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Research Articles

Development and Validation of HPLC Method for the Determination of Flurbiprofen in Pharmaceutical Preparations

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ABSTRACT:
In this study, a new and rapid high-performance liquid chromatography (HPLC) method was developed for the determination of flurbiprofen in pure and pharmaceutical preparations. The method was developed on the Ace C_{18} column using a mobile phase of acetonitrile-0.05 M potassium dihydrogen phosphate solution (60:40, v/v) adjusted to pH 3.5 with phosphoric acid. The eluent was monitored by UV detection at 254 nm. The analysis was performed in less than 6 min with a flow rate of 1.0 mL min^{-1}. Calibration curve was linear over the concentration range of 0.10-5.0 µg mL^{-1}. Intra- and inter-day precision values for flurbiprofen were less than 4.56, and accuracy (relative error) was better than 4.00%. The mean recovery of flurbiprofen was 99.8% for pharmaceutical preparations. The limits of detection (LOD) and quantification (LOQ) were 0.03 and 0.10 µg mL^{-1}, respectively. Also, the method was applied for the quality control of two commercial flurbiprofen dosage forms to quantify the drug and to check the formulation content uniformity.

Keywords: Flurbiprofen, HPLC, pharmaceutical preparation, validation

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1.INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed agents worldwide to treat a variety of pain-related conditions, including arthritis and other rheumatic diseases. In addition, epidemiological studies have shown that long-term use of NSAIDs reduces the risk of developing Alzheimer's disease and delays its onset [1-3]. Flurbiprofen (Figure 1) is used for the treatment of rheumatoid arthritis, degenerative joint disease, osteoarthritis, ankylosing spondylitis, acute musculoskeletal disorders, low back pain and allied conditions [4-7]. It contains a fluorine atom in its molecular structure, producing better effects at a lower therapeutic dose and with fewer adverse effects compared with similar drugs.

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Figure 1. Chemical structure of flurbiprofen.

Several methods have been reported for the determination of flurbiprofen including high performance liquid chromatography (HPLC) [8-19] and liquid chromatography-mass spectrometry (LC-MS) [20]. Over the last 20 years, several HPLC methods using UV or fluorescence detection have been reported for the estimation of flurbiprofen either alone or together with their metabolites in plasma/serum [8-13], in urine [14-18] and in ocular fluids [19].To date, no method is reported till date for the determination of flurbiprofen by HPLC in pharmaceutical preparations. Therefore, we report an HPLC with UV method for the determination of flurbiprofen in pharmaceutical preparations. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to International Conference on Harmonization (ICH) guidelines. The method uses a simple mobile phase composition and a rapid run time of 6 min. Hence, this method can be used for the analysis of a large number of samples.

2.MATERIAL AND METHODS

2.1.Chemicals and reagents

Flurbiprofen was obtained from Sigma (St. Louis, MO, USA). Methanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Majezik and Frolix tablets containing flurbiprofen were obtained from Pharmacy (Erzurum, Turkey). HPLC-grade organic solvents were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade. Distilled water was prepared as required by using aquaMAX™ ultra, Young instrument (Korea) ultra water purification system.

Instrumentation

An Agilent 1260 Elmer series 200 HPLC system equipped with programmable UV/Vis detector and OpenLAB ChemStation software was used. The HPLC mobile phase was composed of acetonitrile-0.05 M potassium dihydrogen phosphate solution (60:40, v/v) adjusted to pH 3.5 with phosphoric acid. Separation was achieved using an Ace C_{18} column (5 µm, 4.6×250 mm i.d.) with a flow rate of 1.0 mL/min. The eluent was monitored by UV detection at 254 nm.

2.2.Preparation of the standard and quality control solutions

The stock standard solution of flurbiprofen was prepared with methanol to a concentration of 50 μg mL^{-1} and stored at 4 ^{0}C under refrigeration. The six standard solutions from 0.1 to 5.0 μg mL^{-1} (0.1, 0.25, 0.5, 1.0, 2.0, 4.0 and 5.0 μg mL^{-1}) in methanol were made by a serial dilution. Three quality control (QC) samples at the concentrations of 0.75, 3.0 and 5.0 μg mL^{-1} were prepared from the stock standard solution.

2.3.Procedure for pharmaceutical preparations

The average tablet mass was calculated from the mass of Majezik and Frolix tablets. They were then finely ground, homogenized and a portion of the powder was weighed accurately, transferred into a 100 mL brown measuring flask and diluted to scale with methanol. The mixture was sonicated for at least 15 min to aid dissolution and then filtered through a Whatman No 42 paper. An appropriate volume of the filtrate was diluted further with methanol so that the concentration of flurbiprofen in the final solution was within the working range and then analyzed by HPLC.

2.4.Data analysis

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 10.0. Correlations were considered statistically significant if calculated P values were 0.05 or less.

3.RESULTS AND DISCUSSION

3.1.Method development and optimization

The development of the RP-HPLC method for the determination of drugs has received considerable attention in recent years because of its importance in the routine quality control analysis. An RP-HPLC method was proposed as a suitable method for the estimation of flurbiprofen in the pharmaceutical dosage form. A good separation was achieved using an Ace C_{18} column (5 μm, 4.6×250 mm i.d.). The chromatographic conditions were adjusted to provide a good performance of the assay. The method involved a mobile phase consisting of acetonitrile-0.05 M potassium dihydrogen phosphate solution (60:40, v/v) adjusted to pH 3.5 with phosphoric acid accomplished at 254 nm. The retention time was 5.4 min at a flow-rate of 1 mL min^{-1} and the injection volume was 10 μl. The total run time for an assay was approximately 10 min. The integrator attenuation was 10 and the chart speed was 0.3 cm min^{-1}. The mobile phase was chosen after several trials with other solvent combinations. Mobile phase selection was based on peak parameters (symmetry, tailing), run time, ease of preparation and cost. Figure 2 shows a typical chromatogram obtained from the analysis of a standard flurbiprofen using the proposed method. As shown in Figure 2, flurbiprofen was eluted forming a

symmetrical peak and well separated from the solvent front. The observed retention time allowed a rapid determination of the drug.

