Süleyman Demirel Üniversitesi
Sağlık Bilimleri Dergisi
Cilt 14, Sayı 2, 292 - 299, 2023

Suleyman Demirel University
Journal of Health Sciences
Volume 14, Issue 2, 292 -299, 2023

In vitro Testing to Investigate Effects of Excipients in Drug Formulation

Yardımcı Maddelerin İlaç Formülasyonundaki Etkilerini Araştırmak İçin *In vitro* Testler

Hulya YILMAZ [1]*, Fatma SERTGOZ [2], Ayse CINKILIC [2], Mustafa CULHA [1]

[1] Sabanci University Nanotechnology Research and Application Center, Istanbul, Turkey
[2] Department of Genetics and Bioengineering, Yeditepe University, 34755 Istanbul, Turkey

ABSTRACT

The formulation of drugs requires active pharmaceutical ingredients (API), which can be combined with some excipients, which are not considered pharmacologically active and could have different roles in a drug formulation. Glukofen, a generic name as a biguanide antidiabetic, is used for treating type 2 Diabetes Mellitus, mainly in overweight patients when dietary management and exercise do not help in adequate glycemic control. In Glukofen formulation, metformin-HCl as an API and some excipients such as povidone, magnesium stearate, hypromellose, and titanium dioxide are combined. The goal of this study is *in vitro* investigation of the effects of Glukofen and metformin-HCl on the human bronchial epithelial cell line (Beas-2b). To observe and compare their toxic effects and uptake into cells, cytotoxicity assay and flow cytometry experiments were carried out. The obtained results from this study showed that any toxic effects of both Glukofen and metformin-HCl on Beas-2b cells were not obtained. In addition, uptake of Glukofen into cells was observed more than metformin-HCl. It is evident that the incorporation of excipients to metformin-HCl results in a significant rise in cell proliferation and uptaking into cells.

Keywords: Glukofen, metformin, *in vitro*, Beas-2b, excipients, toxicity

Alınış / Received: 16.07.2023 Kabul / Accepted: 06.08.2023 Online Yayınlanma / Published Online: 15.08.2023

DOI: 10.22312/sdusbed.1328230
ARAŞTIRMA MAKALESİ

*Corresponding author: yilmazchulya@gmail.com

ÖZ

İlaçların formülasyonu, farmakolojik olarak aktif kabul edilmeyen ve bir ilaç formülasyonunda farklı rollere sahip olabilecek bazı eksipiyanlarla birleştirilebilen aktif farmasötik bileşenler (API) gerektirir. Bir biguanid antidiyabetik olarak jenerik bir isim olan Glukofen, diyet yönetimi ve egzersiz yeterli glisemik kontrole yardımcı olmadığında, özellikle aşırı kilolu hastalarda tip 2 Diabetes Mellitus tedavisinde kullanılır. Glukofen formülasyonunda, bir API olarak metformin-HCl ve povidon, magnezyum stearat, hipromelloz ve titanyum dioksit gibi bazı yardımcı maddeler birleştirilir. Bu çalışmanın amacı, Glukofen ve metformin-HCl'nin insan bronşiyal epitel hücre dizisi (Beas-2b) üzerindeki etkilerinin in vitro araştırılmasıdır. Toksik etkilerini ve hücrelere alımlarını gözlemlemek ve karşılaştırmak için sitotoksisite testi ve akış sitometrisi deneyleri yapıldı. Bu çalışmadan elde edilen sonuçlar, hem Glukofen hem de metformin-HCl'nin Beas-2b hücreleri üzerinde herhangi bir toksik etkisinin elde edilmediğini göstermiştir. Ayrıca Glukofen'in hücrelere alımı, metformin-HCl'den daha fazla gözlendi. Yardımcı maddelerin metformin-HCl'ye dahil edilmesinin, hücre çoğalmasında ve hücrelere alınmasında önemli bir artışa yol açtığı açıktır.

