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Acknowledgments

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All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence their work. If no conflict exists, the authors should include the "The authors declare no conflict of interest." statement under this section.

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Kaynak MS, Celebier M, Akgeyik E, Sahin S, Altınoz S. Application of HPLC to investigate the physicochemical properties and intestinal permeability of ketoprofen. Curr Pharm Anal. (2017); 13(1): 72-79. https://doi.org/10.2174/1573412912666160422151409

Başaran E, Yenilmez E, Berkman MS, Büyükköroğlu G, Yazan Y. Chitosan nanoparticles for ocular delivery of cyclosporine A. J Microencapsul. (2014); 31(1): 49-57. https://doi.org/10.3109/02652048.2013.805839

Book

Fotaki N, Klein S. In vitro drug release testing of special dosage forms. New Jersey: John Wiley & Sons; (2019). ISBN:1118341473

Wilson CG, Crowley PJ. Controlled release in oral drug delivery. New York: Springer; (2011). ISBN:1461410045

Book Chapter

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Patent

Wong HL, Narvekar M, Xue HY, inventors; Temple University, assignee. Nanospheres for therapeutic agent delivery. United States patent no 9724304. (2017).

Thesis

Arora HC. Doxorubicin-nanocarriers enhance doxorubicin uptake and clathrin-mediated endocytosis in drug-resistant ovarian cancer cells [Ph.D.]. Illinois: Northwestern University; (2012).

Finn NA. Role of redox systems in doxorubicin metabolism and doxorubicin-mediated cell signaling: a computational analysis [Ph.D.]. Atlanta: Georgia Institute of Technology; (2011).

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RESEARCH ARTICLE

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Journal of Pharmaceutical Technology

Development and validation of an HPLC method for determination of quercetin

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ABSTRACT

This study validated a high performance liquid chromatography (HPLC) method for the determination of quercetin (QE) in microemulsion (ME) based gel formulations. The analyses were performed on a C18 column (150x4.6 mm, 5 µm particle size) at room temperature with UV detection at 254 nm. The mobile phase was composed of methanol:water (65:35, v/v, 2% acetic acid) mixture, and flow rate was set to 1 mL/min. The method was validated according to the international guidelines with respect to linearity range, stability, limit of quantitation and detection, precision and accuracy. The method was linear within 5 – 100 µg/mL range with a correlation coefficient of 0.9995. The intra- and inter-assay precisions presented RSD values lower than 2%. The method reported is a fast and reliable HPLC method useful for QE determination in ME based gel formulations.

1. INTRODUCTION

QE is readily available in fruits and vegetables and is preferred because of its unique biological properties that can improve mental/physical performance and reduce the risk of infection [1]. Flavonoids are very promising drug candidates because of their ability to scavenge radicals. Considering its anti-inflammatory, antiviral and antioxidant activities and its ability to inhibit lipid peroxidation, platelet aggregation and capillary permeability, its potential benefits for general health and disease resistance are revealed [2].

QE has been formulated and characterized in many dosage forms such as nanoemulsion, liposome, lipid nanoparticle, nanostructured lipid carrier and solid lipid nanoparticle [3-7]. Poor solubility and stability properties of QE occurs difficulties during formulation design. Due to lipophilic cyclic structure of QE, its partition coefficient is 1.82 ± 0.32 [8, 9]. However, despite this log P, penetration into the skin is limited due to quercetin's polar hydroxyl groups. In order to increase the absorption of QE through the skin, various dosage forms have been tried and evaluated with in vitro-in vivo characterizations. Its poor water solubility suggested the presence of a lipid phase to increase the solubility of QE in the formulation. On the other hand, QE polar heads favor water presence, so quercetin can localize at the interface [8]. Microemulsion (ME) is defined as a dispersion consisting of oil, surfactant, cosurfactant and aqueous phase, which promising thermodynamically stable liquid systems with a droplet diameter usually within the range of 10-100

nm. However, the liquid form and low viscosity of MEs limitates adhere to the surface of the skin and maintain long time on the application area [10, 11]. In order to overcome these limitations and prolong the functioning time on the skin, gelling agents were used to obtain proper viscosity for topical application. MEs provide significant advantages on improving drug delivery through transdermal and dermal routes of administration; as the have solubilizing ability for poorly water soluble drugs, convenient particle size and permeation ability across skin membrane [12]. However, since the viscosity plays an important role in promoting drug retention at the application site, microemulsions have a considerable limitation [13]. Microemulsions are low-viscous liquids and are subject to dosing errors during application due to their vicosity, beside allowing limited drug retention at the skin [14]. To overcome these limitations, incorporating microemulsions into gelling systems, enhances the application. In order to increase viscosity, improve application on the skin and to prolong the drug retention at the application site, Carbopol based gels provide great advantages as reported in the literature [15].

Up to now, HPLC and spectrophotometric methods have been the most common techniques which have been utilized for the determination of quercetin [16-19]. In this study, a simple and reliable HPLC method was developed and validated for in vitro determination of QE from the ME based gel formulations.

2. MATERIALS AND METHODS

2.1. Materials

QE was obtained from Sigma Aldrich (USA). Ethanol, disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) were purchased from Merck (Germany). Deionized water was obtained from Mili-Q water system, that is used for preparation of buffers and standard solutions. All other chemicals were reagent grade and used without further purification.

2.2. Methods

2.2.1. Instrumentation

Chromatogrphic system was carried out using isocratic mode on Shimadzu Nexera 2. C18 column 250 x 4,6 mm, particle size 5 µm was used (GL Sciences). The mobile phase consisting 65% methanol: 35% purified water(v/v) mixture with 2% acetic acid. The flow rate was set at 1 mL/min with 20 µL as injection volume. Detection wavelength was 254 nm at 25°C and retention time was 6.5 min [19].

2.2.2. Standard Solutions

The stock solution of quercetin (100 µg/mL) was prepared in ethanol. pH 5.5 phosphate buffered saline:ethanol (60:40) (v/v) mixture was used to obtain standard solutions within the concentration range of 5-100 µg/mL

2.2.3. Method Validation

Method validation studies of Quercetin was carried out occording to the International Council on Harmonization (ICH) Guideline Q2(R1). [20] The described method was validated as linearity, accuracy, precision (intra-day precision, inter day precision), sensitivity and stability.

2.2.4. Linearity

The linearity of the method was determined by selected eleven concentration points which were 5, 10, 15, 20, 30, 40, 80 and 100 µg/mL. The calibration equation evaluated by determination coefficient, slope and intercept.

2.2.5. Precision

The inter- day precision studies were carried out for three different concentrations (5, 40, 80 µg/mL) within calibration range analysed 3 consecutive days. Intra-day precision studies also carried out with same 3 concentrations within the same day. All results evaluated by mean, standard deviation (SD) and relative standard deviation (RSD %) values.

2.2.6. Accuracy

The accuracy of the method was assessed by performing the recovery study. It was conducted by

adding known amount of quercetin. Three different concentrations of quercetin (5, 40, 80 µg/mL) added to placebo formulation as final concentration of active substance will be 0.5%.

2.2.7. Sensitivity

Limit of detection (LOD) is the minimum concentration of analyte which can be detected in a sample. Limit of quantitation (LOQ) is the lowest amount of analyte in a sample which can be quantitatively determined with acceptable precision and accuracy under the specified conditions, to evaluate the sensitivity of method.

Besides, LOD (the lowest QE concentration that can be detected but not quantitated in the sample) and LOQ (the lowest QE concentration in the sample that can be measured with suitable precision and accuracy) were calculated using the following equations (σ= the standard deviation (SD) of the intercept; S=the slope of the calibration curve).

$$LOD = 3.3x\left(\frac{\sigma}{S}\right) \quad \text{Equation 1}$$

$$LOQ = 10x\left(\frac{\sigma}{S}\right) \quad \text{Equation 2}$$

2.2.8. Stability

The stability of samples at three different concentrations (5, 40, 80 µg/mL) were evaluated during test conditions. Results evaluated by mean, SD and RSD % compared to initial concentrations.

3. RESULTS AND DISCUSSION

3.1. Optimization of Chromatographic Conditions

It was determined that QE has a maximum absorbance in 254 nm wavelength. Column selectivity for the separation of all related substances is critical. QE was well retained and separated with comparatively sharp peaks using the C18 column (4.6 x 250 mm, 5 µm particle size) (Figure 1). The optimum conditions for the HPLC method were given in Table 1.

3.2. Validation of the Method

3.2.1. Linearity

Linearity of the method for quercetin evaluated by calibration equation and determination coefficient. According to calibration curves the method was found linear wirthin the concentration range of 5-100 µg/mL. Determination coefficient was over 0.999 as demonstration of linearity.

Table 1. *Optimum conditions for HPLC analysis*

Column	C18 (4.6 x 250 mm, 5 µm, GL Sciences)
Mobile phase	Methanol:Water (65:35) (v/v) + 2% acetic acid
Wavelength	254 nm
Temperature	25°C
Injection volume	20 µL
Flow rate	1 mL/min
Retention time	6.5 min

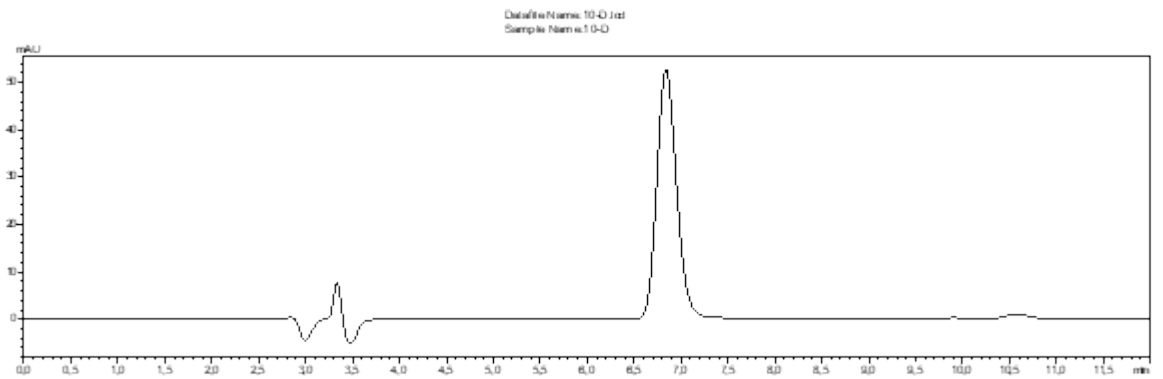

Figure 1. *HPLC chromatogram of QE (10 µg/mL)*

Table 2. *The linearity data of the developed method (n=6)*

Calibration equation	y = 78242x + 49282
Determination coefficient (r2)	0.9995
Linearity range (µg/mL)	5-100
Number of points	8
LOQ (µg/mL)	10.65
LOD (µg/mL)	3.52

3.2.2. Sensitivity

The sensitivity of the analytical method was evaluated by determining LOD; (signal to noise ratios of 3:1) and LOQ; (signal to noise ratios of 10:1). The LOD value of 3.52 µg/mL and LOQ value of 10.65 µg/mL were verified the sensitivity of the analytical method (Table 2). LOQ was taken as lowest concentration of QE that could be quantitively determined with acceptable accuracy and precision

3.2.3. Accuracy

The accuracy of the method was evaluated at 3 different concentrations (5, 40, 80 µg/mL) as a result of recovery calculation. The RSD % values which is less than 2% was taken as an indication of sufficient accuracy of the developed method.