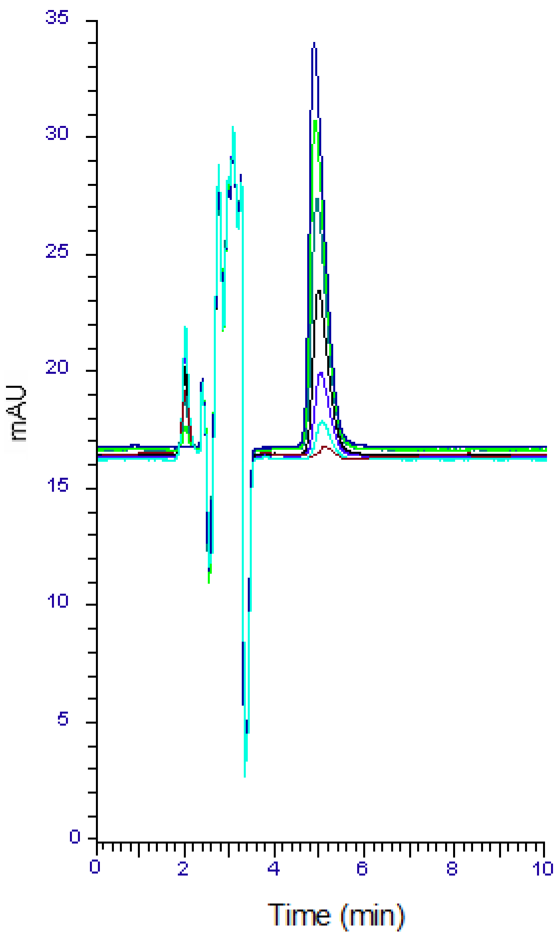

Figure 2. HPLC-UV chromatograms of flurbiprofen (0.1, 0.25, 0,5, 1.0, 2.0, 4.0 and 5.0 µg mL^{-1}).

3.2.Validation of the method

3.2.1.System suitability

A system suitability test of the chromatography system was performed before each validation run. Five replicates injections of a system suitability/calibration standard and one injection of a check standard were made. Area relative standard deviation, tailing factor and efficiency for the five suitability injections were determined. The check standard was quantified against the average of the five suitability injections. For all sample analyses, the tailing factor was ≤ 1.04, efficiency ≥ 2318 and %RSD ≤1.26%.

3.2.2.Linearity

The calibration curve was constructed for the flurbiprofen standard by plotting the concentration of compound versus peak area response. Standard solutions containing 0.10, 0.25, 0.5, 1.0, 2.0, 4.0, and 5.0 μg mL^{-1} of flurbiprofen were prepared and 10 μL was injected into the HPLC column. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. The regression equations were calculated from the calibration graphs, along with the standard deviations of the slope (Sb) and intercept (Sa) on the ordinate (Table 1).

Table 1. Linearity of flurbiprofen

Method	Range (μg mL^{-1})	LR[a]	Sa	Sb	R	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
HPLC	0.10-5.0	y=536.4x+76.13	10.24	11.28	0.9996	0.03	0.10

[a]Based on three calibration curves, LR:Linear regression, Sa: Standard deviation of intercept of the regression line, Sb:Standard deviation of the slope of regression line, R: Coefficient of correlation, x: flurbiprofen concentration, LOD: Limit of detection, LOQ: Limit of quantification y: peak area

3.2.3.Accuracy and precision

Accuracy of the assay method was determined for both intra-day and inter-day variations using the six time analysis of the QC samples. The precision of the assay was determined by repeatability (intra-day) and intermediate precision (interday). Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time that was evaluated by assaying the QC samples during the same day. Intermediate precision was assessed by comparing the assays on different days (3 days). Accuracy and precision calculated for the QC samples during the intra- and inter-day run are given in Table 2.

Table 2. Precision and accuracy of flurbiprofen

Method	Added (μg mL^{-1})	Intra-day			Inter-day		
		Found ± SD	Accuracy	Precision RSD%[a]	Found± SD	Accuracy	Precision RSD%[a]
HPLC	0.75	0.72 ± 0.025	-4.00	3.47	0.74 ± 0.021	-1.33	2.83
	3.0	3.07 ± 0.136	2.33	4.43	2.97 ± 0.071	-1.00	2.39
	5.0	5.08 ± 0.214	1.60	4.21	5.09 ± 0.232	1.80	4.56

SD: Standard deviation of six replicate determinations, RSD: Relative standard deviation, [a]average of six replicate determinations, Accuracy: (%relative error) (found-added)/addedx100

The accuracy ranged from 1.33% to 2.67% and precision from 2.39% to 4.56%. All the values were within the acceptance criteria of 5.0 %.

3.2.4.Sensitivity

Limits of detection (LOD) and quantification (LOQ) were estimated from the signal-to-noise ratio. The detection limit was defined as the lowest concentration level resulting in a peak height of three times the baseline noise. The quantitation limit was defined as the lowest concentration level that provided a peak height with a signal-to-noise ratio higher than 10, with precision (% RSD) and accuracy (% bias) within ±10%. LOD and LOQ values of the HPLC method were determined to be 0.03 and 0.10 μg mL^{-1}, respectively.

