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DOPE–oleic acid–Ca^{2+} as DNA condensing agent

Original Paper

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Abstract Phospholipid-based non-viral carriers composed of neutral phospholipid dioleoylphosphatidylethanolamine (DOPE) and the binary mixture DOPE–oleic acid (OA) are examined as potential DNA delivery vectors. The process of DNA condensation in the presence of Ca^{2+} ions has been monitored through changes in emmision intensity of fluorescent probe ethidium bromide. The decline in fluorescence intensity with increasing Ca^{2+} concentration at two different time intervals was correlated with the binding capacity of complexes and possible release of DNA from the complex. The microstructure of DOPE–OA mixtures at different OA/DOPE molar ratios and that of DOPE–OA–DNA–Ca^{2+} complexes were determined using synchrotron small angle X-ray diffraction (SAXD). We identified inverted hexagonal phase H_{II} as the dominant structure. OA affects the lattice parameter of H_{II} formed by DOPE. With the increasing OA/DOPE molar ratio, the lattice parameter decreases, which results in significantly lower fraction of DNA bound to the OA-enriched complexes.

Keywords *DOPE – oleic acid – calcium – DNA – SAXD*

INTRODUCTION

Gene therapy provides an unique approach to threat diseases by delivering a genetic material into the nucleus in order to correct the loss of function or to express the deficient gene product at physiological level (Glover et al. 2005). Owing to concerns about using viral gene transfer vehicles non-viral vectors represent promising alternative disposing multiple benefits such as biosafety, less immunotoxicity, low cost and ease of production (Ramamoorth and Narvekar 2015). Chemical non-viral nucleic acid delivery systems are, amongst others, cationic lipid–DNA complexes called lipoplexes. For this purpose, hundreds of lipids have been developed for gene transfer; however, lipoplexes still face a lack of transfection efficiency. In our laboratory, lipidic carriers for DNA have been studied previously (Hubčík et al. 2014, 2015, Lengyel et al. 2011, Liskayová et al. 2017). The structure of lipid-based systems has been investigated and reviewed extensively for the past two decades (Uhríková et al. 2007, Uhríková et al. 2005, Pullmannová et al. 2012, Puri et al. 2009).

Dioleoylphosphatidylethanolamine (DOPE) was reported to stabilise lipoplexes and enhance *in vivo* cationic lipid-dependent delivery of plasmid DNA to the mouse brain (Hassani et al. 2005). Free fatty acids (FFAs) have the effect on the organisation and local structures of membranes (Seddon et al. 1997, Templer et al. 1998). They also show the ability to modulate cell functions (Pérez et al. 1997). The effect of oleic acid (OA) on phosphatidylethanolamine (PE) membranes was studied previously (Funari et al. 2003, Prades et al. 2003, Gillams et al. 2014). It was found that OA induces important alterations in the supramolecular organisation of PE derivatives and also the thermal sensitivity of DOPE is altered by the presence of OA. OA modulates the membrane structure, inducing negative membrane curvature strain on DOPE caused probably by the lateral pressure of OA on fatty acids of DOPE.

In this study, we have prepared complexes made of OA–DOPE and studied their capability of DNA compaction in the presence of Ca^{2+} ions as one of the essential steps before DNA delivery. Fluorescence study provides valuable information about the binding capacity of lipid-based carriers for DNA (Geall and Blagbrough 2000). We have examined DNA-binding affinity and its

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condensation using ethidium bromide exclusion assay. We have used the small-angle X-ray diffraction (SAXD) to study the structure of lipoplexes as a function of their composition. Lipoplexes presented in this study have not been reported to be prepared and investigated yet and these new biophysical data may contribute to the knowledge of physico-chemical properties at designing non-viral lipid-based delivery vectors for the transfer of nucleic acids.

METHODS

Materials

Neutral phospholipid DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) was purchased from Avanti Polar Lipids, Inc., USA. Highly polymerised DNA (sodium salt) type XIV from herring testes (average Mr of nucleotide = 308) and OA (analytical standard) were purchased from Sigma Aldrich, USA. NaCl, NaOH and 35% HCl of analytical purity were obtained from Lachema, Brno, Czech Republic. Ethidium bromide (EtBr) and calcium chloride dihydrate for analysis were purchased from Merck, Germany. The chemicals were of the analytical grade and used without further purifications. The aqueous solutions were prepared with redistilled water.

Preparation of DNA solution/UV-VIS measurements

DNA was dissolved in 0.15 mol/l NaCl. The precise value of DNA concentration was estimated spectrophotometrically (Agilent 8453 Diode array spectrophotometer), according to $c_{DNA} = A_{260}\ 4.7 \times 10^{-5}$ [g/ml], where A_{260} is the absorbance at wavelength $\lambda = 260$ nm. The concentration of DNA is referred as the molar concentration of DNA bases. The purity of DNA was checked by measuring the absorbance A_λ at $\lambda = 260$ and 280 nm and evaluating A_{260}/A_{280}. UV-vis was used to determine the amount of DNA bound in the complexes of samples for SAXD experiments by applying the method described in Lengyel et al. 2011 and Rajnohova et al. 2010.

Preparation of complexes for fluorescence experiments

DOPE and OA–DOPE mixtures were dissolved in chloroform and mixed to obtain a mixture with the required OA/DOPE molar ratio. Lipid mixtures were dried under a stream of gaseous nitrogen and the residue of chloroform was removed under vacuum. Each sample contained 1.2 mg of DOPE and the corresponding amount of OA with respect to the OA/DOPE molar ratio. The dry mixtures were hydrated using the solution of DNA in 150 mmol/l NaCl at the ratio of DOPE/DNA = 8 (mol/base) and the solution of $CaCl_2 \cdot 2H_2O$ to obtain the required molar concentration of calcium cations. The volume of samples was adjusted to 2.5 ml using 150 mmol/l NaCl. Afterwards, samples were homogenised (by vortexing and freezing–thawing). The pH of samples was measured before the fluorescence measurement. We recorded a slight decrease in pH with increasing calcium concentration. In the sample with 0 mmol/l calcium concentration, pH = 7.1, whereas at 40 mmol/l calcium concentration pH = 6.5. EtBr as fluorescence probe was added in the last step of sample preparation to obtain the EtBr/DNA molar ratio of 4 mol/base, and the final volume of the sample was 3 ml.

Preparation of complexes for X-ray diffraction experiments

OA–DOPE mixtures and DOPE–OA–DNA–Ca^{2+} complexes were prepared by hydrating the dry lipid film with the solution of DNA in 150 mmol/l NaCl at the DOPE/DNA ratio of 8 (mol/base) and the solution of $CaCl_2 \cdot 2H_2O$ to obtain the molar concentration of calcium cations of 15 mmol/l. The final volume of the sample was 1 ml. Homogenisation of the sample was carried out analogously as described in the previous section. A few minutes after the preparation, a sediment was formed. pH of samples was checked and was determined to be ~6 for both the binary mixtures and the DOPE–OA–DNA–Ca^{2+} complexes. At the OA/DOPE molar ratio of 1, the pH of samples was adjusted by adding 50 mmol/l HCl solution to slightly acidic (~4.8) or by adding 10 mmol/l NaOH solution to raise the pH to ~7.6. The supernatant was gently removed using a Pasteur pipette and the sediment was transferred into a capillary made of special glass with a diameter of 1.5 mm. Filled capillaries were centrifuged for 1 min at 500 RPM using Rotofix 32 A Hettich Centrifuge. The residue of the supernatant was removed using a syringe. Capillaries were sealed and prepared for X-ray diffraction experiments. The amount of DNA bound in the complexes was determined from the supernatant using UV-vis spectroscopy.

Fluorescence experiments

The fluorescence of samples was measured using the Fluoromax-4 spectrofluorometer (Jobin Yvon, France) at the laboratory temperature. The emission fluorescence intensity of EtBr was measured at $\lambda_{em} = 596$ nm using excitation wavelength $\lambda_{ex} = 510$ nm. The samples were measured 60 min after the complexes´ preparation and repeatedly after 30 days. They were constantly stirred during the measurement. The emission intensity of each sample was corrected for the background fluorescence of EtBr in the absence of DNA and then normalised to

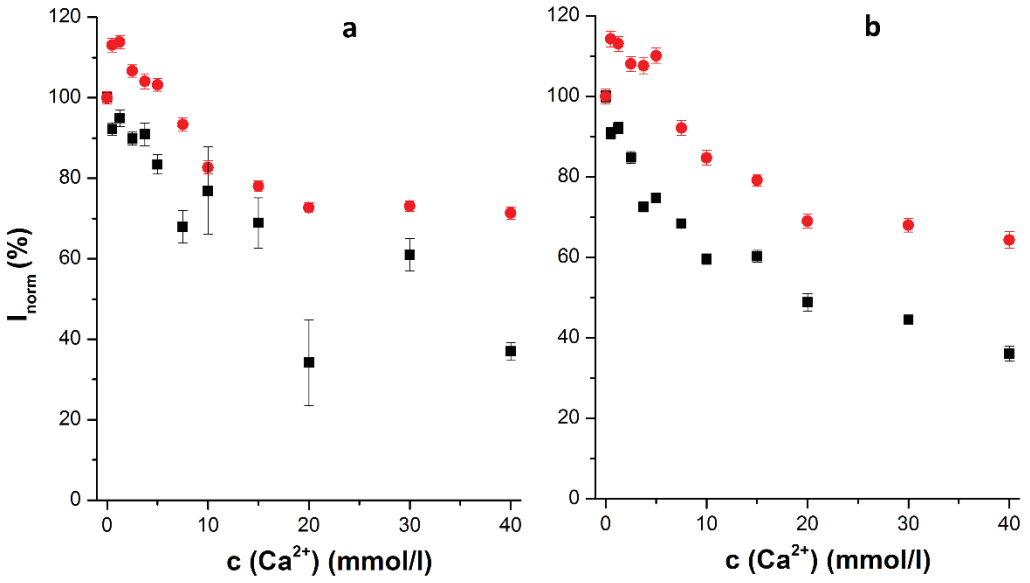

Figure 1. Dependence of nomalised intensity I_{norm} of DNA–EtBr–DOPE–Ca^{2+} and DNA–EtBr–DOPE–OA–Ca^{2+} complexes on in the presence of calcium cations without OA ■ and with OA at OA/DOPE molar ratio of 1 mol/mol · measured at 60 min (a) and 30 days after preparation (b)

the EtBr fluorescence of sample containing DNA without calcium cations according to equation 1:

$$I_{norm} = \frac{I_{DNA+DOPE+Ca^{2+}+EtBr} - I_{NaCl+EtBr}}{I_{DNA+DOPE+EtBr} - I_{NaCl+EtBr}} \quad (1)$$

Small-angle X-ray diffraction experiments (SAXD)

Small-angle synchrotron radiation diffraction experiments were performed at the NCD-BL11 beamline, ALBA Synchrotron, Barcelona (Spain) using radiation of wavelength λ = 0.124 nm. The sample in vertically placed capillary was equilibrated at the required temperature before being exposed to the radation. CCD (Couple-charged device) camera Quantum 210r was supplied by ADSC (Quantum 210r CCD) for SAXD detection. Raw data were normalised against the incident beam intensity. The SAXD detector was calibrated using silver behenate. Diffractograms were evaluated with the intensity of the incident beam following an exponential model. Diffraction peaks of SAXD region were fitted with a Lorenztian curve above a nonlinear background.

RESULTS

DNA condensation

DNA condensation was indicated by a decrease in the emission intensity of EtBr. Figure 1 shows the dependence of the emission intensity of EtBr on the concentration of calcium cations at 25 °C in DNA–DOPE–Ca^{2+} and DNA–DOPE–OA–Ca^{2+} complexes at OA/DOPE molar ratios = 1 mol/mol. The concentration of DNA and EtBr was kept constant, whereas the concentration of calcium cations varied from 0 to 40 mmol/l. The normalised emission intensity of EtBr decreases with increasing concentration of calcium cations in both time intervals. In Figure 1, we can see that the I_{norm} decreases to ~37% (ΔI_{norm} = 63 %) in the presence of DOPE–DNA–Ca^{2+} complexes but only to ~71% in the system with OA. After 30 days, ΔI_{norm} did not change significantly within the experimental error in DOPE–DNA–Ca^{2+} complexes. In case of DOPE–OA–DNA–Ca^{2+} complexes, ΔI_{norm} was increased by ~7% after 30 days as shown in Figure 1b.

X-ray scattering

We studied the structure of OA–DOPE mixtures and DNA–DOPE–OA–Ca^{2+} complexes as a function of OA content. Fully hydrated DOPE (Fig. 2A) forms at 20 °C an inverted hexagonal phase H_{II} showing diffraction peaks at reciprocal distances $q_{hk} = 4\pi(h^2 + k^2 - hk)^{1/2}/(a_H*\sqrt{3})$ nm^{-1}, where h and k are Miller indices. The lattice parameter a_H ($a_H = 4\pi / q*\sqrt{3}$ nm) is determined from the position of $H_{II}(10)$ peak's maximum characterising distance between the axes of two adjacent cylinders forming a columnar hexagonal phase (see Fig. 3).

Figure 2a shows the diffractogram of fully hydrated DOPE in the environment of 150 mmol/l NaCl at 20 °C. We determined the lattice parameter a_H as 7.54 nm. The

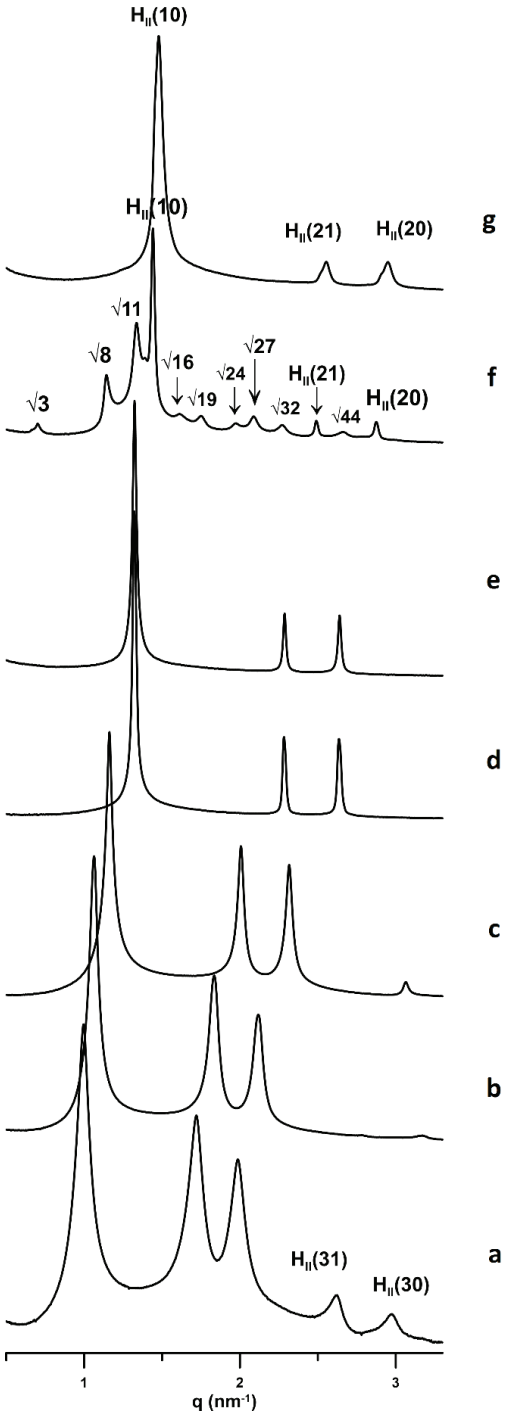

Figure 2. SAXD patterns of DOPE at 20 °C (a), OA–DOPE mixtures at 20 °C at OA/DOPE molar ratios of 0.2 (b), 0.4 (c) and 1 (d) (mol/mol), DOPE–OA–DNA–Ca^{2+} complexes at 20 °C (e), DOPE–OA–DNA–Ca^{2+} complexes at 80 °C at pH of ~4.8 (f) and ~7.6 (g). Cubic phase assignment follows $q_{hkl} = 2\pi(h^2+k^2+l^2)^{1/2}/a_F$, where h, k and l are Miller indices. All samples were prepared in the environment of 150 mmol/l NaCl at 15 mmol/l Ca^{2+} concentration.