3.2.4. Precision

Inter-day and intra-day precision studies evaluated by mean, SD and RSD % values. The RSD % values which is less than 2% was taken as an indication of sufficient precision of the developed method.

3.2.5. Stability

Stability of standard solutions during analyses were evaluated at 3 concentrations (5, 40, 80 µg/mL). Results evaluated by evaluation of mean, SD and RSD % compared to initial concentrations.

Table 3. *Accuracy results*

Added Concentration (5, 40, 80 µg/mL)	**Measured Concentration (µg/mL)**			**Percentage (%)**		
	5.15	40.03	80.12	103.00	100.08	100.15
	5.09	40.10	80.09	101.80	100.25	100.11
	5.11	40.03	80.11	102.20	100.08	100.14
	5.10	40.07	79.91	102.00	100.18	99.89
	4.96	40.09	80.01	99.20	100.23	100.01
	4.98	40.11	80.12	99.60	100.28	100.15
Mean	5.07	40.07	80.06	101.30	100.18	100.08
SD	0.08	0.03	0.08	1.53	0.09	0.11
RSD(%)	1.51	0.09	0.11	1.51	0.09	0.11

Table 4. *Intra day precision results*

Concentration (µg/mL)	**5**	**40**	**80**
	5.11	40.03	80.03
	5.12	40.07	80.08
	5.09	40.12	80.10
	4.92	40.12	80.01
	5.05	40.03	79.98
	5.11	40.14	80.08
Mean	5.07	40.09	80.05
SD	0.08	0.05	0.05
RSD (%)	1.50	0.12	0.06

Table 5. *Inter day precision results*

Concentration (µg/mL)	**5**	**40**	**80**
	5.11	40.12	80.15
	5.13	40.13	80.15
	5.15	40.14	79.89
	5.09	39.91	80.05
	5.12	39.97	80.10
	5.16	40.14	80.15
Mean	5.13	40.07	80.08
SD	0.03	0.10	0.10
RSD (%)	0.50	0.25	0.13

Table 6. *Stability Results*

Time (h)	Concentration (µg/mL)		
	5	40	80
0	5.04±0.03 (0.60)*	40.04±0.02 (0.05)*	80.03±003
24	5.01±0.04	40.03±0.03	80.01±0.04
48	4.98±0.06	40.00±0.05	79.98±0.08

*RSD % values

3.2.6. Application of the method

Pseudo-ternary phase diagrams of microemulsion area were drawn with oleic acid, Kolliphor EL, Transcutol P and water mixtures with surfactant/cosurfactant ratio of 2:1, 1:1, 1:2 [21, 22]. In order to prepare microemulsion based gels, Carbopol 980 1 % was dispersed in purified water with constant stirring to obtain gel base, then the pH was adjusted to 6-6.5 using triethanolamine. Microemulsions were mixed with the gel in 1:1 (w/w) ratio to obtain homogeneous formulation [23].

The particle size and polidispersity index (PDI) of 1:2 ME, 1:1 ME and 2:1 ME were 183,7 nm (PDI:0,292), 179,8 nm (PDI: 0,518) and 441,1 nm (PDI:0,445) respectively [24]. According to in vitro characterization studies, surfactant:cosurfactant ratio 1:1 microemulsion and microemulsion based gel (1:1)(w/w) formulations compared to the pure quercetin during in vitro release studies. Cumulative percentage of quercetin released from formulations determined by the validated HPLC method at predetermined time intervals and carried out for 24 hours. All that results showed that the validated HPLC method can be easily used for the in vitro characterization of QE from microemulsion based gel formulations.

4. CONCLUSION

A new HPLC method was developed and validated for determination and quantification of QE in microemulsion based gel formulations. The method was successfully validated, and all the results obtained confirmed linearity, sensitivity, precision and accuracy of the proposed method. All validation results revealed that it is a preferred method for obtaining reliable data in further experiments such as formulation development and quality control studies for QE.

ETHICAL APPROVAL

The study was approved by the XXX (Protocol no. XXX / Month DD,YYYY).

AUTHOR CONTRIBUTION

Concept: TÇ, DO, UMG, ED; Design: TÇ, DO, UMG, ED; Supervision: TÇ, DO, UMG, ED; Materials: TÇ, DO, UMG, ED; Data Collection and/or Processing: TÇ, DO, UMG, ED; Analysis and/or Interpretation: TÇ, DO, UMG, ED; Literature Search: TÇ, DO, UMG, ED; Writing: TÇ, DO, UMG, ED; Critical Reviews: TÇ, DO, UMG, ED.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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REVIEW ARTICLE

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A review on collagen as a food supplement

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ABSTRACT

One of the most abundant proteins formed in the human body is collagen. It is responsible for the strength of body tissues in all cellular systems by establishing support networks. Collagen fibers are generally opaque and white, due to this reason they are easily recognized in the tissues. Collagen fibers, which are viscoelastic, have low extensibility and high tensile strength. The isoelectric point is around pH 5.8. Collagen is widely used as it helps blood coagulate, remodeling tissue. Although there are no known side effects, there is some concern about its role in inflammation, group-to-group variability, and possible disease transfection, given that animal-derived (natural) collagen is used in many clinical applications. Not every protein is of equal value, so not every collagen is of equal value. Thanks to its low molecular weight, hydrolyzed collagen is quickly digested by our body and enters our blood circulation. According to study, the most suitable type of collagen is the one in powder or liquid form for collagen peptides that can be easily absorbed and used by our body. It is necessary to rely on the most appropriate analysis technique to evaluate the quality feature. Techniques such as SEC, MS, HPLC and NMR are used to characterize complex peptide mixtures. Classical fibril-forming collagen includes collagen types I, II, III, V, and XI. These collagenes are characterized by their ability to aggregate into highly oriented supramolecular aggregates, forming fibrillar arrays with diameters between 25 and 400 nm. Being a natural protein and its technical characteristics, it is expected that development and consumption of collagen hydrolyzate will increase in the coming years. In this review, physical and chemical properties of collagen, types, pharmacology properties, quantity determination methods are briefly described.

1. INTRODUCTION

One of the most abundant proteins formed in the human body is collagen. It is responsible for the strength of body tissues in all cellular systems by establishing support networks. Therefore, many companies are making tremendous efforts to integrate this biomolecule into many products in different pharmaceutical forms such as tablets, luqiud forms, capsules, semisolid topical forms etc. In addition, collagen hydrolyzate has been shown to exhibit properties such as antioxidant, antihypertensive activity, lipid lowering activity, as well as restorative properties in damaged skin. It has also been proposed in recent animal studies and human trials that oral

administration of collagen or its hydrolysates has beneficial effects on skin aging. The question of whether collagen can be absorbed and moreover, in what manner, must be explained in order to speculate on the mechanism of the physiological effects. It has been documented that the concentration of hydroxyproline (Hyp) in human blood increased dramatically and reached a maximum level after oral ingestion of collagen hydrolysates within 0.5-2 h [1]. However, there is little data on the bioavailability of collagen, a high molecular weight scleroprotein (about 300 kDa) with a special triple helix structure and an unbalanced amino acid profile [2] .

In addition, after oral ingestion of collagen hydrolysates, many collagen peptides (Pro-Hyp, Pro-Hyp-Gly, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Leu-Hyp, Ile-Hyp, Phe-Hyp, Hyp-Gly) have been identified from the blood [3].

2. PHYSICOCHEMICAL PROPERTIES

Collagen fibers are generally opaque and white, due to this reason they are easily recognized in the tissues. Collagen fibers, which are viscoelastic, have low extensibility and high tensile strength. The isoelectric point is around pH 5.8 [4]. Based on the molecule's position in the human body, its chain link, and its structure until now, it is divided into 26 different species grouped into eight families. Classifications may include fibril-forming, basement membrane, microfibrillar, anchoring fibrils, hexagonal network, discontinuous triple-helix [FACIT], transmembrane and fibril-associated collagen with multiplexes. A left-handed helical conformation is adopted by each collagen chain, and the three strands intertwine with a right-handed superhelical twist exhibiting a molecular weight as high as ~ 300 kDa [5]. As a natural polymer, collagen molecules appeared to entangle and accumulate in solution, resulting in a remarkable viscoelastic behaviour. For instance, the storage modulus of collagen at 2.1% wt was 750 Pa tested in oscillatory rheological measurements (at a constant frequency of 1 Hz and a constant strain of 5%), while the storage modulus of poly(vinyl alcohol) (2.1%) was only 0.2 Pa at the same experimental conditions. Furthermore, the complex viscosity values of collagen (0.8%) was 171.6 Pa s at 0.1 Hz, far higher than that of hydroxypropyl methylcellulose (3.2 Pa s) [6]. Some physicochemical properties of polymers have been found to be associated with their aggregation behavior in solution, such as optical properties, colloidal stability and tensile strength [7]. It was therefore important to explore the aggregation behavior of polymers in solutions to provide some information on their use or processing. As for the aggregation activity of collagen, several studies have been performed. Shi et al., for instance, examined collagen aggregation in acetic acid, using the 3-methoxy-4'-N,N-dimethylamino flavone derivative intramolecular charge transfer compound as a fluorescence probe and fluorescence polarization. Collagen was thought to be self-associated in acid aqueous solution by increasing its concentration and its critical aggregation concentration (CAC) was 0.5 mg/mL [8]. Wu et al. used the intrinsic fluorescence of aromatic amino acid residues in the collagen molecule to further analyze the aggregation of collagen in the acid solution; and it has been shown that collagen aggregates have occurred in the range of 0.30 - 0.45 mg/mL, while the more complex collagen aggregates have appeared in the range of 0.75 - 1.05 mg/mL [9]. Collagen as a protein was heat sensitive and would be denatured at a temperature called denaturation temperature (T_d) of approximately 40°C. Interestingly, it was found that the denaturation temperature of collagen decreased with increased concentrations (5, 8, 12, 16 and 20 mg/mL) suggesting that the molecular state of collagen was different in collagen solutions with different concentrations [10]. The reaction of the collagen molecules to temperature was therefore closely linked to the aggregation behavior of the collagen. Since collagen-based materials were often used as solutions or involved in the processing of collagen solution, it was important to explain the relationship between the aggregation behavior of the collagen and the temperature. İn the research to investigate the aggregation behavior of the polymer, was studied through fluorescence techniques including ultra-sensitive differential scanning calorimeter (US-DSC), atomic force microscopy (AFM) and intrinsic fluorescence measurements and the use of external fluorescence probe, pyrene. In this study, collagen solutions at concentrations of 0.25, 0.50 and 1.0 mg/mL were prepared by dissolving freeze-dried collagen in 0.1 mol/L acetic acid solutions. In the present work, researchers have mainly used fluorescence