3.2.5. Stability

Stability studies indicated that the samples were stable when kept at room temperature, 4 and -20 ^{0}C refrigeration temperature for 8 h (short-term) and refrigerated at 4 and -20 ^{0}C for 72 h (long-term). The results of stability studies were given in Table 3 and no significant degradation was observed.

Table 3. Stability of flurbiprofen in solution

Stability (%)		Room temperature stability (Recovery % ± RSD)		Refrigeratory stability, +4 °C (Recovery % ± RSD)		Frozen stability, - 20 °C (Recovery % ± RSD)	
Method	Added (μg mL^{-1})	8 h	24 h	24 h	72 h	24 h	72 h
	0.50	99.7 ± 3.17	101.2 ± 3.84	99.1 ± 3.47	101.2 ± 4.26	97.4 ± 4.25	101.2 ± 3.92
HPLC	2.5	101.4 ± 3.64	102.3 ± 4.68	101.4 ± 5.06	98.2 ± 4.27	101.3 ± 4.78	99.4 ± 3.74
	4.5	98.3 ± 3.24	98.6 ± 4.09	101.3 ± 3.62	99.6 ± 5.84	99.2 ± 3.49	98.2 ± 4.98

RSD: Relative standard deviation of six replicate determinations

3.2.6.Recovery

Recovery studies by spiking different concentrations of pure drug in the pre-analyzed tablet samples within the analytical concentration range of the proposed method. The added quantities of the individual drugs were estimated by the above method. The results of recovery studies were found to be satisfactory and the results are presented in Table 4.

Table 4. Recovery of flurbiprofen in pharmaceutical preparations by HPLC method

Pharmaceutical preparation	Added (µg mL^{-1})	Found ± SD	Recovery (%)	RSD [a] (%)
Majezik	0.5	0.51 ± 0.018	102.0	3.53
tablet	2.5	2.52 ± 0.102	100.8	4.04
(1.0 µg mL^{-1})	3.5	3.51 ± 0.109	100.3	3.10
Frolix	0.5	0.50 ± 0.020	100.0	4.00
tablet	2.5	2.49 ± 0.129	99.6	5.18
(1.0 µg mL^{-1})	3.5	3.48 ± 0.112	99.4	3.20

SD: Standard deviation of six replicate determinations, RSD: Relative standard deviation, [a]average of six replicate determinations, Accuracy: (%relative error) (found-added)/addedx100

3.2.7.Comparison of the methods

Flurbiprofen is a non-steroidal anti-inflammatory agent, one of the propionic acid group, which has significant anti-inflammatory, analgesic and antipyretic properties. In this study, a fast and simple HPLC method is employed in the analysis of commercial preparations in the drug industry. The proposed method is used so much because it is a method easy to apply. Also, Pharmacopoeias [21-23] have reported titrimetric and liquid chromatographic methods for the analysis of flurbiprofen in pure form and pharmaceutical formulations. Titrimetric method involves dissolving about 0.5 g of accurately weighed flurbiprofen in 100 mL of alcohol (previously neutralized with 0.1 M sodium hydroxide versus to the phenolphthalein endpoint) and then, titrating the same (after adding phenolphthalein) with 0.1 M sodium hydroxide versus till the first appearance of faint pink colour that persists for not less than 30 seconds. Each ml of 0.1 M sodium hydroxide is equivalent to 24.43 mg of flurbiprofen. Another method has recommended the HPLC method for analysis of related substances in pure flurbiprofen and assay of flurbiprofen in pharmaceutical dosage form (tablet and ophthalmic drop). The methods recommended using a mobile phase of water-acetonitrile-glacial acetic acid (60:35:5, *v/v*) at a flow rate of 1 ml min^{-1}, using UV detection (254 nm) on a stainless steel column (4 µm, 3.9×15 cm i.d.).

The present work describes the validation parameters stated either by USP 26 [21] or by the ICH guideline [24] to achieve HPLC method for the determination of flurbiprofen. The proposed method is very effective for the assay of flurbiprofen in two different tablets. The validity of the proposed method was presented by recovery studies using the standard addition method. For this purpose, a known amount of reference drug was spiked to formulated tablets and the nominal value of

drug was estimated by the proposed method. Each level was repeated six times. The results were reproducible with low SD and RSD. No interference from the common excipients was observed. The RSD for intra- and inter-day variation was less than 4.56% for the HPLC method, which falls well below the acceptance criteria described by Shah et al. [25].

In comparison with earlier reported and official methods for estimation of flurbiprofen in pharmaceutical formulations the proposed HPLC method gave a lower LOD and LOQ at 30 and 100 ng ml^{-1} when compared to 100 ng ml^{-1} and 1 mg ml^{-1} of the earlier two proposed methods [26,27]. The proposed methods also gave a comparable or in most cases lower range of the calibration plot. Unlike reported methods, the proposed method does not utilize a special extraction step for recovering the drug from the formulation excipients matrices thereby decreasing the degree of error and time in estimation. The proposed methods of estimation of flurbiprofen is, therefore, more accurate and precise, rugged, reproducible and easier compared to other reported methods. Also, the sample recoveries in all formulations were in good agreement with their respective label claims and thus suggested the validity of the methods and non-interference of formulation excipients. The results show the high reliability and reproducibility of the method.

4.CONCLUSIONS

A rapid and simple isocratic HPLC method for the determination of flurbiprofen has been developed and validated. This chromatographic assay fulfilled all the requirements to be identified as a reliable and feasible method, including accuracy, linearity, recovery and precision. The chromatographic run time of 6 min allows for the analysis of a large number of samples in a short period. Therefore, the method is also suitable for analysis of sample during accelerated stability studies, routine analysis of formulations and raw materials.