Anahtar Kelimeler: Glukofen, metformin, *in vitro*, Beas-2b, yardımcı maddeler, toksisite

1. Introduction

In pharmaceutical industry, drug formulations are created with utmost precision, ensuring that active pharmaceutical ingredients (API) and other essential components are included in optimal concentrations to achieve maximum efficacy [1]. Maintaining the safety and efficacy of drugs is crucial in drug therapy, as both the APIs and impurities play a significant role in determining the drug's safety. The pharmacological-toxicological profile of APIs and impurities determines the drug's overall safety. In order to achieve this, the pharmaceutical industry adheres to strict safety standards while treating diseases to protect public health.

A drug's chemical properties can significantly affect its pharmacokinetics and pharmacodynamics properties [2]. For a drug to be absorbed and distributed to its intended site of action or elimination, it must be able to dissolve in aqueous solutions, be released from its formulation, and cross several hydrophobic and hydrophilic barriers [3]. However, most APIs are not well water-soluble, so excipients are added to help increase solubility. Additionally, excipients play a crucial role in maintaining the drug's stability, shelf-life, and dosage uniformity, ensuring maximum therapeutic efficacy by providing reproducibility of absorption, dissolution, and bioavailability [4]. Although excipients were traditionally not considered pharmaceutically active, they are now crucial in improving the drug's performance [5]. Various excipients, including diluents, binders, disintegrates, glidants, lubricants, tablet coatings, films, and coloring agents, are incorporated into the dosage form to ensure the drug's safety and efficiency [6].

Figure 1 shows the chemical structure of metformin hydrochloride (HCl), the active ingredient of Glukofen, produced by Sandoz drug company. Its IUPAC name is 3-(diamino methylidene)-1,1-dimethylguanidine hydrochloride. Metformin, a type of guanidine from the biguanide class, contains two methyl substituents at position 1. Glukofen treats type 2 diabetes mellitus and can be used with another oral antihyperglycemic agent or insulin. Glukofen works through three active pathways, namely, i) reducing hepatic glucose production by inhibiting gluconeogenesis, ii) improving peripheral glucose uptake and usage by increasing insulin sensitivity in muscle, and iii) retarding intestinal glucose absorption [7].

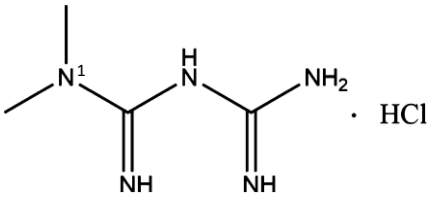

Figure 1. Chemical structure of metformin-HCl

The tablet form of Glukofen includes the excipients listed in Table 1, which shows the specific properties of the formulation.

Table 1. Excipients in Glukofen and their properties in the formulation

Excipients	IUPAC name	Functions	Mechanism	References
Hypromellose	Cellulose hydroxypropyl methyl ether	Film coating-Tablet binder	The grades of hypromellose differ in the extent of substitution and viscosity. In capsules and tablets, high-viscosity grades can be utilized to delay the release of drugs from the matrix.	[8]
Titanium Dioxide	Dioxotitanium	Film coating	White pigment in film-coating suspensions, gelatin capsules, and sugar-coated tablets	[9]
Povidone	(1-Ethenyl-2-pyrrolidinone homopolymer)	Solubilizer-Tablet core	Not absorbed by mucous membranes or the gastrointestinal tract, it might be considered substantially nontoxic when consumed orally.	[10]
Magnesium stearate	Octadecanoic acid magnesium salt	Lubricant-Tablet core	Due to the hydrophobicity, it might delay the dissolution of the drug from a solid-dosage form. When administered orally, it is usually considered nontoxic.	[11, 12]

Studies suggest that long-term use of metformin is linked to decreased levels of serum vitamin B12. This factor can impact respiratory muscle function and increase the likelihood of Chronic Obstructive Pulmonary Disease exacerbation and hospitalization [13]. In light of this information, an investigation of the effects of metformin on lung cells is a vital issue. This study aims to explore how the tablet version of metformin-HCl affects Beas-2b cells. The absorbance of Glukofen was measured to compare to the absorbance of metformin-HCl by using Ultraviolet-visible (UV-vis) spectrophotometry. Next, we conducted studies on the cell viability and uptake of Glukofen and metformin-HCl in Beas-2b cells and observed the morphological changes through white light images. Based on the findings, one can conclude that while metformin does not exhibit any toxicity towards Beas-2b cells, the presence of excipients causes increased cell proliferation.

