	11	24	25	25	25	1,24

F - фактор на прифатливост I - прв петриев сад II -втор петриев сад

Табела 6. Фактор на прифатливост при определување микробиолошка чистота на Кафетин® таблети со суспензија од *Aspergillus niger* ATCC 16404

тест- материјал		директно нанесување на хранлив медиум						
	вид на медиум	24h cfu	48h cfu	72h cfu	96h cfu	пет дена cfu	седум дена cfu	F
пептонска вода + суспензија од *Aspergillus niger*	I 1,0 ml na Sabouraud	0	35	35	35	споени	споени	
	II 1,0 ml na Sabouraud	0	35	35	36	споени	споени	
Суспензија од Кафетин® таблети + суспензија од *Aspergillus niger*	I 1,0 ml na Sabouraud	0	38	38	38	споени	споени	0,92
	II 1,0 ml na Sabouraud	0	34	35	35	споени	споени	1,02
тест – материјал		мембранска филтрација						
	вид на медиум	24h cfu	48h cfu	72h cfu	96h cfu	пет дена cfu	седум дена cfu	F
пептонска вода + суспензија од *Aspergillus niger*	I 1,0 ml na Sabouraud	0	21	25	25	споени	споени	
	II 1,0 ml na Sabouraud	0	23	23	23	споени	споени	
Суспензија од Кафетин® таблети + суспензија од *Aspergillus niger*	I 1,0 ml na Sabouraud	0	38	38	38	споени	споени	0,65
	II 1,0 ml na Sabouraud	0	31	31	31	споени	споени	0,74

F - фактор на прифатливост I - прв петриев сад II - втор петриев сад

Табела 7. Споредба на факторите на прифатливост за тест микроорганизмите за Кафетин® таблети на TSA и *Sabouraud* хранлив медиум со директна инокулација и со мембранска филтрација

тест-микроорганизам		директна инокулација	мембранска филтрација
		F	F
Escherichia coli	I	1,71	1,50
	II	2,10	1,75
Pseudomonas aeruginosa	I	1,30	1,47
	II	2,26	1,53
Staphylococcus aureus	I	1,54	3,22
	II	2,00	2,50
Candida albicans	I	1,25	1,37
	II	1,48	1,24
Aspergilus niger	I	0,92	0,65
	II	1,02	0,74

F- фактор на прифатливост I - прв петриев сад II -втор петриев сад

Заклучок

Кафетин® таблетите не поседуваат антимикробно дејство и при определување на микробиолошката чистота нема потреба од инактивација.

Методот што е применет за определување на микробиолошката чистота на Кафетин® таблетите е соодветен и одговара на бараните перформанси за микробиолошка чистота на цврсти фармацевтски препарати.

Тест микроорганизмите и хранливите медиуми што се користени при валидирање на методот покажуваат соодветност со барањата на современите фармакопеи.

Сите резултати во трудот покажаа дека факторот на прифатливост се движи во рамките на компатибилноста, да не е поголем од фактор 5, што значи дека методите што се користат за определување на микробиолошката чистота, се валидирани според важечките критериуми за фармацевтско производство.

Литература

1. Denyer,S. and Baird, R. (1990), Guide to Microbiological Control in Pharmaceuticals pp. 236-240.
2. European Pharmacopoeia, 3th Edition (1997), Council of Strasbourg, pp. 45-55.
3. The United States Pharmacopoeia 24 / The National Formulary 19 (1995), United States Pharmacopoeial Convention Inc., 1752-1753; 1809-1823.
4. Государственнаја Фармакопеја СССР XI. (1987), Медицина, Москва
5. Goidelines for Validation (1990), MPMD, Merck & Co.Inc.
6. Collins and Lynes (1989), Microbiological Methods, Butterworth & Co. (Publishers) Ltd.

Summary

Validation of the method for determination of microbiological purity of the Caffetin® tablets

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Key words: microbiological purity, validation of the method, Caffetin® tablets.

A validation of the method for determination of the microbiological purity of Caffetin® tablets has been done. For this purpose the test microorganisms: *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans* and *Aspergillus niger* from the collection ATCC have been used. A non-specific nutritious medium for aerobic microorganisms and specific nutritious media for adequate test microorganisms: ENDO, Cetrimid, Baird-Parker and Sabouraud nutritious agar medium have been used. The method was performed in two modes: a direct inoculation into a nutritious medium and a membrane filtration. At the same time, a Challenge test was as well used the test of counting the growing colonies (CFU/ml). A calculation of the factor has been done, which represents relationship between growing microorganisms of the inoculated nutritious medium with and without adding to the examined preparation, as a criterion for acceptance of the achieved analytical results. The achieved values for the factor as a criterion for acceptance have shown satisfying values. It can be concluded that this method can be used for determination of the microbiological purity of Caffetin® tablets.

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Стручен труд

Определување селен во помошни лековити средства со примена на AAS

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Апстракт

Определувањето селен во помошни лековити средства беше извршено со атомска апсорпциона спектрометрија со примена на графитна печка и хидриден систем.

Подготовката на примероците беше направена со микробраново разорување. За графитна техника е користена смеса од модификатори што содржи раствори на $Pd(NO_3)_2$ со масена концентрација 1,5 g/l и $Mg(NO_3)_2$ со масена концентрација 1g/l во раствор на HNO_3 со концентрација 2,3 mol/l. За хидридниот систем користен е раствор на $NaBH_4$ со масен удел 1,5% во раствор од NaOH со масен удел 0,5%. Методот беше валидиран преку определување: линеарност (во концентрационо подрачје од 15 до 75 μg/l за графитна техника и 5 до 25 μg/l за хидриден систем), точност, прецизност, лимит на детекција (4 μg/l за графитна техника и 1 μg/l за хидриден систем) и квантификација (13 μg/l за графитна техника и 3 μg/l за хидриден систем). Методот беше применет за определување селен во три препарати во кои селенот е во комбинација со: цинк, бакар и витамините А, С и Е. Добиените резултати покажуваат дека предложените методи за определување селен во фармацевтски препарати се осетливи, прецизни, точни и репродуцибилни.

Клучни зборови: селен, електротермичка атомска апсорпциона спектрометрија, атомска апсорпциона спектрометрија со хидриден систем

Вовед

Интензивните оксидативни процеси во организмот доведуваат до појава на т.н. оксидативен стрес кој е последица на оксидативни оштетувања на ткивата и појава на нивна патофизиолошка функција. Човековиот организам поседува т.н. антиоксидативен одбранбен систем за неутрализација на слободните кислородни радикали и спречување на оштетувањата што тие може да ги предизвикаат. Важен дел во тој систем зазема селенот. Намалувањето на капацитетот и функцијата на антиоксидативниот систем доведува до забрзано стареење на организмот, намалување на природниот имунитет и појава на голем број заболувања, вклучувајќи ја и карциногенезата (1, 2).