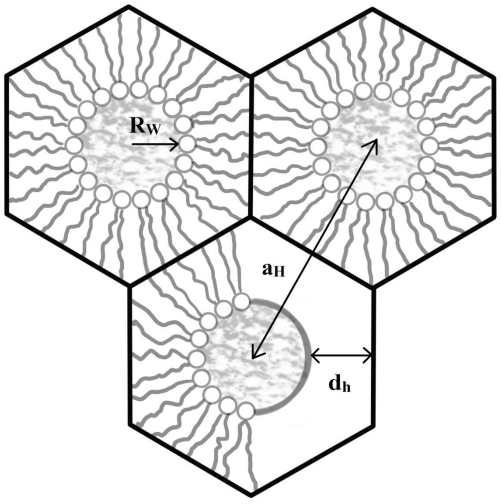

Figure 3. Illustration of the inverse hexagonal phase H_{II} in cross section. The shaded regions represent water. Tubes of water of radius R_w are arranged on a hexagonal lattice, surrounded by a lipid layer.

addition of OA resulted in the decrease of a_H value and was dependent on the OA/DOPE molar ratio. The greater the OA content, the smaller is the lattice parameters in the OA–DOPE systems. The diffractograms of OA–DOPE mixtures at individual molar ratios are shown in Fig. 2b–d. At the OA/DOPE molar ratio of 1, the a_H was reduced up to 5.50 nm. The obtained a_H values are summarised in Table 1.

The structure of DOPE–OA–DNA–Ca^{2+} complexes was studied at 15 mmol/l Ca^{2+} concentration prepared in the environment of 150 mmol/l NaCl at the OA/DOPE molar ratio of 1. The diffractogram of the complexes at 20 °C shows hexagonal arrangement of the mixture (Fig. 2e). The lattice parameter was maintained at the same values as in the OA–DOPE system at the OA/DOPE molar ratio of 1 (see Table 1).

OA/DOPE mixtures as well as DOPE–OA–DNA–Ca^{2+} complexes were heated in the temperature of 20–80 °C with 10 °C temperature increment. Heating sequences were recorded at two different pH values, ~4.8 and ~7.6. The lattice parameter of hexagonal phases for both the mixture OA–DOPE and the complexes DOPE–OA–DNA–Ca^{2+} decreased with rising temperature. The most significant structural polymorphism was recorded at OA/DOPE = 1 mol/mol. In addition to peaks related to hexagonal phase, diffractograms show new peaks corresponding to a cubic phase. Peaks assigned to positions √3, √8, √11, Ö16, √19, √24, √27, √32 and √44 fit well an inverse micellar cubic phase of the Fd3m space group. The lattice parameter of the Fd3m phase, a_F = 16 nm, was determined from the slope of the plot of the peak positions versus $\sqrt{(h^2 + k^2 + l^2)}$ passing by the

Table 1. Samples' composition and lattice parameters of hexagonal (a_H) and cubic phase (a_F) at 20 and 80 °C at different pH values

Composition	OA/DOPE (mol/mol)	t (°C)	pH	a_H (nm)	a_F (nm)
DOPE	0	20	6	7.54 ± 0.01	-
DOPE–OA	0.2	20	6	6.88 ± 0.01	-
DOPE–OA	0.4	20	6	6.27 ± 0.01	-
DOPE–OA	1	20	6	5.50 ± 0.01	-
DOPE–OA–DNA–Ca^{2+}	1	20	6	5.50 ± 0.02	
DOPE–OA–DNA–Ca^{2+}	1	80	4.8	5.00 ± 0.01	15.70 ± 0.01
DOPE–OA–DNA–Ca^{2+}	1	80	7.6	4.93 ± 0.02	-

origin (0,0). For OA/DOPE, Fd3m phase appeared at ~60 °C at moderately acidic pH and was shifted to higher temperatures at pH of ~7. However, DOPE–OA–DNA–Ca^{2+} complexes prepared at neutral pH do not arrange into Fd3m phase at all, as hexagonal phase alone was identified (Fig. 2f and g). Lattice parameters of both phases at selected temperatures are summarised in Table 1.

DISCUSSION

Fatty acids (FAs) are important components of the plasma membrane, in which the core is formed by moieties of FAs as a part of phospholipid molecules. PE constitutes ~5–50% of total lipids in membranes (Cevc 1993), so that the study of FA–PE mixtures is of biological relevance. FAs affect membrane structure (Ibarguren et al. 2014), cell physiology (Lu et al. 1996) and their levels have also been associated with pathological states (O'Connor et al. 1999). The present study is investigating the physicochemical characteristics of potential non-viral nucleic acid vectors composed of the OA–DOPE mixture in the presence of Ca^{2+}.

DNA itself can condense into polydispersed rod-like, spheroid and toroid structures in a first-order phase transition that is induced by multi-valent cations (Hud and Vilfan 2005). In the bulk solution, condensation cannot be used to shape DNA-based nanomaterials by design because of the lack of predetermined spatial organisation, the abruptness of the transition and the high stability of the condensed structures (Iwataki et al. 2004, Mel'nikov et al. 1995). However, DNA condensation can be controlled by layering DNA using cationic lipid membranes (Koltover et al. 2000). In our experiment the electrostatic interaction between the neutral phospholipid DOPE and polayanionic DNA is mediated by divalent calcium cations. Owing to the interaction, DNA can be packed into structures formed by the lipid. Fluorescence spectroscopy is a suitable method to follow DNA condensation by a decrease in the emission intensity of fluorescence probe EtBr. Free molecules of EtBr in a solution follow a nonradiative decay pathway that involves donation of an amino group proton to the solvent. When EtBr is intercalated into DNA, the ethidium cation is isolated from the solvent and the proton transfer pathway between EtBr and the solvent is blocked. This leads to about 20-fold increase in the fluorescence intensity (Izumrudov et al., 2002) because of the electrostatic attraction between cationic agent and negatively charged phosphate groups of DNA. Neutralisation of the negative charge of DNA phosphate groups leads to compaction and condensation of the DNA molecules and their condensation (Eastman et al., 1997). The condensation of DNA leads to displacement of intercalated EtBr, that presents itself as the decrease in the fluorescence intensity. Eastman et al. (1997) suggested that the observed decrease in fluorescence is directly related to the amount of DNA bound in the formed aggregate. The greater the decrease in fluorescence intensity of EtBr, the more EtBr molecules have been displaced from the DNA base pairs because of the DNA condensation and thus the forming of DNA–phospholid complexes. In the DOPE–DNA–Ca^{2+} complexes, ~63% of DNA was bound, but it was only 29% after the incorporation of OA to the complexes at the OA/DOPE molar ratio of 1. Complexes were also measured 30 days after the preparation to see if more DNA would encapsulate or, on the other hand, the DNA starts to release from the complex after one month. In the DOPE–DNA–Ca^{2+} system, the amount of DNA bound in the complexes did not change. The binding capacity of DOPE–OA–DNA–Ca^{2+} complexes was improved by ~7% after 30 days. Practically, no release of DNA was recorded in the system without OA. This was found in other cationic lipid-based system forming reverse hexagonal mesophase (Amar-Yuli et al. 2011). The presence of cationic substance leads to such a strong electrostatic confinement of DNA at the water–lipid interface that the release into the excess water is prevented.

OA is a molecule with an aliphatic chain and a carboxylic group whose ionisation is dependent on the solution pH.

Subsequently, intermolecular interactions depend on its ionisation state. The pKa of the carboxylic group is around 4.8 (Cistola and Small 1991). For medium- and long-chain fatty acids, this value increases to a higher apparent pKa (Cistola et al. 1988). The apparent pKa value of OA in the aggregated phase is somewhat ill-defined and difficult to estimate (Salentinig et al. 2010, Peterlin et al. 2009) and, depending on the used method, can vary between 7.5 at low concentrations inserted in a PC bilayer and up to 9.85 in pure monolayers. The pKa^{app} value of OA, determined by titration, was reported to be between 8.0 and 8.5 by Cistola et al. (1988) and 9.85 by Kanicky and Shah (2002). pH of samples in our EtBr accessibility experiment was ~6.5–7. We assume that the most part of OA present in the complexes is in its unionised form; however, the presence of the ionised form of OA in the system and subseqently the interation of Ca^{2+} with negatively charged molecules of OA cannot be excluded. In Figure 1, one can see that the initial increase in the normalised EtBr fluorescence intensity takes the values of more than 100 at low calcium concentrations in the complexes with OA. In the DOPE–OA–DNA–EtBr system, small amounts of DNA can be captured in the tubules of the hexagonal structure even in the absence of calcium cations. Because of high volume fraction of OA (OA/DOPE = 1 mol/mol) in this experiment, we cannot exclude that Ca^{2+} may 'bridge' two molecules of OA. The formed salt of FA may result in the exclusion of DNA from the tubules, resulting in the increase in intensity as follows from the properties of EtBr used as a probe. We did not observe such behaviour at OA/DOPE < 1 mol/mol (not shown). With further increase in the calcium concentration, the complex formation is the predominant event and the emission intensity of EtBr decreases.

Fully hydrated DOPE at laboratory temperature arranges into nonlamellar, inverted hexagonal phase H_{II} with lattice parameter a_H of ~7.5–7.6 nm. The radius of water cylinders (R_w) inside the tubules was determined by Tate and Gruner (1989); they reported R_w = 2.2 nm at 20 °C, the thickness of the lipid layer d_h = 1.62–2.20 nm (as illustrated in Fig. 3). Inverse hexagonal phase H_{II} was also found in our OA–DOPE mixture at different OA/DOPE molar ratios, but with significantly lower lattice parameters. As we can see in Table 1, the addition of OA to DOPE remarkably decreases the lattice parameter. Note, at the OA/DOPE molar ratio of 0.2, we determined a_H = 6.88 nm, and the lattice parameter was reduced up to 5.50 nm at OA/DOPE = 1 mol/mol. Gillams et al. (2014) studied phase behaviour of OA/DOPE mixtures as a function of OA mole fraction. Pure water was used as hydrating medium. The authors report that OA increases the propensity of bilayer lipid membranes to curve, up to level of inverse micellar cubic phase formation. In their study, H_{II} phase was observed up to mole fraction of OA x_{OA} = 0.6. Similar to our finding, the authors observed the decrease in the lattice parameter a_H up to 5 nm at x_{OA} = 0.6. Their analysis indicates that OA affects the elasticity of lipid/OA monolayers resulting in the reduction of the radius (R_w) of water cylinders. Whilst changes in the lipid layer thickness (d_h) can be considered of little importance, the variance in d_h with respect to R_w is less than 2% (Gillams et al. 2014 and references therein).

Interestingly, DOPE–OA–DNA–Ca^{2+} complexes prepared at OA/DOPE = 1 mol/mol in 15 mmol/l of $CaCl_2$ show the same value of a_H = 5.5 nm as determined for the OA–DOPE mixture itself. Let us analyse this finding: It was reported that DNA can be ‚accommodated' in the water cylinders of inverted hexagonal phase formed by DOPE in the presence of divalent cations such as calcium or magnesium. For example, condensed inverted hexagonal phase H_{II}^c was observed at 20 °C in DOPE–DNA–Mg^{2+} complexes (Uhríková et al. 2006). The coexistence of both H_{II} and H_{II}^C phases was reported in the DOPE–DNA–Ca^{2+} system at 20 °C (Lengyel 2010). Lattice parameters (a_H) of H_{II}^c phase range were ~7.1 nm in both the experiments mentioned earlier. In these studies, 5 mmol/l NaCl was used as the hydrating medium. The difference between lattice parameters of H_{II} formed by DOPE and those H_{II}^C observed for DOPE+DNA+ion^{2+} is small (~0.5 nm). In this system, Ca^{2+} mediates DNA–phospholipid binding, and the tendency of DNA to be wrapped by the lipid results in R_w reduction, R_w = 1.7 nm. It is evident that hydrated DNA strands with diameter of 2.5 nm (Lieberman 2005) can arrange readily into water cylinders of H_{II}^C phase. As we see, the lattice parameters of DOPE+DNA+ion^{2+} are increased by ~1.6 nm in comparison to a_H value (a_H = 5.5 nm) found in our DOPE–OA–DNA–Ca^{2+} system. In view of discussion above and results of Tate and Gruner, if we assume the thickness of the lipid layer, d_h = 1.62 nm, one gets the radius of the water core R_w = 1.13 nm. Thus the structure shrinks, the water core with radius R_w = 1.13 nm in OA-enriched complexes can be seen as limit or even insufficient for DNA accommodation. However, our data from fluorescence spectroscopy indicate that up to ~29% of total DNA in the sample was bound in the complexes. Additional UV-VIS measurements on complexes prepared for SAXD experiments indicate that ~10–13% of total DNA bound four months after the complex preparation.

