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Validate a Stability Indicating RP- HPLC Method for Simultaneous Estimation of Univestin and UC-II in Bulk and Pharmaceutical Formulations

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***Abstract*: A simple, rapid, precise, sensitive and reproducible reverse phase high performance liquid chromatography (RP-HPLC)(1) method has been developed for the quantitative analysis of Univestin and UC-II in pharmaceutical dosage form. Chromatographic separation of Univestin and UC-II was achieved on Waters Alliance-e2695 by using Chiral Cell ODH 150x4.6mm, 5µ column and the mobile phase containing Hexane + THF and 0.1% Formic Acid in the ratio of 80:20% v/v. The flow rate was 1.0 ml/min; detection was carried out by absorption at 308nm using a photodiode array detector at ambient temperature. The number of theoretical plates and tailing factor for Univestin and UC-II were NLT 2000 and should not more than 2 respectively. % Relative standard deviation of peak areas of all measurements always less than 2.0. The proposed method was validated according to ICH guidelines. The method was found to be simple, economical, suitable, precise, accurate & robust method for quantitative analysis of Univestin and UC-II study of its stability.**

***Keywords*: HPLC, Univestin and UC-II.**

1. Introduction

DRUG PROFILE

1) Drug profile of univestin (2)

Category: Anti-Inflammatory Agents

Description: Univestin is a blend of extracts of Scutellaria Baicalensis and Acacia Catechu which is used to relieve the pain, stiffness or discomfort in the joints. It improves the range of motion, enhances the flexibility and supports joint health.

Uses of Univestin: Univestin is used to prescribe for the treatment of:

- Pain due to arthritis
- Joint inflammation Storage: Store at room temperature

Away from heat and direct light Side Effects

Report to the physician immediately if the patients are having any of these following symptoms:

- Abdominal pain
- Nausea
- Dark urine
- Indigestion
- Increased Liver Enzymes
- Contraindications of Univestin

Univestin must not be used in the following conditions:

- Hypersensitivity to the active ingredient(s) or to any of the excipients of the medicine

2. DRUG PROFILE OF UC-II

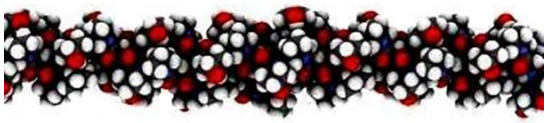

Fig. 1. Molecular structure of UC-II

Table 1
Drug profile of UC-II

Description	UC-II contains a patented form of undenatured type II collagen derived from chicken sternum. Previous preclinical and clinical studies support the safety and efficacy of UC-II in modulating joint discomfort in osteoarthritis and rheumatoid arthritis.
Solubility	Water soluble

1) Mechanism of Action:

Collagens are extracellular matrix molecules used by the cells for structural integrity and a range of further functions. Numerous hypotheses were suggested to clarify the precise mechanisms by which the collagen products enhance the articular cartilage health. UC-II appears to exert joint-health benefits by oral tolerance, based on pre-clinical research. Oral tolerance is an immune process the body uses to distinguish between innocuous compounds (e.g., dietary proteins, intestinal bacteria) and potentially harmful foreign invaders. It takes place in the gut-associated lymphoid tissue (GALT). The GALT is mostly made up of mesenteric lymph nodes and patches of lymphoid tissue neighboring the small intestine (Peyer's patches). Peyer's patches take in and screen compounds from the gut lumen and, depending on the compound, switch the