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Селенот претставува есенцијален олигоелемент. Најголем дел од дневните потреби кои за возрасни изнесуваат 50-200 μg се задоволуваат преку исхраната. Имајќи го предвид антиоксидативното дејствување како и улогата во превенцијата на дегенеративни и малигни заболувања, се почеста е примената на селенот во облик на препарати што се користат како додатоци во исхраната или како помошни лековити средства (3, 4).

Според литературните податоци, квантитативното определување на селен може да се врши со примена на атомската апсорпциона спектрометрија, (5-9), со примена на спектрофотометриски метод (10-12), со високоефикасна течна хроматографија (13-15) или со примена на капиларна електрофореза (16, 17).

За примена на спектрофотометрискиот метод, високоефективната течна хроматографија и капиларната електрофореза неопходно е комплексирање на селенот со соодветни комплексирачки органски молекули: 2,3-диаминонафтален, 3,3-диаминобензидин, 1,2-диамин-4-нитробензен и др. (10-17).

Целта на овој труд е развивање метод за квантитативно определување на селен во дозирани фармацевтски форми, таблети и капсули, што спаѓаат во групата на помошни лековити средства со атомска апсорпциона спектрометрија (AAS) со примена на графитна техника (ETAAS) и со хидриден систем (HGS).

Експериментален дел

Испитувани препарати

Определувањето на селен е вршено во дозирани фармацевтски форми што претставуваат комбинација селен со витамините A, C и E, и тоа: капсули со содржина на селен (комплекс со квасец) од 50 и 100 μg/капсула и во ефервесцентни таблети што се комбинација на витамини и на олигоелементи со содржина на селен од 25 μg/таблета.

Електротермичка атомска апсорпциона спектрометрија (ETAAS)

Апаратура: Користен е Perkin Elmer AAS 4100 систем опремен со графитна печка HGA 700, автосемплер AS - 70, EDL Se -ламба и континуиран деутериумов коректор на зрачењето на фонот. AAS системот е контролиран преку софтверска програма AAWin Lab.

Работни услови:	
бранова должина:	196 nm
отвор на процепот:	2,0 nm
јачина на струја во ламбата:	310 mA
температура на предатомизација:	1100 °C
температура на атомизација:	2100 °C
волумен на инјектирање:	20 μl (стандард, анализа) 10 μl модификатор

Подготовка на стандардни раствори: За подготовка на стандардните раствори користен е стандарден раствор на селен, 1000 mg/l-Merck и 5% HNO_3-Merck- trace pure.

За конструирање на калибрациониот дијаграм за определување селен користена е серија од работни стандардни раствори на селен со концентрација од 15-75 μg/l, а за утврдување на лимитот на детекција и квантификација серија од работни стандардни раствори со концентрација од 4-12 μg/l. Работните стандардни раствори се приготвени со додавање HNO_3 така што нејзината концентрација да изнесува 2,3 mol/l.

Подготовка на примероците: Минерализацијата на примероците е вршена во микробранова печка PAAR – PHYSICA. Определена маса од испитуваниот примерок што одговара на едно просечно полнење (капсули), односно една просечна маса (таблети) е минерализирана со додаток на 2,0 ml 30% H_2O_2-Merck-trace pure и 4,0 ml 65% HNO_3-Merck-trace pure. Со разредување на минерализираниот примерок подготвени се работни пробни раствори во 2,3 mol/l HNO_3 со концентрација 50 μg/l.

Како слепа проба користен е раствор на HNO_3 со концентрација 2,3 mol/l.

Смесата на модификатори содржи $Pd(NO_3)_2$ со масена концентрација од 1,5g/l и $Mg(NO_3)_2$ со масена концентрација 1g/l во раствор на HNO_3 со концентрација 2,3 mol/l. За подготовка на смесата користен е раствор на $Pd(NO_3)_2$ со масена концентрација 10g/l, Merck- trace pure и раствор на $Mg(NO_3)_2$ со масена концентрација 10g/l, Merck- trace pure.

Метод на стандардни додатоци: Пробите користени за методот на стандардни додатоци се добиени со додавање стандарден раствор на селен во испитуваните препарати пред минерализација во количества од 40%, 60% и 100% од декларираната маса на Se (25 μg Se/таблета); 30%, 60% и 100% од декларираната маса на Se (50 μg Se/капсула) и 25%, 50% и 75% од декларираната маса на Se (100 μg Se/таблета).

Хидриден систем (HGS)

Апаратура: Користен е Perkin Elmer AAS 372 систем опремен со MHS - 10 и EDL ламба.

Работни услови	
бранова должина:	196 nm
отвор на процепот:	2,0 nm
смеса на гасови:	ацетилен/воздух
интеграционо време:	15 секунди
волумен на инјектирање:	10 ml

Подготовка на стандардни раствори: За подготовка на стандардните раствори користен е стандарден раствор на селен (1000 mg/l Merck).

За калибрациониот дијаграм на селен користена е серија од работни стандардни раствори на селен со концентрација од 5-25 μg/l, а за утврдување на лимитот на детекција и квантификација серија од работни стандардни раствори со концентрација од 0,5-4,5 μg/l. Работните стандардни раствори се приготвени со деминерализирана вода.

Метод на стандардни додатоци: Растворите користени за методот на стандардни додатоци се приготвени на ист начин опишан во делот за графитна техника.

Подготовка на примероците: Минерализацијата на примероците е вршена на ист начин опишан во делот за ETAAS. Со разредување на минерализираниот примерок со деминерализирана вода изработени се работни пробни раствори со концентрација од 15μg/l.

Приготвените работни раствори пред воведување во уредот за генерирање на хидрид се закиселуваат со 37 % HCl, така што нејзината концентрација изнесува 1,2 mol/l.

За слепа проба користен е раствор на HCl со концентрација 1,2 mol/l.

Раствор на натриум тетрахидроборат: раствор на $NaBH_4$ со масен удел 1,5% и на NaOH со масен удел 0,5%. За негова подготовка користени се NaOH (Merck trace pure), $NaBH_4$ (Merck trace pure).

Резултати и дискусија

Определување селен со електротермичка атомска апсорпциона спектрометрија (ETAAS)

Примената на електротермичката апсорпциона спектрометрија за квантитативно определување на селен е лимитирана од физичките својства на овој елемент. Тоа, пред си се однесува на ниската температура на вриење на Se и неговите соединенија. Поради тоа е неопходна примена на модификатори на матриксот што го преведуваат селенот во селенид (18).