techniques to research the reaction of collagen molecules to temperature. Collagen molecules have been shown to be sensitive to temperature. As a result, the stability of the collagen helix appeared to decrease as the concentration of collagen increased. It was also recognized that thermal denaturation of collagen was an endothermic method. Thus the thermal denaturation of collagen may be influenced by the rate of heat transfer. Although many types of collagen have been discovered: base membrane, microfibrillar, anchoring fibrils, hexagonal mesh, discontinuous triple helix [FACIT], the species most commonly used for corrective purposes today are also fibril-forming species. In the cosmetics industry, collagen is a selectable raw material for cosmetic formulations due to its availability, biocompatibility and biodegradability [3]. Collagen hydrolysates obtained from enzymatic hydrolysis of collagen are a mixture of low molecular weight (LMW) peptides [11]. Collagen is a high molecular weight extracellular protein that is insoluble in water [12]. On the other hand, the mixture of peptides derived from collagen digestion is highly water soluble and active peptides can be detected in the bloodstream and in the skin within a few minutes after oral administration [13] .

Types I, II and III, fibrill-forming collagens, form structural networks that restrict tissue deformation and provide a structure that defines tissue size and shape. Collagen type I is associated with structural fibrils greater than 100 nm in diameter, while collagen type II and type III are associated with fibrils of 10 and 30 nm in diameter, respectively. It is well known biochemically that type III is less soluble at pH acid than type I, because type III requires lower concentrations of NaCI to 'salt out of solution' than Collagen type I. Physical properties of pepsin-solubilized collagen types I, II, III and V have been calculated in 10°C acid solution. Results indicate that collagen molecules of type I, II and III undergo a monomer-aggregate balance in solution, while molecules of type V tend to attract each other but do not undergo a similar monomer-aggregate balance. Interstitial collagen monomers (types I, II and III) have molecular weights between (M_2^{*b}) $280x10^3$ and $289x10^3$, translational diffusion coefficients between ($D^{\circ}_{20,w\,(cm^2\,s^{-1})}$) 0.820 x 10^{-7} and 0.845 x 10^{-7} cm^2s^{-1} and particle dispersion factors at an angle of 175.5° and wavelength of 633 nm between (P(175.5°)) 0.430 and 0.460 [14].

The characteristic of the extracted collagen are given in the Table 1. Although each of the three collagen extracted according to Table 1 is the same color, there are differences in other properties.

2.1. Collagen structure

Collagen is a complex molecule, the structure of which has been revised over the years. Type IV collagen with more flexible triple helix (Figure 1) is combined in meshes confined to basic membranes [15]. Microfibrillar type VI collagen is highly disulfide crosslinked and contributes to a network of beaded filaments intertwined with other collagen fibrils. Fibril-associated collagen with discontinuous triple helixes (FACITs) such as type IX, XII and XIV

Table 1. *The table shows the collagen values by extracting the hydrogels in different molar acids. Each value is the mean ± SD of three independent measurements.*

Characteristics of hydrogels	Calf collagen	Fish collagen extracted with 0.5 M acetic acid	Fish collagen extracted with 0.1 M HCI
1. Appearance	Translucent gelatinous	White gelatinous	White opaque
2. Color	White-yellow	White-yellow	White-yellow
3. Humidity [%]	13.5±0.1	15.5±0.1	15.0±0.2
4. Dry matter [%]	1.75±0.02	1.55±0.04	1.2±0.03
5. Ash600-800°C [%]	1.5±0.1	0.7±0.2	0.8±0.2
6. Total nitrogen mg (Khieldal) [%]	16.5±0.1	10.9±0.2	8.8±0.3
7. Proteic substance [%]	90.5±0.2	86.5±0.1	78.5±0.3
8. pH	2.9±0.1	3.5±0.3	2.5±0.2
9. Collagen contact [%]	92.2±0.1	90.1±0.2	82.3±0.4

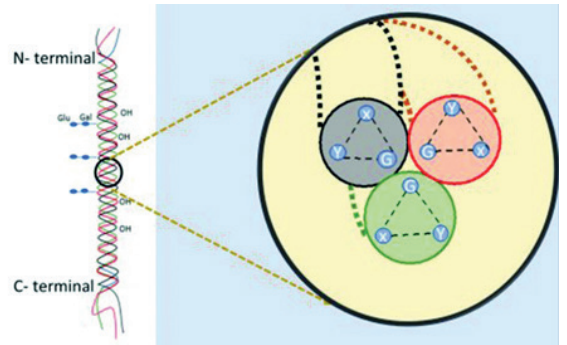

Figure 1. *Triple helical structure of collagen [15].*

collagen is the only one with large collagen fibrils. They assemble into molecules and possibly play a role in regulating the diameter of the collagen fibrils. Collagen type VIII and X form hexagonal networks while others (XIII and XVII) encompass cell membranes. These can be formed by three identical chains (homotrimers) as in collagen type II, III, VII, VIII, X and others, or by two or more different chains (heterotrimers) such as collagen types I, IV, V, VI, IX XI [1].

According Figure 1, the creation of collagen starts in the nucleus where different exons of a gene are consubstantiated to generated messenger RNAs (mRNAs) of diverse collagen kinds. Disulfide bonds which assist to keep the properly chains aligned to each other consubstantial the triple helix chains, following which a molecule called procollagen is formed.

The function of water and hydroxyproline (Hyp) in the collagen stability mechanism remains debatable. The purpose of this research was to consider at the effects of Hyp and water on the molecular stability of collagen. Four collagen-like peptide (CLP) models were compared in terms of conformational energy and hydrogen bonding types. The CLP1 model represents a normal collagen structure without water molecules, while the CLP2 model represents a collagen structure that does not contain water and a Hyp residue. CLPW1 and CLPW2 are models CLP1 and CLP2 with water molecules around them. The total steric energy is listed as follows: CLP2> CLP1> CLPW2> CLPW1, indicating that CLPW1 is the most stable collagen model. The effects of HYP and bound water on collagen stability were investigated using four CPL models in this study. Models have slightly different angle values from some of the studies previously published [16-19]. This discrepancy may be due to the use of crystallographic experiment data and the model optimization method of geometry. This finding supports the fact that the overall structure of the models is sufficiently suitable for the study of the stability of collagen [20].

2.2. Biosynthesis of collagen

The biosynthesis of collagen starting with gene transcription of the genes in the nucleus is a complex multi-step process until the collagen heterotrimers aggregate into large fibrils. Since most of our knowledge of these mechanisms is based on fibril-forming collagen, this discussion will mostly focus on type I collagen. It is possible that the basic mechanisms of triple helix formation and processing also apply to other types of collagen. Regarding fibril collagen, the most detailed research on collagen biosynthesis has been conducted. The process is complex and involves many intracellular and extracellular steps, all of which contribute to the structure and biomechanical properties of the final fibrils. The first event after the synthesis of procollagen chains on ribosomes is their introduction into the rough endoplasmic reticulum. They underwent a series of post-translational modifications there, which led to the assembly of procollagen molecules [21].

These steps include modification of proline residues to hydroxyproline, modification of lysine to hydroxylysine, N and O linked glycosylation, trimerization, disulphide bond, prolyl cis-trans isomerization and triple spiral folding. The molecules then pass through the Golgi network, where they are packaged into secretory vesicles before being exported to the extracellular matrix. Procollagen processing occurs during or shortly after secretion, and then the fibrils are assembled. Finally, the fibrils are stabilized by the formation of covalent cross-links [22].

3. COLLAGEN TYPES

3.1. Collagen types I, II, III, V and XI - fibril-forming collagen

Classical fibril-forming collagen includes collagen types I, II, III, V, and XI. These collagenes are characterized by their ability to aggregate into highly oriented supramolecular aggregates, forming fibrillar arrays with diameters between 25 and 400 nm. In electron microscopy, fibrils are identified by a characteristic banding pattern with a periodicity of about 70 nm, based on a gradual arrangement of individual collagen monomers . It is the type I collagen that is the most widely found and studied type of collagen. It makes up more than 90% of organic bone mass and is the main collagen of tendons, skin, ligaments, cornea and many interstitial connective tissues, with the exception of very few tissues such as hyaline cartilage, brain, and vitreous body. Triple helical fibers are incorporated in the composite in vivo, often containing either type III collagen (in the skin and reticular fibers) or type V collagen (in bone, tendon, cornea). In most organs, and particularly in tendons and fascia, type I collagen provides tensile stiffness and defines important biomechanical properties in bone, particularly after calcification, related to load bearing, tensile strength, and torsional stiffness [23].

Fibril-forming type II collagen is the characteristic and predominant component of hyaline cartilage. However, it is also found in the vitreous body, corneal epithelium, notochord, nucleus pulposus of intervertebral discs and embryonic epithelium. The triple helix of type II collagen consists of a homotrimeric molecule similar in size and biomechanical properties to type I collagen. Collagen fibrils in cartilage represent heterofibrils containing type XI and IX collagen that are expected to limit the fibril diameter to about 15-50 nm and other non-collagen proteins, in addition to dominant collagen type II. Compared to type I collagen, type II collagen chains show a higher hydroxylisine content as well as glucosyl and galactosyl residues that mediate interaction with proteoglycans, another typical component of the highly hydrated hyaline cartilage matrix. Type III collagen is homotrimary and is widely distributed in tissues containing collagen type I except bone. It is an important component of reticular fibers in the interstitial tissue of the lungs, liver, dermis, spleen, and vessels. This homotrimeric molecule also frequently contributes to mixed fibers with type I collagen and is also abundant in elastic tissues [24].