In summary, both methods, fluorescence and UV-VIS spectroscopy measurements, confirmed that ~10–29% of DNA was bound in the complexes. Generally, it is supposed that at hexagonal arrangement of lipoplexes, in condensed inverted hexagonal phase H_{II}^C, DNA occupies interior of water-filled cylinders formed by lipid monolayers of inverted hexagonal phase. The symmetry of arrangement of DNA is dictated by symmetry of the lipid arrangement. As such, the difference between SAXD patterns of H_{II} and H_{II}^C is not obvious. The greater electron density of DNA with respect to water should lead to the relative suppression of the (21) and (20) Bragg

peak intensities compared with that in the lipid H_{II} phase (Koltover et al. 1998). Comparing patterns of OA–DOPE and DOPE–OA–DNA–Ca^{2+} (Fig. 2d and e, respectively), relative changes in the intensities of (21) and (20) are rather small. This fact together with closeness of the obtained lattice parameters raises the question: where is the detected DNA located in our complexes? From the physical point of view, our complexes are the so-called 'polycrystalline' samples with random orientation of microdomains (with internal order) of colloid mixture. As such, the lattice parameter derived from SAXD is an average value of lattice parameters of microdomains exposed to X-ray in the moment of an image collection. Our SAXD data show no difference between the lattice parameters of hexagonal phases formed by OA–DOPE and DOPE–OA–DNA–Ca^{2+}. However, we found up to ~29% of DNA bound. We suppose that a part of DNA can be localised in the interior of water cylinders of H_{II} phase; however, DNA strands can also be trapped by the structure itself at the lipid–water interface. Temperature measurements of the complexes support our assumption. High temperature stimulates the formation of micellar cubic phase Fd3m. In our studied system, Fd3m was observed at 60 °C first in OA–DOPE mixtures at both moderately acidic and neutral pH. After the addition of DNA to OA–DOPE mixture in the presence of Ca^{2+} at neutral pH, we did not detect any peak related to the cubic phase. DNA stabilises the inverse columnar hexagonal arrangement in the system DOPE–OA–DNA–Ca^{2+}, because the formation of H_{II} only was observed at pH of ~7.6 in the whole studied temperature range (see Fig. 2f). The disruption of hexagonally packed cylinders of H_{II} and their rearrangement into a phase of three-dimensional symmetry (Fd3m) depends strongly on external conditions, such as temperature and ionic strength. For example, Gillams et al. (2014) observed coexistence of H_{II} and Fd3m phases for OA/DOPE = 1 mol/mol already at 20 °C. Pure water was used as hydrating medium in this study. At the same composition of OA–DOPE mixture, we detected the presence of cubic phases at ~60 and ~70 °C in dependence on pH of the solution (pH of ~4.8 and 7.6, respectively). The high concentration of the salt (150 mmol/l NaCl) used in our experiment might be responsible for this difference. Recent knowledge documents no significant effect of NaCl on the bilayer structure for ion concentration up to 1 mol (Petrache et al. 2006, Pabst et al. 2007). Pabst et al. (2007) reported a decrease in bilayer elasticity and shift of main transition temperature in the presence of NaCl and $CaCl_2$. Reduced elasticity of the bilayer because of a tight binding of sodium cations to the carbonyl oxygens of the phospholipid was also found elsewhere (Böckmann et al. 2003). This rigidification and ‚stabilisation' of the structure because of high salt content is highly responsible for H_{II} phase as the only one observed in our system. Moreover, DNA trapped in DOPE–OA–DNA–Ca^{2+} complexes prepared at neutral pH stabilises hexagonal packing as well, up to high temperature as documented in our experimental data.

CONCLUSIONS

The study was aimed to design nucleic acid delivery vectors based on phospholipid. A mixture of zwitterionic DOPE and OA was used as lipid matrix. Fatty acids are natural components of biological membranes and are known as effective modulator of polymorphic behaviour of phospholipids. SAXD experiments revealed that OA induces structural changes in the inverted hexagonal phase H_{II} of DOPE. We have found that increasing the content of oleic acid, as additive, reduces the lattice parameter of H_{II} formed by DOPE. The effect is marked and recognised as crucial obstacle in efficient DNA binding into water cylinders that form the core of OA–DOPE tubules arranged in hexagonal symmetry. The amount of DNA bound in complexes (~10–29%) was determined using fluorescence and UV-vis spectroscopy. After 30 days, either the binding capacity of the complexes or the DNA release from the hexagonal phase into excess water was not affected. High content of OA and increase in temperature promote formation of an inverted micellar cubic phase of Fd3m space group. At neutral pH, DNA bound in complexes prevents disintegration of the structure and hexagonal phase was observed up to 80 °C. The findings obtained under this study contribute to knowledge about DNA complexation into supramolecular structure formed by lipid bilayer, what is the essential feature at gene delivery vectors designing.

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The estimation of competitive positions of non-steroid anti-inflammatory drugs on pharmaceutical market of Ukraine

Original Paper

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Abstract The aim of our analysis is the estimation of non-steroid anti-inflammatory drugs (NSAID) competitiveness level, calculation of capacity, share and saturation of studied segment of market that allow to improve the competitive strategies of pharmaceutical manufacturing enterprises.

MATERIALS AND METHODS: According to the results of content-analysis of foreign and native economic literature, we elaborated the methodology of estimation of NSAID competitive positions that consists of six main stages. The offered model of estimation of competitiveness of the studied group of medicines is based on the construction of competition map of medicines on the example of non-selective NSAID as the largest group by the number of presented trade names (TN) using the calculation of market share of medicines for 2015-2016 and dynamics of its growth.

RESULTS: The results of NSAID TN distribution in groups according to the market share volume testify that only 8 TN of medicines among 74 are the leaders of market and 10 TN are characterized with strong competitive position. At the same time 51 NSAID TN are outsiders and 5 TN have weak competitive position.

The results of NSAID TN distribution in groups according to the change of their competitive position allow state that 16 TN among 74 have fast worsening competitive position, 43 TN belong to the group with worsening competitive position and only 5 TN has competitive position, characterized by fast improvement.

CONCLUSION: The analysis of position, occupied by NSAID TN in matrix, demonstrated that 8 TM of medicines are the leaders of the market. At the same time 51 medicines TN are outsiders. The results of research demonstrated that 59 NSAID TN are characterized with worsening and fast worsening competitive positions.

Keywords *pharmaceutical market – non-steroid anti-inflammatory drugs – market share – competition map*

INTRODUCTION

One of the most important indicators of effectiveness of enterprise activity that determines the success of work, financial status and position of an enterprise in the market is the competitiveness of pharmaceutical products (Dolzhansky & Zagorna, 2006).

The competitiveness of each commodity in the market is determined by the totality of many factors and is considered as an advantage of this commodity as compared with the others (of analogous importance). The competitiveness of medicines is determined by the totality of different characteristics that determine their comparative positions in the market.

Under the conditions of rigid competitive environment that has been formed in the native market of medicines, the urgent and timely acceptance of managerial-productive decisions, directed on the search and mastering of prospective market niches, formation of competitive positions and unique advantages of medicines, coordination of assortment-selling strategies are the determinative components of successful activity of pharmaceutical firms, enterprises and organizations (Levy & Weitz, 2004; Kotler & Keller, 2006; Kotvitska & Kostiuk, 2016).

Thus, the aim of our analysis is the estimation of non-steroid anti-inflammatory drugs' (NSAID) competitiveness level, calculation of capacity, share and saturation of studied segment of market that allow to improve the competitive strategies of pharmaceutical manufacturing enterprises.

MATERIALS AND METHODS

According to the results of content-analysis of foreign and native economic literature, we elaborated the methodology of estimation of NSAID competitive positions that consists of six main stages, including the succession of arrangements at each stage and the use of correspondent effective indices (Fig. 1) (Berman & Telen,2004; Dzhuparova et al., 2010; Kaune, 2005; Mnushko et al., 2009).

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The offered model of estimation of competitiveness of the studied group of medicines is based on the construction of competition map of medicines, on the example of non-selective NSAID as the largest group by the number of presented trade names (TN), using the calculation of market share of NSAID for 2015–2016 and the dynamics of its growth.

The first stage includes the analysis of assortment and determination of modern tendencies of NSAID market formation.

It is necessary to note that the methodological base of estimation of competitive positions in the market share. That is why, *the second stage* provides the estimation of competitive situation on NSAID market, realized by calculation of the market share of each formulation of the presented TN of studied segment of medicines. Thus, the calculation of market share for each NSAID TN was carried out according to the formula 1 (Berman &Telen,2004):

$$D_i = \frac{T_i}{T_{gen.}} \times 100\% \quad (1)$$

where, *D* – market share of i-th TN NSAID, %

T_i – sales volume for i-th TN NSAID in DDDs sold per year

$T_{gen.}$ – general volume of NSAID sales in DDDs sold per year.

Depending on the volume of market share, medicines can be leader or outsider in the market. To differentiate the groups of medicines – outsiders, medicines with weak, strong competitive position and leaders of the market., within *the third stage,* it is necessary to determine the mean-square deviation of NSAID TN market share and also its minimal, maximal and mean arithmetical value. Thus, we realized the determination of limits of classification groups of studied NSAID using the law of variation of individual values of sign ('three-sigma rule').

The directions of *fourth stage* of methodology that are the estimation of NSAID competitive positions provide determination of dynamics of growth, mean value and dispersion of dynamics of growth of NSAID TN market share. Thus, the calculation of mean dynamics of the market share growth for each formulation of medicines TN for the studied period (2016) relative to the base period (2015) and its mean-square deviation was realized according to the formula 2 (Burtseva et al., 2005):

$$T_i = \frac{D_i - D_0}{D_0} \times 100\% \quad (2)$$

where, T_i – dynamics of growth of market share of i-th NSAID TN, %

Di – market share of i-th TN in the studied period, %

D_0 – market share of i-th TN in the base period, %.

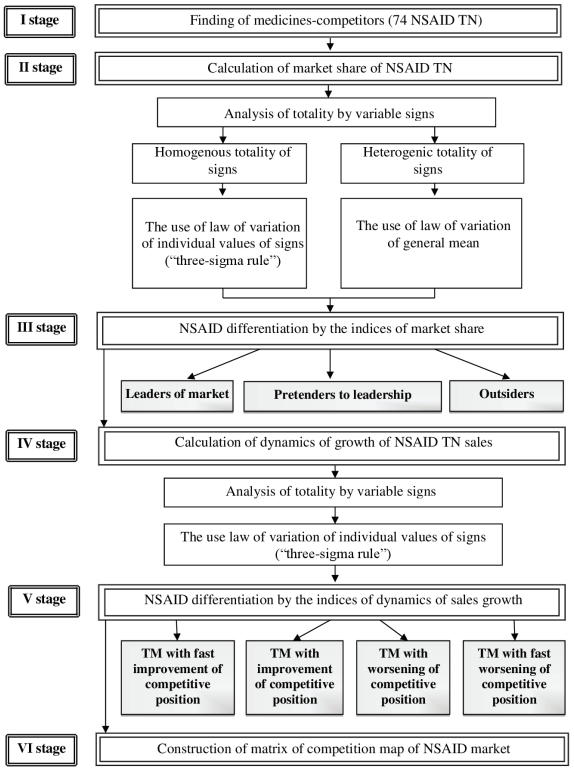

Figure 1. The methodology of estimation of NSAID competitive positions on pharmaceutical market of Ukraine

The estimation of medicines' competitive positions within the chosen NSAID segment is *the fifth stage* of offered methodology that is expedient to be realized for the revelation of prospective market niches for elaboration of medicines and introducing them in production. For the estimation of degree of change of competitive position of the studied medicines, we separated four groups of medicines by the dynamics of their marker share growth (Mnushko et al., 2009):

- medicines with fast improvement of competitive position;
- medicines with improvement of competitive position;
- medicines with worsening of competitive position;
- medicines with fast worsening of competitive position.

At *the sixth stage*, the matrix of competition map of the market is constructed, taking into account the received results of estimation of the market share distribution and degree of change of competitive position of the studied NSAID TN. It is based on the cross classification of NSAID TN by the indices of market share and its dynamics, as a result of which, we use the 16 types of strategic positions of medicines that differ by the degree of use of competitive advantages and potential possibility to resist the competitors' pressure are determined (Mnushko et al., 2009). The competition map of medicines allows us to determine their status and systematize the competitive advantages.

Table 1. The criteria of reference of TN of non-selective NSAID to classification groups

Classification groups	Formulas of calculation of classification groups limits, %	Results of calculation of market limits	Characteristic	Number of NSAID TN
I	$D_{max}; D_{mean}+3\times\sigma_2/\sqrt{n}$	15.19;…2.69	TN – leaders of market	8
II	$D_{mean}+3\times\sigma_2/\sqrt{n}; D_{mean}$	2.69;…1.35	TN with strong competitive position	10
III	$D_{mean}; D_{mean}-3\times\sigma_1/\sqrt{n}$	1.35;…1.20	TN with weak competitive position	5
IV	$D_{mean}-3\times\sigma_1/\sqrt{n}; D_{min}$	1.20;…0.00*	TN - outsiders	51

**the absolute value is 0.00002*

Table 2. The results of estimation of the degree of change of NSAID TN competitive positions

Classification groups	Formulas of calculation of classification groups limits %	Results of calculation of market limits	Characteristic	Number of NSAID TN
I	$T_{max};T_{mean}+3\times\sigma/\sqrt{n}$	1579.32;…92.03	TN with fast improvement of competitive position	5
II	$T_{mean}+3\times\sigma/\sqrt{n}; T_{mean}$	92.03;…21.91	TN with improvement of competitive position	10
III	$T_{mean}; T_{mean}-3\times\sigma/\sqrt{n}$	21.91;…-48.21	TN with worsening of competitive position	43
IV	$T_{mean}; T_{mean}-3\times\sigma/\sqrt{n}$	-48.21;…-99.93	TN with fast worsening of competitive position	16

RESULTS

At *the first stage* of the offered methodology, according to the results of the data of Public register of medicines, which includes all medicines approved for marketing by the Ministry of Health of Ukraine, it was established that NSAID assortment is presented by 13 pharmacotherapeutic groups: M01AB01 – *indometacin*, M01AB05 – *diclofenac*, M01AB15 – *ketorolac*, M01AB16 – *aceclofenac*, M01AC01 – *piroxicam*, M01AC02 – *tenoxicam*, M01AE01 – *ibuprofen*, M01AE02 – *naproxen*, M01AE03 – *ketoprofen*, M01AE09 – *flurbiprofen*, M01AE14 – *dexibuprofen*, M01AE17 – *dexketoprofen*, M01AG01 – *mefenamic acid* (Ministry of Health of Ukraine, 2015).

The analysis at *the second stage* included 74 TNs of medicines, divided into 2 sectors in correspondence with the occupied share. The first sector included medicines, for which the market share was more than the mean arithmetic value of market shares of the studied NSAID; the other one united medicines, for which the market share was less than the mean arithmetic value (1.35%).

Thus, the first sector was formed of 18 NSAID TNs with mean volume of market share 4.51%, and other 56 TNs with mean volume of market share 0.34%.

Within *the third stage*, the calculations of mean-square deviation of NSAID TN market share, that in totality with minimal and maximal values allows us to make distribution of the studied medicines in 4 classification groups, were realized in each sector (Table1).

The results of NSAID TN distribution in groups according to the market share volume testify that only 8 TNs of medicines among 74 are the leaders of market and 10 TNs are characterized with strong competitive position. At the same time, 51 NSAID TNs are outsiders and 5 TNs have weak competitive positions.

It is well-known that the estimation of tendencies of change of competitive position is realized using the index of mean dynamics of market share growth. Thus, the calculated mean dynamics of growth of the studied NSAID segment is 21.91% (*the fourth stage of methodology*).

NSAID TN classification by the degree of change of competitive position is presented in Table 2 (*the fifth stage* of offered methodology).