Изборот на модификаторот претставува еден од најзначајните моменти за квантитативна атомизација на испитуваниот елемент. Според литературните податоци за определување селен, како модификатори се користат $Ni(NO_3)_2$, $Cu(NO_3)_2$, $Pd(NO_3)_2$ и $Mg(NO_3)_2$, поединечно или во смеса (18-20). Во нашиот труд е користена смеса од $Pd(NO_3)_2$ и $Mg(NO_3)_2$ во раствор на HNO_3 со концентрација 2,3 mol/l, со масена концентрација 1,5 g/l $Pd(NO_3)_2$ и $Mg(NO_3)_2$ со масена концентрација 1g/l. Смесата на модификатори обезбедува целосно преведување на селенот во соодветни селениди. Тоа овозможува примена на температура на предатомизација од 1100 °C, наместо 350 °C која вообичаено се користи за селен, без да настанат губитоци заради испарување (19).

Во еден од испитуваните примероци, од олигоелементите, покрај селен се присутни и цинк и бакар. Брановата должина на максимална апсорпција на бакарот е 324,8 nm, а на цинкот 213,9 nm. Високата температура на предатомизација на селенот овозможува елиминација на цинкот во оваа фаза, бидејќи неговата температура на предатомизација е 700 °C. Со тоа се отстрануваат можните интерференции од страна на цинкот во фазата на атомизација. Температурата на предатомизација на бакарот е блиска со онаа на селенот (1300 °C), меѓутоа постои значителна разлика во брановата должина на максимумот на апсорпција (21).

Селенидите на паладиум и магнезиум се нерастворливи во вода, а се добро растворливи во киселина. Како растворувач најчесто се користи HNO_3 (18-20). Нашето експериментално искуство покажа дека примената на 2,3 mol/l HNO_3 како медиум за растворање на минерализираниот примерок овозможува комплетно растворање на селенидите.

AAC определување селен со атомизација со хидриден систем

AAS методот со примена на хидриден систем се базира на својството на селенот во оксидационен број +4 во кисела средина и присуство на $NaBH_4$ да гради лесно испарливи хидриди. Хидридите образувани од селен во оксидационен број +6 даваат речиси немерливи сигнали. Поради тоа хлороводородната киселина присутна во растворот има двојна функција: овозможува редукција на Se(IV) и обезбедува кисел медиум за одвивање на хемиската реакција (21). При нашата работа користен е медиум на HCl со концентрација од 1,2 mol/l.

Концентрацијата на растворот на $NaBH_4$ е втор значаен момент. Според литературните податоци, концентрацијата варира зависно од очекуваната концентрација на Se^{4+} во растворот (18, 19, 21). Од извршените испитувања беше утврдено дека раствор на $NaBH_4$ со масен удел 1,5% во раствор од NaOH со масен удел 0,5%, проток од 10 ml/минута и интеграционо време од 15 секунди обезбедува квантитативно преведување на селенот од испитуваниот раствор во селен хидрид.

Предложените методи за определување на селен со ETAAS и AAS со систем за хидридно генерирање се валидирани преку испитување на линеарност (Табела 1), лимит на детекција и квантификација(Табела 2), точност и прецизност.

Табела 1. Параметри на калибрационите дијаграми за утврдување линеарност на методите со ETAAS и AAS со хидриден систем

	ETAAS	HGS
конц. област (μg/l)	15 – 75	5 – 25
a*	0,0093	0,0132
b*	0,0039	0,0354
SD*(a)	$\pm 3{,}4 \times 10^{-3}$	$\pm 6{,}4 \times 10^{-3}$
SD*(b)	$\pm 8{,}9 \times 10^{-4}$	$\pm 1{,}9 \times 10^{-3}$
r^2 *	0,9998	0,9998

a–пресек, b–наклон на регресиона права, SD–стандардна девијација, r–коефициент на корелација

Табела 2. Параметри на калибрационите дијаграми за утврдување лимит на детекција и лимит на квантификација на методите со ETAAC и AAC со хидриден систем

	ETAAS	HGS
конц. област (μg/l)	4 - 12	0,5 - 4,5
a*	0,0003	0,0013
b*	0,0046	0,0377
SD*(a)	$\pm 4{,}8 \times 10^{-3}$	$\pm 5{,}4 \times 10^{-3}$
SD*(b)	$\pm 7{,}3 \times 10^{-4}$	$\pm 6{,}0 \times 10^{-2}$
r^2 *	0,9996	0,9996

*a - пресек, *b - наклон на регресиона права, SD* - стандардна девијација, r* - коефициент на корелација

Лимитот на детекција и квантификација на селен беа определени со анализа на калибрационите дијаграми во подрачјето на апроксимативниот квантификационен и детекционен лимит.

Лимитот на детекција и лимитот на квантификација за селен со примена на ETAAS изнесува:

LD = 3,3 x SD/b LD = 3,3 x 0,005238/0,003942 = 4,38 μg/l ≈ 4,0 μg/l

LK = 10 x SD/b LK = 10 x 0,005238/0.003942 = 13,28 μg/l ≈ 13,0 μg/l

Лимитот на детекција и лимитот на квантификација за селен со примена на AAS со хидриден систем изнесува:

LD = 3,3 x SD/b LD = 3,3 x 0,0130/0.0377 = 1,13 μg/l ≈ 1,0 μg/l

LK = 10 x SD/b LK = 10 x 0,0130/0.0377 = 3,44 μg/l ≈ 3,0 μg/l

(SD– стандардна девијација на слепа проба, b - наклон на регресиона права)

Точноста и прецизноста на двата метода (ETAAS и AAS со систем за хидридно генерирање) се утврдени преку определување на содржината на селен во испитуваните препарати (Табела 3), а се потврдени со методот на стандардни додатоци (Табела 4).

Добиените вредности за релативната стандардна девијација, 0,96-3,40% за ETAAS и 1,06-3,85% за AAS со хидриден систем и вредностите за аналитички принос, 100,32-102,8% za ETAAS и 99,42-102,38% за AAS со хидриден систем, укажуваат дека примената на двете техники овозможува висок степен на прецизност, репродуцибилност и точност на резултатите добиени од определувањето селен во фармацевтските препарати. Вредноста на t-коефициентот добиена со "STUDENT t-test" покажува дека не постои статистички значајна разлика меѓу резултатите добиени со примена на двете техники.

Точноста на методот со примена на двете техники беше потврдена со методот на стандардни додатоци.

Добиените вредности за аналитички принос од методот на стандардни додатоци, 98,93-102,32% за ETAAS и 98,94-102,10% за AAS со хидриден систем го потврдува фактот дека методите со примена на ETAAS и со примена на AAS со хидриден систем се квантитативни и точни.