In type V and XI collagen, they form a heterotrimer molecule. It is remarkable that only the degree of glycosylation and hydroxylation differs from type XI collagen type II . Although not finally resolved, there appears to be a combination between different collagen types of V and XI chains in various tissues . Thus, collagen types V and XI form a subfamily within fibril-forming collagen, although they share similar biochemical properties and functions with other members of this family. As noted previously, type V collagen typically forms heterofibrils with type I and III collagen and contributes to the organic bone matrix, the corneal stroma and the interstitial matrix of the muscles, liver, lungs, and placenta. Type XI collagen is distributed largely to the articular cartilage by type II collagen. Because the triple helix areas are immunologically masked in the tissues, they are thought to be located in the center of the fibrils rather than their surface. In the “gut-sac” experiments in this study, peptides within the MW range were detected from 1 kD to 10 kD on the serosal side of the intestine after application of gelatin hydrolysate, suggesting that gelatin is also absorbed to some degree in a high molecular form. In summary, this study demonstrates that gelatin hydrolysate is absorbed from the intestine and ideally deposited in cartilage. These findings indicate that the specific amino acid and peptide profile of gelatin could be responsible for previous reports of the therapeutic effectiveness of oral gelatin in musculoskeletal degenerative conditions [25].

Figure 2 shows the appearance of different types of collagen under the microscope: A, fibril forming (collagen type I) [26], B, basement membrane (collagen type IV) [27], C, microfibrillar (collagen type VI) [28], D, microscopy of the most common collagen groups showing binding fibrils (collagen type VI) [29], E, FACIT (collagen type XIII) [30], F, transmembrane (collagen type XIII) [31, 32].

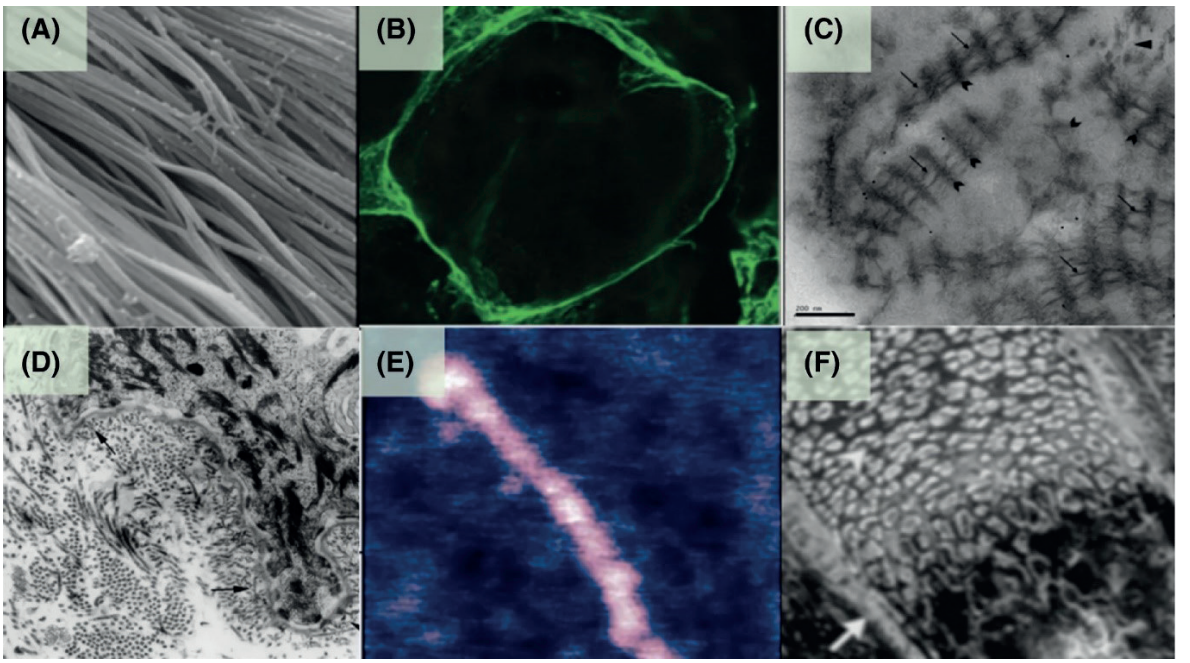

Figure 2.

3.2. Collagen types IX, XII and XIV - The FACIT collagen

Collagen types IX, XII, XIV, XVI, XIX and XX belong to Fractured Triple-helix Fibril Associated Collagen (FACIT collagenes) [33]. The structures of these collagenes are characterized by "collagen domains" interrupted by short non-helical areas, and trimeric molecules are associated with the surfaces of various fibrils. Collagen type IX is distributed together with type II collagen in the cartilage and vitreous body. Type IX collagen molecules are periodically located in the antiparallel direction along the surface of type II collagen fibrils. This interaction is stabilized by cross links. In addition, collagen type XVI is found in hyaline cartilage and skin. Type XII and type XIV collagen are similar in structure. Both molecules associate or coexist with type I collagen in the skin, perichondrium, periosteum, tendons, lung, liver, placenta and vessel walls [34].

According table 2 the collagen superfamily comprises 28 members numbered with Roman numerals in vertebrates (I–XXVIII). The Table 2 includes some members of the family. The common structural feature of collagens is the presence of a triple helix that can range from most of their structure (96% for collagen I) to less than 10% (collagen XII). As described in the following discussion, the diversity of the collagen family is further increased by the existence of several a chains, several molecular isoforms and supramolecular structures for a single collagen type, and the use of alternative promoters and alternative splicing.

4. IDENTIFICATION AND QUANTIFICATION METHODS

It is necessary to rely on the most appropriate analysis technique to evaluate the quality feature. Techniques such as SEC, MS and NMR are used to characterize complex peptide mixtures. Size exclusion chromatography (SEC) is a well-known technique widely used to determine the polydispersity of collagen hydrolysates as a quality feature [36]. The detailed SEC profile can identify subtle differences and the acceptable range of differences between batches to prove the consistency of the manufacturing process. However, the most advanced techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) are more sensitive and highly capable methods for determining the composition and properties of components found in complex mixtures [13]. DSC is a well-developed analytical method used to measure

Table 2. *The collagen family [35].*

The collagen family		
Collagen type	α Chains	Molecular species
Collagen I	α1(I), α2(I)	[α1(I)]2, α2(I), [α1(I)]3
Collagen II	α1(II)	[α1(II)]3
Collagen III	α1(III)	[α1(III)]3
Collagen IV	α1(IV), α2(IV), α3(IV), α4(IV), α5(IV), α6(IV)	[α1(IV)]2, [α5(IV)]2, α2(IV), α3(IV), α4(IV), α5(IV), α6(IV)
Collagen V	α1(V), α2(V), α3(V), α4(V)a	[α1(V)]2, α2(V) [α1(V)]3 [α1(V)]2α4(V)
Collagen VI	α1(VI), α2(VI), α3(VI), α4(VI), α5(VI), α6(VI),	
Collagen VII	α1(VII)	[α1(VII)]3
Collagen VIII	α1(VIII)	[α1(VIII)]2, α2(VIII), α1(VIII), [α2(VIII)]2 [α1(VIII)]3 [α2(VIII)]3

transitions in polymers and is typically used to study collagen thermal stability [37].

In this study that Sonja Gamsjaeger et al. was to validate Raman spectroscopic parameters describing one of the major mineralizing type I trivalent cross-links, namely pyridinoline (PYD). To this end, a series of collagen cross-linked peptides with known PYD content (as determined by the HPLC analysis), human bone, porcine skin, predentin and dentin animal model tissue were analyzed by Raman microspectroscopy. The results of this study indicate that it is possible to track the spectroscopic analysis of PYD trivalent collagen cross-links in mineralized tissues by Raman, exclusively through the Raman band ~1660 wavenumbers. This enables the determination of the relative PYD content in undecalcified bone tissue with a spatial resolution of ~1 μm, thus enabling correlations with histological and histomorphometric parameters. In conclusion, the findings of the present study indicate that the detection of the amide I underlying peak ~1660 cm^{-1} (based on the second derivative spectra) in the Raman spectra indicates the presence of PYD collagen cross-links in mineralized tissue samples; in addition, the resolution area underlying the 1660 cm^{-1} peak is significantly associated with the PYD content and may therefore be used in analytics [38].

In our review, we have summarized the studies on collagen in Table 3. Some of the studies include collagen, some of its dispersion products, and some studies about collagen types. Identification, characterization and quantification of collagen were performed with the devices we specified. The purpose and results of the studies indicated in Table 3 will be helpful in guiding such future studies, especially since there are few studies on quantification in the studies.

5. PHARMACOLOGY

5.1. Mechanism of Action

As a result of its capacity to stop bleeding, it to use as a hemostat or coagulation element. Animal-derived (natural) collagen is used in many clinical applications, but there is some concern about its role in inflammation, group-to-group variability, and possible disease transfection. Some synthetic nanomaterials that can mimic their properties have been developed to avoid immune problems. Accordingly, collagen with the trade name KOD™ was developed. KOD collagen, anticoagulant fractions, can increase platelet activation and adhesion. It also binds the platelets of the form clots, activates them and promotes healing without promoting inflammation [51].

Table 3. *A brief summary some studies on collagen is dcscribed in the following table.*

Investigated fragment	Analysis made / Equipment	Identification Characterization/ Quantification	Purpose	Result	Object or Subject	Reference
Pyridinoline Trivalent Collagen	HPLC and Raman	Identification Characterization	The objective of the present study was to validate Raman spectroscopic parameters describing one of the major trivalent type I mineralization cross-links, namely pyridinoline (PYD).	In conclusion, the findings of the present study indicate that the detection of the amide I underlying peak ~1660 cm−1 (based on the second derivative spectra) in the Raman spectra indicates the presence of PYD collagen cross-links in mineralized tissue samples; in addition, the resolution area underlying the 1660 cm−1 peak is significantly associated with the PYD content and can therefore be used in analytics.	Healthy pediatric human bone, Porcine dermis, Porcine teeth (dentin and predentin)	[38]
Collagen Type I m/z 773.9 III m/z 533.09.	HPLC–MS	Identification Characterization	In this research, the collagen in ACM from different tissues of pig were determined by detection of marker peptides. The marker peptides of Type I and III collagen were identified from the digested collagen standards using ions trap mass spectrometry (LCQ).	The collagen detection method was developed on the basis of HPLCMS and marker peptides, which could detect the contents of type I and III collagen in the tissues. The number of collagen type I and III was found to be similar to the total collagen content and collagen content in Type I and Type III in various tissues corresponded to the distribution of collagen to the body.	Fresh small intestinal submucosa (SIS), Achilles tendon and dermiş of pig	[39]
Collagen	FTIR UV-Vis analysis ζ - Potential Analysis SEM Analysis	Identification Characterization	In this analysis, the collagen was isolated from non-commercial fish skin waste and cryogelation methods were used to create scaffolds from this collagen. The SEM and UV-Vis analysis have shown morphology of the pores and protein structure of the collagen. Zeta potential analysis(FTIR) has identified the iso-electrical point of the protein.	This study successfully insulated acid-soluble collagen (ASC) from the fish-industry, with a yield of 14,53% and a pepsin-solutionsoluble collagen (PSC) with a yield of 2.41%, based on SEM, UV-Vis, Zeta potential and FTIR examination. The recipes using various quantities of collagen and glutaraldehyde, such as interconnection of porosity, mechanical stability, spongy and elastic structure, developed the properties of the grout.	Shark skin	[40]