The results of NSAID TN distribution in groups according to the change of their competitive position allow us to state that 16 TNs among 74 have fast worsening competitive position, 43 TNs belong to the group with worsening competitive position and only 5 TNs have competitive position, characterized by fast improvement.

The received results of estimation of distribution of market share and the degree of change of competitive position of the studied NSAID TN allowed us to construct the matrix of competition map of the market (Table 3) within *the sixth stage* of methodology.

The results of analysis of NSAID TN position on the competitive map testifies that among the studied segment of medicines, 7 TN-leaders are characterized by worsening

Table 3. The matrix of formation of competition map of the native market of NSAID

Classification of groups by the market share / NSAID TNgroup by the change of market share	TN – leaders of market	TN with strong competitive position	TN with weak competitive position	TN - outsiders
Fast improvement of competitive position	---	---	---	5 NSAID TN
Improvement of competitive position	1 NSAID TN	3NSAID TN	---	6 NSAID TN
Worsening of competitive position	7 NSAID TN	7 NSAID TN	5NSAID TN	24NSAID TN
Fast worsening of competitive position	---	---	---	16NSAID TN

competitive position; 1 TN-leader has competitive position, characterized by improvement; 3TNs that pretend to leadership have improving competitive position; 12 TNs that pretend to leadership have worsening competitive position; 5TN-outsiders have fast improving competitive position; 6 TN-outsiders have improving competitive position; 24 TN-outsiders are characterized with worsening competitive position; and 16 TN-outsiders have fast worsening competitive position.

DISCUSSION

In modern practice of applied marketing research, considerable attention is paid to the analysis of market environment. The study of market processes involves determining the dynamics of market conditions. Each company faces the task of assessing its own competitiveness, which is due to the quality management of supply processes. The most important for enterprises are the indicators that reflect the dynamics of consumption of goods, the volume of wholesale and retail trade. Also, in the conditions of limited information for manufacturers, it is relevant to calculate the capacity and market share, to assess the level of competitiveness of products.

The results of the conducted research provide comprehensive and reliable information on both the dynamics of pharmaceutical market and trends in its structural segments, trademarks. Using the obtained results, the manufacturing companies are able to monitor market conditions, its capacity and development trends. Also, it is possible to conduct market segmentation by commodity and market characteristics to conduct their comparative analysis. Searching market niches and choice of perspective directions of optimization of commercial policy; conducting competitive analysis and establishing market segments with the lowest competitive activity becomes more reasonable and appropriate.

Thus, the competitive map of medicines serves to determine their status and to systematize their competitive advantages. In 2016, in the Ukrainian market of NSAIDs, there is a situation where there are no TN-leaders of market and TN that pretend to leadership with rapid improvement of the competitive position.

The offered methodology of estimation of NSAID competitive positions, from our point of view, can be used by pharmaceutical enterprises for choosing the grounded approaches to elaboration of competitive strategy, preventing the negative tendencies in production-selling processes of enterprises. The received results that are the real reproduction of the modern market situation, allow enterprises to determine the features of development of competitive situation on the market and to reveal the closest competitors.

For manufacturers of medicines-leaders of market, according to the market share, the main directions of competition strategies are the stabilization of competitive position by maintaining the achieved level of profitability and by creating a more flexible pricing policy.

In order to achieve competitiveness, the following strategic alternatives are recommended for the manufacturers of medicines with a strong competitive position: search for a vacant niche with weak competition, adapting to the selected target market, following the behaviour of the leader.

Manufacturers of medicines with weak competitive position must focus on reducing the cost of medicines.

The position of medicines-outsiders of the investigated segment of the market requires pharmaceutical companies to reorganize their marketing strategies (with a significant reduction in costs or, conversely, an increase in the medicine prices in the case of inelastic demand).

LIMITATIONS OF RESEARCH

Market researches on the competitiveness in pharmacy on various thematic areas of competitive analysis are widely represented in the scientific works of Ukrainian scientists. But detailed analysis of publications shows that mainly the competitiveness of manufacturing companies, pharmacies and limited analysis of the competitive advantages of medicines were investigated.

At the same time, the lack of available state statistical information on sales volumes makes it impossible to carry out a full-fledged analysis of the market situation in the domestic pharmaceutical market, which determines the use of special methods of calculation and additional sources of information. The additional sources of information include data on the

intensity of goods consumption, information on primary and additional sales and information obtained from the results of the retail audit.

CONCLUSIONS

According to the results of NSAID TN differentiation by competitive position, it was established that 16 medicines among 74 have fast worsening competitive position, 43 TNs are included in the group with worsening competitive position and only 5 TNs have competitive position characterized with fast improvement.

The matrix of formation of competition map of the market, that is, an instrument of determination of status and competitive advantages, was constructed; the NSAID TN positions on the native pharmaceutical market were determined.

The analysis of position, occupied by NSAID TN in matrix, demonstrated that 8 TNs of medicines are the leaders of the market. At the same time, 51 medicines TN are outsiders. The results of research demonstrated that 59 NSAID TNs are characterized with worsening and fast worsening competitive positions.

Thus, the obtained results are a real reproduction of the current market situation and allow developing a competition strategy more reasonably, to avoid or prevent the negative trends in the production and sales processes of the enterprises. Taking into account the obtained results, enterprises have the opportunity to operate information about the competitive status, to know the features of the development of a competitive situation on the market and to identify the closest competitors.

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Support of medication adherence by community pharmacists in Czech and Slovak Republics: a questionnaire survey study

Original Paper

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Abstract **Introduction:** Intervention of pharmacists in medication adherence can meaningfully contribute to achieving therapeutic outcomes. Exploring the real-life readiness and opportunities of pharmacists may result in the adoption of measures, which could be seen through improvement of patients´ adherence to pharmacotherapy.
Aim: The aim of the paper was to make a survey on community pharmacists´ potential in medication adherence support in its connectivity to technical and personnel factors, which underline the capacities of pharmacies in dealing with medication adherence.
Methods: The questionnaire survey was conducted from October to December 2014 and involved 158 pharmacists from 117 Czech (CZ) and 41 Slovak (SK) community pharmacies. The structured questionnaire surveyed both technical and personnel factors, including provision of consultancy services related to medication adherence. Non-adherence risk reduction was evaluated by adopting Morisky Scale modified from the pharmacist's perspective. Questionnaires outcomes were summarised in contingency tables and analyzed for associations between respective categorical variables using χ^2 or exact tests and association coefficients. All results are reported as significant at $P \leq 0.05$.
Results: The average score of adherence support (CZ/SK 1.95/1.93) was significantly higher as compared to that of persistence or concordance ($P<0.001$). Reduction of non-adherence risk reached the score of a medium degree ($P=0.73$, average 2.29 in CZ and 2.22 in SK). These findings were significantly associated with personnel capacities (provision of consultancy, preference for the use of recommended procedures in CZ ($P<0.001$), number of years of practice in SK ($P=0.029$)), while significant association with technical equipment (consultancy room) in the SK ($P=0.037$).
Conclusion: The pharmaceutical care is developing towards the improvement of medication adherence in both countries - assuming a medium degree of adherence support. Further progress may be observed in strengthening the pharmacists' personnel capacities, and accelerated mainly using information technologies, i.e. through technical capacities.

Keywords *community pharmacy – consultancy – medication adherence – Czech and Slovak Republics*

INTRODUCTION

Modern public healthcare accentuates the importance of professional capacities of pharmacists from the perspective of their engagement in integrated and evidence-based pharmaceutical practice. Such practice is expected to expand towards and consolidate existing expertise in the areas of responsible use of medicines, innovation, disease prevention and treatment. This will also involve cooperation with other healthcare practitioners, and above all, with patients (FIP, 2011).

With the onset of the development of modern pharmaceutical care, the findings garnered within the field of clinical pharmacy have been progressively applied to the practice of community pharmacies. This means that, along with hospital and clinical pharmacists, the pharmacists practicing in community pharmacies are also continuously becoming more engaged in dealing with drug related problems (DRPs) (PCNE, 2006). The most common problems include drug interactions, adverse effects and dosage regimes, but the pharmacists' attention is also continuously shifting towards issues of medication adherence. Adherence of patients to treatment is a fundamental requirement for achieving therapeutic effect. Not complying with a prescribed therapeutic regime leads

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to therapeutic failure and increased costs for the treatment of diseases (Kriška et al., 2015, Spinewine et al., 2012). It is a well-known worldwide problem that not only affects acute illnesses, but more frequently chronic diseases. According to the WHO (2003), up to 50% of chronically ill patients do not comply with the recommended treatment regime. Therefore, innovative and more effective multidisciplinary approaches are being sought that would improve patients´ adherence, built upon an equal relationship with patients and the systematic improvement of patients' health literacy (Spinewine et al., Taitel et al., 2012). A promising approach is to link adherence with pharmacovigilance, which has become an indispensable part of the responsible use of medicines (Leporini, 2014, Sun et al., 2014). In practice, interventions are used to improve medication adherence; recently, with the help of wireless and mobile technologies, for instance, to improve dosage regimes (Středa & Hána, 2016). Essentially, there is an increasing array of evidence that non-adherence can be effectively tackled by two complementary approaches: firstly, via a multidisciplinary approach, and secondly, via electronic and computerized healthcare systems (e-Health). If pharmacists are to be a part of this, monitoring and systemic support of medication adherence should become one of the priorities of their practice and of drug dispensation related activities.

Despite continuous scientific evaluation of pharmacists' involvement in DRPs, medication adherence including, treatment satisfaction and patient education programs, only a limited number of studies have been performed concerning the role of community pharmacists in DRPs in the Czech (CZ) and Slovak (SK) Republics (Masaryková et al., 2014, Vlček et al., 2009). A review of the existing literature reveals that there has been no research whatsoever on medication adherence support or on the reduction of non-adherence risks by community pharmacists (this is also the case specifically with regards to the technical and personnel capacities of community pharmacies). Therefore, further investigations conducted in this area could rectify this lacuna. This work has a character of a pilot study. It might contribute to the evaluation of hypothesis on a non-adherence risk reduction by pharmacists and detection in which form and with what intensity medication adherence could be supported by community pharmacists in CZ and SK. In addition, we have also focused on counselling and consultations provided by pharmacists in order to determine personnel and technical factors of pharmacies that may correlate with support for adherence in both countries.

METHODS

Study design and participants

Questionnaire survey on adherence support was provided by 158 community pharmacists from CZ and SK community pharmacies in a period from November till December of 2014. The CZ data set was composed of 117 community pharmacists (one pharmacist from one community pharmacy), that represented 56.5% of 207 pharmacies grouped in the association of independent pharmacists and in cooperation with the Faculty of Pharmacy in Hradec Králové, Charles University. From a geographical perspective, all 14 regions of CZ were covered.

The SK data set was composed of 41 community pharmacists (one pharmacist representing one community pharmacy) out of 128 (31.8%) that we approached via students of the Faculty of Pharmacy, Comenius University in Bratislava) undertaking their mandatory practice there. From a geographical perspective, 7 out of 8 regions of SK were covered.

We are conscious of the fact that there is no specification of the Good Pharmacy Practice, which would be available exclusively for the selected data sets of CZ and SK pharmacists-responders, as compared to other CZ and SK community pharmacists, and could therefore represent a systematic error in this regard.

We used a structured questionnaire in the Czech and Slovak languages to estimate the pharmacists' support for medication adherence. The questionnaire was addressed to the heads of pharmacies or to their deputies, who answered about their practice in a specific pharmacy. It contained 15 questions divided into 5 categories that enabled us to map the basic characteristics of pharmacies, such as a geographic distribution, size, as well as specific technical and personnel factors. The aim was to examine the intensity and extent of pharmaceutical patient-oriented care from the perspective of the pharmacists taking part in this survey, regarding the surveyed medication adherence promotion provided in a pharmacy. The questionnaire was available online (Fig. 1).

Structure of the questionnaire

I. Geographic distribution of pharmacies in 5 categories according to the number of inhabitants in the local region: up to 4,999, from 5,000 to 9,999, from 10,000 to 49,999, from 50,000 to 99,999 and above 100,000 inhabitants;
II. Technical equipment of pharmacies, meaning the spatial, technical and technological capacities of pharmacies, which are determined according to the legal requirements on pharmaceutical care provision in CZ (Decree No 92/2012) and SK (Decree No 129/2012). In the questionnaire, we have focused on spatial capacities–space for confidential conversation with the patient designated for counselling and consultations;
III. Personnel capacities of community pharmacies, meaning the number of professional employees, and the number of years of professional experience of the respondent and his or her qualification; engagement of pharmacists into counselling and consultancy;
IV. Counselling and consultations provided by pharmacists to resolve DRPs. In this context, these activities are

Please answer the questions in the suggested form:

a) closed question – if applicable, use number **1**
b) open questions - please provide the answers citing relevant data and information

1. Type and size of the pharmacy:

1.1 Type
a) community pharmacy which is part of an association, alliance or cooperative
b) community pharmacy which is part of a pharmacy network (one brand)
c) community pharmacy which is not part of an association, alliance or cooperative or a network
e) non-state hospital pharmacy
d) state-owned hospital pharmacy

1.2 Number of professional employees (pharmacists and lab workers) employed on part- and full-time basis:
a) 1-5
b) 6-10
c) > 10

2. Municipality/town/region in which the pharmacy is located:
a) municipality/town
b) region

3. Number of years of professional experience (1 person per pharmacy):
a) 0-9
b) 10-19
c) 20-29
d) > 29

4. Which professional/scientific area(s) are you specialized in? Please specify:
a) I am specialized in:
b) I do not have a specialty

5. Is your pharmacy equipped with an appropriate space for consultations and therapy assessment, or a specialized workplace where information on medicines is provided? (in accordance to CZ Decree No 92/2012 Coll. & SK Decree No 129/2012 Coll.)

5.1 Consultation space or room
a) yes
b) no

5.2 Discrete zone
a) yes
b) no

5.3 How often do you provide consultations to patients beyond the usual drug dispension-related activities?
a) daily
b) 1-3 times per week
c) 1-3 times per month
d) other (please specify)

6. Please provide information on your 2 or 3 most frequently used sources of information related to medicines and recommended procedures of the Czech Pharmacists´ Association and how frequently you use them:

Source/form/frequency of information related to medicines
1
2
3

Recommended procedures
1
2
3

7. Do you cooperate with a medical or healthcare practitioner in your pharmacy regarding DRPs?
a) yes
b) no

Note: **DRPs** means Drug Related Problems (e.g. interactions, incorrect use, attitude and behavior of patients, etc.)