Conflict of Interest

Author has no personal financial or non-financial interests.

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Review

Effectiveness of Propolis in the Treatment of Covid-19

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ABSTRACT:
Propolis is one of the ways to protect the colony health and immunity of honey bees, and it is now known that honey bees can protect the colony from many microorganisms by forming a propolis layer inside the nest. This antimicrobial property of propolis and the fact that honey bees use propolis in their nests to kill bacteria and viruses have led scientists to investigate whether it can be used in the treatment of COVID-19. In this review, studies investigating the effectiveness of propolis in the treatment of COVID-19 in the literature were compiled by scanning the PubMed internet database with the terms of propolis, COVID-19, SARS-CoV-2 and novel coronavirus. As a result of the search, nine reviews, five research articles, one case report, two preliminary studies and one randomized controlled study were found. From these studies, a study found that it can be used as a promising treatment method by developing a liposomal formulation against COVID-19 using corn propolis. Another study showed that the propolis prevents and inhibits the adhesion of the COVID-19 virus to the ACE2 protein, which was its main target to infect the host cell. They explained that this was due to the fact that the components of caffeic acid, quercetin, kaempferol and myricetin in propolis had a strong interaction with the ACE2 protein. In a study on the enzymatic inhibition of the SARS-CoV-2, it was determined that molecular compounds of propolis showed therapeutic efficacy. As a conclusion, studies have shown that propolis has promising therapeutic efficacy and can be used in the treatment of COVID-19. However, since most of the studies were experimental, non-clinical studies and reviews, there is a need for clinical studies with a high level of evidence.

Keywords: Bee product, COVID-19, propolis, review, treatment effectiveness

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1. INTRODUCTION

The novel coronavirus disease (COVID-19) which was first detected in Wuhan, China in December 2019 and then spread all over the world, is a respiratory disease that is transmitted from person to person through air droplets [1]. The coronavirus (SARS-CoV-2) uses the ACE2 (Angiotensin-converting enzyme-2) receptor to enter cells, similar to the other coronaviruses associated with the severe acute respiratory syndrome [2]. Therefore, it is important to prevent the connection of coronaviruses to the ACE2 receptor or targeting to reduce the binding potential to

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prevent the infection and accelerate the healing process. For this purpose, propolis, one of the popular bee products, has been the subject of researches in different studies in the treatment of COVID-19, due to its antiviral and antioxidant effects. In these propolis studies, it was targeted that propolis may have promising efficacy in the treatment of the COVID-19. In this review, we aimed to investigate these studies in the literature to identify the effectiveness of propolis in the treatment of the COVID-19.

2. METHOD

Different studies examining the effectiveness of propolis in the treatment of COVID-19 are available in the literature. In order to search the studies on this topic, the PubMed internet database was scanned with the term of propolis, COVID-19, SARS-CoV-2 and novel coronavirus. In the literature review, within the scope of the effectiveness of propolis in the treatment of COVID-19; one study examining by in-vitro and in-silico molecular method, one liposomal formulation development study, one enzymatic inhibition study, one host cell entry receptor (ACE2) inhibition study, one clinical study and one case report were included and compiled.

3. STUDIES INVESTIGATING THE EFFECTIVENESS OF PROPOLIS

In a study in Turkey, ethanol extracts of propolis were obtained from different cities in Turkey, from Van, Rize, Zonguldak, Muğla, Antalya, Diyarbakır and Giresun, and a commercial propolis extract sample was included in the study [3]. In the laboratory analyses of the propolis samples; they found that propolis was rich in caffeic acid, p-coumaric acid, ferulic acid, t-cinnamic acid, hesperetin, chrysin, pinocembrin and caffeic acid phenethyl ester (CAPE). The binding energies of these polyphenols to both the spike protein in COVID-19 and the ACE2 receptor in our cells were separately calculated by a molecular docking study. Based on the in-silico modeling and absorption, distribution, metabolism and excretion behavior of polyphenols, the propolis compounds were found to exhibit the potential to act effectively as new drugs. This study suggests that propolis has a high inhibitory potential against the COVID-19 virus, so it can be used as a natural food supplement to protect from COVID-19 and accelerate the treatment process.

Another study in Egypt showed that some components of Egyptian propolis had a higher binding affinity to the 3-chymotrypsin-like protease enzyme (3-CLpro) and spike protein of COVID-19 virus, compared to favipiravir, hydroxychloroquine and remdesivir [4]. An optimized liposomal formulation was also created in that study to increase the anti-viral effect of propolis against COVID-19, and this resulted in better delivery of propolis to both target cells and facilitated the entry of encapsulated propolis into cells.

In a study conducted in Indonesia, the effect of propolis on the enzymatic inhibition of the new coronavirus was investigated [5]. Compounds in Sulawesi propolis, produced by Tetragonula sapiens, a stingless bee genus, was found to

