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CHEMOMETRICS ASSISTANT RP - HPLC METHOD DEVELOPMENT AND VALIDATION OF COLLAGEN TYPE II AND UNIVESTIN

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ABSTRACT

The current investigation was pointed at developing and progressively validating novel, simple, responsive and stable RP-HPLC method for the estimation of UC-II and Univestin.The chromatographic strategy utilized Chiral Cell ODH 150x4.6mm, 5µ, using isocratic elution with a mobile phase of Hexane + THF and 0.1% Formic Acid (80+20). A flow rate of 1 ml/min and a detector wavelength of 308 nm utilizing the PDA detector were given in the instrumental settings.Validation of the proposed method was carried out according to an international conference on harmonization (ICH) guidelines.LOD and LOQ for the two active ingredients were established with respect to test concentration. The calibration charts plotted were linear with a regression coefficient of $R^2 > 0.999$, means the linearity was within the limit. Recovery, specificity, linearity, accuracy, robustness, ruggedness were determined as a part of method validation and the results were found to be within the acceptable range. The proposed method to be fast, simple, feasible and affordable in assay condition.

Key words: UC-II,Univestin, RP-HPLC, Development, Validation.

INTRODUCTION

Glycosylated (Crich, 2010; Jaeken, 2013) undenatured type II collagen is found in UC-II, a natural component. Small dosages of UC-II have been found in previous research to alter joint health in both OA and RA. Consuming microgram doses of undenatured type II collagen reduces the levels of circulating inflammation (Ferrero-Miliani et al., 2007; Serhan, 2008) related cytokines (Reche, 2019; Leonard, 2001), potentially reducing both the incidence and severity of arthritis (Deane et al., 2017). Oral tolerance is the ability to change immunity by consuming a meal or antigen (Lindenmann et al., 1984). As part of a normal physiological process, the digestive system (Kong et al., 2008) is continuously protected against immunological harm. IL-10 and TGF- are released by different types of T-regulatory cells (Rayner et al., 2018), according to research on their mechanism of action. Food or antigen must be consumed regularly to sustain the tolerogenic state (Morelli et al., 2001). This, together with our existing understanding of the role of cytokines in joint function, led us to hypothesise that supplementing healthy subjects' joints will ease joint discomfort and restore joint function to normal levels of function. UC-II has been shown to be effective in the treatment of arthritis in previous investigations. In a clinical research including healthy patients supplemented with UC-II and experiencing temporary knee joint pain (Reider et al., 1981), a statistically significant improvement in knee joint function over placebo was also documented. After 120 days of dosage, these same people likewise took longer to feel discomfort. To test if UC-II is equally effective as placebo and GC, a commonly available joint pain reliever (Mallinson, 2017; Mehlisch, 2002).

A mix of Scutellaria baicalensis (Zhang et al., 2011; Feng et al., 2002) and Acacia catechu extracts, Univestin relieves joint pain, stiffness (Baumgart et al., 2000) and discomfort by reducing inflammation. Enhance flexibility and joint health by increasing range of motion and flexibility. It is derived from Scutellaria Baicalensis and Acacia catechu. Univestin is prescribed to people with arthritis, inflammation, and other illnesses (Johnson, 2002; Hanne et al., 2007) that cause them discomfort. Clinical studies have shown that Univestin relieves joint pain and stiffness. Inflammation-causing enzymes are inhibited, and range of motion and flexibility are enhanced. Scutellaria baicalensis and Acacia catechu plant extracts are used to make Univestin. Stiffness is normally relieved in 3 days, whereas joint discomfort is usually relieved in 5. Depending on the ailment or purpose for which it is being used, the dosage will vary. This drug has been confirmed both safe and effective, according to the FDA.

Materialsand Method

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Chemicals: Acetonitrile, HPLC-grade formic acid,water,were purchased from Merck India Ltd, Mumbai, India. APIs of UC-II,Univestinstandards were procured from Glenmark,Mumbai.

The Instrumentation: Waters alliance liquid chromatography (model 2695) monitored with empower 2.0 data handling system and a detector of photo diode array (model 2998) was used for this study.

Method optimization: To optimize the chromatographic conditions, different ratios of phosphate buffer and the acetonitrile in the mobile phase with isocratic mode was tested. However the mobile phase composition was modified at each trial to enhance the resolution and also to achieve acceptable retention times. Finally Hexane + THF and 0.1% Formic Acid (80+20) with isocractic elution was selected because it results in a greater response of active pharmacy ingredient. During the optimization of the method various stationary phases such as C_8, C_{18} and amino, phenyl columns were tested. From these trials the peak shapes were relatively good with Chiral Cell ODH 150x4.6mm, 5µ with a PDA detector. The mobile phase flow rate has been done at 308nm in order to obtain enough sensitivity. By using above conditions we get retention times of UC-II and Univestin were about 7.337 min and 2.709 min with a tailing factor of 0.98& 1.08. The number of theoretical plates for UC-II and Univestin were 8986, 4852 which indicate the column's successful output the % RSD for six replicate injections was around 0.18% and 0.53%, the proposed approach suggests that it is extremely precise. According to ICH guidelines, the method established was validated.