7.1 If yes, how often do you do so?
a) daily
b) 2-3 times per week
c) 1-2 times per month
d) 5-6 times per month

8. Do you take records of interventions in your pharmacy (DRPs)?
a) yes
b) no

8.1 If yes, in which form do you take such records?
a) electronic form
b) paper form

9. Do you have a system in your pharmacy that counts the number of patients and their revisits?
a) yes
b) no

9.1 If yes, please make an estimate of proportions of loyal vs random patients in your pharmacy:
a) loyal patients (v %)
b) random patients (v %)

10. In which manner do you offer support/motivate patients towards higher compliance in the recommended therapy? (multiple choice possible)
a) educating patient with the use of available information materials
b) suggesting a cheaper alternative in the alternative form of generic substitution
c) offering consultations (individual counselling)
d) support programs for a larger group of patients - e.g. group discussion, audio-visual programs
e) other (please specify):

Note: **Compliance** means that the patient's behavior follows the treatment recommendation by a healthcare professional (medicine practitioner, nurse, etc.)

11. Do you provide any consultations to patients, or individual counselling on use of medicine?
a) yes
b) no

11.1 If yes, how often do you do so?
a) daily
b) 2-3 times per week
c) 1-2 times per month
d) 5-6 times per month

12. How do you usually identify the risk of non-adherence? What questions do you usually ask the patient? The questions related to: (multiple choice is possible)
a) forgetfulness
b) missing dosage of medicine in case of adverse or side effects occur
c) early termination of therapy

13. In what manner do you usually offer support for adherence? (multiple choice is possible)
a) we provide information about use of medicine
b) we provide the information related to the use of medicine and verify if the patient understands the information
c) we recommend to patient his/her appropriate system to monitoring and recording use of medicines
d) other (please specify)
e) we do not offer such support
Note: **Adherence** means an affirmative attitude on behalf of patient towards treatment, acting in accordance with the pharmacotherapy recommended by the healthcare practitioner. It significantly influences the therapeutic value of the prescribed medicine.

14. In what manner do you offer support for persistence – continuity of treatment – throughout the recommended period of therapy? (multiple choice is possible)
a) we engage the patient by asking questions related to his or her readiness to undertake a long-term treatment
b) we agree with the patient on a continuous cooperation
c) we communicate with a medical practitioner in case we have suspect that the patient deliberately adjusts the length of the recommended pharmacotherapy
d) other (please specify)
e) we do not offer such support
Note: **Persistence** – means continuity throughout the entire treatment (i.e. patient uses medicine throughout the entire therapy).

15. How do you increase trust in patients in order to achieve their concordance? (multiple choice is possible)
a) we provide personal counselling related to the selected drug form, application and dosage
b) we consult with patient the recommended medication dose titration (e.g. due to skin irritation)
c) we identify with the patient his or her options for a routine use of medicines in accordance with the maintenance therapy recommended by a medicine practitioner (e.g. system of notifications, personal monitoring and recording)
d/ we engage the patient in different way (please specify):
e) we do not deal with such issue

Note: **Concordance** means a partnership between patient and pharmacist that leads to an agreement on why and how to use the relevant medicines. Unlike compliance, concordance represents an agreement based on a patient's own conviction, reached as a result of an individual approach and it is based on the provision of adequate information, including an explanation of the expected therapeutic effect and adverse effects, the costs of treatment and other relevant factors.

Figure 1. Questionnaire to community pharmacists.

understood as going beyond the scope of primary (necessary) information provided by individual pharmacists about safe and appropriate use of dispensed medicines.

V. Offering support by pharmacists to follow a recommended pharmacotherapy in order to achieve:

a. Compliance, adherence, persistence and concordance. The terms and definitions refer to a literature review by Vrijens et al. (2012);

b. Reducing the risk of non-adherence – we used four item Morisky Medication Adherence Scale (Morisky et al., 1986), which we modified from the pharmacist's perspective, who responds according to his/her experience and common practice in communication with patients on medication use (Fig. 1, question 12/a-d).

Statistical methodology

To process the results of the questionnaires, we used methods of descriptive and inductive statistics. The core of the questionnaire was formed around the questions of a dichotomic or polytomic character. The final quantities were summarized in a contingency table. To test associations, we used the chi-squared test (χ^2) or exact tests (Fisher and Fisher-Freeman-Halton tests). The differences between groups were tested by the t-test and, in relevant cases, by non-parametric methods (Mann-Whitney test for analysis of variations). We analysed the degree of associations with correlation coefficients: Cramér's V and Goodman and Kruskal's gamma. For certain comparisons, we introduced an odds ratio (OR) with the respective confidence interval, 95% (95% CI). In all

Table 1. Consultancy and resolving DRPs by community pharmacists.

Consultations on DRPs	DRPs		CZ score	P	Cohen Kappa (95% CI)	DRPs		SK score	P	Cohen Kappa (95% CI)
	NO	YES	SUM			NO	YES	SUM		
Yes	19	62	81	0.796	-0.074 (-0.241 to 0.092)	4	34	38	0.00004	0.554 (0.183 to 0.926)
No	6	30	36			3	0	3		
Total	25	92	117			7	34	41		

cases, we tested at a significance level of alpha ≤0.05. We performed the analyses with StatsDirect 2.8.0 (StatsDirect Ltd., Cheshire, UK) and Microsoft Office Excel 2010 (Microsoft Corporation).

Study's limitations

The legal framework for personal data protection of patients, their unavailable medication history and non-existence of the e-Health in CZ and SK limited our study with regards to community pharmacist's support on specific patient, his/her specific type of problem/s and pharmacotherapeutic outcome/s. Therefore, collected data from the pharmacists' responses give an estimate on the medication adherence promotion provided in respective pharmacies participating in the questionnaire survey.

RESULTS

Consultations, counselling and DRPs

The majority of pharmacists in our data set stated that they provide consultations or counselling to patients (CZ: 69.2%, SK: 92.7%). We investigated whether these activities could be associated with resolving DRPs. Unlike CZ, in SK there was a significant and high concurrence in answers (answer type yes-yes) that showed that if SK pharmacists provide consultations, they do so in order to help patients in finding solution to DRPs and, on the other hand, those pharmacists that do not provide consultations, do not engage with DRP-solving activities (answer type no-no), P < 0.0001 (Table 1).

In registering DRPs, we found a statistically significant difference in favour of SK pharmacists (P = 0.008). Pharmacists do register DRPs, although there is no e-Health currently at place in either country. Such registrations therefore tend to be non-systematic and rare. Due to the small number of responses, we could not assess to what extent DRPs are registered in either paper or electronic form. In both countries, pharmacists deal with DRPs at a similar rate of frequency, usually either daily or 2 to 3 times per week (P = 0.052).

Just about half of the pharmacists (50.8%) in both countries register loyal patients and their revisits. Loyal patients represented more than 50% of all patients visiting pharmacies. We did not find a correlation between revisits of patients and resolving DRPs in the data sets.

Support of medication adherence

Each evaluated activity of pharmacists aimed at an improvement of medication adherence, persistence or concordance, was scored with one point. The overall relative score for all activities combined could range from 0 to 4 points. The more intensive the support of a pharmacist was, the higher the score. We did not observe significant differences between CZ and SK in support of the adherence, persistence or concordance. Support for adherence reached the average level with the highest scoring value in both countries. Support for adherence (CZ: 1.95; SK: 1.93) was significantly higher than support for persistence (CZ: 1.18;SK: 1.07), and was also higher than support for concordance (CZ: 1.28;SK: 1.27), (Fig. 2). We used the adopted Morisky scale to estimate with what intensity non-adherence risk could be reduced. We evaluated this from the perspective of the pharmacist, who addresses one or more critical areas of a patient's attitude and behaviour, which can help to uncover patient's compliance with a prescribed pharmacotherapy. In CZ, we measured that the overall average score was marginally higher than for SK. It reached the medium degree of support in both data sets [CZ: 2.29 and SK: 2.22; P = 0.739; 95% CI for the difference in mean scores went from (-0.35) to 0.49]. The difference was attributed to a higher score achieved by CZ pharmacists concerning the reduction of any risk that patient would not comply with the recommended time of medication use (P = 0.032; the lower and upper limits of the 95% CI for the odds ratio were 1.02 and 5.09). Other risk factors were comparable with SK. These include forgetfulness, missing medication or termination of medication use due to adverse reaction or, on the contrary, because therapeutic effect has already been attained (Table 2).

Relationship analysis between estimated support for medication adherence and technical and personnel characteristics of pharmacies

The survey demonstrated that *geographic* parameters of location (in relation to the number of inhabitants) or *size* of pharmacies by the *number of professional employees*, namely,

Table 2. Non-adherence risk reduction from pharmacists-responders´ perspective in the most frequent areas.

Reduction of risk of non-adherence	Country CZ	SK	TOTAL	p	OR 95% CI
1. Forgetfulness					
Yes	64	24	88	0.717	0.855 0.387 to 1.861
No	53	17	70		
2. Not complying with the recommended timing of usage of medicines					
Yes	85	22	107	0.032	2.294 1.022 to 5.091
No	32	19	51		
3. Missing dosages of medicines in case of occurrence of adverse or side effects					
Yes	67	27	94	0.361	0.694 0.304 to 1.541
No	50	14	64		
4. Early termination of treatment after achieving the desired therapeutic effect					
Yes	52	18	70	> 0.999	1.022 0.470 to 2.241
No	65	23	88		
Total score value	2.29	2.22		0.739	95% CI -0.350 to 0.492
n	117	41	158		

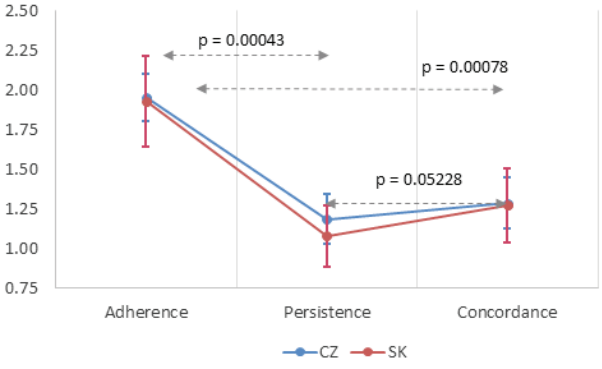

Figure 2. Support of patients' attitude to follow recommended pharmacotherapy.

did not correlate with the estimated degree of support for medication adherence.

The CZ and SK sets of pharmacies are equipped with technical equipment, that is, a *discrete zone given by law and eventually, a consultation room– dedicated for consultancy purpose by law in CZ, not regulated in SK*. The SK pharmacists, while using consultation rooms, were engaged more with adherence-related issues as compared to CZ (P = 0.037; Cramér's V = 0.37). On the other hand, the CZ pharmacists use the consultancy rooms – if they are equipped with – more frequently than SK pharmacists, although at the margin of significance (P = 0.058).

Majority of the survey's respondents had a *qualification* in pharmacy (CZ: 88%; SK: 83%). As far as the number of *years of professional experience (practice)* of the pharmacists (i.e., the respondents) is concerned, our data showed that the respondents mostly had between 20 and 29 years of practice in both data sets. When it comes to the number of years of professional experience, we found a difference between SK and CZ – there is a stronger relationship between the number of years of experience and adherence support in SK (p = 0.029), whereas we could not establish a correlation in CZ (P = 0.175). Moreover, in the SK data set, we also found a strong linear relationship between the number of years of professional experience and medication adherence support (P = 0.0496) (Table 3). We also found a strong correlation between the preferential *source of information* for pharmacists as the Recommended Procedures authorized by the Czech Chamber of Pharmacists (2010) and support for adherence by CZ pharmacists (P = 0.005), in comparison to SK pharmacists, where a similar source of information was not available. We observed, that majority of pharmacists *cooperated with healthcare practitioners* in solving the DRPs in both countries (CZ: 78.6%; SK: 82.9%; P = 0.51; 95% CI 0.253 to 2.023) (Table 4). A close relationship between adherence and *DRPs* was confirmed in the CZ data set (P = 0.00015) as compared to the SK data set, despite Cramer's V showing a weak association (0.334). It is possible, that if data sets were larger, we would not observe the difference between CZ and SK data sets. (Table 5).

DISCUSSION

Results of the questionnaire survey matched with our prediction that pharmacists had been involved in the medication adherence support, as similarly published by other authors (Lau et al., 2010, Santschi et al., 2012, Vlček, et al., 2009). We assume that pharmacists can support patient adherence and they are able to reduce the risk of patients´ non-adherence, reaching in average the medium degree. We found that support for medication adherence has been provided to a greater degree than support for other forms of patients' adherence attitudes towards treatment, such as persistence and concordance. (Herein, we refer to

Table 3. Association between the number of years of pharmacists´ practice and support for medication adherence.

	Support to medication adherence (n = number of pharmacists-responders)								
Number of practice (years)	We inform patient about use of medicines (n)	We provide information and verify if patient understands it (n)	We recommend that patient creates his or her own system of monitoring of use of medicines (n)	Other (n)	We do not provide support to adherence (n)	Sum (n)	Fisher-Freeman-Halton exact P	Good man-Kruskal gamma	P
CZ									
0 – 9	1	3	7	3	4	18	0.294	0.135 (0.06 to 0.33)	0.175
10 – 19	5	5	17	9	4	40			
20 – 29	2	7	8	11	7	35			
> 29	2	0	9	10	3	24			
Total	10	15	41	33	18	117			
SK									
0 – 9	1	4	0	2	0	7	0.024	0.348 (0.04 to 0.66)	0.029
10 – 19	1	1	8	2	1	13			
20 – 29	1	1	0	3	2	7			
> 29	1	3	2	3	5	14			
Total	4	9	10	10	8	41			

Table 4. Interdisciplinary teamwork between community pharmacists and healthcare practitioners.

Cooperation between pharmacist and a healthcare practitioner on DRPs (n = number of pharmacists-responders)						
Cooperation	CZ		SK			Fisher exact
	n	%	n	%	Total	
Yes	92	78.6	34	82.9	126	P = 0.51
No	25	21.4	7	17.1	32	95% CI: 0.253 to 2.023
Total	117	100.0	41	100.0	158	

Table 5. Adherence support and resolving DRPs by community pharmacists.

	Association between adherence support and DRPs resolving by pharmacists (n = number of pharmacists-responders)							
Adherence and DRPs	We do not provide support to adherence We inform patient about use of medicines (n)	Forgetfulness (n)	Not complying with the recommended timing of usage of medicines (n)	Missing dosages of medicines in case of occurrence of adverse or side effects (n)	Early termination of treatment after achieving the desired therapeutic effect (n)	Sum (n)	Fisher-Freeman-Halton exact P	Cramér's V
CZ								
Yes	3	14	28	30	17	92	0.00015	0.4553
No	7	1	13	3	1	25		
Total	10	15	41	33	18	117		
SK								
Yes	4	7	7	10	6	34	0.3561	0.3345
No	0	2	3	0	2	7		
Total	4	9	10	10	8	41		

terms and definitions on medication adherence used in the questionnaire by Vrijens et al. (2012)). As the issues of persistence and concordance are more complicated – the processes are more complex and demanding (professionally, in terms of time or work organization). We estimate that pharmacists supported patients only at a low degree of intensity in these activities. In general, this degree of support could change if pharmacists were more actively, or even pro-actively, engaged in the cooperation with patients and healthcare practitioners in both countries. It is most probably associated with education and further training, their motivation, as well as availability of specific intervention package/s motivational interviews with patients, patients' education, home medicines review, dose administration aid, medication use review, etc.), enabling the pharmacists to improve patient adherence (Lau et al., 2010, Salvo & Cannon-Breland, 2015).