2. Materials and Methods

Materials

Human Bronchial Epithelial cells (Beas-2b) were purchased from ATCC. Dulbecco's Modified Eagle Medium (DMEM, D6421-500 mL), Fetal Bovine Serum (FBS), Phosphate Buffered Saline (10X PBS with calcium-magnesium), and 0.25% Trypsin-EDTA (1X) were purchased from Gibco, USA. The well plates (Corning™ Costar™, Sweden), serological pipettes (SPL Life Science, Korea), Hemocytometer (Bright Line, Germany), T-flask, automatic pipette (IsoLab, Germany), falcon tubes and Eppendorf tubes (Isolab) were obtained. Glukofen 850 mg retard (Sandoz, Switzerland) contains Polyvinylpyrrolidone (K90), magnesium stearate, polyethylene glycol, hydroxypropyl methylcellulose, and opadry II white as excipients, was obtained from the pharmacy to test in the study. Metformin-HCl and Dimethyl sulfoxide (DMSO) were supplied from Sigma Aldrich. Guava easyCyte™, a Microplate reader from BIO-TEK ELx800, USA, with the software program of KCjunior (Winooski, VT), UV-vis spectrophotometry was used.

Methods

Five technical and three biological replicates were conducted for each experiment. 10% DMSO was used as a positive control. Whereas the cells in the media were used as a negative control, the media was used as a blank. Figure 2 shows a schematic representation of *in vitro* experimental models.

Preparation of standard stock solutions: Increasing concentrations of (0.5, 1, 2.5, 5,10, and 20 µM) Glukofen and metformin-HCl in DMEM-High glucose were prepared by diluting each drug from stock solutions in a sufficient quantity of media in separate. First, ten tablets of 850 mg Glukofen were ground up by a mortar and pestle to obtain the powder, mixed to homogenize, and weighed. Stock solution (100 µM, 25 mL) as a concentrate for each was prepared to be diluted and stored in 2 mL Eppendorf tubes at -20°C to keep it stable.

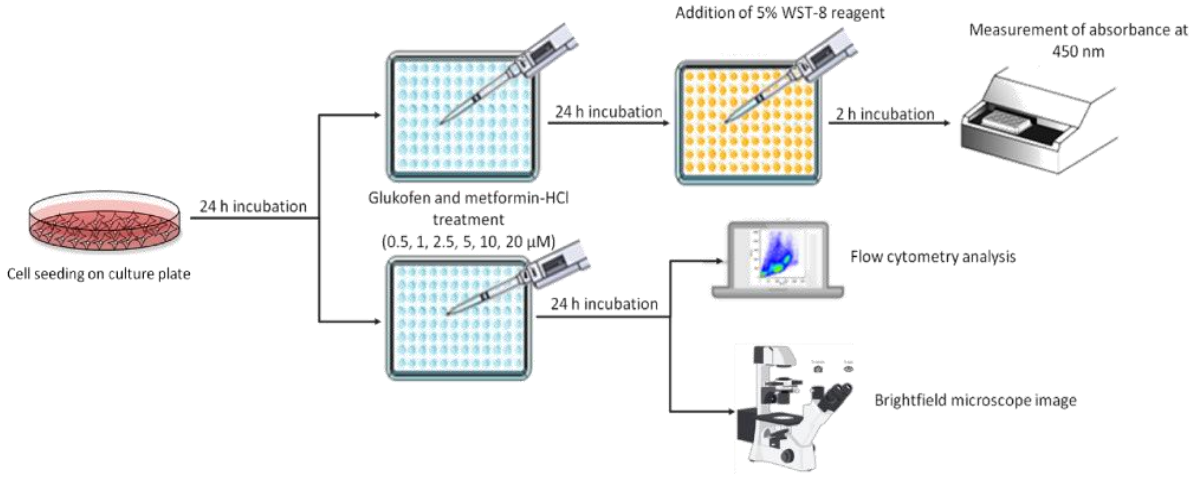

Figure 2. Schematic representation of the *in vitro* experimental procedure

Cell viability: WST-8 Assay was conducted according to the supplier's protocol to test the viability of cells. Initially, $12x10^3$ Beas-2b cells/well were cultured in DMEM-High Glucose medium with 5% FBS and incubated at 37°C and 5% CO_2 conditions by reaching a confluence of 80%. Afterward, the prepared six doses (0.5, 1, 2.5, 5, 10, and 20 µM) of Glukofen and metformin-HCl by dilution from stock solutions were exposed to the cells. After 24 hours of incubation, 5% WST-8 reagent with completed media was exposed to the cells and left incubator for 2 hours, and then 96 well-plates were analyzed at 450 nm by ELISA reader.