have the potential to inhibit SARS-CoV-2 key protease activity. It was determined that two compounds called broussoflavonol and gliasperin A, which are among the active ingredients in Sulawesi propolis, interact with the main protease catalytic sites of COVID-19 and have the ability to bind with the main protease.
According to a study in Brazil, it was determined that propolis inhibits the binding of COVID-19 to the ACE2 receptor, which is the main target for host cell invasion [6]. It was stated that propolis components, especially CAPE, rutin, quercetin, kaempferol and myricetin, have a strong in-silico interaction with ACE2. It was found that propolis reveals a decrease in interleukin-6 (IL-6), interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) levels, thus supporting the immunoregulation of pro-inflammatory cytokines. This immunoregulation includes monocytes and macrophages, as well as Janus kinase 2/signal transduction and activator of transcription 3 (Jak2/STAT3), Nuclear Factor kappa B (NF-kB) and inflammatory pathways, and reduces the risk of cytokine storm syndrome, which is an important death factor in advanced COVID-19 disease. In addition to these positive effects, it was stated that propolis does not interact with major liver enzymes or other key enzymes. It has also been reported that propolis can be used together with main drugs without the risk of potentiation or inactivation according to the criteria accepted by the World Health Organization (WHO).
In a randomized controlled clinical study conducted in Brazil, the efficacy of propolis in the treatment of COVID-19 was investigated by giving Brazilian green propolis as an adjunct treatment to 124 hospitalized COVID-19 patients [7]. In this single-center study, hospitalized adult COVID-19 patients were given a standard green propolis extract supplement as adjunctive therapy. In the study, a control group that received standard treatment and two separate propolis treatment groups that were treated with different doses of propolis treatment in addition to the standard treatment were formed. In the first group consisting of 40 patients from the propolis treatment groups, 400 mg of green propolis orally every day for seven days; the second group consisting of 42 patients was given 800 mg of green propolis orally. Standard treatment was applied to the control group consisting of 42 patients. After admission, the patients were followed-up for 28 days by applying group-specific treatment. It was observed that the length of hospital stay was shorter in both propolis groups than in the control group. The recovery period of the first group receiving 400 mg propolis supplement was seven days; The recovery time of the second group who received 800 mg propolis supplement was measured as six days, whereas, it was measured as 12 days in the control group who received standard treatment. It was determined that the use of propolis did not significantly affect the need for oxygen supplementation of the patients. However, it was observed that both propolis groups had much lower rates of acute kidney injury than the control group; and particularly, it was noted that the rate of acute kidney injury decreased significantly in patients who received high-dose

(800mg/day) propolis. In addition, propolis treatment was not discontinued in any of the patients during the treatment due to any side effects related to propolis. All the results showed that the use of propolis during the treatment of COVID-19 would accelerate the healing process and have positive effects in preventing acute kidney damage that may occur during the disease.

In a case report from Turkey, a 38-year-old male patient was admitted to the hospital with a complaint of tickling in his throat and the reverse transcription polymerase chain reaction (RT-PCR) test was positive [8]. It was stated that the routine blood values and Thorax computed tomography (CT) results when the patient was admitted to the hospital were normal. The patient, whose COVID-19 test was positive, developed a cough complaint 3 days after starting the medical treatment recommended in the Ministry of Health guidelines, and on the 5th day, this complaint became more severe. A repeat control thorax CT showed that bilateral large areas of ground glass were formed. After this stage, moxifloxacin was added to the patient's treatment. However, after 72 hours, it was decided to follow up the patient in the intensive care unit, whose general condition deteriorated and respiratory failure started in addition to the increasing complaints. Continuous positive airway pressure (CPAP) support was started by adding tocilizumab to his treatment. Although 2 days passed in the intensive care treatment, the patient's oxygenation and clinical condition did not improve. When a positive response to the treatment is not obtained despite the complete implementation of standard treatments; it was added Anatolian Propolis as a natural supplement to support the treatment, and Anatolian Propolis extract was started to be given to the patient as a 30% ethanol extract as 80 drops every day. At the end of the third day of propolis supplementation, a remarkable improvement was observed in the patient's oxygenation, blood parameters and radiological findings. The patient was discharged on the 10th day of hospitalization.

As a result of these reviewed studies, it was considered that it would not be correct to attribute the remarkable acceleration of the recovery processes of patients receiving COVID-19 treatment after starting regular propolis supplementation to propolis alone, but we suggest that propolis can be used as a strong natural supplement in addition to treatment in the light of these studies.

4. CONCLUSION

As natural support to the healing process, people who do not have a special allergy to bee products can be supplemented with propolis. We think that propolis, which has proven antioxidant and antiviral effects, will contribute positively to the treatment process of patients receiving COVID-19 treatment. But still further studies are required in this regard.

Conflict of Interest

Author has no personal financial or non-financial interests.

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Review

Visfatin as a Promising Marker of Metabolic Risk

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ABSTRACT:
Beyond energy storage, adipose tissue is an active endocrine organ in which adipocytokines are secreted, which have specific effects on various biological processes such as metabolic homeostasis, immunity and inflammation. However, excess energy accumulated in the body changes the size, distribution and cellular composition of adipose tissue, causing deterioration in its structural and functional functions. In this situation, beneficial adipocytokines are down-regulated, while leptin and other proinflammatory adipokines are highly up-regulated. Visfatin, also known as nicotinamide phosphoribosyl transferase (Nampt), is a proinflammatory cytokine intensely expressed in adipose tissue. Accumulating evidence focuses on the potential role of visfatin in the pathogenesis of metabolic diseases and related complications, suggesting that it may be a promising molecule. However, the contradictory results of the studies show that our understanding of visfatin is still speculative, but its secretion mechanism and physiological role are not fully understood. In this review, the role of this promising molecule in metabolic diseases is explained by presenting comprehensive information about visfatin/Nampt.

Keywords: Metabolism, obesity, risk factor, visfatin

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1. BACKGROUND

The function, molecular targets, and potential clinical significance of recently identified visfatin expressed in adipose tissue have not yet been adequately clarified due to conflicting findings. In addition to being a prominent biomarker in inflammation, clinical evidence supporting its role in various metabolic diseases is increasing. It is an adipocytokine with beneficial effects as an insulin-mimetic, which also has glucose-lowering properties. Elevated serum visfatin has been associated with increased adipose tissue mass in obesity, PCOS, and metabolic syndrome. Considering the increasing morbidity and mortality rates in metabolic diseases; Further research is needed regarding the effect of visfatin and its therapeutic target potential.