We found that the location of a pharmacy and the number of inhabitants in a location does not influence support for medication adherence in either country (for pharmacies located in larger cities or smaller municipalities). As far as technical factors were concerned, we focused on discrete zones and consultation rooms and their use. In SK, where discrete zones are mandatory components of pharmacies (Decree No 129/2012), consultation rooms are additional spaces not required by law. The outcomes suggested that there is an association between support for adherence (DRPs) and the usage of consultation rooms, which has been stated by Masaryková et al., 2015 too. In CZ, only pharmacies that actively provide consultations and counselling have such consulting rooms (Decree No 92/2012). Despite strong differences in terms of the legislative requirements in the two countries, our finding showed that CZ pharmacies use consultation rooms more frequently - though the frequency was only marginally higher than SK pharmacies. This finding indicates that legislation is not the unique factor that influences the extent to which pharmaceutical care is provided. In this respect, other factors, both personal and professional, also have significant importance. The difference in the legislative and societal development of pharmaceutical care in the two countries reveals potential that we could elaborate upon to increase support for medication adherence by pharmacists.

From the perspective of personnel characteristics of pharmacy, the degree of support for adherence was not dependent upon the number of professional workers. However, in the SK data set, we found that the number of years of professional experience increase the probability that the pharmacists approach patients, discuss a prescribed treatment and try to help to resolve patients' DRPs. We assume, that SK pharmacies, in comparison to CZ pharmacies, provide such a kind of consultations that are closely linked to resolving DRPs in collaboration with healthcare practitioners.The importance of interdisciplinary cooperation in DRPs (most notably effective interaction between community pharmacist and healthcare practitioner/clinic setting) has been often highlighted at scientific symposia and discussed by more authors (Pape et al., 2011, Rojaz-Fernandez et al., 2014). According to recently published data from SK pharmacies by Masaryková et al., the DRPs most frequently relate to poor treatment efficacy and therapy costs (2015). Non-adherence very likely represents the common denominator of these problems. The risk of non-adherence should be detected and resolved responsibly, whereas cooperation between pharmacists and healthcare practitioners gives a solid ground for furnishing effective and prompt solutions to the identified problems. To examine other possibilities of how to increase support for patient adherence, the specific profile of risk patients could be considered, which the pharmacists could focus on.

Our findings indicated that there is a close association between support for medication adherence and personnel capacities (factors) of pharmacy. These factors mostly concern the professional experience of pharmacists and the quality of consultation and counselling, which might be efficiently supported by training and/or authorized sources of recommendations for pharmacists such as Recommended Procedures of the Czech Pharmacists' Association available since 2010. Recently, the Association opened new courses in DRPs, enabling additional services, which include recommendations in terms of dosage and therapeutic regime. The Association is planning to extend its recommendations into other areas of pharmaceutical professional consultancy. Since 2013, the Czech Chamber of Pharmacists offers a mobile application mapping the pharmacies with consultation rooms. All these initiatives, as mentioned hereby, represent useful electronic tools that could enable the pharmacists to address patients' adherence and vice-versa, in more effective way.

In general, we can assume that the support for medication adherence is provided in both CZ and SK pharmacies. It reaches a medium degree and develops in the right direction – towards concordance in the pharmacist-patient relationship. The responses to our survey suggest that the development of interdisciplinary cooperation with other healthcare professionals, mostly medical practitioners, remains challenging (non-systematic and rare registration of DRPs). The e-Health system will soon be introduced in both countries, and is expected to become an important factor in this dimension, as it will enable connection with relevant data and information. It is also recognizable that recommended practice, education in DRPs and life-long education of pharmacists represent important pre-requisites for further development of pharmaceutical care in medication adherence support.

CONCLUSION

We analysed the current state of provision of counselling and consultation services aimed at supporting medication adherence by community pharmacies through a questionnaire

survey in CZ and SK. Based on the pharmacists' responses, we assumed a medium degree of support for patient adherence, predominantly provided in order to resolve DRPs in both countries. We can also point out that education and experience for pharmacists could be one of the crucial factors in ensuring support for adherence. Its progress might also be accelerated through the use of information technologies, as research in multiple countries has also demonstrated recently. We conclude that our findings can offer a solid base for further investigations aimed at evidence-based interventions in this area, and so to contribute to pharmaceutical care development.

DECLARATIONS

List of Abbreviations

CI: Confidence Interval **CZ:** Czech or Czech Republic **DRPs:** Drug-related problems e-**Health:** Electronic and computerized healthcare system **FaF UK:** Faculty of Pharmacy, Comenius University in Bratislava or Faculty of Pharmacy in Hradec Králové, Charles University **OR:** Odds Ratio **SK:** Slovak or Slovak Republic **WHO:** World Health Organization **χ^2:** chi-squared test

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Restriction on the re-export of medicinal products and the supervision of compliance with it by public administration bodies

Original Paper

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Abstract After years of mainly expert discussions (not only in the Slovak legal environment), the concept of and legislation on re-export offer a sort of solution provided for in the Act no. 306/2016 Coll. Said act amends the key legislation in this field, namely the Act no. 362/2011 Coll. on Medicinal Products and Medical Devices and on the amendment of certain acts, as amended (hereinafter referred to as the "Act on Medicinal Products") and the Act no. 363/2011 Coll. on the Scope and Conditions of Payments for Medicinal Products, Medical Devices and Dietetic Foods from Public Health Insurance and on the amendment of certain acts, as amended (hereinafter referred to as the "Act on Payments"). The topic of the paper belongs in the area of medicinal products and pharmaceutical services, it offers, however, significant overlaps in the area of the constitutional, administrative and European law and is aimed at multidisciplinary research into the issue of the reverse export (re-export) of medicinal products. Besides these laws, also the Constitution of the Slovak Republic ("SR") and the sources of the European Law have to be taken into account in relation to the subject in question. The main aim of legislation in this area of law was restriction on the re-export of selected products and protection of patients from adverse impacts of such business activity. The aim of the paper is the authors' effort to analyse the issue of the re-export of medicinal products within the context of the adopted Act no. 306/2016 Coll., whose legislative solution is inevitable for the protection of life and health of the population of the Slovak Republic.

Keywords *Re-export of medicinal products – pharmaceutical services – protection of life and health – right to free enterprise – administrative law*

METHODS

Paper contains analysis and comparison of the legislation – Slovak law and European Union law - in the field of medicinal products.
Paper defines problems of legislation in terms of legal practice.
Paper gives synthesis of the findings, summarisation and drawing of conclusions (in order to protect a public interest (patient)).

INTRODUCTION

The organisation of health care, provision of health care or the assurance of the right to health from the point of view of the comparative constitutional law constitutes only a rare subject of legislation in the member states of the European Union. If the Constitution of the Slovak Republic contains provisions that prove that its proposers did not fully understand social changes that were supposed to have taken place after the November of 1989 as a natural and inevitable

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transition from the socialist to the capitalist society, then such provisions especially include the blunt wording of Article 40 of the Constitution of the SR. If in the phase of interpretation and application of the law the SR has legal provisions on fundamental rights and freedoms that are far from being applied, thus indicating shortcomings in the fulfilment of the model of material rule of law, it is again the provisions of Article 40 of the Constitution of the SR. When the Constitution of the SR was being adopted, the then Slovak National Council contented itself with a wording granting everybody the right to the protection of health. On the basis of health insurance, the right to free health care and to medical devices is only guaranteed to citizens of the SR under conditions set out in the law. Pharmaceutical care, i.e. especially the availability of medical products, forms an integral part of this right guaranteed by the constitution. (Drgonec, 2012)

However, via Article 55 (2) of the Constitution of the SR the Slovak Republic protects and supports competition, which is an inevitable part of every market economy. However, the behaviour of undertakings is not always in conformity with ethical principles. The right to conduct free enterprise is also interpreted very extensively by many distributors of medicinal products, who aim to maximise their profits and seek to export selected types of medicinal products abroad after their import into to the territory of the SR instead of supplying them to pharmacies and hospitals. Available information shows that more medicinal products were re-exported in 2015 than in 2014, when this figure exceeded one million packages. These alarming figures were provided by the State Institute for Drug Control (Štátny ústav pre kontrolu liečiv - ŠÚKL), to which planned exports have to be reported. Its figures show that 589 medicinal products numbering nearly 1.4 million packages were exported from the Slovak Republic in 2015, which is nearly 400 thousand more than in 2014. In 2016 these figures grew again, with 700 medicinal products in more than 1.2 million packages in the first two quarters of 2016. The exporters' target was countries of Western Europe, where their price is incomparably higher. (TERAZ.SK, 2016)

The State Institute for Drug Control was authorised to prohibit the export of a medicinal product pursuant to already ineffective legislation, i.e. only until 31 December 2016, however, only if it threatened to be unavailable on the domestic market. For comparison, according to the opinions of the ŠÚKL, on the basis of import, export and planned export figures, it prohibited the export of 27 medicinal products in 2014 with 182 decisions, which represented 57,446 packages and in 2016 it prohibited the export of a medicinal product as many as 1,700 times. (Dôvodová správa, 2016)

The Ministry of Health of the SR identified several problems causing the unavailability of medicinal products. These especially include bad logistics between the pharmacy and the distributor, problems in the production of a medicinal product or in the distribution chain, as well as the undeniable fact that imported medicinal products are re-exported abroad. The re-export of medicinal products, which substantially contributed to the unavailability of certain medicinal products in Slovakia, especially includes medicinal products for treatment of serious diseases such as epilepsy, schizophrenia, Parkinson's disease or several medicinal products for treatment of oncologic diseases.

Also the Slovak Chamber of Pharmacists has been pointing to this negative trend for years, saying that significantly lower prices in comparison to other EU states, the ability of pharmacies to sell medicinal products also to entities other than patients, as well as the ability of distributors to export medicinal products abroad were the basis for the re-export of medicinal products, which were subsequently unavailable to Slovak patients. It is possible to agree with the opinion of the Association of Innovative Pharmaceutical Industry (Asociácia inovatívneho farmaceutického priemyslu - AIFP), which says that this situation was caused mainly by the fact that prices of medicinal products in Slovakia are some of the lowest in the world, which makes our market attractive for distribution firms. Member companies of the AIFP do not take part in the re-exports and always import a sufficient quantity of medicinal products to Slovakia as determined by statistical estimates of morbidity.

The Slovak Republic was the only one of member states of the European Union to legislatively introduce the possibility to prohibit the export of medicinal products, which is why their export has been monitored since 2013. The European Commission believes that this legislative measure is contrary to the legal order of the European Union, which led to the launch of infringement proceedings against the Slovak Republic. By letter of March 2015 the European Commission asked the SR to provide an answer to justify its viewpoint and demonstrate the compliance of the Slovak legislation with the EU law, especially in relation to the rules on free movement of goods. (Dôvodová správa, 2016)

LEGISLATIVE MEASURES

Despite the opposition by the European Commission the Ministry of Health of the SR developed a draft law amending:

- the Act no. 362/2011 Coll. on Medicinal Products and Medical Devices and on the amendment of certain acts, as amended (hereinafter referred to as the "Act on Medicinal Products") and
- the Act no. 363/2011 Coll. on the Scope and Conditions of Payments for Medicinal Products, Medical Devices and Dietetic Foods from Public Health Insurance and on the amendment of certain acts, as amended (hereinafter referred to as the "Act on Payments").

The aim of this law is to prevent the re-export of medicinal products and remove the reservations of the European Commission, thus bringing the Slovak legislation regarding medicinal products for human use into compliance with the Treaty on the Functioning of the European Union. Said

draft law was submitted by the Government of the SR to the National Council of the SR for approval as part of its legislative initiative. The Parliament approved this draft law, which was subsequently promulgated under document number 306/2016 in the Collection of Laws of the Slovak Republic on 23 November 2016 with an effective date from 1 January 2017 (hereinafter referred to as the "amendment"). The vacation legis, i.e. the time period for the addressees to acquaint themselves with the content of this new act, is 38 days, which is sufficient given the extensive nation-wide discussion (Mrva et al., 2009)

CONTENT AND SIGNIFICANCE OF THE AMENDMENT

Right at the outset the amendment supplements the Act on Medicinal Products with the new term "medical prescription in an anonymised form". It is a copy of an issued medical prescription through which a medicinal product for human use included in the list of categorised medicinal products was prescribed to a patient and on which the holder of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy or a person authorised by a holder of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy anonymises the patient's personal information. The pharmacy subsequently attaches this copy of a medical prescription to the order addressed to the registration holder via an information system providing automated electronic placement, reception and confirmation of orders of medicinal products for human use included in the list of categorised medicinal products created and operated by the registration holder.

The Act toughens sanctions regarding when after a repeated infringement of an obligation imposed by this act a licence issued to a holder of a licence for the wholesale distribution of medicinal products for human use and to a holder of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy is obligatorily legally revoked. Further, the words "exporter of a medicinal product" were deleted from the Act on Medicinal Products, because only a holder of a licence to manufacture medicinal products may be an applicant for the issue of a certificate that the manufacturer of a medicinal product is a holder of a licence to manufacture medicinal products and that the holder of a licence to manufacture medicinal products meets applicable provisions of the World Health Organization relating to the manufacture of medicinal products intended for export to third states. It is specified more precisely, to which entities a holder of a licence for the wholesale distribution of medicinal products for human use may supply medicinal products for human use included in the list of categorised medicinal products. The obligation to deliver an ordered medicinal product to a pharmacy within 24 hours imposed on a holder of a licence for the wholesale distribution of medicinal products for human use has been deleted, too. This obligation is only imposed on the holder of the registration of the ordered medicinal product.

New obligations are imposed on a holder of a licence for the wholesale distribution of medicinal products for human use. A holder of a licence for the wholesale distribution of medicinal products for human use is, just as a holder of the registration of a medicinal product, obliged to supply a categorised medicinal product only to a holder of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy or to another holder of a licence for the wholesale distribution of medicinal products for human use. Should it supply a categorised medicinal product to another holder of a licence for the wholesale distribution of medicinal products for human use, it is obliged to supply the medicinal product to it only for the purposes of final delivery to a holder of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy.

As part of resale, a holder of a licence for the wholesale distribution of medicinal products for human use is authorised to return excessively categorised medicinal products to the holder of their registration, which constitutes the only exception when these medicinal products may be directed elsewhere than to a pharmacy or another wholesale distributor. Upon request, a holder of a licence for the wholesale distribution of medicinal products for human use is obliged to submit to the Ministry of Health of the SR records of the receipt of medicinal products for human use included in the list of categorised medicinal products and their deliveries:

- to holders of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy,
- to other holders of a licence for the wholesale distribution of medicinal products for human use and
- to holders of the registration of these medicinal products in the case of their resale

or data from these records in an electronic format allowing automated processing. Furthermore, a holder of a licence for the wholesale distribution of medicinal products for human use is obliged to keep and retain documentation showing to which qualified entities it has supplied categorised medicinal products and is obliged to submit this documentation or requested data therefrom upon request to the Ministry of Health of the SR in an electronic format. These new obligations imposed on a holder of a licence for the wholesale distribution of medicinal products for human use, relating to categorised medicinal products, are meant to prevent the export of these medicinal products and are in compliance with Article 81 of the Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use. We believe that this provision does not prevent the application of stricter requirements laid down by the member states in relation to the wholesale distribution of: narcotic drugs or psychotropic substances in their territory, medicinal products manufactured from blood, immunological medicinal products and radioactive medicinal products.