Табела 3. Статистичка процена на добиените резултати при определувањето на содржината на селен во фармацевтски препарати со примена на ETAAS и хидриден систем (HGS)

Фармацевтски облик	Метод	Θ (μg/дозирана единица)	Аналитички принос (%)	RSD (%)	t
Таблети	ETAAS	24,45	92,72-100,12	3,40	
25 μg Se	HGS	24,29	92,08-100,52	3,85	0,2857
Капсули	ETAAS	50,13	98,26-100,86	1,31	
50 μg Se	HGS	50,19	97,06-103,96	2,80	0,0862
Таблети	ETAAS	101,43	100,32-102,08	0,96	
100 μg Se	HGS	101,54	99,42-102,38	1,06	0,1691

Табела 4. Резултати од методот на стандардни додатоци (н=6)

Метод	Декларирано (μg/дозирана единица)	Пресметано (μg/дозирана единица)	Определено (μg/дозирана единица)	Аналитички принос (%)
ETAAS	25	-	23.99	-
		33,99	33,87	99,67
		38,99	39,31	100,84
		48,99	44,89	99,78
	50	-	49,86	-
		64,86	64,69	99,75
		79,86	80,93	101,35
		99,86	98,79	98,93
	100	-	101,88	-
		126,88	129,30	101,91
		151,88	155,40	102,32
		176,88	179,09	101,25
HGS	25	-	23,80	-
		33,80	33,73	99,82
		38,80	39,32	101,35
		48,80	48,28	98,94
	50	-	48,95	-
		63,95	64,68	101,15
		78,95	78,57	99,53
		98,95	99,75	100,81
	100	-	101,15	-
		126,15	128,28	101,69
		151,15	154,32	102,10
		176,15	177,75	100,91

Заклучок

Предложените методи за определување селен со електротермичка AAS и AAS со систем за хидридно генерирање се брзи, едноставни, точни и репродуцибилни и можат да се применат за рутински анализи при определувањето селен во дозиран фармацевтски препарат.

AAS методот со примена на атомизација со хидриден систем е специфичен за определена група хемиски елементи (As, Se, Bi, Sb, Sn, и Te) од кои единствено селенот се среќава како активна компонента во голем број помошни лековити средства. Методот се карактеризира и со поголема осетливост. Тоа дава можност да му се даде предност на овој метод во однос на графитната техника за квантитативно определување селен во помошни лековити средства во кои е присутен во ниски дози и во комбинација со други олигоелементи.

Литература

1. O. Lozanov-Stankov, M. Demajo, I. Djujic and M. Mandic, *J. Environ. Path., Toxic. Oncol.,* **17**, 251-157 (1998).
2. H. D. Holben and A. M. Smith, *J. Am. Diet. Ass.*, **7**, 836-843 (1999).
3. S. J. Fairweather-Tait, *European J. Clin. Nutr.*, **51,** S20-S23 (1997).
4. L. A. Daniels, *Biol. Trace Elem. Res.,* **54**, 185-199 (1996).
5. A. Lozak and Z. Fijalek, *Chem. Anal.*, (Warsaw) , **43** (1), 1-7 (1998).
6. A. Lozak and Z. Fijalek, *Chem. Anal.*, (Warsaw) , **43** (6), 1003-1010 (1998).
7. A. Lozak and Z. Fijalek, *Acta Pol. Pharm. – Drug Research*, **53** (2), 83 – 85 (1996).
8. J. Isegava , K. Yamamoto, K. Terashima and M. Sato, *Biomed. Res. trace Elements*, **3** (2), 225 – 226 (1992).
9. G. Rotaru and D. Napredean, *Farmacia* (Bucharest), **44** (7-8), 71-75 (1996).
10. J. Stachoviak, *Rocz. Akad. Roln. Poznaniu*, **281**, 43-47 (1995).
11. W. Wanzheng, *Biomed. Res .Trace Elements*, **2** (7), 65-67 (1995).
12. K. N. Ramachandran and G. S. Kumar , *Talanta*, **43** (10), 1711-1714 (1996).
13. Li Huizhi and Z. Xinwei, *Yejin Fenxi*, **12** (4), 18-21 (1992).
14. K. Gottfried, K. Kurt and I. Kurt, *Appl. Organomet. Chem*., **7** (7), 443 – 466 (1993).
15. L.Yuanying, B. Zhiyi, L. Junbun and L. Wenyuan, *Fenxi Ceshi Tangbao*, **11** (6), 54-7 (1992).

16. A.Colon Luis and B. Eugene, *J. High Resolut. Chromatogr*., **14** (9), 608-612 (1991).
17. S. Sri Juari, S. Noriyuki and T. Shigeru, *Anal. Sci*., **9** (5), 657-62 (1993).
18. W. Andrea, B. Phyllis, J. Petr, J. R. William and H. L. Allan, *J. Chromatogr*., **593** (1-2), 289-295 (1992).
19. B. Welz, *Atomic Absorption Spectrometry*, 2th ed, Verlagsgesellshaft mbH, Weinheim, Germany, 1985, p.p. 148-163
20. D. A. Skoog and J. J. Leary, *Principles of Instrumental Analysis*, 4th Edition, Saunders College Publishing, 1992, p.p. 211-223
21. R. D. Beaty and J. D. Kerber, *Concepts, Instrumentation and Techniques in Atomic Absorption Spectrophotomrtry,* The Perkin-Elmer Corporation, Norwalk, CT, USA, 1993.

Summary

AAS method for determination of selenium in therapeutical nutritions

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Key words: selenium, therapeutical nutrition, graphite furnace atomic spectrometry, atomic spectrometry with hydride system

An AAS method with graphite furnance and hydride system was developed and validated for determination of selenium in pharmaceutical dosage forms used as therapeutical nutritions. The method was applied for determination of selenium in three therapeutical nutritions after microwave digestion. Acid samples with Pd/Mg nitrate as a modifier were used for graphite furnace and acid samples with sodium borhydride alkaline solution for hydride system. Validation of applied method was carried out by determination of linearity (in concentration range from 15 to 75 μg/l for graphite furnace technique and from 5 to 25 μg/l for hydride system) as well as accuracy, precision, detection and quantification limit. The results obtained have shown that the proposed AAS method with graphite furnace and hydride system for determination of selenium in therapeutical preparations are sensitive, accurate and reproducible.

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Стручен труд

Споредни ефекти на амјодарон врз структурите на тиреоидната жлезда кај млади Wistar стаорци

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Апстракт

Испитувано е ткивото на тиреоидната жлезда на млади Wistar стаорци по триесетдневна апликација на двојно повисоки дози отколку терапевтските, на кардиотоникот амјодарон. Заклучивме дека овој лек кај повеќето опитни животни предизвикува деструктивни, дегенеративни и инволутивни промени на тиреоидните фоликули, особено изразени кај младите Wistar стаорци. Фоликуларниот епител и колоидот се подеднакво засегнати со овие процеси. Сврзното ткиво разраснува во интерстициските простори и го притиска функционалниот паренхим на жлездата.

Клучни зборови: тиреоидна жлезда, Wistar стаорци, амјодарон, хистолошка анализа, светлосна микроскопија

Вовед

Хистотоксичните реакции на тиреоидната жлезда како резултат на влијанието на различни лекови и нивните метаболити врз неа го ангажирале вниманието на многу автори кои на проблемот му приоѓале од различни аспекти: хистопатолошки, физиолошки, фармаколошки, биохемиски.