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body's immune response on or off. When consumed, UC-II® undenatured type II collagen is believed to be taken up by the Peyer's patches, where it activates immune cells . It transforms naive T-cells into T regulatory (Treg) cells that specifically target type II collagen. Treg cells then migrate through the circulation. When they recognize type II collagen in joint cartilage, Treg cells secrete anti-inflammatory mediators (cytokines), including the transforming growth factor-beta (TGF-beta), interleukin 4 (IL-4) and interleukin 10 (IL-10) [50, 51]. This action helps reduce joint inflammation and promotes cartilage repair. Undenatured type II collagen contains active epitopes that are able to interact with Peyer's patches and induce oral tolerance. The key structural macromolecules of the cartilage tissue in the mammals are collagen and proteoglycans (aggrecan) [2, 46]. Glucosamine, hyaluronic acid, and chondroitin sulfate are vital basic natural constituents of cartilage and synovial fluid. Denatured type II collagen, by contrast, lacks these essential structural components. Preclinical studies support oral tolerance as the mode of action of UC-II® undenatured type II collagen and confirm that the undenatured form of type II collagen is critical for joint-health benefits: In an animal model (mouse) of RA, only undenatured type II collagen protected against joint damage, an action attributed to oral tolerance [52]. In an animal model (rat) of RA, undenatured type II collagen provided symptom relief, an action attributed to oral tolerance and modulating inflammatory pathways [51]. In a cell study, Treg cells specific for type II collagen secreted anti-inflammatory cytokines, which play a chief role in the cells' ability to induce oral tolerance [53]. In a cell study with human chondrocytes (cells that make up cartilage), the anti-inflammatory action of IL-10 protects against damage from tumor necrosis factor-alpha (TNF-α), a pro-inflammatory mediator elevated in osteoarthritis [54]. Clinically validated lab assays confirm active epitopes in UC-II® undenatured type II collagen resist digestion and retain the undenatured 3D-structure needed to interact with Peyer's patches and induce oral tolerance [49]. This process initiates anti-inflammatory and cartilage protective pathways that prevent the immune system from injuring its joint cartilage while promoting cartilage repair and regeneration. On the other hand, immunohistochemical staining and gene expression of proteins linked to cartilage metabolism, such as collagen type II and X, matrix metallopeptidase 13 (MMP-13), sex-determining region Y-box 9 (SOX9), and connective tissue growth factor (CCN2) expressions, were suggested to be performed in the rat models of OA [17].

2) Uses

Previous studies have shown that undenatured type II collagen (UC-II) is effective in the treatment of rheumatoid arthritis, and preliminary human and animal trials have shown it to be effective in treating osteoarthritis (OA).

3) Side effects

Since collagen type II contains chondroitin and glucosamine, large doses might lead to the same side effects as those seen with chondroitin and glucosamine supplements. These side effects include nausea, heartburn, diarrhea and constipation, drowsiness, skin reactions, and headache.

3. Materials and Requirements

1) Instrument

HPLC, make: Waters alliance e-2695 chromatographic system consisting of quaternary pump, PDA detector2996 and chromatographic software Empower-2.0 was used.

Reagents: Hexane + THF and 0.1% Formic Acid (80+20).

2) Mobile Phase Preparation

- Mobile Phase-A: Hexane + THF
- Mobile Phase-B: 0.1% Formic Acid
- Diluent Preparation: Mix Mobile Phase-A and Mobile phase-B in 80:20 v/v.

3) Optimization of mobile phase

Different trials have done, different buffers and different mobile phases were used to develop the method. In all trials peaks are not separated properly. Finally for the proposed method all the peaks are separated and the entire suitability conditions are within the limit.

4) Isocratic Programme:

Mobile phase-A and Mobile phase B = 80:20

5) Chromatographic conditions: Optimized chromatographic conditions

Table 2

Parameters	Observation
Instrument used	Waters HPLC with auto sampler and PDA detector.
Injection volume	10µl
Mobile Phase	Hexane + THF and 0.1% Formic Acid (80+20)
Column	Chiral Cell ODH 150x4.6mm, 5µ
Detection Wave Length	308nm
Flow Rate	1 mL/min
Runtime	10min
Temperature	Ambient(25□ C)
Mode of separation	Isocratic mode

S.no	Parameter	Univestin	UC-II
1	Retention time	2.709	7.337
2	Plate count	4852	8986
3	Tailing factor	1.08	0.98
4	Resolution	----	17.56
5	%RSD	0.534	0.183

6) Standard Solution

Accurately weigh and transfer 25.8 mg of Univestin, 5 mg of UC-II working standard into a separate 10 ml clean dry volumetric flasks add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Pipette out 4ml of the UC-II solution into a 10 ml volumetric flask and make up to the mark with diluents (Stock solution). Further pipette 1 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent. (258ppm of Univestin, 20ppm of UC-II).