EXPORT OF A MEDICINAL PRODUCT FOR HUMAN USE INCLUDED IN THE LIST OF CATEGORISED MEDICINAL PRODUCTS

Legal philosophy notes that no power may reach further than its purpose requires. The power holder - in this case the state - must respect the dignity of every person and its fundamental civil rights, i.e. also the right to conduct business, which may be statutorily restricted but cannot be abolished (Peráček 2014). This doctrine was respected also by the legislator and the provision of Section 19a of the Act on Medicinal Products was substantially changed by the amendment with the aim to regulate the issue of the export of a medicinal product for human use with a new wording deleting provisions which the European Commission criticized most as discriminatory and unfair. Pursuant to Section 19a of the Act on Medicinal Products, the export of a medicinal product for human use included in the list of categorised medicinal products from the Slovak Republic is understood to mean the export of a medicinal product for human use included in the list of categorised medicinal products to another member state or to a third state. However, the legislator also defined this concept negatively enshrining in the legislation that the resale of a medicinal product for human use included in the list of categorised medicinal products or the return of a medicinal product for human use included in the list of categorised medicinal products, namely due to the assertion of claims for defects of the medicinal product for human use delivered or due to the withdrawal of a medicinal product for human use from the market, is not considered as export. It would constitute disproportionate interference with the buyer's rights, especially in the case of liability for the seller's, i.e. the distributor's defects. A medicinal product for human use included in the list of categorised medicinal products may only be exported by the holder of a licence to manufacture medicinal products that has manufactured the medicinal product being exported, the holder of the registration of this medicinal product or a holder of a licence for the wholesale distribution of medicinal products for human use may also only export if it has been authorised to export a medicinal product for human use included in the list of categorised medicinal products by the holder of the registration of this medicinal product via a written power of attorney. From the point of view of the civil law theory, power of attorney constitutes a unilateral legal act addressed to third persons which certifies the existence and scope of the authorised representative's authorisation to act on behalf and for the person represented. (Cirák et al., 2009) Act is issued separately for each export of a medicinal product for human use included in the list of categorised medicinal products and must contain the name of the medicinal product for human use, its code assigned by the State Institute, the size, the number of packages of the medicinal product for human use and the batch number, the name of the state into which the medicinal product for human use is being exported and the date by which the export is supposed to take place. Furthermore, the holder of the registration of the medicinal product for human use is obliged to notify its export in an electronic format to the State Institute not later than seven days after the export takes place. After the reception of this notification, the State Institute immediately publishes the export of the medicinal product for human use included in the list of categorised medicinal products on its website. A special situation applies to the export of a medicinal product for human use included in the list of categorised medicinal products for the needs of the Armed Forces of the Slovak Republic, Armed Security Corps and the Fire and Rescue Corps to ensure the fulfilment of their tasks outside the territory of the Slovak Republic under specific legislation.

The resale of medicinal products, which pharmacies normally used to perform as one of their activities, namely to any holder of a licence for the wholesale distribution of medicinal products for human use, is deleted from the pharmaceutical care. The medicinal product may only be resold to the licence holder the pharmacy purchased the medicinal product from.

The exchange of medicinal products for human use from the list of categorised medicinal products is only possible between holders of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy, but only for the purposes of their issue in a public or hospital pharmacy.

The aim of these measures delimiting the scope of activities carried out in a public or hospital pharmacy is to prevent the export of categorised medicinal products, which a holder of registration supplied to the market in the Slovak Republic for the purposes of their issue to the patient in said pharmacies. The problem was that many holders of a licence for the wholesale distribution of medicinal products for human use purchased categorised medicinal products from holders of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy and subsequently exported them.

New obligations are imposed on a holder of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy. The most important of them is the issue of a categorised medicinal product to a patient. The following are also allowed:

- the resale of these medicinal products to a holder of a licence for the wholesale distribution of medicinal products for human use who supplied them to a holder of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy,
- the exchange of medicinal products for human use included in the list of categorised medicinal products between holders of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy for the purposes of their issue in a public or hospital pharmacy.

Another obligation imposed on a holder of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy is the obligation to order medicinal products for

human use included in the list of categorised medicinal products from the holder of their registration via an information system providing automated electronic placement, reception and confirmation of orders of medicinal products for human use included in the list of categorised medicinal products if it requires the holder of the registration of a medicinal product for human use to deliver a medicinal product for human use included in the list of categorised medicinal products within 24 hours after the order reception. An anonymised medical prescription has to be attached to the order.

A holder of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy is obliged to maintain for 10 years records of holders of a licence for the wholesale distribution of medicinal products for human use and holders of the registration of medicinal products for human use from whom it acquired medicinal products for human use included in the list of categorised medicinal products and submit these records or data therefrom upon request to the Ministry of Health of the SR in an electronic format allowing automated processing of these data.

The registration holder has some more obligations e.g. the creation and operation of an information system for ordering medicinal products for which it holds registration. The database provides the holder of the registration of medicinal products for human use with an overview of holders of a licence to provide pharmaceutical care in a public or hospital pharmacy to which it supplied medicinal products included in the list of categorised medicinal products within 24 hours on the basis of an anonymised medical prescription. It is obliged to maintain this information system in operable condition and in case of its outage it is obliged to receive and confirm orders of medicinal products for human use included in the list of categorised medicinal products also in a different manner that will allow it to ensure the delivery of the medicinal product to the pharmacy within 24 hours.

It is further required to deliver medicinal products for human use included in the list of categorised medicinal products within 24 hours to holders of a licence to provide pharmaceutical care in a public or hospital pharmacy on the basis of an order via an information system with an anonymised medical prescription attached. This obligation does not apply to a holder of the registration of a medicinal product for human use who has claims against a holder of a licence to provide pharmaceutical care in a public or hospital pharmacy for delivered medicinal products included in the list of categorised medicinal products after double expiration of the contracted payment due date. The registration holder is obliged to designate as well as notify to the Ministry of Health of the SR a person responsible for the delivery of medicinal products for human use included in the list of categorised medicinal products residing or having its registered office in the territory of the Slovak Republic, if the holder of the registration of medicinal products for human use does nor reside or does not have its registered office in the territory of the Slovak Republic.

The registration holder is obliged to maintain records of holders of a licence for the wholesale distribution of medicinal products for human use and of holders of a licence to provide pharmaceutical care in a public pharmacy or hospital pharmacy to which it supplied medicinal products included in the list of categorised medicinal products. It is obliged to retain also these records for 10 years and submit these records or data therefrom upon request to the Ministry of Health of the SR in an electronic format allowing automated processing of these data. The registration holder is obliged to supply a mandatory medicinal product for human use included in the list of categorised medicinal products to a holder of a licence for the wholesale distribution of medicinal products for human use solely for the purposes of final delivery to a holder of a licence to provide pharmaceutical care in a public pharmacy or in a hospital pharmacy.

The Act grants the Ministry of Health of the SR new powers to publish on its website the name, surname and address of the person responsible for the delivery of medicinal products within 24 hours if it is a natural person or the name or business name and registered office of the person responsible for the delivery of medicinal products within 24 hours if it is a legal person and its contact details consisting of the e-mail address and the mobile phone number.

SUPERVISION BY ADMINISTRATION BODIES AND IMPOSITION OF SANCTIONS FOR INFRINGEMENT OF THE ACT

The application of the Act added several subject matters of different administrative offences for the infringement of new obligations imposed on the registration holder contained in Section 138 of the Act on Medicinal Products. The Ministry of Health of the SR has new powers to impose fines for different administrative offences regarding the new obligations imposed on a holder of a licence for the wholesale distribution of medicinal products for human use. The procedure for accountability for a different administrative offence is based on the Act on Medicinal Products (special act) in combination with proceedings pursuant to the Code of Administrative Procedure (general legislation) (Horvat, 2014).The Act no. 363/2011 Coll. on the Scope and Conditions of Payments for Medicinal Products, Medical Devices and Dietetic Foods from Public Health Insurance and on the amendment of certain acts has been amended by the Act no. 460/2012 Coll. and the Act no. 265/2015 Coll. has been amended with the aim to bring sanctions pursuant to this act into compliance with different administration offences pursuant to the Act on Medicinal Products.

The Act on Medicinal Products provided significant room for the exercise of the powers of state administration bodies in the field of health care even before the adoption of the Act. The exercise of state administration in the field of human pharmacy is performed by the Ministry of Health of the SR and the State Institute for Drug Control and the state

administration in the field of human pharmacy in a self-governing region is performed as its transferred exercise also by the self-governing region. The Act contains a fairly broad range of sanctions for offences in its eighth part. Proceedings on offences in the field of human pharmacy and their hearing are covered by the Act no. 372/1990 Coll. on Offences, as amended. Besides, public administration bodies may impose sanctions (a fine) up to EUR 1 million for different administrative offences according to the gravity of the misconduct using the procedure laid down in the Act no. 71/1967 Coll. on Administrative Proceedings (Code of Administrative Procedure), as amended. However, within the administrative judicial system, persons concerned may require the court to review the lawfulness of the decision to impose a sanction. (Ficová et al., 2010)

BRINGING THE ACT INTO COMPLIANCE WITH EUROPEAN UNION LAW

One of the fundamental principles of the functioning of the European Union is the development of social policy also in member states. This idea is contained in the Treaty of Rome of 1957 establishing the European Economic Community (Vojtech & Levický, 2016). Pursuant to the second sentence of Article 7 (2) of the SR Constitution, legally binding acts of the European Communities and European Union take precedence over the laws of the Slovak Republic, which was also respected by the legislator, which is why the wording of the Act is fully in accordance with European Union law (Gregušová & Peráček, 2015). The free movement of goods, which includes medicinal products, constitutes the first of the four fundamental freedoms of the internal market, which derives its legal basis from Articles 26, 28 to 37 of the Treaty on the Functioning of the European Union. It is guaranteed especially by the abolition of the customs duties and quantitative restrictions as well as by the prohibition of measures having equivalent effect. To support the completion of the internal market, principles of mutual recognition, abolition of physical and technical constraints and standardisation support were added. The adoption of the new legislative framework in 2008 significantly reinforced the marketing of products, the free movement of goods, the system of surveillance over the European Union market and the CE marking. The right to the free movement of goods originating in member states and third countries that are in free circulation in member states is one of the fundamental principles of the Treaty (Article 28 of the TFEU). Originally, the free movement of goods was considered to be part of the customs union between the member states, which included the abolition of customs duties, quantitative restrictions and measures having equivalent effect and the introduction of a common customs tariff of the Community. Later, attention was focused on the abolition of all remaining constraints of free movement with the aim to create an internal market - space without internal borders in which goods could move just as freely as on the national market (Nováčková & Milošovičová, 2011).

CONCLUSION

Legal opinions of the issue of the limitation of the free movement of medicinal products within member states are not and will never be uniform. It is possible to agree with the view that freedom to conduct business cannot be understood as an absolute right without any limitation. (Mucha et al., 2016) It is because this freedom also has its own content, which may be affected by a state intervention which may not necessarily be unlawful or discriminatory from the point of view of the Community law. From the point of view of exporters of medicinal products, it is evident that they understand such intervention by the legislator as a denial of the foundation of free enterprise when they are stripped of the possibility to export medicinal products from the Slovak Republic to another EU member state. Such intervention may, however, only be justified by legitimately invoking the general nation-wide interest because the task of a fair society and its institutions, on which it is based, is to develop a framework where individuals will be guaranteed conditions inevitable for them to achieve what they themselves consider good, i.e. the right to available health care (Chovancová, 2009). The right to conduct business in the area of import and export of medicinal products is not generally being prohibited by the legislator; it is only being limited in certain situations as the Slovak Republic guarantees everybody the right to the protection of health pursuant to the first sentence of Article 40 of the SR Constitution. This right, which applies both to the citizens of the Slovak Republic and to foreigners regardless of their nationality, must always take precedence over anybody's right to conduct business and make profit regardless of possible consequences (Peráček et al., 2016).

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Legislation

– zákon č. 71/1967 Zb. o správnom konaní (správny poriadok) v znení neskorších predpisov
– zákon SNR č. 372/1990 Zb. o priestupkoch v znení neskorších predpisov
– ústavný zákon SNR č. 460/1992 Zb. Ústava Slovenskej republiky v znení neskorších predpisov
– zákon č. 306/2016 Z. z., ktorým sa mení a dopĺňa zákon č. 362/2011 Z. z. o liekoch a zdravotníckych pomôckach a o zmene a doplnení niektorých zákonov v znení neskorších predpisov a ktorým sa mení zákon č. 363/2011 Z. z. o rozsahu a podmienkach úhrady liekov, zdravotníckych pomôcok a dietetických potravín na základe verejného zdravotného poistenia a o zmene a doplnení niektorých zákonov v znení neskorších predpisov,
– zákon č. 362/2011 Z. z. o liekoch a zdravotníckych pomôckach a o zmene a doplnení niektorých zákonov v znení neskorších predpisov,
– zákon č. 363/2011 Z. z. o rozsahu a podmienkach úhrady liekov, zdravotníckych pomôcok a dietetických potravín na základe verejného zdravotného poistenia a o zmene a doplnení niektorých zákonov v znení neskorších predpisov.

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Stability Of Sildenafil Citrate Oral Suspension With Syrspend® Sf

Original Paper

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Abstract Aim: Sildenafil citrate is a drug used to treat erectile dysfunction (ED) and pulmonary arterial hypertension (PAH). As for both clinical applications, the only available dosage form is tablets; there is a clear need for a safe oral liquid, especially for children. The objective of this study was to determine the stability of sildenafil citrate in SyrSpend® SF PH4, a suspending agent containing neither sorbitol nor alcohol.
Material/Methods: The studied sample was compounded into a 2.5-mg/mL suspension and stored in low-actinic plastic bottles at temperatures between 2 and 8°C and at room temperature conditions. Six samples were assayed at each time point out to 92 days by a high-performance liquid chromatography (HPLC) method. The method was validated for its specificity through forced-degradation studies.
Results: The samples remained within 90–110% of the initial concentration throughout the course of the study.
Conclusions: On the basis of the data collected, the beyond-use date of this product is at least 92 days when protected from light at both refrigerated and room temperature storage conditions.

Keywords *Compatibility – Stability – Compounding – Oral suspension – SyrSpend – Sildenafil*

CONFLICT OF INTEREST

This work has been conducted under the sponsorship of Fagron.