Во своите истражувања авторите нотираат абнормалности во функцијата и во цитоморфологијата на тиреоидната жлезда во зависност од експерименталните услови или патолошките процеси. Широката примена на медикаменти коишто во себе содржат различни количини на јод, а се аплицираат за нетиреоидни заболувања, потоа при разни дијагностички снимања (контрастните средства), наметнува потреба да се проследи тиреоидната жлезда и можните ефекти врз неа.

Амјодаронот (Cordarone®) е антиаритмик што има и антиангинозно и антифибрилаторно дејство. Овој лек во својата структура содржи два атома на јод. Пресметано е дека е тоа квантитативно 75 mg, органски јодид (37%) на 200 mg активна супстанца-1 таблета, а со текот на биотрансформацијата во организмот од таа количина лек се ослободуваат 6 mg слободен јод.

Хистолошки кај пациенти подложени на тиреоидектомија заради AIT (amiodarone-induced thyrotoxicosis) (1) најдени се инволутивни промени во тиреоидејата, дегенеративни и деструктивни фоликуларни лезии, фибротични лезии и неспецифична хронична инфламација со инфилтрација од макрофаги.

Тиреоидна хистопатологија кај обдуцирани пациенти претходно третирани со амјодарон без претходно евидентирано тиреоидно заболување забележале Brenan *et al.* и Mulligan *et al*. (2,3).

Амјодаронот се применува и при лекување млади пациенти-деца и адолесценти. Во литературата постојат извештаи за употребата на амјодаронот кај овие пациенти (4-6). Во најекстензивниот труд (4), авторите известуваат дека со амјодарон се лекувани преку сто пациенти на возраст под 16 години. Успехот е оценет како многу поволен, но присутни се и почести нуспојави, во зависност од дозата и начинот на апликацијата на лекот.

Заради тоа бевме мотивирани да воспоставиме соодветен експериментален модел кој ќе ни послу-

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жи да ги испитаме,да ги утврдиме и да ги истакнеме евентуалните промени во структурите на тиреоидната жлезда под влијанието на амјодаронот врз стаорци чијашто возраст одговара на детската кај човекот.

Експериментален дел

Како опитни животни користени се машки Wistar стаорци, чијашто возраст на почетокот на експериментот одговараше на детската кај човекот.

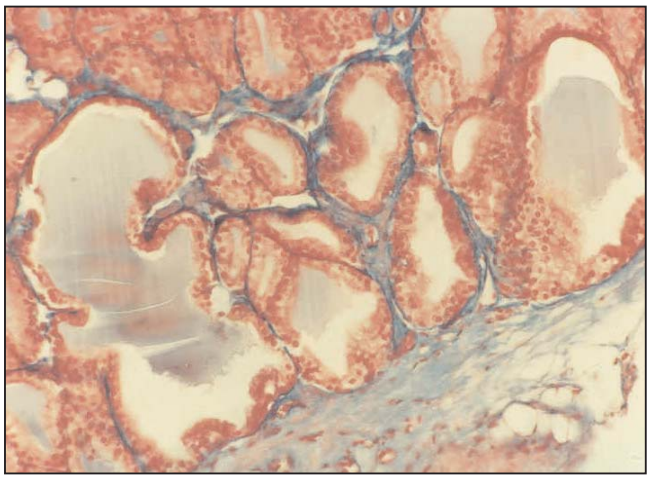
Амјодарон хлорид користен во експериментите е производ на фирмата "Krka" Ново Место. Лекот беше растворуван во водата за пиење чијашто количина беше одредена пред и во текот на експериментот строго водејќи сметка секој ден да преостане минимално количество вода во којашто е растворен лек. Wistar стаорците пиеја 0,1% раствор (двојно повисока доза од терапевтската) амјодарон хлорид, ad libitum во тек на 30 дена.

По жртвувањето на стаорците ткивото на тиреоидната жлезда беше подготвено со вообичаените хистолошки техники и боења за квалитативна анализа со светлосен микроскоп.

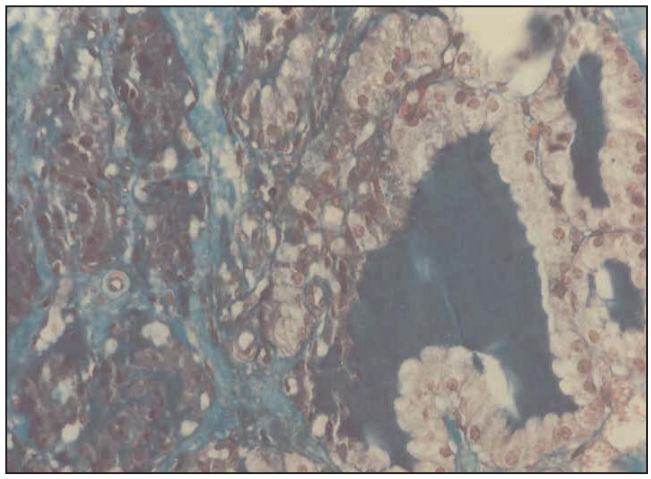
Резултати и дискусија

При панорамичната глетка врз тиреоидната жлезда кај овие експериментални групи уочливи се дилатирани фоликули во периферните делови на жлездата, чиј изглед сугерира на раскинување на ѕидовите на соседните фоликули и создавање комуникација помеѓу нив (Сл.1).

Фоликуларниот епител, во непроменетите фоликули, враснува во вид на папили во колоидот. Повеќето папили се со строма од сврзно ткиво околу која е обложен фоликуларниот епител во еден ред на

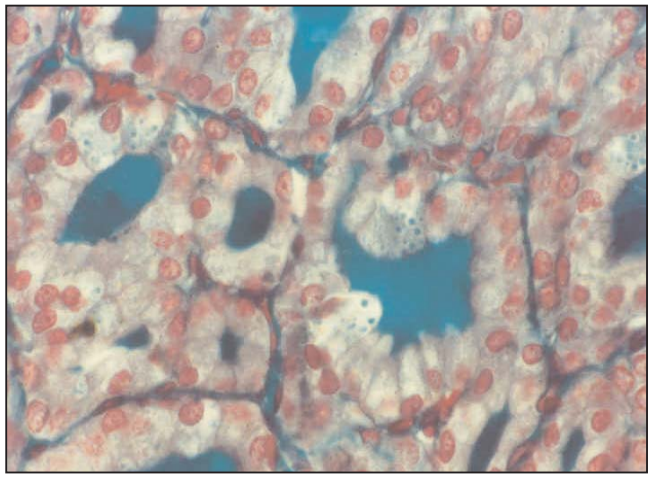


Слика 1. Дилатирани фоликули во периферните делови на жлездата (400 x, Azan)