Table 3. *A brief summary some studies on collagen is dcscribed in the following table.*

Investigated fragment	Analysis made / Equipment	Identification Characterization/ Quantification	Purpose	Result	Object or Subject	Reference
Collagen Hydrolysate	LC-MS/MS	Quantification	Here, after the ingestion of high tripeptide containing collagen hydrolysate in human beings, we have analyzed plasma amount of collagen-derived peptides. We have progressively identified 17 forms of collagen-derived peptides with a specific gly-pro-hyp enrichment.	In our research, we have demonstrated the passing increase of the levels of Gly-Pro-Hyp (Gly-Pro-hyp), which have a higher trpeptide concentration in the blood of humans and mice, through oral administration of HTCcol. We observed that HTC-col was not only transported into the skin with Pro-Hyp but also with other collagen dependent peptides.	Mouse plasma, Human skin	[41]
Elastin	LC/ESI-ion trap mass spectrometry Nanoelectrospray-qTOF mass spectrometry	Identification Characterization	This research analyzed peptides extracted from the digestion of pepsin and thermitase in human skin elastine. Peptide characterization was carried out by two additional mass techniques: LC/ESI-ion trap and nano-ESI-qTOF MS.	The proteases have been shown to be appropriate with site-specific enzymes for the hydrolysis of insoluble Elastine. Thus, the data base search and the combined sequencing of novo with the data base method unmistakably detected 155 different peptides of the two digesters.	Human skin	[42]
Collagen,	UPLC−ESI-MS	Identification Characterization	This research examined the bioavailable and bioavailability of collagen in rats after oral administration. Collagen is relative and absolute bioavailability, which was indirectly tested in collagen by the bioavailability of Hyp with a pharmacokinetic approach respectively, 57.8 per cent and 49.6 per cent.	Finally, the bioavailability and bioavailable sources of collagen have been studied in this study following oral administration to rats. Instead of being free amino acid, the majority of digested collagen was absorbed in peptides and the absorbed amino acid relied on the collagen contents thereof.	Rats	[43]

Table 3. *A brief summary some studies on collagen is dcscribed in the following table.*

Investigated fragment	Analysis made / Equipment	Identification Characterization/ Quantification	Purpose	Result	Object or Subject	Reference
Collagen Type I Collagen Type II Collagen Type III Collagen Type IV Collagen Type V	HPLC-MS/MS	Quantification	This method is based on collagen fragmentation by cyano gen bromide followed by trypsin digestion. After that, HPLC-MS/MS (HPLC coupled to an IT mass spectrometer) analyses of the resulting peptide mixtures (peptide maps) were performed. Specific peptides for each collagen type were selected. According to online databases, these peptides are present in human, bovine, and rat collagens. As a result, this method can be potentially applied to other species' tissues as well, such as human tissues, and provides a universal and simple method of quan- tifying collagen types. The applicability of this method for analyzing collagen types was demonstrated on rat tissues (skin, tendon, and aorta).	HPLC-MS/MS is an important method not only for identifying peptides and proteins, but also for quantifying them. While we have previously employed the application of marker peptides in the aorta, skin and tendon [1] to identify and measure the types of collagen I and III, the quantification of other types of collagen (in particular, II and IV) remained an issue. We also expanded the use of HPLC-MS/ MS to quantify other forms of collagen by changing the sample preparation procedure using marker peptides.	Rat placenta	[44]
Collagen Type I	RP-HPLC	Quantification	In this study we have compared the quantities and structures from three different sources of the collagen type I of food-derived gelatine peptides in human blood. According to the findings, Hyp is absorbed as both an amino acid (free form) and a peptide, with a profile similar to that described by Iwai et al. As a result, the biological activity of orally administered collagen, gelatin, or their hydrolysates is thought to be dependent on the type or source of collagen.	This finding showed that the structure and amount of food-derived peptides in human blood differed depending on the type of collagen and the source of the collagen. As a result, the biological activity of orally administered collagen, gelatin, or their hydrolysates is believed to vary depending on the type of collagen or the source of the collagen. However, there is a scarcity of data for comparing the structures and quantities of Hyp-containing peptides in human blood. As a result, this study compared the amount and structure of Hyp-containing peptides in human blood to three types of type I collagen.	Human plasma	[45]

Table 3. *A brief summary some studies on collagen is dcscribed in the following table.*

Investigated fragment	Analysis made / Equipment	Identification Characterization/ Quantification	Purpose	Result	Object or Subject	Reference
Hydroxyproline	LC-MS.	Quantification	We recently developed "SIcollagen," a stable isotope-labeled collagen that can be used as an internal standard in various types of collagen analyses using liquid chromatography mass spectrometry (LC-MS). We created stable isotope-labeled Hyp-containing peptides from SI-collagen by mimicking the protein degradation pathways in the body with trypsin/ chymotrypsin and plasma proteases. Using a protease digest of SIcollagen as an internal standard mixture, we were able to achieve highly accurate simultaneous quantification of Hyp and 13 Hypcontaining peptides in human blood using LC-MS.	The quantitative precision of LCMS analysis of plasma samples with and without an internal standart was first investigated. Following oral ingestion of gelatin hydrolysate, plasma levels of both free and peptide-form Hyp increased significantly. Our method can help with the investigation of the biological activity of collagen-derived peptides and the production of functionally characterized gelatin hydrolysate by quantifying the level of absorption and metabolism of orally ingested gelatin hydrolysate.	Blood	[46]
10. Collagen Peptides	UPLC-MS/MS	Identification Characterization	This research will look at the amino acid profiles and molecular weight distributions of collagen peptides. Collagen peptides' anti-inflammatory role can include increased synthesis of key components of extracellular matrix (ECM) and inhibition of chondrocyte apoptosis. UPLC-MS/MS defined the peaks' components as GPRGPPGPVGP and VAIQAVLSLYASGR. Because of their antioxidative stress and disruption of the catabolism and anabolism processes in arthrodial cartilage, the detected collagen peptides provide a possible therapeutic strategy for the treatment of osteoarthritis (OA).	To investigate the spectrum-effect relationship of collagen peptides, HPLC fingerprints and a series of in vitro antioxidant and anti-inflammatory assays were combined in this research. These identified peptides could be used as drugs or functional foods to treat or prevent osteoarthritis . This study provides a new platform for defining the functional components of collagen peptides using the spectrum-effect relationship, which could contribute to the creation of new directions in the potential use of bioactive peptides.		[47]

Table 3. *A brief summary some studies on collagen is dcscribed in the following table.*

Investigated fragment	Analysis made / Equipment	Identification Characterization/ Quantification	Purpose	Result	Object or Subject	Reference
11. Collagen	DSC XRD UV FTIR	Identification Characterization	To obtain their key characteristics, collagenous biopolymers can be studied utilizing physic-chemical, densitometric, and spectroscopic research, with the aim of biotechnological manipulation. As a result, the aim of this work is to provide a summary of extraction processes, characterization techniques, and future research with a focus on aquaculture and fisheries sources.	Given the need to respond to current circumstances and contribute to the mitigation of environmental degradation, the use of collagen derived from fishery resource residues has emerged as a valuable asset for preservation and impact reduction. Finally, this study discusses the collagen global industry trends. Through this paper, we hope to promote the use of collagen from aquatic sources in new research that will help to secure this biopolymer's place in the global collagen industry, given how close their physical, biochemical, and densitometric spectroscopic extracted collagen is to that of mammals.	Fish skin	[48]
12. Hydroxyproline	HPLC LC-MS	Quantification	The concentration of 4-hydroxy-l-proline (hydroxyproline) can be useful in determining the diagnosis and forecast of diseases affected by collagen metabolism disorders. The aim of this analysis was to use liquid chromatography-mass spectrometry (LC-MS) to determine the concentration of hydroxyproline.	The LC-MS calculated concentration of hydroxyprolines in lung and liver tissues was compared to values obtained with a colorimetric approach and a high-performance fluorescence chromatography system (HPLC) in our community from earlier studies. These findings indicated that calculating hydroxyproline concentrations using the LC-MS system would be useful for diagnosing diseases associated with impaired collagen metabolism.	Rats	[49]
13. Hydroxyproline	HPLC	Quantification	This chapter covers in vivo methods for estimating collagen synthesis and degradation rates, as well as the proportion of freshly synthesized collagen that degrades rapidly intracellularly. The techniques are focused on radiolabeled proline being incorporated into collagen as hydroxyproline and measuring it in intact protein and collagen breakdown items. In addition, for in vivo and in vitro studies, an effective and highly sensitive high-performance liquid chromatography method for measuring hydroxyproline is defined.	This gradient profile was created to maximize hydroxyproline isolation while minimizing the time between sample runs. The profile may be modified to isolate and measure other amino acids, such as proline, if needed.	Rats	[50]

Gelatin is a heterogeneous mixture of polypeptides formed by collagen hydrolysis. In addition to the fact that gelatin hydrolysate is absorbed from the intestine, an examination of the consistency of gelatin absorption is of great importance. It is generally believed that peptides are hydrolyzed in the gastrointestinal tract prior to absorption so that free amino acids primarily enter the circulation. However there is ample evidence that peptides or even macromolecules may also be absorbed intact. Earlier investigations indicated that all peptides and macromolecular proteins can be absorbed from the intestine and maintain some biological function.

Peptides formed by hydrolysis of a large molecule of collagen can have great health benefits and can enhance the skin's properties. Using "gut sac" studies, Oeser et al. studied the molecular weight of hydrolyzed collagen consumed in the intestinal tract. Techniques such as high-performance liquid chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis have shown that peptides in the 1–10 kDa molecular weight range can be absorbed. In summary, this study demonstrates that gelatin hydrolysate is absorbed from the intestine and ideally deposited in cartilage. These findings indicate that the specific amino acid and peptide profile of gelatin could be responsible for previous reports of the therapeutic effectiveness of oral gelatin in musculoskeletal degenerative conditions [25]. Scientific research have shown that the first effect, which is emphasized after absorption of collagen hydrolyzate, is an antioxidant and the second effect is biological activity. Collagen hydrolyzate is a bioactive component that has the effect of improving epidermis moisture content, preventing aging of the skin and regenerating joints and connective tissues. Collagen is digested by the enzyme collagenase [52]. After oral intake, more than 90% of collagen hydrolyzate is digested and quickly absorbed [53]. The digestion of collagen hydrolyzate has been found to be healthy as a food product [54].