7) Sample Solution

Accurately weighed and transfer 535mg of sample into a 100mL clean dry volumetric flask add Diluent and sonicate it up to 30 mins to dissolve, and centrifuge for 30min. to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45 micron Injection filter. (Stock solution). Further pipette 1 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark

Table 4

S.no	Univestin		UC-II	
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area
1	64.50	604895	5.00	244515
2	129.00	1254526	10.00	494575
3	193.50	1858347	15.00	737484
4	258.00	2462478	20.00	986563
5	322.50	3092594	25.00	1225894
6	387.00	3624456	30.00	1431436
Regression equation	y= 9444.47x+ 14965.36		y =48207.53x + 8382.36	
Slope	9444.47		48207.53	
Intercept	14965.36		8382.36	
R^2	0.9997		0.9996	

1) Accuracy

Accuracy results of Univestin by RP-HPLC method

%Concentration(at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1254687	129	131.05	101.6	99.9
100%	2442554	258	255.12	98.9	
150%	3680641	387	384.44	99.3	

The Accuracy results for UC-II by RP-HPLC method

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	494872	10	10.03	100.3	100.0
100%	989194	20	20.04	100.2	
150%	1471753	30	29.82	99.4	

Discussion: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.9% and 100.0% for Univestin and UC-II respectively.

2) Precision Method Precision

Method Precision was investigated by the analysis of six separately prepared samples of the same batch. From these six separate samples solution was injected and the peak areas obtained used to calculate mean and percentage RSD values.

Intermediate Precision.

Table 5

Method Precision for Univestin & UC-II by RP-HPLC method

S.no	Area for Univestin	Area for UC-II
1	2431871	988714
2	2464952	986542
3	2479874	983441
4	2452478	991578
5	2484736	982719
6	2447893	989617
Average	2460300	987101
Standard Deviation	20125.73	3518.11
%RSD	0.82	0.36

3) Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%.

4) LOD and LOQ

Sensitivity parameters (LOD & LOQ) by RP-HPLC

Table 6

Name of drug	LOD(µg/ml)	LOQ(µg/ml)
Univestin	0.322	1.062
UC-II	0.025	0.082

with diluent. (258ppm of Univestin, 20ppm of UC-II).

4. Results and Discussion

1) Validation of proposed method

The method was validated for parameters like system suitability, specificity, and linearity, LOD, LOQ, Precision, Accuracy, Robustness and Ruggedness as per ICH guidelines [17, 18].

2) System Suitability

The HPLC system was stabilized for 60min to get a stable baseline. Six replicate injections of standard solution were injected. The results are summarized below table 1.

Table 7
Robustness

Robustness results of Univestin by RP-HPLC

Parameter	Univestin					
	Condition	**Retention time(min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**
Flow rate Change (mL/min)	Less flow (0.8ml)	3.365	2628948		1.17	3877
	Actual (1ml)	2.719	2489082		1.12	3072
	More flow (1.2ml)	2.260	2138948		1.45	2382
Organic Phase change	Less Org (72:28)	3.413	2582691		1.45	3574
	Actual (80:20)	2.712	2467503		1.13	3069
	More Org (88:12)	2.302	2273958		1.70	3245

Robustness results of UC-II by RP-HPLC

Table 8

Parameter	UC-II					
	Condition	**Retention time(min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**
Flow rate Change (mL/min)	Less flow (0.8ml)	9.176	1041464	20.98	1.09	12358
	Actual (1ml)	7.337	987078	19.13	1.04	10999
	More flow (1.2ml)	6.138	957894	17.60	1.23	9614
Organic Phase change	Less Org (72:28)	9.205	996921	21.05	1.13	12341
	Actual (80:20)	7.334	985521	19.15	1.06	10987
	More Org (88:12)	6.188	973567	19.56	1.19	9821