Слика 2. Папила изградена од строма и епител, врасната во колоидот (400 x, Florentin)

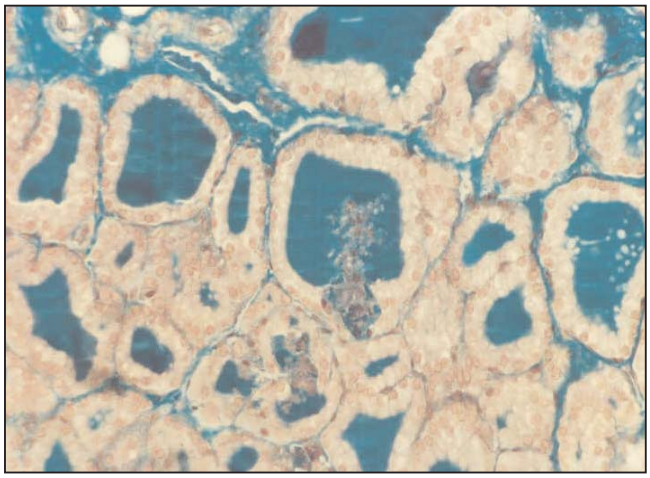
клетки или во повеќе редови на клетки (Сл.2). Ваквото папиларно враснување на епителот присутно е кај 30% на експерименталните животни.



Слика 3. Гранули во цитоплазмата на тиреоцитите (1000 x, Florentin)

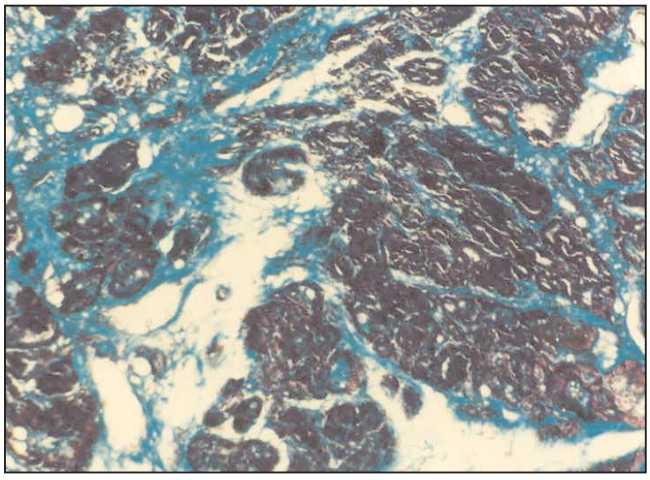
Клетките на фоликуларниот епител што е сочуван содржат гранули во цитоплазмата (Сл.3). Според литературата формирањето на интрацелуларни колоидни капки укажува на почетен процес на тиреоидна хормонска секреција (7).

Во тиреодната жлезда кај експерименталните животни присутни се и фоликули со површно дегенериран фоликуларен епител, при што делови од неговите клетки и цели клетки отпаѓаат во колоидот. Овие промени најчесто се локални - се среќаваат на едно одредено место во ѕидот на фоликулот каде што се деструира и отпаѓа фоликуларниот епител се до базалната мембрана (Сл.4). На некои места од жлездата овие промени стануваат дифузни.



Слика 4. Локална деструктивна промена(250 x, Florentin)

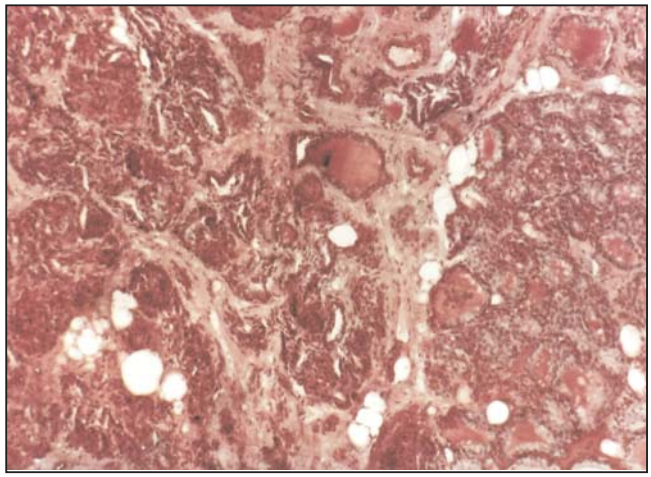
Дегенеративни и деструктивни фоликуларни лезии, како и фибротични лезии се обемно распространети низ паренхимот на тиреоидната жлезда кај 80% од младите Wistar стаорци (Сл.5).



Слика .5. Дегенеративни и деструктивни фоликуларни лезии и фибротични лезии (100 x, Florentin)

Во периферните делови на жлездата, а исто така и во централните регии, постои разраснување на сврзното ткиво (Сл.6).

Патогенезата на зоните на фиброза и нивната содржина од изобличени фоликули и групи од епителни клетки кај амјодарон третираните групи се нејасни, меѓутоа (1) го сугерираат следниов начин на развиток. По променливата дегенерација и деструкција на еден до друг поставените фоликули - некои тотално разрушени, други делумно погодени, постои ослободување на колоид, ликвефекација и апсорпција на продуктите на целуларната дегенерација и постои преживување на некои фоликуларни клетки. Муралните лезии често се придружени



Слика 6. Буење на сврзното ткиво во централните делови на жлездата (40 x, H E)

со целуларен инфилтрат од плазма клетки или хистиоцити и лимфоцити лоциран перифоликуларно. Интерфоликуларно се среќаваат зони на фиброза. Кондензацијата на фиброваскуларната строма ги опкружува разрушените фоликули или индивидуалните фоликуларни клетки опфатени во овие полиња на фиброза кои можат да се реконституираат како фоликули или да се регенерираат и да формираат нови.

Заклучок

Во овој труд кон проблематиката придовме од хистолошки аспект. Под наведените експериментални услови, хистолошкиот наод укажува дека се атакувани хистоцелуларните сруктурни елементи на тиреоидната жлезда.

Констатираните ефекти од апликацијата на амјодарон-от врз структурата на тиреоидната жлезда сугерираат неопходна претпазливост при ординирањето на лекот во клиничката практика, пред се, преку дефинирање и следење на тиреоидниот статус пред и во тек на терапијата со овој препарат.