INTRODUCTION

Sildenafil citrate is used to treat erectile dysfunction (ED) in adult males and pulmonary arterial hypertension (PAH) in both children and adults. National Institute of Health (NIH) has defined ED as the permanent inability of a man to attain and/or maintain penile erection sufficient to permit satisfactory sexual intercourse (NIH, 1993). Abdo et al. (2006) defined it as a condition clearly compromising the quality of life. The main treatment for ED is the use of oral phosphodiesterase type 5 (IF5) inhibitors, amongst which sildenafil citrate is best known and most widely used (Toledo, 2013; Afif-Abdo, 2007). IF5 inhibitors increase relaxation of smooth muscle and dilation of the sinusoids body, resulting in an increased blood flow, allowing penile erection (Porst et al., 2012; Montorsi et al., 2010). PAH is characterised by progressive destruction of the pulmonary vasculature and associated with high morbidity and mortality, especially in children (Tissot & Beghetti, 2009). Sildenafil is most widely used in PAH by relaxing the blood vessels in the lungs and reducing blood pressure and has contributed to improved survival in this group, from a historical less-than-1-year survival in untreated children with severe PAH in the 1980s to a 97% 5-year survival rate (Tissot & Beghetti, 2009; Martínez et al., 2003; Humpl et al., 2005; Keller et al., 2004; Kothari & Duggal, 2002; Ladha et al., 2005; Leibovitch et al., 2007; Namachivayam et al., 2006; Barnett & Machado, 2006). However, it must be conceded that most of the agents mentioned are used off-label in children

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and the use is based mainly on experience in adults with PAH (Dodgen & Hill, 2015). For both ED and PAH, the only commercially available dosage form of sildenafil citrate is tablets. Sildenafil is available as a tablet only and the dosage in newborns ranges from 0.3 to 8 mg/kg/day (Huddleston et al., 2009). In addition, up to 22.4% of the adult population have swallowing difficulties, which highlights the importance of liquid formulations with adequate quality and stability for both paediatric patients and adults (Lau et al., 2003; Schirm et al., 2003).

The objective of this study was to determine the stability of sildenafil citrate in SyrSpend® SF, a suspending agent containing neither sorbitol nor alcohol or other excipients to be avoided in children (Gershanik et al., 1982). Currently, the stability of a large number of other active pharmaceutical ingredients (APIs) has already been shown in SyrSpend® SF (Vu et al., 2008; Geiger et al., 2012a, 2012b; Sorenson & Whaley, 2013; Geiger et al., 2013a, 2013b; Sorenson et al., 2012; Whaley et al., 2012a, 2012b; Voudrie & Allen, 2010; Voudrie et al., 2011; Geiger et al., 2015; Ferreira et al., 2016; Polonini et al., 2015; Polonini et al., 2016a, 2016b, 2016c). Sildenafil stability in SyrSpend® SF (2.5 mg/mL) was assessed throughout a 92-day period at both controlled refrigerated (2–8°C) and room temperature.

METHODS

Chemical Reagents

Sildenafil citrate raw powder was received from Fagron US (Lot 0911227111; St. Paul, Minnesota). SyrSpend® SF PH4 (liquid) was received from Fagron US – formally Gallipot (Lot 1110358V14; St. Paul, Minnesota). High-performance liquid chromatographic (HPLC) grade acetonitrile (Lot DG353; Honeywell Burdick and Jackson, Muskegon, Michigan), dibasic sodium phosphate heptahydrate (Lot 115824; Fisher, Fairlawn, NJ) and phosphoric acid (Lot 40350080404; CCI, Columbus, Wisconsin) were used in this study. HPLC-grade water was supplied by filtering deionised water from a Millipore Elix through a Millipore Simplicity (Billerica, Massachusetts).

Equipment and Chromatographic Conditions

Two different types of HPLCs were used. The first, used for validation and the stability study, was a Perkin Elmer 200-Series (Waltham, Massachusetts) equipped with a quaternary gradient solvent delivery system, a dual wavelength UV/VIS detector, a 100-vial programmable autosampler with a Peltier tray, 200-µL sample loop and a 250-µL syringe. The second HPLC system, used for forced degradation studies, was a Varian Prostar (Palo Alto, California) equipped with a tertiary gradient solvent delivery system, a photodiode array detector (PDA), an 84-vial programmable autosampler with a 100-µL sample loop and a 250-µL syringe. The Perkin Elmer HPLC was operated and the data was collected using Perkin Elmer Totalchrom chromatography software, whilst the Varian HPLC used Galaxie chromatography software. The mobile phase for the HPLC method was prepared by adding 5.3 g of dibasic sodium phosphate heptahydrate to 650 mL of HPLC-grade water and 350 mL of HPLC-grade acetonitrile. The mobile phase was adjusted to pH 6.5 with 85% phosphoric acid and delivered at 1.5 mL/min. Chromatographic separation was achieved using a 150 mm × 4.6 mm Phenomenex (Torrance, California) Gemini C8 column with 5-µL particle packing. The mobile phase was used as solvent to dilute the standard and assay preparations to 25 µg/mL sildenafil citrate. The assay was monitored following a 100-µL injection.

Validation of Forced-Degradation Studies to Determine the Characteristics of the HPLC Method

Sildenafil citrate samples were stressed and assayed at 293 nm to determine the specificity of the HPLC method to any possible degradation product produced during the storage of an oral suspension. Sildenafil citrate was diluted to 25 µg/mL in solutions of acid (0.1M HCl), base (0.1M NaOH) and hydrogen peroxide (3.5%), in addition to exposure to ultraviolet light at 365 nm and heat at 70°C for 3 h. Any extraneous peaks found in the chromatogram were labelled and the resolution was determined between the degradant and the sildenafil citrate. Purity calculations were performed in Galaxie on the sildenafil citrate peak using the controlled unstressed standard as a reference.

For determining the linearity of the method, the test was conducted by the plotting three standard curves in the range of 2.49–99.55 µg/mL and a determination coefficient higher than 0.99 was considered adequate. Precision of the method was assessed through repeatability: it was determined by consecutively analysing six replicates by a single analyst in a single day. An injection precision of <5% relative to the coefficient of variation (CV) was considered acceptable. Accuracy measurements were performed by the same analyst by injecting the chromatographic samples to which the matrix was added (at the same concentration levels performed for the linearity test (n = 3 for each concentration level)). The result was expressed as a percentage of recovery and compared with the analytical curve obtained from linearity.

Preparation of Sildenafil Citrate Suspension Samples

The sildenafil citrate suspension was prepared by adding 250 mg of sildenafil citrate to a low actinic prescription bottle. An aliquot of SyrSpend® SF PH4 (liquid) was added to the bottle to achieve a final volume of 100 mL. The final concentration was 2.5 mg/mL sildenafil citrate. The suspensions were stored at temperatures between 2 and 8°C and at room temperature storage conditions for the duration of the study.

Stability Study

The sample of sildenafil citrate suspended in SyrSpend® SF PH4 (liquid) at a concentration of 2.5 mg/mL was submitted for stability. The initial submitted sample was assayed and then split into two containers. The sample was packaged in low actinic plastic prescription bottles and stored at refrigerated storage conditions between 2 and 8°C and at room temperature storage conditions. Time points for the study were initial (T = 0), 7 days (T = 7), 14 days (T = 14), 30 days (T = 30), 61 days (T = 61) and 92 days (T = 92). The evaluation parameter was percent recovery assay and also appearance, taste, odour and pH. The stability of the sildenafil citrate in suspension was defined by the percent recovery with respect to T = 0 using the validated HPLC method. The sample stock was prepared six times by adding 100 µL of suspension to a 10-mL volumetric flask and diluting to volume with mobile phase. The average and coefficient of variation of all replicate injections (n = 3) at each time point were used to calculate the percent recovery.

RESULTS AND DISCUSSIONS

As a first step of our work, the HPLC method was monitored in order to verify its applicability to the main objective of this study, which is the inference for the stability of sildenafil citrate in SyrSpend® SF PH4 (liquid). A summary of the HPLC method parameters can be found in Table 1, which provides the data for peak tailing, range of the analytical curve, its coefficient of determination (linearity), precision and accuracy (in terms of percentage of recovery). All data are within international guidelines acceptance criteria (Council of Europe, 2015; ICH, 2005), which are coefficient of determination higher than 0.99 for linearity, coefficient of variation lower than 5% for precision (in terms of repeatability, intra-assay variation) and percentage of recovery within 98.0% and 102.0% of the target value for accuracy. Figure 1 depicts typical chromatograms of standard and simple, confirming specificity of the method. With a peak tailing lower than 2.0 and a theoretical plate number over 4,000, good separation capacity for the HPLC column was demonstrated.

Evaluation of possible degradation was also conducted to identify the decomposition of the APIs and assure sufficient separation by chromatographic analysis. The decomposition profile of the API varied for different stressing conditions. Sildenafil citrate was stable to acid, base, light and heat, but oxidiser created significant degradation. The degradants present were all completely separated from the analyte with acceptable resolution (>1.5).

The suspension compounded with SyrSpend® SF PH4 (liquid) was prepared and its stability profile was traced. The initial concentration of the suspension was 2.59 mg/mL, which was set as the baseline for all other time points tested. The results were expressed in terms of percentage of recovery (Table 2). The assay results varied between 2.57 (T = 30) and 2.59 mg/mL (T = 0) for the room temperature storage condition and between 2.56 (T = 7) and 2.61 mg/mL (T = 61) for the refrigerated storage condition. All sample preparations at each time point were within specification, with a maximum variability in the assays of CV = 4.21% (T = 30) for room temperature conditions and 4.91% (T = 92) for refrigerated conditions. All time points showed a similar chromatographic profile and clear degradant separation. In addition to the assay, appearance, taste and odour were evaluated and remained exactly the same as T = 0 throughout the whole study. Initial pH was measured as 4.21 and also remained constant during the study.

By the exposed, the sildenafil citrate suspension compounded with SyrSpend® SF PH4 (liquid) presented equal to or better physicochemical stability than other vehicles. Roque et al. (2013) developed two different oral liquid formulations of

Table 1. Summary of the HPLC parameters used in the stability study of sildenafil citrate in SyrSpend® SF PH4 (liquid).

Parameter	Result
Peak tailing	1.31 (CV = 0.23)
Theoretical plates	4126.82 (CV = 0.59)
Linearity	
Range	2.49–99.55 mcg/mL
Determination coefficient	0.9996
Precision (repeatability, n = 6)	CV = 1.06%
Accuracy	Recovery = 99.74%

CV, coefficient of variation.

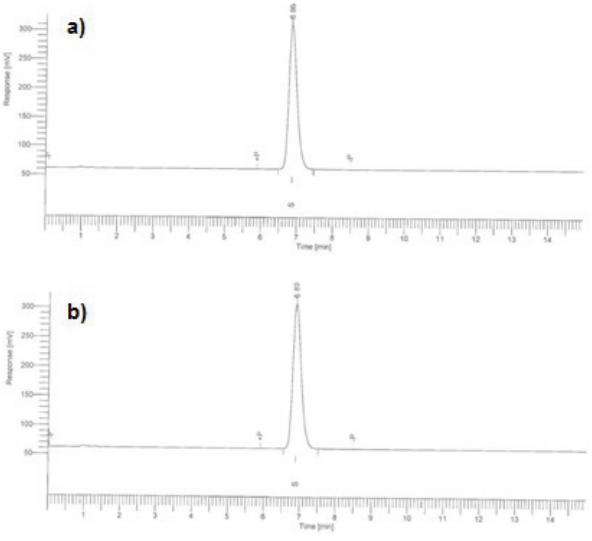

Figure 1. Typical chromatograms of sildenafil oral suspension in SyrSpend® SF PH4 obtained with the HPLC-validated method: (a) sildenafil standard; (b) oral suspension sample. Retention times of the chromatographic peaks show suitability of the method.

Table 2. Stability of sildenafil citrate in SyrSpend® SF PH4 (liquid).

Elapsed Time (days)	% Recovery	
	Room Temperature	Refrigerated Temperature (2–8 ºC)
T = 0	100.00	100.00
T = 7	99.94 ± 3.14	98.82 ± 1.45
T = 14	99.41 ± 2.10	99.41 ± 2.47
T = 30	99.13 ± 4.21	99.55 ± 1.36
T = 61	99.38 ± 2.85	100.72 ± 3.19
T = 92	99.57 ± 1.28	100.52 ± 4.91

Values expressed as the average of three replicates ± relative standard deviation (= coefficient of variation)

sildenafil citrate for paediatric use without preservatives, and the shelf-life of both formulations was three months (91 days); however, upon opening, aqueous solutions should be used within 10 days and kept refrigerated, and syrup solutions should be used within 14 days – which are significantly lower than what was obtained with our study.

A study by Nahata et al. (2006), for their turn, verified stability of two different extemporaneously prepared sildenafil formulations, prepared from crushed tablets and using two different suspending agents (1:1 mixture of Ora-Sweet® and Ora-Plus® and a 1:1 mixture of methylcellulose 1% and Simple Syrup NF). Both formulations were evaluated for physicochemical stability over a three-month period (91 days) under refrigerated conditions (4 and 8°C). No changes in pH, odour or physical appearance were observed throughout the study period, and the products remained stable after 91 days of preparation. Ora-Sweet® and Ora-Plus®, however, contain ingredients to be generally avoided in children, including glucose (Hill EM, Jijo A), carrageenan (Bhattacharyya S), glycerin (Maclaren NK), parabens (Rowe RC, European Commission, Weil, E) and sorbitol (Johnston KR, Payne ML, Pawar S and Pecar A). In addition, theses suspensions needed to be kept in refrigerator and were prepared from commercial tablets, not high-quality pharmaceutical raw materials (Jooste, 2011).

Lastly, Provenza et al. (2014) developed two oral liquid formulations of sildenafil citrate for paediatric use from pure powder: a suspension (containing citrate buffered solution, 'excipient for syrup' and distilled water) and a solution (containing citrate buffered solution, 'excipient for syrup sugar free' and distilled water). They showed that the suspensions presented better stability (90 days at 4 and 25 °C, whilst the solution was stable for 30 days when stored at 25 and 40 °C and 15 days at 4 °C, because of the formation of non re-dispersible sediment).

All these studies confirm that the sildenafil citrate suspension compounded with SyrSpend® SF PH4 (liquid) posses better physicochemical stability than other vehicles reported on the literature and ingredients that are safe for use in children.

CONCLUSION

When compounded from the raw powder, sildenafil citrate was stable in SyrSpend® SF PH4 (liquid) for 92 days at both room temperature and refrigerated conditions. The samples were still within specification at day 92 so the beyond-use date is concluded to be 92 days. The findings of this study show that SyrSpend® SF PH4 (liquid) is an acceptable oral syrup and suspending vehicle for preparing individually compounded sildenafil citrate suspensions. The formulations would be viable alternatives to commercially available tablets when that dosage form is found to be inappropriate whilst remaining alcohol-, sorbitol- and sugar-free.

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