Uptake Studies: Beas-2b was seeded as $4.2x10^4$ cells/well in 24 well plates. After 24h, increasing concentrations of Glukofen and metformin-HCl, like in the cell viability study, were applied to the cells for 24 hours. After incubation of cells with Glukofen and metformin-HCl, each well was washed with PBS, and then, the cells were treated with 0.25% trypsin-EDTA. Afterward, 400 µL complete DMEM-High was added to each well, and these mixtures were transferred into Eppendorf tubes separately. They were centrifuged at 1500 rpm for 5 minutes. The supernatants were discarded, and the pellets were dissolved with 200 µL PBS. The samples were analyzed by flow cytometer.

Brightfield Microscope Image: The cells were seeded as $4.2x10^4$ cells/well on 24 well plates. After one day of the cultured cells on the wells, six doses (0.5, 1, 2.5, 5, 10, and 20 µM) of both Glukofen, metformin-HCl were prepared. The plates were placed in the incubator and kept for 24 hours. After incubation of cells for 24 hours, the media on each well were discarded, and 500 µL PBS was added into each well. Then, their images were taken for both Glukofen and metformin-HCl treatments at 40X objective by brightfield microscope.

Statistical Analysis: The two-paired Student's t-test was used, and P-values are calculated and given by asterisks, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. * and ** were given on the graph to avoid redundancy. (n=5, mean±SD).

3. Results and Discussion

Figure 3 shows the UV/Vis spectra of Glukofen and Metformin-HCl, and, as seen, λ_{max} is 236 nm, confirming the literature value [14]. From the spectra, it is clear that Glukofen spectrum is dominated by metformin.

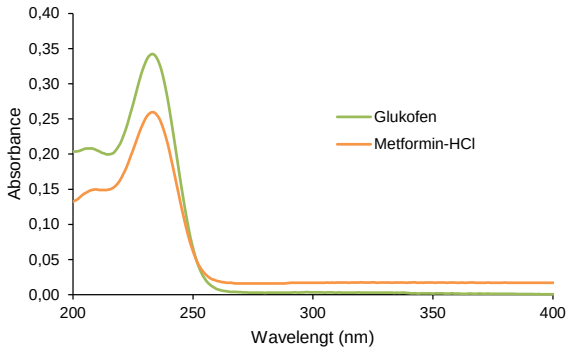

Figure 3. Absorbance of Glukofen and metformin-HCl in diH_2O

In vitro, cytotoxicity assays conducted can aid in identifying cell proliferation or direct cytotoxic effects resulting in cell death when treated with external substances such as drugs [15]. WST-8 reagent was used to determine the percentage of metabolically active cells. Figure 4 shows the viability percentage of Beas-2b cells exposed to Glukofen and metformin-HCl at increasing concentrations. The graph shows that neither Glukofen nor metformin-HCl was toxic on Beas-2b cells. A recent study has also shown that metformin can protect against the acute effects of radiation by reducing the senescence of bronchial-epithelial cells [16]. Glukofen has also influenced the proliferation of cells positively. It can be explained that its combination with excipients may metabolically activate the cells.