Литература

1. C. T. Smyrk, M. D. Goellner, D. M. Brennan and J. A. Carney, *Am. Jour. Surg. Path.,* **11** (3),197-204 (1987).
2. M. D. Brennan, D. Z. Erickson, J. A. Carney and R. S. Bahn, *Thyroid,* **5** (3), 177-83 (1995).
3. D. C. Mulligan, C. R. Mc Henry, W. Kinney and C. B. Jr. Esslstyn, *Surgery,* **114** (6), 1114-9, (1993).
4. P. Conmel and J. Fidelle, *Am. Heart J* **100** (6, Part 2),1063-9 (1980).
5. A. Garson, *Am. Call. Cardiol,* **4**,749-755 (1984).
6. B. R. Keeton, C. A. Bucknall, P. V. L. Curry, M. C. Josph and G. R. Sutherland, *Br. J. Clin. Pract*., **40** 115-20 (1986).
7. K. Yamamoto, T. Onaya, T. Yamada and M. Kotani, *Endocrinology,* 90**,** 986 (1972).

Summary

Side effects of amiodarone upon the structures of thyroid gland in young Wistar rats

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Key words: thyroid gland, Wistar rats, Amiodarone, histological analysis, light microscopy

Thyroid gland tissue of young Wistar rats after the application of double more doses than therapeutic ones, of cardiotonic amiodarone in a 30 day period was examined.

We concluded that amiodarone in most animals induced destructive, degenerative and involutive changes in thyroid follicles, particularly in young rats.

The epithelium and the colloid were equally affected with these processes. Connective tissue grows in the interstitial space and makes pressure upon the functional parenchymal tissue of the thyroid gland.

УПАТСТВО ЗА АВТОРИТЕ

Македонскиот фармацевтски билтен е официјално гласило на Македонското фармацевтско друштво. Во списанието се објавуваат оригинални научни трудови, куси соопштенија, ревијални трудови и стручни трудови од сите фармацевтски дисциплини и сродни подрачја на работа кои се од интерес за фармацијата (фармацевтска и медицинска хемија, имунологија со имунохемија, молекуларна биологија, фармацевтски анализи, контрола на лекови, фармацевтска технологија, фармакоинформатика, биофармација, фармакологија, применета ботаника, фармакогнозија, токсикологија, клиничка фармација, храна и исхрана, физичка фармација, органски синтези, социјална фармација, историја на фармација и др.).

Во Македонскиот фармацевтски билтен се објавуваат и други прилози (пораки и соопштенија, извештаи и календари на научни и на стручни манифестации во земјата и во странство, прегледи на книги). Составен дел на Билтенот се и разни рубрики.

Оригиналните научни трудови ги содржат резултатите на сопствени, дотогаш необјавени научни истражувања кои претставуваат заокружена целина, а даваат придонес кон развојот на соодветната научна област. Бројот на страниците (заедно со сликите и со табелите) не треба да биде поголем од 15.

Кусите соопштенија содржат резултати од начни истражувања презентирани во помал обем, чиј број на страници не треба да биде поголем од пет (дозволени се една табела и една слика).

Прегледите се пишуваат на покана од Уредувачкиот одбор, а претставуваат поопширен приказ на истражувањата и сознанијата на повеќе автори во определена научна област, за која компентноста на авторот на прегледот е потврдена преку неговите објавени истражувања.

Стручните трудови известуваат за корисни резултати кои овозможуваат податоците од оригиналните научни истражувања да бидат применети во практичната работа. Стручните трудови можат да се темелат и на обработка на теоретски податоци. Бројот на страниците (заедно со сликите и со табелите) не треба да биде поголем од 10.

Оригиналните научни трудови, кусите соопштенија и прегледите треба да бидат напишани на англиски јазик, додека стручните трудови и сите останати прилози можат да бидат напишани и на македонски јазик.

Прифаќањето на трудовите за печатење во Билтенот ги обврзува авторите истите резултати да не ги објавуваат во друго списание.

Подготовка на ракописот:

Ракописите треба да се достават во три примероци испечатени на компјутерски печатач, едностарно со проред од 1,5 со маргина од 3,0 cm од секоја страна, на хартија со формат А4 на адреса на главниот уредник:

Проф. д-р Светлана Кулеванова
Фармацевтски факултет,
Водњанска 17, 1000 Скопје,
Република Македонија

Страниците и прилозите треба да бидат нумерирани.

При подготвување на ракописот за печатење треба да се води сметка тој да биде максимално концизен, без непотребни повторувања.

При пишување на ракописот задолжителна е употреба на следните единици за: должина (m, cm, mm, mm, nm, E); маса (kg, g, mg, mg, ng, pg); волумен (l, ml, μl); време (s, min, h, d); температура (^oC, K); радијација (Bq, Ci, dpm, Gy, rad): концентрација (M, mol/l, N, mg/ml, %, %(v/v), %(w/v), ppm).

Имињата на супстанциите треба да се во согласност со препораките и правилата дадени од IUPAC или, ако такви не постојат, според практиката на Chemical Abstracts.

Табелите и сликите (фотографии, дијаграми и скици), како и структурните формули треба да бидат дадени секоја на одделен лист (во текстот да е обележано нивното место). Табелите и сликите се нумерираат со арапски бројки (на пр. Табела 1., Сл.1).

Ракописот треба да содржи: наслов, апстракт, клучни зборови, вовед, експериментален дел, резултати и дискусија, заклучок, благодарност (по потреба), литература и резиме.

На **насловната страница** треба да стои насловот на ракописот. Во посебни редови треба да стои полно име и презиме на авторите, назив и адреса на институцијата на авторите. Кога авторите потекнуваат од повеќе различни институции треба да се означи потеклото на секој автор со [1, 2, 3].... на крајот од неговото име. Со ѕвездичка треба да биде означено името на авторот за коресподенција. Во долниот лев агол од насловната страница се наведува неговата e-mail адреса, телефонскиот број и број на факс.

Апстрактот треба да биде до 250 збора (научни и стручни трудови), односно до 100 збора (куси соопштенија), а треба да ги содржи целта на работата, методологијата и позначајните резултати. Во продолжение треба да бидат застапени три до шест клучни збора распоредени според редоследот на важност.

Во **воведниот дел** треба накусо да се наведат само најважните податоци од поранешните истражувања поврзани со обработуваната проблематика и да се објасни целта и важноста на работата.

Експерименталниот дел треба да биде издвоен и да содржи податоци за употребуваните материјали и опис на применетите методи на начин што ќе овозможи репродуцирање на постапката, но без детално опишување на веќе познатото.

Трудовите што се однесуваат на теоретски истражувања, наместо експериментален дел треба да имаат соодветно поглавје со поднаслови во кое ќе бидат изнесени подробности неопходни за проверка на презентираните резултати.

Резултатите и дискусијата треба да бидат дадени во едно поглавје. Дискусијата треба да содржи анализа на резултатите и на заклучоците што притоа можат да се извлечат.

Заклучокот што произлегува од изложените резултати и дискусија се издвојува во посебно поглавје.

Литературата се наведува во посебно поглавје, при што библиографските единици се нумерираат по оној ред по кој се појавуваат во текстот. Ако во текстот се спомнуваат автори, се наведуваат само презимињата на авторите (ако ги има еден или два), а во случај на повеќе автори се пишува, на пример, *Vetter et al.* (1).