Iwai et al. showed that a large amount of hydrolyzed collagen derived from hydroxyproline existed in the blood of healthy human volunteers who ate, after 12 hours of fasting, cartilage hydrolyzed collagen, chicken legs and porcine skin. After consumption of collagen, the amount of hydroxyproline-containing peptides in the blood increased to a peak after 2 hours, followed by a decrease to half the maximum level at 4 hours after ingestion. A small peptide, proline-hydroxyproline (Pro-Hyp), was detected in the blood following ingestion of hydrolyzed collagen. The amount of Pro-Hyp present in human plasma was found to be 25–60 nmol/mL after ingestion of 9.4–23 g of hydrolyzed collagen. The higher levels of Pro-Hyp found in blood may be partially explained by the higher amount of Pro-Hyp in collagen. Studies by Iwai et al15 indicate that Pro-Hyp may be considered an indigestible peptide because more than 75% of Pro-Hyp has been shown to remain in the blood for 24 hours after an in vitro human serum reaction [53].

Chen et al. have studied the effect of various concentrations of hydrolyzed collagen extracted from fish on fibroblasts and keratinocytes. They looked, in particular, at the proliferation and development of collagen. Collagen concentrations of 48–97 μg/mL were found to result in optimal proliferation (191%) [55].

Ohara et al. performed a single-blind crossover analysis comparing the structure and quantity of food-derived gelatin hydrolysates in human blood from three collagen type I sources. Five stable male volunteers ingested collagen type I gelatin hydrolysate from fish scales, fish skin, or porcine skin after 12 hours of fasting. Approximately 30 per cent of hydroxyproline-containing peptides have been found in the blood for a period of 24 hours [45].

There is however, significant evidence that peptides can be hydrolyzed in the gastrointestinal tract until they are consumed so that primarily free amino acids can enter the circulation. Hydroxyproline is absorbed in two ways, i.e. the combination of amino acid and peptide [56].

The mechanism of absorption through the intestinal membrane has been extensively studied. Epithelial cells are critical sites for the absorption of various nutrients. There are three ways in which the intestinal transport of oligopeptides can take place: PEPT1-mediated transport of dipeptides and tripeptides mediated by PEPT1 [57]; transport of macromolecules such as proteins through the transcytotic route [58]; and transport of peptide

absorption through the passive intracellular route. The complete role of these pathways in intestinal oligopeptide absorption is not yet completely understated [59].

Transcellular transmission of these peptides through intestinal epithelial cells is a two-step process involving the transport of peptides across two different membranes, i.e. the absorption of epithelial cells through the borderline membrane of the brush and the absorption into the bloodstream through the basolateral membrane. The first stage is the introduction of hydrogen ion-coupled peptide transporters, namely PEPT1 and PEPT2. PEPT1 serves as an enantioselective transporter of monovalent, polyvalent and neutral charged peptides. Collagen-derived peptides (Pro-Hyp and Glycine-Pro-Hyp) have been shown to be absorbed through the PEPT1 transporter [60].

Collagen peptides are digested and spread across the body after ingestion. Watanabe-Kamiyama et al examined the delivery of collagen peptides to the skin and other tissues through an in vivo experiment in which 14C-labelled proline or collagen peptides were administered to Wistar rats. Radioactivity was assessed in various tissues 0–6 hours after ingestion of collagen peptides and 14 days afterwards. The findings were very positive in terms of time spent in the skin and showed that radioactivity stayed elevated in the skin tissue for up to 14 days. This shows the capacity of collagen peptides to penetrate the skin of the dermis where their key advantage is observed [61].

5.2. Absorption, distribution, metabolisation and elimination

The first effect that is illustrated after absorption of collagen hydrolyze has been shown by experimental studies to be an antioxidant and the second to be biological activity [62]. Studies show that CH is detected in the portal vein and blood after oral administration, and the peptides are absorbed from the gut as larger peptides in vivo. Some studies show that the peptide derived from CH is absorbed from the gut after oral administration of CH and many tissues can be reached with systemic administration. There is some research on the absorption of collagen-derived tripeptides and their transport into the bloodstream in their intact form. In these studies, studies have shown that collagen tripeptide is rapidly absorbed into the blood and transferred to tissues easily since the collagen tripeptide's average molecular weight is far smaller than that of the conventional collagen peptide. According to the results of the research, it has been suggested that the application of collagen as tripeptides enables the absorption of functional peptides [63].

5.3. Absorption and Distribution

Collagen is long used in medicinal applications but it is not well known the absorption mechanism. Peptides are typically digested proteolytically in the gastrointestinal tract until absorption. Nevertheless, Oesser et al. suggested that collagen hydrolysate administered orally can be absorbed from the bowels and accumulated preferably in the cartilage tissue by the mice [25]. We have previously stated that after oral ingestion of collagen hydrolysates many peptides derived from collagen can be detected in human serum and plasma [53].

Collagen, the main macromolecule extracellular matrix, is commonly used for medical purposes. Examined by Kamiyama et al. the absorption process of low molecular weight collagen hydrolysate (LMW-CH) in rats and its effects on osteoporosis. When applied to Wistar rats, LMW-CH rapidly increased plasma radioactivity with [^{14}C] Proline (Pro Group) or glycyl [^{14}C] prolyl-hydroxyproline (CTp group). It was absorbed as LMW-CH peptide in the blood of Wistar rats. In plasma and kidney, glycyl-prolyl-prolyl hydroxyproline tripeptide continued. In this study, researchers examined the absorption, using radioactive tracers, of low-weight molecular collagen hydrolysate (LMW-CH). Researchers measured radioactive distribution in the body and analyzed the plasma's radioactive peptides. Kamiyama et al. also examined whether LMW-CH is spontaneously hypertensive to bone composition of ovary-extracted (OVX) stroke-prone rats (SHRSPs). Here researchers described new findings on the mechanism of absorption and beneficial effects of collagen hydrolyse on osteoporosis.Wistar rats with either [^{14} C] proline (Pro group) or glycyl-[^{14} C]prolyl-hydroxyproline (CT group), LMW-CH

rapidly increased plasma radioactivity. LMW-CH was absorbed into the blood of Wistar rats in the peptide form. Glycyl-prolyl-hydroxyproline tripeptide remained in the plasma and accumulated in the kidney. İn both groups, radio activity was retained at a high level in the skin until 14 days after administration. Additionally, the administration of LMW-CH to ovariectomized stroke-prone spontaneously hypertensive rats increased the organic substance content and decreased the water content of the left femur [61].

In the study by Sneha et al. examined that peptides containing Hyp, such as Gly-Pro-Hyp (GPH) and Pro-Hyp (PH), are more effectively absorbed and hit a higher plasma level after oral administration of CTP (collagen tripeptide) in rats than high molecular weight collagen peptides (H-CP). GPH and PH were found to be stable in gastrointestinal fluid and rat plasma for 2 hours. The capacity of GPH and PH to permeate biological membranes was also investigated using an in vitro model of human intestinal mucosa (Caco-2 cell monolayer) [64].

Anne J. et al. analyzed four collagen hydrolysates in different matrices (solvent, TIM dialysate, and human serum) using non-targeted LC-MS to compare the state of the hydrolysates before and after absorption, either in vitro or in vivo. The (data) analytical workflow presented here is particularly useful for investigating the behavior of protein hydrolysates. Their structure is often highly complex prior to ingestion, and during digestion and absorption, hydrolysate components will be exposed to many enzymes (with broad specificity) and chemical hydrolysis. Many hydrolysate components would be produced from precursors and degraded at the same time, reducing the usefulness of recording intermediate concentrations. Researchers demonstrated how mixing non-targeted and targeted data analysis can provide a general overview of the data collection. [65].

According Table 4, lists the numerous kinds of collagen, as well as their function and distribution in various orodental tissues [66]. As can be seen from the table 4, each of the 19 collagen types has different characteristics. This difference was also seen in the distribution in orodental tissue. While many types of collagen are dispersed in cartilage, bond, and basal lamina, very few of them are distributed in cell surface, pulp, and dentin.

5.4. Metabolization and Elimination

Unfortunately, little is known about the regulation of collagen synthesis, secretion, deposition, and turnover in connective tissues. Changes in the cell environment are known to lead to changes in collagen forms, and it is recognized that simple changes such as an increase in ions of K+ will lead to an increase in cell proliferation and extracellular matrix synthesis [67].

All of these processes include regulatory changes in the forms of collagen synthesis, and these changes are caused or triggered by changes in the environment of mesenchymal cells or immature fibroblasts. Identical variations arise in the formation of a cartilaginous fracture callus, ectopic bone formation, and several repair processes. The amount of collagen synthesized has also been shown to be affected by a variety of factors [68]. Ascorbate, for example, has been shown to increase collagen synthesis with or without an increase in prolyl hydroxylase activity [69]. Prostaglandins El and Fl induce collagen synthesis in the skin and bone of chick embryos, and activated macrophages release a soluble factor that stimulates the synthesis of collagen and other proteins in granulation tissues. Since these prostaglandins are increased in inflammatory lesions, these findings may help to explain why inflammation stimulates collagen synthesis. Prostaglandin E2 is a relatively specific inhibitor of collagen synthesis by osteoblasts, according to numerous reports, and other connective tissues produce one or more small basic proteins that mimic hyaluronate production but inhibit collagen production. Nonetheless, these findings are all preliminary, and many more good basic studies of the control of collagen transcription, translation, and posttranslational modifications, as well as the extracellular enzymes necessary for collagen deposition, stabilization, and degradation in all connective tissues, are urgently needed [70]. There are few studies on the metabolism and elimination of collagen.

Table 4.

Type of collagen	Special features	Distribution in orodental tisses
I	Most abundant	Bone, dentin, cementum, tendon and ligaments
II	Heterofibril with IX and XI	Cartilage
III	Most in elastic tissues	Pulp, blood vessels, lymphoid tissue and embryonic tissue
IV	Interacts with laminin, integrin, nidogen and type IV collagen	Basal lamina
V	1.Forms core of collagen I 2.Binds to DNA, heparin sulfate, heparin, thrombospondin and insülin	Basal lamina, dentin, skin, blood vessels, periodontal ligament
VI	Disulfid bonds	Skin, ligamnet skin
VII	Dimers in anchoring plagues	Epithelium
VIII		Endothelium
IX	Associated with cartilage glycosaminoglycans	Cartilage
XI	Core of type II	Cartilage
XII		Abundant in connective tissue
XIII	Transmembrane domain and collagenous domain.	Cell surface, focal adhesions, epidermis
XIV		Abundant in connective tissue
XV	Antiangiogenic factors	Epithelium and Endothelium
XVI	Interruptions present	Perineural endothelium epithelial basal lamina, muscle
XVII	Hydrophobic transmembrane	Hemidesmosome
XVIII	Antiangiogenic factors	Epithelium and Endothelium
XIX		Perineural endothelium epithelial basal lamina, muscle
XX		Tendon cartilage

The amino acid composition of collagen was identified in this review, as well as the current state of knowledge about the synthesis of its specific amino acids, hydroxyproline and hydroxylysine. When studying collagen or its metabolism in the whole animal, the paper emphasizes the importance of understanding the function of histoarchitecture and interactions of collagen with other compounds [71].