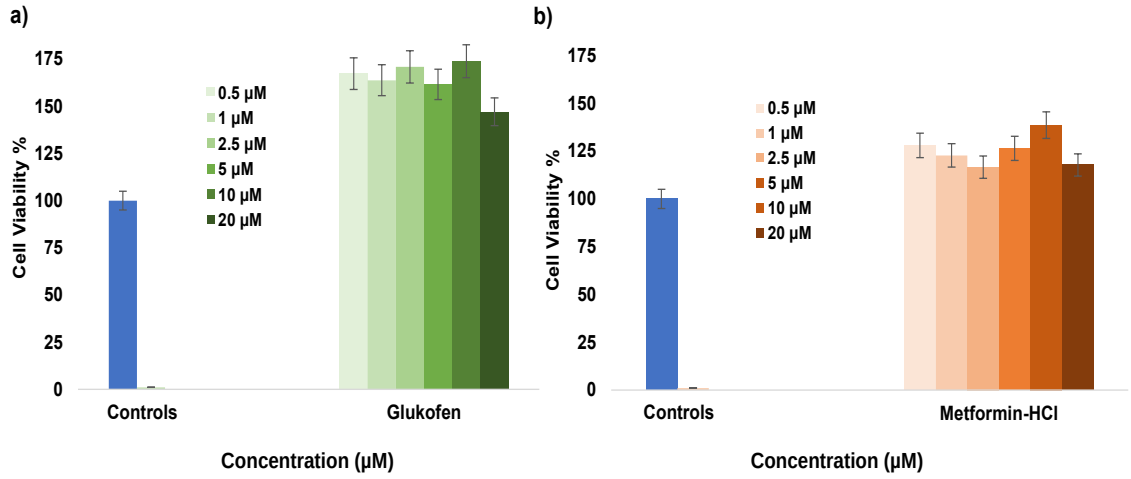

Figure 4: Results of Beas-2b cells cytotoxicity assay for a) Glukofen and b) metformin-HCl

Substances such as drugs can only pass through cell membranes through specific transporters or carriers in the plasma membrane. This is due to their inability to diffuse across the membrane independently. When a substance is taken up by a cell, it increases the cell content/granularity. Metformin, for example, is transported into cells by various organic cation transporters, which have been shown to facilitate the cellular uptake of metformin [17, 18]. In this study, flow cytometry was utilized to monitor the uptake of Glukofen and metformin-HCl into cells comparatively. The laser in flow cytometry has two paths, one along the axis and the other at a 90° angle. The scattered light at two different angles is collected and referred to as forward angle light scattering (FCS) and side angle light scattering (SSC). While the FCS provides information about the cell size, the SSC gives information about the cell granularity (Internal complexity) by comparing it to the control. Therefore, the increasing SSC value on

the histogram means that the cells have higher granularity. Figure 5a shows an SSC shift in the Glukofen treatment with increasing doses. The highest SSC shifts were observed at 10 and 20 µM concentrations, which is a significant finding compared to the control. This indicates that as the Glukofen concentration increased, the cells' granularity also increased. On the Beas-2b cell line, it was observed that the molecules/organelles in the cells also increased with the uptake of Glukofen at increased doses. Therefore, it can be concluded that Glukofen uptake was successfully achieved with these doses on Beas-2b cells. However, in contrast, metformin-HCl uptake by cells was unsuccessful based on the data obtained in this study. As seen in Figure 5b, the SSC shift of metformin-HCl decreases as the concentration increases, in contrast to its drug form, Glukofen. However, no significant difference is observed compared to the control indicating no granularity increase. This observation could be due to the positive charge of metformin at the physiological pH resulting in its strong binding to the proteins in the culture media and preventing them from easily uptaken by the cells. However, when metformin is in the drug formulation, several expedients help it to remain stable and uptaken by cells easily [19].