Table 9
System precision table of Univestin & UC-II

S. No	Concentration Univestin (µg/ml)	Area of Univestin	Concentration of UC-II (µg/ml)	Area of UC-II
1.	258	2489082	20	987078
2.	258	2467503	20	985521
3.	258	2470656	20	988502
4.	258	2451863	20	986639
5.	258	2461588	20	984957
6.	258	2479914	20	989764
Mean	247	0101	987	077
S.D	1318	1.25	1808	.76
%RSD	0.5	34	0.1	83

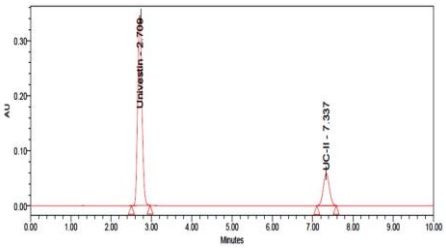

Fig. 2. Chromatogram of standard

3) Specificity

Discussion: Retention times of Univestin and UC-II were 2.712 min and 7.334min respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

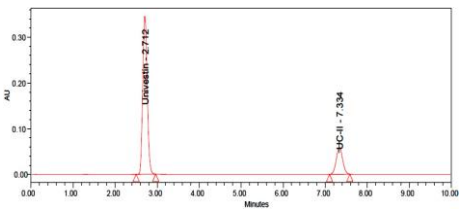

Fig. 3. Optimized chromatogram

4) Results of linearity for Univestin and UC-II

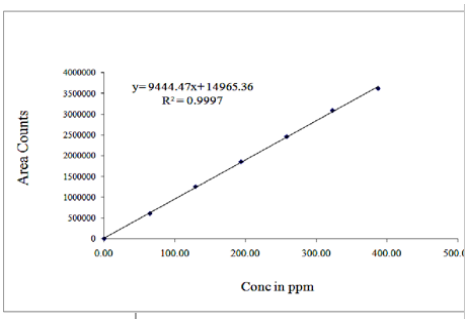

Fig. 4. Calibration curve for UC-II at 308nm

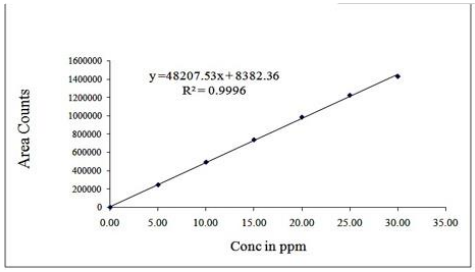

Fig. 5. Calibration curve for Univestin at 308 nm

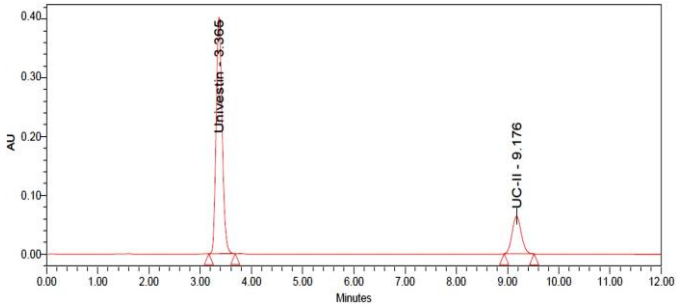

Fig. 6. Chromatogram for less flow rate (0.8 ml)

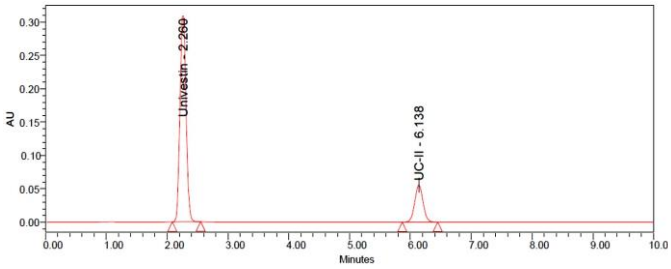

Fig. 7. Chromatogram for more flow rate (1.2mL)

5. Conclusion

The present work concluded that stability indicating assay method by RP-HPLC was simple, accurate, precise, and specific and has no interference with the placebo and degradation products. Hence these can be used for routine analysis of Univestin and UC-II.

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