This analysis will discuss various basic steps in collagen anabolism and catabolism as well as the particular types of collagen found in the skin, tendon, bone, blood vessels, cartilage, and cell membranes and addresses illnesses linked to excesive collagen accumulation leading to different types of fibrosis [72].

This analysis focuses on recent findings regarding the function of proline (Pro) in collagen biosynthesis and cellular metabolism. It seems apparent that one of the primary substrates for collagen biosynthesis, Pro, is needed for the formation of a collagen molecule. The review raises the question of whether Pro for collagen biosynthesis is synthesized “de novo,” derived directly from degraded proteins, or transformed from other amino acids. Recent research found that extracellular Pro (added to culture medium) had an important but minimal effect on collagen biosynthesis in fibroblasts (the primary collagen synthesized cells) cultured in the presence of glutamine (Gln). Extracellular Pro, on the other hand, significantly improved collagen biosynthesis in cells cultured in Gln-free medium. This study discusses the proposed mechanism of this

method as well as the potential implications of this understanding in the pharmacotherapy of connective tissue diseases [73].

Proline and hydroxyproline, formed by the breakdown of collagen, are important amino acids (AAs) for cell structure and function. During the life cycle, there are significant differences in proline metabolism and requirements among vertebrate animals. Proline, on the other hand, seems to be a significant regulator of cell metabolism and physiology, according to new research. In this study, chemical structures and functions of proline and hydroxyproline, developmental changes of proline and hydroxyproline in body proteins and physiological fluids, and proline metabolism are studied [74].

Transepidermal elimination of abnormally stained collagen bundles is seen on histopathological analysis of the lesions. The cause of extrusion of collagen through the epidermis is little known. Several investigations have revealed that the removed collagen is structurally normal; this suggests that more subtle chemical changes are occurring that are not compromising the structural integrity of the collagen molecules. The immune system may subsequently detect the changed collagen as alien, and a humoral immune response may initiate the process of transepidermal elimination [75].

5.5. Side Effects

Collagen is widely used as it helps blood coagulate, remodeling tissue. Although there are no known side effects, there is some concern about its role in inflammation, group-to-group variability, and possible disease transfection, given that animal-derived (natural) collagen is used in many clinical applications. Products containing collagen hydrolysates have been used for many years without reporting serious side effects. However, it is also important to realize that different collagen hydrolyzate formulations are marketed and can exert an unparalleled effect on safety. Also, safety was evaluated in vitro using CaCo-2 and HepG2 cell lines, which are routinely used in the pharmaceutical industry to control drug toxicity [76].

6. COLLAGEN PRODUCTS

6.1. Collagen Supplements

Not every protein is of equal value, so not every collagen is of equal value. Thanks to its low molecular weight, hydrolyzed collagen is quickly digested by our body and enters our blood circulation. According to study, the most suitable type of collagen is the one in powder or liquid form for collagen peptides that can be easily absorbed and used by our body. When we ingest this type of collagen via food, it reaches the bloodstream that will be used by the body in a very short time. It has a high bioavailability and is therefore the most favored collagen in this type. In terms of bioavailability, the collagen pill type provided in tablet or capsule form is lower than the others. It is more difficult for the collagen in the capsule to be digested in our stomach and to completely enter the bloodstream. Collagen capsules also have other disadvantages. Although it may seem easy to use, it can actually be misleading. You may need to swallow 10-20 of these capsules a day to get the daily amount of collagen you need. In addition to collagen, we also take into our bodies the ingredients such as thickener and emulsifier used in the production of these capsules [77].

Taking the collagen needed by our body only with nutrition under normal conditions can cause various digestive problems or weight problems. Collagen sachet dosage form is prepared with the amount of collagen needed with a formula that does not contain any extra ingredients. It does not contain any additives and is presented in a form that you can easily add to your food and drinks. Another advantage of collagen sachet products is that you can easily consume it wherever you are at work, at home or at school. Being in single-use packages creates ease of use [78].

Collagen and its fractions have shown a major function as valuable nutritional fibers and protein source in composing human diets due to the features of moisture absorption [79]. As human beings grow older, the synthesis of collagen will decrease and the tissues will become thinner, weaker and less flexible. Collagen supplements are designed to protect users' skin , hair , nails and body tissues. By attracting fibroblasts that generate the synthesis of new

collagen, the collagen metabolites assemble bone, skin and ligaments. It develops the diameter and cohesion of the dermal collagen fibers of collagen fibrils in the dermis. The thickness, flexibility and resilience of the tissues, as well as hydration, will therefore be improved. The function of skin dermis and epidermis may be advanced by collagen.By increasing the capacity of the outermost skin layer to absorb water. Skin tissue hydration is directly related to smoothness and reduces wrinkling. Supplementing collagen can increase lean muscle gain, decrease recovery time, rebuild damaged joint structure, and boost cardiovascular performance. This is achieved by promoting natural creatine, an essential amino acid in new muscle growth after workouts, by collagen [80].

6.1.1. Liquid collagen

Luqiud collagen forms have similar properties to powder collagen forms. Since it is a hydrolyzed collagen supplement, its absorption is faster and easier. However, collagen in liquid form contains aroma. The majority of collagen supplements are available in powder or liquid form, and they can be flavored or unflavored. Many people prefer the unflavored kind since it can be mixed into dishes and beverages without altering the flavor. At the same time, it is easy to consume in daily life as its portion and mixture are ready.

6.1.2. Oral collagen in solid dosage forms

Powder form

Collagen supplements, which are in powder form, are absorbed from the moment they enter the body, before they reach the digestive system organs such as stomach and intestines. In this way, higher efficiency is obtained. Collagen supplements in powder form are easier to use, they can be easily drunk by adding them to coffee, water or the liquid you will consume. One research, discovered that using oral collagen supplements improved skin elasticity, hydration, and collagen density [81].

Collagen tablets

Collagen tablets contain less hydrolyzed collagen than liquid and powder forms. It also takes longer to break down and absorb than other forms.

6.2. Collagen as an additive to food

Collagens are used as food additives that enhance the rheological properties of sausages and frankfurters, as well as ensure sufficient quantities of animal nutritional fibers. Technological and rheological characteristics of meat containing raw material added with collagen or its fractions may be enhanced. The addition of collagen to liverwurst or paste increases the consistency of the products and decreases the rate of incidence of fat caps. Santana et al . (2011) indicated that collagen fiber treated with heat has a good potential for use as an emulsifier in the application of food , especially in acidic products [80].

6.3. Collagen in beverages

Collagen-infused beverages are another global market development nowadays. Many goods, such as soy collagen, cocoa collagen, cappuccino collagen, collagen juice and bird nest drink with collagen, are published by producers. Proposed a collagen-infused energy drink to help promote the natural capacity of the body to produce fatty tissues [82]. The collagen drink usually claims to activate the body's collagen producing process , which in turn will promote the tissues of the body and reduce the wrinkles and sagging of the skin. As a consequence, the drink "Vitagen Collagen" was developed to promote the production of beneficial gut bacteria and profoundly radiate beauty from outside the skin. In addition, Avon has also developed the Avon Life Marine Fish Peptide Collagen Drink, a groundbreaking drink made from salmon fish skin, vitamin C and fructooligosaccharides made from pure and high-quality fish peptide collagen [80].

6.3.1. Coffee

With the growing popularity of collagen supplements has come a slew of new trends, such as the addition of collagen peptides to coffee. Many people believe that this is an excellent method to get extra collagen into their diets. Because of its neutral flavor, the unflavored form may be simply added to meals and beverages without dramatically altering their flavor.

When it comes to adding collagen to coffee, the biggest issue may be the influence of higher temperatures on the supplement's quality. Proteins are generally denatured when exposed to high temperatures or acidic and alkaline solutions, resulting in a minor change in their structure.

This is significant because collagen peptides are often produced by exposing animal skins to an acidic or alkaline solution in order to release the collagen. The hides are then boiled in water at temperatures as high as 190°F (88°C) to extract more collagen peptides. [83]

Even so, if collagen proteins are subjected to even greater temperatures, a process known as degradation can occur, further degrading the protein. At this time, the protein may no longer work properly, rendering the supplement ineffective.

One research found that when collagen proteins were exposed to temperatures ranging from 302–788°F (150–420°C), their initial breakdown occurred at 302°F (150°C) [84].

Coffee, on the other hand, is normally brewed at 195–205°F (90–96°C) - a far lower temperature range. As a result, as long as your coffee is below 302oF (150oC) when you add your collagen supplement, the powder's quality should be unaffected [85].

7. CONCLUSION

In the food and beverage sectors, collagen has proved to be a significant ingredient. Collagen has been used as protein dietary supplements, meat processing carriers, edible film and product coatings, and food additives to enhance the consistency of products. In addition, collagen can improve the product's health and nutritional value.

As an identity test of collagen hydrolysates, we took advantage of the abilities of the analytical techniques SEC, MS, and NMR to determine the characteristic peptide patterns.

Developing new functional food formulations using collagen hydrolyzate and optimizing production conditions technologically is critical for the food industry. Studies have supported the beneficial effects of collagen hydrolyzate on health and skin, as well as high levels of bioavailability as a result of oral intake of collagen hydrolyzate. Collagen hydrolyzate has been identified in food , cosmetics, photography, medicine and pharmacy for a broad range of uses. Being a natural protein and its technical characteristics, it is expected that development and consumption of collagen hydrolyzate will increase in the coming years.

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Evaluation the plausibility of repurpose of levamisole and niclosamide in treatment of Covid-19

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ABSTRACT

Since the world health organization declared the pandemic of covid 19, many drugs have been tested and re-evaluated to find effective treatment for the novel corona virus infection. Niclosamide and Levamisole which are FDA approved anthelmintic drugs have been evaluated by many researchers and agencies to repurpose of these drugs as additional options for existing treatment strategy used for patients with Covid-19. Hence we are trying in this review to introduce most reports that evaluated the use of Niclosamide and Levamisole for treatment of patients infected with Covid 19. We concluded that the encouraging studies regarding the repurpose of the two drugs may highlighting for further studies that can widening the options for existing treatment strategy used for patients with COVID-19.