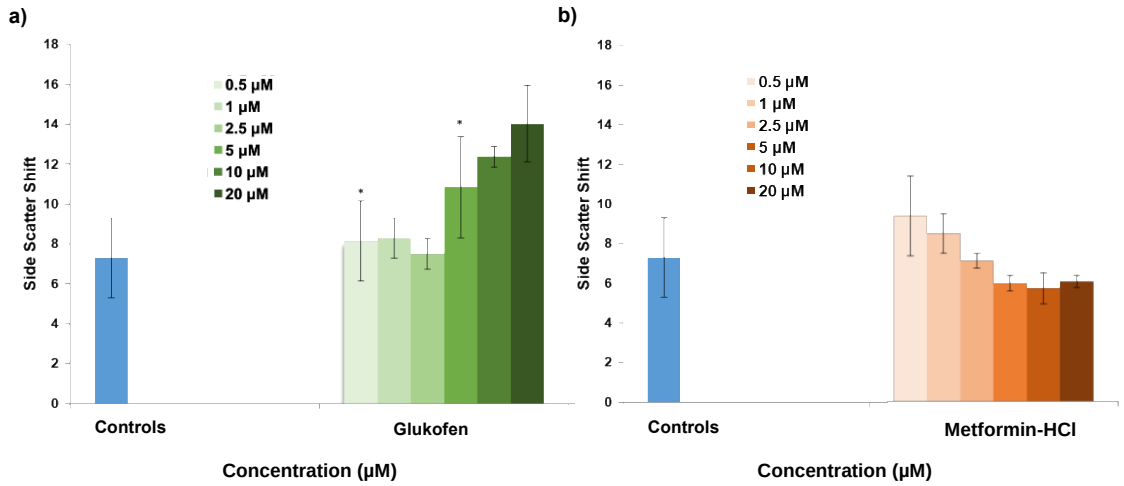

Figure 5. Uptake of a) Glukofen and b) metformin-HCl into Beas-2b cells

Cell morphology is another significant issue for the investigation of the effects of a drug. The morphologies of Beas-2b exposed with Glukofen and metformin-HCl were visualized via brightfield microscopy equipped with a 40X objective after 24 hours of treatment with Glukofen and metformin-HCl. Figure 6 shows adherent Beas-2b cells have a more regular shape, like polygonal. In the control group, the cells are left only in the culture medium without any exposure. When comparing Glukofen and metformin-HCl, it is apparent that as the dose of Glukofen increases, the cells' content expands more than the control group. However, with metformin-HCl, the expansion of cell content is not dependent on the dose increment. Moreover, upon examining the morphology of cells in the control group, it was observed that their shape was altered upon treatment with Glukofen and metformin-HCl. Although the cells remained connected and retained a polygonal shape, they appeared swollen and irregular. As the concentration of the treatments increased, the irregularities in the cell morphology became more pronounced. It can be inferred that the presence of excipients influences the action mechanism of metformin hydrochloride. This is possibly due to the binding of metformin to other excipients influencing the ionization status to help it to be uptaken easier by the cells. However, it is challenging easy to elaborate on how and to what degree metformin interacts with the molecular and ionic species in the tablet (see the experimental section). One can easily realize the role of the other molecular and ionic species present in the formulation for cell uptake.

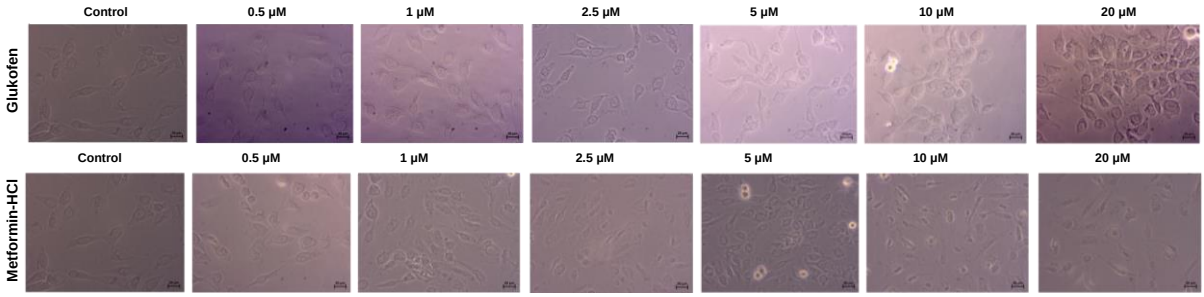

Figure 6. The brightfield microscope images of Glukofen, metformin-HCl, and controls by 40X objective

4. Conclusions

This study is aimed to compare the effects of Glukofen and metformin-HCl on Beas-2b cells *in vitro*. Beas-2b cells were exposed to Glukofen and metformin-HCl at increasing concentrations to determine comparatively which from had a more significant impact on cell viability and uptake. The WST-8 assay and flow cytometry were used for evaluation. While we found that both treatments positively impacted cell viability, Glukofen significantly increased cell proliferation. Both Glukofen uptake and cell viability were raised with the increasing concentration. The metformin-HCl uptake was less with increasing concentration compared to Glukofen. Therefore, it can be concluded that the combination of Metformin-HCl and excipients in Glukofen increases cell viability and its cellular uptake in Beas-2b cells. Further studies on the influence of tablet formulation content on other cellular processes, such as cell cycle and apoptosis, should be investigated to clarify the role of expedients in metformin-based drugs.

Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

References

[1] Katny, M., Frankowski, M. 2016. Impurities in Drug Products and Active Pharmaceutical Ingredients. Critical Reviews in Analytical Chemistry, 47 (3), 187-193

[2] Jain, D., Basniwal, P. 2013. Forced degradation and impurity profiling: Recent trends in analytical perspectives. Journal of Pharmaceutical and Biomedical Analysis, 86, 11-35.

[3] Bardal, S., Waechter, J., Martin, D. 2011. Pharmacokinetics. In S. Bardal, J. Waechter, D. Martin, Applied Pharmacology (pp. 17-34). St. Louis, Missouri: Elsevier Saunders.

[4] Fortunak, J. M., de Souza, R. O., Kulkarni, A. A., King, C. L., Ellison, T., Miranda, L. S. 2014. Active pharmaceutical ingredients for antiretroviral treatment in low- and middle-income countries: a survey. Antiviral Therapy, 15-29.

[5] Fabiano, V., Mameli, C., Zuccotti, G. 2011. Pediatric Pharmacology: Remember the excipients. Pharmacological Research, 63 (5), 362-365.

[6] Hamman, J., Steenekamp, J. 2011. Excipients with specialized functions for effective drug delivery. Expert Opinion on Drug Delivery, 9 (2), 219-230.

[7] Çubuk, G., İnce, S. 2015. Oral Antidiyabetik İlaçlar. Kocatepe Veteriner Dergisi, 95-102.

[8] Li, C., Martini, L., Ford, J., & Roberts, M. 2005. The use of hypromellose in oral drug delivery. Journal of Pharmacy and Pharmacology, 57 (5), 533-546.

[9] Rowe, R. 1984. Materials used in the film coating of oral dosage forms. Critical Reports on Applied Chemistry, 6, 1-36.

[10] Adeyeye, C., Barabas, E. 1993. Excipient Profile: Povidone. In H. G. Brittain (Ed.), Analytical Profiles of Drug Substances and Excipients (Vol. 22, pp. 555-585). London: Academic Press.

[11] Butcher, A., Jones, T. 1972. Some physical characteristics of magnesium stearate. The Journal of Pharmacy and Pharmacology, 1P-9P.

[12] Lerk, C., Bolhuis, G., Smallenbroek, A., Zuurman, K. 1982. Interaction of tablet disintegrants and magnesium stearate during mixing. II. Effect on dissolution rate. Pharmaceutica acta Helvetiae, 282-286.

[13] Yen, FS., Wei, J.CC., Yang, YC. 2020. Respiratory outcomes of metformin use in patients with type 2 diabetes and chronic obstructive pulmonary disease. Scientific Reports, 10, 10298.

[14] Hinge, M A., Patel, KV. 2016. Development and Validation of Spectrophotometric Method for Metformin and Sitagliptin by Absorbance Ratio Method. J Pharm Sci Bioscientific Res. 6(5): 733-739.

[15] Holley, R. W. 1975. Control of growth of mammalian cells in cell culture. Nature, 487-490.

[16] Hansel, C., Barr, S., Schemann, A.V., Lauber, K., Hess, J., Unger, K., Zitzelsberger, H., Jendrossek, V., Klein, D. 2021. Metformin Protects against Radiation-Induced Acute Effects by Limiting Senescence of Bronchial-Epithelial Cells. International Journal of Science. 22, 7064.

[17] Pandey, P., Balekar, N. 2018. Target-specific Delivery: An insight. Drug Targeting and Stimuli Sensitive Drug Delivery Systems, 117-154.

[18] Wood, J., Horton, A., Byrne, J., Pedroso, K., Bisnow, M., Auer, R. 1989. Flow Cytometry: Advanced Research Applications. (A. Yen, Ed.) Boca Raton, FL: CRC Press.

[19] Zhang, R., Qin, X., Kong, F., Chen, P., Pan, G. 2019. Improving cellular uptake of therapeutic entities through interaction with cell membrane components. Drug Deliv. 26(1):328-342.