2. INTRODUCTION

Beyond energy storage, adipose tissue is an active endocrine organ in which adipocytokines are secreted, which have specific effects on various biological

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processes such as metabolic homeostasis, immunity, and inflammation [1]. However, excess energy accumulated in the body changes the size, distribution, and cellular composition of adipose tissue, causing deterioration in its structural and functional functions [2]. In this situation, beneficial adipocytokines are down-regulated, while leptin and other proinflammatory adipokines are highly up-regulated. Visfatin in 1994 by Samal et al. It is an adipocytokine with 491 amino acids, weighing 52 kDa, defined as the "pre-B cell enhancing factor (PBEF)" protein that stimulates B-lymphocyte formation in the bone marrow [3]. The protein, also known as nicotinamide phosphoribosyl transferase (Nampt), was later determined to be secreted predominantly in visceral adipose tissue and was changed to "visceral fat protein" visfatin. Apart from adipose tissue, it is effective in various biological activities in a paracrine or endocrine manner expressed in hepatocytes, myeloblasts, immune cells, heart and pancreas [4]. Accordingly, studies have focused on the potential role of visfatin in the pathogenesis of metabolic diseases and related complications. It has been reported that increased visfatin serum levels are associated with diseases such as obesity, diabetes and insulin resistance [5]. Despite the contradictory results of recent studies showing that our understanding of Visfatin is still speculative, its secretion mechanism and physiological functions are not fully understood.

3. DISCUSSION

Visfatin is a versatile molecule that plays an important role in many physiological and pathophysiological processes, especially in maintaining metabolic homeostasis and inflammation [3]. Studies have focused on the potential role of visfatin in the pathogenesis of metabolic diseases and related complications. While various studies indicate that plasma visfatin levels are increased in obesity, type 2 diabetes and metabolic syndrome, on the contrary, some studies have claimed that plasma visfatin levels are unchanged or even lower in obesity and type 2 diabetes compared to healthy controls [4,5]. In addition, circulating Visfatin levels and body mass was investigated in the study. A significant correlation between index and body fat percentage was found [6]. Jin et al. In their study, it was determined that visfatin levels were significantly increased in obese adolescents [7]. We sought to elucidate the underlying molecular mechanisms associated with increased visceral adipose tissue insulin resistance and type 2 diabetes [8].

Similar to insulin, visfatin stimulates glucose uptake in adipocytes and myocytes, while inhibiting glucose release from hepatocytes [3]. It has been suggested that visfatin, which has a glucose-lowering effect by binding to the insulin receptor, may play a role in the pathogenesis of diabetes.

In the study investigating the relationship between serum visfatin and insulin and magnesium in patients with type 2 diabetes without insulin resistance, it was determined that visfatin level was significantly higher in the study group than in healthy controls [9].

Polycystic ovary syndrome (PCOS) is a common hormonal disorder associated with insulin resistance and obesity among women [10]. Some of the studies evaluating the correlation between serum visfatin levels and some metabolic parameters in non-obese women with PCOS and controls found serum visfatin levels in PCOS patients and controls to be similar [10, 11] while other studies found higher visfatin levels in PCOS patients [12].

Conflict of Interest

Author has no personal financial or non-financial interests.

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Review

Chemical Structure and Synthesis of Baloxavir Marboxil, A Novel Endonuclease Inhibitor For The Treatment Influenza : An Overview

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ABSTRACT: One drug target that was identifed as a promising candidate for infuenza antiviral drugs is the infuenza virus polymerase complex. The three-component polymerase complex, which is highly conserved and is essential for infuenza virus replication, has received considerable attention as a potential target for infuenza antiviral drugs. Baloxavir marboxil (BXM, Xofuza ®; hereafter referred to as baloxavir), the prodrug of baloxavir acid, is a frst-in-class, small molecule inhibitor of the cap-dependent endonuclease reaction that is conducted by the polymerase acidic (PA) protein subunit of the infuenza virus polymerase complex. In this review, we summarized the chemical structure, synthesis, mechanism of action, pharmacological activity and synthesis of Baloxavir marboxil, a novel FDA-approved endonuclease inhibitor for the treatment of influenza.

Keywords: Antiviral, baloxavir marboxil, endonuclease inhibitor, influenza, synthesis.

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1. INTRODUCTION

The influenza virus (IV) causes an infectious disease associated with 290,000 to 650,000 deaths and 3 to 5 million cases of severe illness worldwide each year [1]. Additionally, pandemics caused by emerging reorder viruses can have a devastating impact globally. Therefore, continuous efforts are needed to improve the vaccines and antiviral drugs used in the treatment [2]. Two classes of antivirals are currently available for clinical use, neuraminidase inhibitors (NAIs: oseltamivir, zanamivir, peramivir) and M2 ion channel inhibitors (amantadine, rimantadine) [3]. However, influenza viruses are now largely resistant to M2 inhibitors. Furthermore, the antiviral potency of NAIs is relatively moderate, and another concern for this class of drugs is the emergence of resistance, as occurred during the 2008 to 2009 season when oseltamivir-resistant H1N1 was prevalent

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[4-7]. Therefore, more effective antiviral agents with a new mechanism of action are required for the treatment and prevention of influenza virus infections [7].
Baloxavir is the first antiviral drug in the cap-dependent endonuclease inhibitors class. Currently, the only FDA-approved indication is the treatment of acute uncomplicated influenza in patients 12 years of age and older who have been symptomatic for more than 48 hours. Treatment consists of an oral dose within the first 48 hours of illness onset [8]. Shionogi discovered and developed baloxavir marboxil and received approval in Japan in February 2018 [8]. Shionogi transferred its rights to Roche / Genentech, Inc. on May 17, 2018, with approval in the United States in October 2018 [8]. The general properties of baloxavir marboxil are given in Table 1 [9].