1. INTRODUCTION

Niclosamide is an oral antihelminthic medicine that included in the essential medicines list of WHO. The drug was approved in 1982 by the FDA as a therapy for tapeworm diseases that infect humans [1]. The drug besides treating safely millions patients with parasitic infections worldwide, its broad clinical applications in the treatment of many diseases and manifestations such as cancer, bacterial, viral infection and various metabolic disorders, have been shown by recent studies [2-4]. Levamisole is an antihelminthic drug that was produced by Janssen and first used in 1969 as an active agent against many nematodes that infect animals and man [5-9]. The drug then was commonly applied to treat different infections caused by parasites, viruses, and bacteria [10]. Levamisole was used in 1970s and 1980s as an antirheumatic therapy for patients with rheumatoid arthritis [9]. In 1990 the drug got the approval by the FDA as an adjuvant treatment for colon cancer [11], since then Levamisole is prescribed together with other medicine as a therapy for various cancers [9,10]. The immunomodulatory effects of levamisole have been evaluated in attempt to study its effect in enhancing protective immune responses. The drug showed positive results with various rates of success in the treatment of various diseases in animals and human [10,12-14]. In 2000 Levamisole was withdrawn from the American market because the possibility to cause serious side effects [9].

Both drugs [Niclosamide and Levamisole] were found to have a role in suppression of viral infections through direct effect or by the immunostimulant effect respectively. Recently many research have been experimented the plausibility of repurpose of these drugs in treatment of patients infected with SARS-CoV-2. Hence we are trying in this review to introduce most reports that evaluated the use of Levamisole and Niclosamide for treatment of patients infected with Covid 19.

2. NICLOSAMIDE (NCS)

2.1. Niclosamide's Mechanism of Action

Niclosamide acts in prevent glucose consumption, oxidative phosphorylation, and anaerobic metabolism in the target worms [15]. Evaluation of the antineoplastic effect of NCS in many human malignant cancers indicated that NCS exerts anticancer activity by suppressing many oncogenic signaling pathways at the same time [4,16,17]. NCS action against viruses shows mainly through its role as a carrier of proton and that may lead to neutralize the acidity of endosomes which prevent endocytosis through influencing on trafficking of endosome and this inhibits the entrance of viruses [18,19,20].

2.2. Niclosamide Role in Viral Infections

Niclosamide has been evaluated against a wide scale of viruses and found to has a role in inhibition the entrance and the replication of many viruses including Herpes Simplex Virus(HSV-2) [18], Dengue Virus (DENV) [19,21], ZikaVirus (ZIKV) [22,23,24], Chikungunya Virus (CHIKV) [25], Lassa and Ebola Viruses [26], Epstein-Barr Virus (EBV) and Kaposi's Sarcoma-associated Herpesvirus (KSHV) [27], and Japanese Encephalitis Virus (JEV) [28]. Evaluation of Niclosamide against Coronaviruses was also studied. At a concentration of 1.56 μM, NCS found to be able in prevention SARS-CoV multiplication and completely terminated antigen formation of the virus. According to the study, the inhibiting activity was noticed even when NCS was added three hours following the inoculation of cells with SARS-CoV [29]. In Vero E6 cells, the cytopathogenic effect of SARS-CoV has been suppressed at 1 μM of NCS. And with an EC50 value of minimal than 0.1 μM, the replication of SARS-CoV was found to be inhibited [30]. Another study found that NCS didn't reveal clear inhibitory potency towards SARS-CoV(3CL protease) even up to 50 μM concentration. The study concluded that the drug may acts through different mechanism against the virus [31]. In Vero B4 cells, the evaluation of 10 μMNCS e against MERS-CoV demonstrated up to 1000-fold inhibition of MERS-CoV replication at 48 h p.i [32].

2.3. Evaluation of Niclosamide on Covid-19

Due to prior studies that described NCS as an encouraging efficient therapy or SARS-CoV infection [29], and since SARS-CoV-2 genome sequences have been found to match with SARS-CoV in about 79.6% [33]. Besides that Covid-19 threat is still push scientists around the world to find more than successful therapy. All these reasons as well to NCS wide effects against viruses gave envision that NCS may be repurposed and developed as effective antiviral drug against COVID-19 if approved experimentally and clinically [34]. Recent study suggested that NCS may have a feasible activity towards COVID-19 probably through prevents the entrance of virus via changing the pH of endosomes and suppresses the multiplication of the virus via autophagy prevention. This plausible mechanism need to be confirmed clinically to evaluate the efficiency of NCS against COVID-19 [35]. Group of researchers in The University of Texas at Austin, Division of Molecular Pharmaceutics and Drug Deliver are attempting to develop a mechanism that make the intake method of NCS reach directly to the lungs. This mechanism could demonstrate effectiveness at management and control the critical manifestations in COVID-19 patients [36]. Recently, an inhaled NCS -Lysozyme formulation has been developed and revealed encouraging results in vitro and in vivo against SARS-CoV-2 [37]. Another developed nanoparticles formulation of NCS demonstrated an in vitro effectiveness against SARS-CoV-2 replication [38]. Another group of investigators in clinical trial which still waiting to be approved hypothesized that the antiviral activity of NCS may be extended to COVID-19 [39]. A recent study suggested that NCS may potentially inhibit the entrance of SARS-CoV2 through the interference with pH-dependent endocytic pathway that the

virus depends on during its entrance [40]. South Korean Daewoong Pharmaceutical Co. Ltd. has collaborated with other companies for conducting Phase I clinical trial of a long-acting intramuscular formulation of NCS. The injectable intramuscular (IM) formulation of NCS demonstrated promising results that successfully removed the virus from lungs in animal tests. Daewoong researchers worked on quick development of the formulation to reach the stage of "First-in-Human" evaluation. The successful completion of this work can immensely facilitate COVID-19 disease treatment [41].

3. LEVAMISOLE (LMS)

3.1. Levamisole's Mechanism of Action

The paralytic effect of the drug is the main antiparasitic action against the target worms. The drug acts as neuromuscular blocking agent (their revocable, noncompetitive depolarization type) which results in persistent contract of the worm somatic muscles leading to paralysis. It is also prevents succinate synthesis [42]. Chemically the immune-stimulant effect of LMS shows through forming a thymopoietin-mimetic tertiary structure. Thymopoietin compound influences several immunological cells such as neutrophils, macrophages, and lymphocytes; hence the enhancement of phagocytosis and regulatory T cells are probably the medicinal effect of this compound [43].

3.2. Levamisole Role in Viral Infections

The role of LMS in viral infection has been evaluated through its action as immune-stimulant agent. The drug found to enhance the host defense mechanisms in10-day-oldrats and gave the rats protection against HSV-2 [44]. Using of LMS alone demonstrated stimulation of antibody formation against influenza A and B viruses. The stimulation is probably due to provoking of immunocompetent memory cells [45]. Studying the ability of LMS to enhance monocyte chemotaxis in vitro, demonstrated that the drug efficiently prevented the chemotaxis dropping induced by influenza virus when incubated with normal monocytes. LMS also showed improving in monocyte chemotaxic reaction in vitro. The cells were taken from confirmed acute influenza cases, which indicate that LMS may be helpful in acute influenza cases through boosting the performance of cellular immunity [46]. LMS also found to enhance production of IFN-γ through its role in macrophages activation which in turn provokes the growing of cells participated in cellular mediated immunity [47,48]. Another study noticed an increase in CD4 (+)/CD8 (+) ratio after one course therapy with liniment LMS, and stated that the drug acts in boosting cellular immune role in chronic hepatitis B individuals [49]. Other published paper found that giving LMS alone to chronic hepatitis B children at 2.5 mg/kg/day per os, three fold weekly for 90 days; didn't show any cellular immunostimulation in them. While a combination of LMS plus HBs Ag vaccine demonstrated a notable increase CD3, CD4 and CD4/CD8 ratio with remarkable depression in CD8 in chronic hepatitis B sets [50]. More studies found the same encouraging results on chronic hepatitis B (CHB) persons and healthy carriers if received LMS and vaccine [51]. For inactivated viral vaccines, humoral and cell-mediated responses found to be improved by using modified LMS adjuvant [52]. LMS and killed-virus-based antiviral vaccines combination may contribute in to promoting Th1-biased immune responses depending on the optimal LMS dosage [53]. A research included serious forms of influenza demonstrated that LMS can induces interferon efficiently, and advised the usage of the drug in combination treatment, particularly for those with a severe course [54]. After repeated regimen administration with LMS, the experimentally infected quails with H9N2 AI viruses showed CMI responses enhancement towards H9N2 AI viruses with reduction in virus discharging duration [55].

3.3. Evaluation of Levamisole on Covid-19

Very limited number of published reports indicated the assessment of LMS on covid -19. As an antiviral agent, LMS found as an effective therapy in treating diarrhea [56], and in improving cough and dyspnea in COVID-19 patients [57]. A study, based on the effect of LMS as an immune-stimulant, concluded that this drug may help the immune system of COVID-19 patients to overcome the hidden virus by establishing a strong response [58]. Few clinical trials in study level that are still waiting to be published is tested the immune-stimulant activity of LMS on patients

with COVID-19. One of these trials suggested that LMS can play a role in enhancing the immunity in covid-19 patients through increasing the number of lymphocytes and can also interferes with virulence factors of the virus [59]. Other trials have been evaluated the prophylactic and the therapeutic role of LMS on COVID-19 patients and the results is still expected [60,61]. Evaluation of LMS as antiviral and immune-stimulant drug for patients with covid-19 needs further studies to recognize the specific role of the drug during infection.

4. CONCLUSION

Considerable and exceptional global researches have been undertaken during the SARS-CoV-2 pandemic. Facilitating cooperation with coordination between scientists and global health professionals and accelerating efforts by WHO has been achieved on a scale not seen before. All these efforts have been succeeded in December 2020 to provide the first vaccine that paved the road to initiate a mass programme of vaccination around the world and until now around 13 types of vaccines are available and taken in different countries [62]. Although up to the 25 January 2022, about nine and half billion vaccine doses have been provided, COVID-19 virus extensively expanded to more than 200 countries, infected around 350 million cases and resulted in more than five and half million deaths [63,64]. The huge increase in numbers of infected and dead people as well the continuous appearance of different variants of the virus is still urges the need to find an effective treatment that eliminate COVID-19 entirely and is also still fuels the researchers around the world to search for promising drugs that can participate in the control of COVID-19 outbreak completely. So we believe that highlighting on the latest encouraging studies of evaluation of Niclosamide and Levamisole and their plausibility of repurpose in treatment of patients with Covid 19, may attract more attention for wider clinical studies on these drugs. Testing each of these drugs alone or in combination with other drugs should also consider in these studies. Providing a promising treatment for COVID-19 from already existing drugs may give additional options for existing treatment strategy used for patients with COVID-19.

AUTHOR CONTRIBUTION

Concept: ABA, SEK; Design: ABA, SEK; Supervision: SEK; Data Collection and/or Processing ABA; Analysis and/or Interpretation: ABA, SEK; Literature Search: ABA; Writing: ABA; Critical Reviews: SEK..

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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