Статии од научни списанија се цитираат на овој начин:

1. P. Premov and D. Dulanov*, Maced. Pharm. Bull.,* **20**, 2-10 (2000).

Кратенките на списанијата се наведуваат според *Chemical Abstracts.*

Книги се цитираат на овој начин:

2. A. Robert, M. Sporn, *Progress in Organic Chemistry,* 3rd ed., Butterworths Scientific Publications, London, 1998, pp 125-154.

Книги со уредник:

3. J. Cook, in *The retinoids,* P. Cornwell, Ed., Academic Press, Orlando, 1996, pp 25-29.

Трудовите напишани на англиски јазик на крајот треба да содржат **резиме** на македонски јазик. Резимето треба да содржи наслов, полно име и презиме на авторот/авторите, назив на институцијата на авторот/авторите, клучни зборови и текстот на апстрактот. Стручните трудови напишани на македонски јазик на крајот треба да содржат резиме напишано на англиски јазик во кое треба да се вклучени истите податоци.

Примените ракописи Уредувачкиот одбор ги испраќа на рецензирање. Рецензираните трудови, заедно со евентуалните забелешки и мислења на Уредувачкиот одбор, се доставуваат до авторите. Тие се должни најдоцна во рок од 30 дена да ги извршат неопходните корекции и финалната верзија на ракописот да ја достават до Уредувачкиот одбор во печатена форма и задолжително и на *PC* форматирана дискета **(Word; Times New Roman, Mac C Times, Macedonian Times** и големина на букви **11).**

Авторите добиваат 20 бесплатни сепарати од секој труд објавен во Билтенот.

INSTRUCTIONS FOR AUTHORS

Macedonian Pharmaceutical Bulletin is an official publication of the Macedonian Pharmaceutical Association. The journal publishes original scientific papers, short communications, reviews and professional papers from all fields of pharmacyand corresponding scientific fields of interest for pharmacy (pharmaceutical and medicinal chemistry, immunology and imunochemistry, molecular biology, pharmaceutical analyses, drug quality control, pharmaceutical technology, pharmacoinformatics, biopharmacy, pharmacology, applied botany, pharmacognosy, toxicology, clinical pharmacy, food and nutrition, physical pharmacy, organical synthesis, social pharmacy, history of pharmacy etc.).

The Macedonian Pharmaceutical Bulletin, also, publishes and other contributions (recommendations and announcements, reports of meetings, important events and dates, book reviews, various rubrics).

Original scientific papers should contain own unpublished results of completed original scientific research. The number of pages (including tables and figures) should not exceed 15.

Short communications also should contain completed but briefly presented results of original scientific research. The number of pages should not exceed 5 (including one table and one figure).

Reviews are written at the invitation of the Editorial Board. They should be surveys of the investigations and knowledge of several authors in a given research area, the competency of the authors of the reviews being assured by their own published results.

Professional papers report on useful practical results which are not original but help the results of the original scientific research to be adopted into practical use. Professional papers might be based on the elaborating of theoretical data. The number of pages should not exceed 10 (including tables and figures).

Original scientific papers, short communications and reviews should be written in

English, while professional papers and all other contributions may be submitted in Macedonian.

Acceptance for publication in the Bulletin obliges the authors not to publish the same results elsewhere.

Preparation of manuscripts:

Manuscripts should be submitted in triplicate, typed (1 S spaced) on A4 paper with margins of 3.0 cm on each side. It is recommended the manuscript be sent on the Editor-in-Chief address's:

Prof. dr. Svetlana Kulevanova
Faculty of Pharmacy
Vodnjanska 17
1000 Skopje
Republic of Macedonia

The pages and appendices should be numbered.

The papers should be written in the shortest possible way and without unnecessary repetition.

Obligatorily, only the follow quantities and units must be used: length (m, cm, mm, mm, nm, E); weight (kg, g mg, mg, ng, pg); volume (l, ml, μl); time (s, min, h, d); temperature (^oC, K); radiation (Bq, Ci, dpm, Gy, rad); concentration (M, mol/l, N, mg/ml, %, %(v/v), %(w/v), ppm).

The names of substances should be in accordance with the IUPAC recommendations and rules or Chemical Abstracts practice.

Figures (photographs, diagrams and sketches) and structural formulae should each be given on a separate sheet (the place to which they belong in the text should be indicated). The tables and the figures should be numbered in Arabic numerals (e.g. Table 1, Fig. 1).

Manuscript should contain: title, abstract, key words, introduction, experimental, results and discussion, conclusion, acknowledgment (if desired) references and summary.

Papers should be preceded by a title page comprising: the title, the complete name(s) of the authors, and the author's affiliations (institution and address). When the authors affiliated from various different institutions the origin of the each author should be indicated with 1,2,3... at the end of his name. The correspodence author should be indicated with * and his e-mail address, telephone and fax number to be supplied in the bottom left part of the title page.

Each paper must begin with an Abstract which should not exceed more than 250 (original scientific and professional papers) or 100 (short communications) words. The abstract should include the aim of the research, methods and the most important results. A list of 3 to 6 key words arranged in the order according to their importance should follow the abstract.

In the introduction only the most important previous results related to the problem in hand should be briefly reviewed and the aim and the importance of the research should be stated.

The experimental section should be written as a separate section and should contain a description of the materials used and methods employed in form which makes the

results reproducible, but without detailed description of already known methods.

Manuscripts which are related to theoretical studies, instead of experimental, should contain a sub-heading and the theoretical background where the necessary details for verifying the results obtained should be stated.

It is often advantageous to combine Results and Discussion in one section. The discussion should contain an analysis of the results and the conclusions that can be drawn.

Conclusion which is in consequence from the presented results and discussion should be given in the separate section.

The list of references should only include works that are cited in the text and that have been published or accepted for publication. References should be numbered in the order in which they appear in the text and listed in numerical order in the reference list.

The surname of one or two authors may be given in the text, whereas in the case of more than two authors they should be quoted as, for example *Vetter et al.* (1).

Papers from scientific journals should be cited as follows:

1. P. Premov and D. Dulanov, *Maced. Pharm. Bull.* **20**, 2-10 (2000).

The names of journals should be abbreviated as in Chemical Abstracts.

Books should be cited as follows:

2. A. Robert, M. Sporn, *Progress in Organic Chemistry*, 3 rd ed., Butterworths Scientific Publications, London, 1998, pp 125-154.

Books with editor:

3. J. Cook, in *The retinoids,* P. Cornwell, Ed., Academic Press, Orlando, 1996, pp 25-29.

Manuscripts written in English should contain a summary in Macedonian at the end of the paper. The summary should contain: title, author(s) full-name(s), surname(s), author's affiliations (institution and address), key words and abstract. Professional papers written in Macedonian should contain a summary in English in which the same data should be included.

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