Table 1. General properties of baloxavir marboxil compound

Generic Name	Baloxavir Marboxil
Trade Name	Xofluza
CAS Number	1985606-14-1
Formula	$C_{27}H_{23}F_2N_3O_7S$
Molecular Weight	571.55 $g \cdot mol^{-1}$
Mechanism of Action	Endonuclease inhibitors
Class	Antivirals; Dibenzothiepins; Esters; Pyridines; Triazine
Application Path	Oral
Recommended Indication	Treatment of acute uncomplicated influenza in patients 12 years of age and older who have been symptomatic for no more than 48 hours

1.1. General Chemical Structure of the Compound

Baloxavir marboxil is a polycyclic carbamoylpyridone derivative compound [10]. The structure of the polycyclic carbamoylpyridone derivatives of the present invention is as in Figure 1. The polycyclic carbamoylpyridone derivative of the present invention functions as an inhibitor of influenza 5' cap-like structure (CAP)-dependent endonuclease activity and can be used to treat a cold caused by influenza virus [10, 11]. The IUPAC name of BXM is ({(12aR)-12-[(11S)-7,8-difluoro-6,11-dihydrodibenzo[b,e]thiepin-11-yl]-6,8-dioxo-3,4,6,8,12,12a-hexahydro-1H-[1,4]oxazino[3,4-c]pyrido[2,1-f][1,2,4]triazin-7-yl}oxy)methyl methyl carbonate [12].

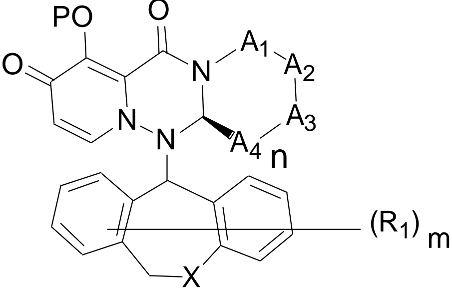

Figure 1. Structure of Polycyclic Carbamoylpyridone Derivatives

Baloxavir acid (BXA,1) is the active metabolite of the prodrug baloxavir marboxil (BXM, 2) which is marketed as Xofluza® (Figure 2). Baloxavir marboxil is a prodrug that hydrolyzes to produce the active drug, baloxavir acid (Figure 2). At the 5 stages of a viral life cycle in the host cell (viral entry, uncoated, viral replication, assembly and budding, and finally viral release), baloxavir targets the third stage of viral replication [13].

Baloxavir Acid (BXA) **Baloxavir Marboxil (BXM)**

Figure 2. Chemical structure of baloxavir acid (BXA, active form) and baloxavir marboxil (prodrug form).

1.2. Mechanism of Action

Influenza virus is a member of the RNA-virus group and humans are infected by three viral strains: IVA, IVB, and IVC. IV infects cells by binding sialic acid residues, which promotes its endocytosis via an enzymatic cascade. This virus, after its penetration and uncoating, RNA is replicated through a relatively complicated process [14]. Eight segments of viral RNA encode for 11 to 12 proteins only, depending on the strains [15]. RNA dependent RNA polymerase and other viral nucleoproteins are a molecular complex called the viral ribonucleoprotein complex [16]. This complex works on both replication of viral RNA to produce new virions and transcription to synthesize proteins. RNA dependent RNA polymerase is formed of different subunits: polymerase acidic protein (PA), polymerase basic protein 1 (PB1), and polymerase basic protein 2 (PB2) [17]. Baloxavir acid inhibits the endonuclease activity of polymerase acidic (PA) protein, one of the subunits of viral RNA polymerase. This endonuclease is a virus-specific enzyme required for viral gene transcription [12]. Transcription of viral mRNA begins via a "cap snatching" mechanism, binds to a capped RNA formed by the host's RNA polymerase II, and the endonuclease of the PA protein cleaves from the 5'-cap of the RNA to produce a primer [18]. This capped primer is then used by viral RNA polymerase for viral mRNA synthesis. The enzyme that produces this essential primer is called capsular endonuclease, and through inhibition of this enzyme, baloxavir can inhibit influenza viral replication for both influenza A and B viruses [19]. The antiviral mechanism of BXM is given in Figure 3 [18].

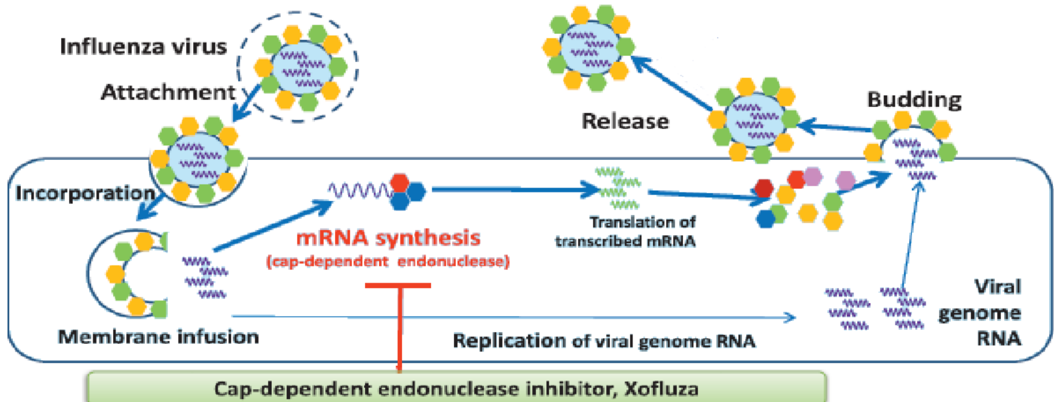

Figure 3. Antiviral mechanism of BXM

1.3. Pharmacokinetic Properties of Baloxavir Marboxil

Following oral administration of baloxavir marboxil, it is converted to the active metabolite, baloxavir, by esterases in the GI lumen, liver, and blood [20]. The mean time to reach plasma concentrations of baloxavir after oral administration of baloxavir marboxil is 4 hours [21]. Baloxavir and its metabolites are distributed in milk in rats, it is not known whether they are distributed in breast milk. The plasma protein binding of BXM is approximately 93-94% [22]. Following oral administration, baloxavir marboxil is rapidly hydrolyzed by esterases to its active metabolite baloxavir [20]. Baloxavir is metabolized via UGT1A3 and CYP3A4 with major contribution from UGT1A3. No clinically relevant effects were observed when baloxavir was co-administered with strong CYP3A and UGT inhibitors such as itraconazole and probenecid [23]. In addition, Baloxavir can chelate with polyvalent cations and therefore co-administration with drugs containing calcium, aluminum, magnesium or iron should be avoided [13, 23].

1.4. Synthesis of Baloxavir Marboxil

Baloxavir consists of two main parts, each with a chiral center. These two parts are shown in Figure 4. Each of these two parts (1,2) must be synthesized to obtain the resultant compound. The coupling and deprotection steps must then be performed [8, 24].

Figure 4. Retrosynthetic Approach to Baloxavir

a) Synthesis of compound 1:

Synthesis of compound 1 started from lactam 3. Protection of 3 with allyl chloroformate yielded compound 4 in 62% yield. The synthesis proceeded with a three-step reaction of morpholino compound 6 (Figure 5). Reduction of DIBAL-H (93% yield) and addition with MeOH under acidic conditions in 87% yield resulted in racemic methoxy compound 6 (Figure 5) [24].

Figure 5. Preparation of Morpholino Compound 6

To prepare the hydrazine fragment (10) (1-amino-3-benzyloxy-4-oxo-1,4-dihydro-pyridine-2-carboxylic acid ethyl ester), the carboxylic acid-bearing compound 7 was esterified with EtI in quantitative yield and then It was reacted with Boc-hydrazine to produce pyridone 9 in 73% yield, followed by quantitative deprotection with anhydrous HCl (Figure 6) [24].

Figure 6. Preparation of Hydrazine Fragment 10

The combination of fragments 6 and 10 was mixed with $SnCl_4$ in MeCN at −25 °C to form the nonsubstituted hydrazine (11) (Figure 7). The Alloc protecting group was removed using Pd catalysis by cyclization to obtain compound 1-rac in 100% yield (Figure 7). Separation of the 1-rac enantiomers was accomplished by forming hydrazide diastereomers, selective crystallization of the desired (R, R)-diastereomer, and then hydrolysis to produce 1-R. Specifically, 1-rac was precipitated with diastereomers (R)-tetrahydrofuran-2-carboxylic acid (12) precipitated from the reaction mixture to form 13A and 13B. Removal of the tetrahydrofuroyl group was accomplished by addition of DBU in EtOH for 0.5 hours at room temperature. Then, by adding diisopropyl ether to the reaction mixture, 1-R was obtained in 90% yield (Figure 7) [24].

Figure 7. Preparation of compound 1

b) Synthesis of tricyclic sulfur (2) compound

The synthesis of the tricyclic sulfur compound (2) was prepared in five steps and in 71% yield (Figure 8). 3,4-Difluorobenzoic acid (14) was metallized at the 2-position between the carboxylic acid and the 3-F group using Lithium diisopropylamide (LDA) in tetrahydrofuran and then treated with DMF to form compound 15. After replacing the solvent with toluene, reaction with D-camphorsulfonic acid-mediated thiophenol gave thioacetal compound 16 as solution in toluene. Compound 16 was then reduced with 1,1,3,3-tetramethylsiloxane and $AlCl_3$ to obtain compound 17. Treatment of compound 17 with pure phenylpropanolamine (PPA) at 120 °C yielded compound 18, which is tricyclic sulfide in 91% yield. Reduction of the ketone was carried out with $NaBH_4$ to give racemic sulfide 2 in 97% yield [8, 24].

Figure 8. Preparation of Tricyclic Sulfite (2) compound

c) Final step for baloxavir marboxil synthesis:

Figure 9. Final step for baloxavir marboxil synthesis

The coupling of the 1-R and 2 fragments was carried out under dehydration conditions of 1-propanephosphonic anhydride (T_3P) and methanesulfonic acid at 70 °C to obtain protected baloxavir 19. Compound 19 was then reacted with 0.6 equivalents of PhBr and K_2CO_3. Debenzylation was then carried out using LiCl in CH_3CONMe_2 to give baloxavir acid in 94% yield. In the final step for the preparation of the prodrug, baloxavir acid was reacted with chloromethyl methyl carbonate in dimethylacetamide in 93% yield to form baloxavir marboxyl. The reaction mechanism for the final step for the synthesis of BXM is given in Figure 9 [8, 24].

CONCLUSION

The approval of baloxavir marboxil as an influenza antiviral drug is a big step forward as it represents a new age class of drugs for the treatment of influenza, such as endonuclease inhibitors. The discovery, synthesis and proof of influenza inhibitory effect of BXM, a polycyclic carbamoylpyridone derivative, is important for the discovery of new endonuclease inhibitor antiviral agents with this special structure.

Conflict of Interest

Author has no personal financial or non-financial interests.

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