Till today there are no HPLC methods were reported in the literature, but only few methods are developed in individual analysis of UC-II and Univestin. Hence we developed method for the simultaneous quantification of UC-II and Univestin. The developed HPLC method was utilized for the estimation of the combined drugs by *in vitro* method.

Validation procedure

The analytical parameters such as system suitability, precision, specificity, accuracy, linearity, robustness, LOD, LOQ were validated according to ICH Q2 (R1) guidelines.

Chromatographic conditions: The HPLC analysis was performed on reverse phase HPLC system with isocratic elution mode using a mobile phase of Hexane + THF and 0.1% Formic Acid (80+20) andChiral Cell ODH 150x4.6mm, 5µcolumn with a flow rate of 1 ml /min.

Preparation of standard stock 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)

Sample Solution Preparation:

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

Resultsand Discussion

The main analytical challenge during development of a new method was to separate active Pharma ingredients. In order to provide a good performance the chromatographic conditions were optimized.

System suitability: In System suitability injecting standard solution and reported USP tailing and plate count values are tabulated in table 1 and the standard chromatogram was shown in figure 1.

Specificity: In this test method placebo, standard and standard solutions were analyzed individually to examine the interference. The below figure shows that the active ingredients were well separated from blank and their excipents and there was no interference of placebo with the principal peak.Hence the method is specific. Figure 2 shows the blank chromatogram.

Linearity: The area of the linearity peak versus different concentrations has been evaluated for UC-II, Univestin, as 10,25,50,100,125,150 percent respectively. Linearity was performed in the range of 5-30 µg/ml of UC-II and 64.5-387 µg/ml of Univestin. The correlation coefficients achieved greater than 0.999 for all. Linearity results were shown in table 2 and the calibration curves were shown in figure 3.

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Accuracy: In this method, Accuracy was conducted in triplicate by analyzing active pharma ingredientstandard solution at three kinds of concentration levels of 50, 100 and 150% of each at a specified limit. For all impurities, percentage recoveries were measured and found to be within the limit. The accuracy and reliability of the developed method were established. The percentage recovery values were found to be in the range of 99.4-100.3% for UC-II and 98.9-101.6% for Univestin. The results are given in table 3.

Intraday precision: Six replicates of a sample solution containing UC-II (20 µg/ml) and Univestin (258 µg/ml) were analysed on the same day. Peak areas were calculated, which were used to calculate mean, SD and %RSD values and the results were shown in table 4 and figure 4 represents method precision chromatogram.

Inter-day precision: Six replicates of a sample solution containing UC-II (20 µg/ml) and Univestin (258 µg/ml) were analysed on a different days. Peak areas were calculated which were used to calculate mean, SD and %RSD values. The present method was found to be precise as the RSD values were less than 2% and also the percentage assay values were close to be 100%. The results are given in table 5.

LOD and LOQ: The LOD concentrations for UC-II are 0.025 µg/ml and s/n values is3 and Univestin0.322 µg/ml and s/n value7. The LOQ concentration for UC-II0.082 µg/ml and their s/n values are 23 and Univestin1.062 µg/ml and s/n value is 28. Table 6 gives the LOD and LOQ concentrations.

Robustness: The conditions of the experiment were designed to test the robustness of established systemintentionally altered, such as flow rate, mobile phase in organic percentage in all these varied conditions.Robustness results for UC-II and Univestin found to be within the limit and results are tabulated in Table 7.

Table 1: Results of system suitability

System suitability parameter	Acceptance criteria	Drug name	
		UC-II	Univestin
USP Plate Count	NLT 2000	8986	4852
USP Tailing	NMT 2.0	0.98	1.08
USP Resolution	NLT 2.0	17.56	
% RSD	NMT 2.0	0.18	0.53

Table 2: Linearity of UC-II and Univestin

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	

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R^2 - Correlation coefficient

Table 3: Results of accuracy

S. No	% Level	UC-II % Recovery	Univestin % Recovery
1	50	100.3	101.6
2	100	100.2	98.9
3	150	99.4	99.3

Table 4: Intraday precision results of UC-II and Univestin

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

Table 5: Inter-day outcomes of accuracy of UC-II and Univestin

S. No.	Area for Univestin		Area for UC-II	
	Day-1	Day-2	Day-1	Day-2
1	2447812	2496569	987816	986651
2	2431841	2442772	981237	983470
3	2474984	2479201	986942	988049
4	2458483	2486545	979817	974962
5	2509357	2443084	989741	985347
6	2494788	2465173	992947	996218
Average	2469544	2468890	986416	985782
Standard Deviation	29216.67	22565.71	5026.23	6890.74
%RSD	1.18	0.91	0.50	0.69

Table 6: LOD and LOQ for UC-II and Univestin

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

Table 7: Robustness data of UC-II and Univestin

Parameter name	% RSD	
	UC-II	Univestin
Flow minus (0.8 ml/min	1.48	0.84
Flow plus (1.2 ml/min)	0.25	1.07
Organic minus (-10%)	0.36	0.55
Organic plus (+10%)	0.31	1.05

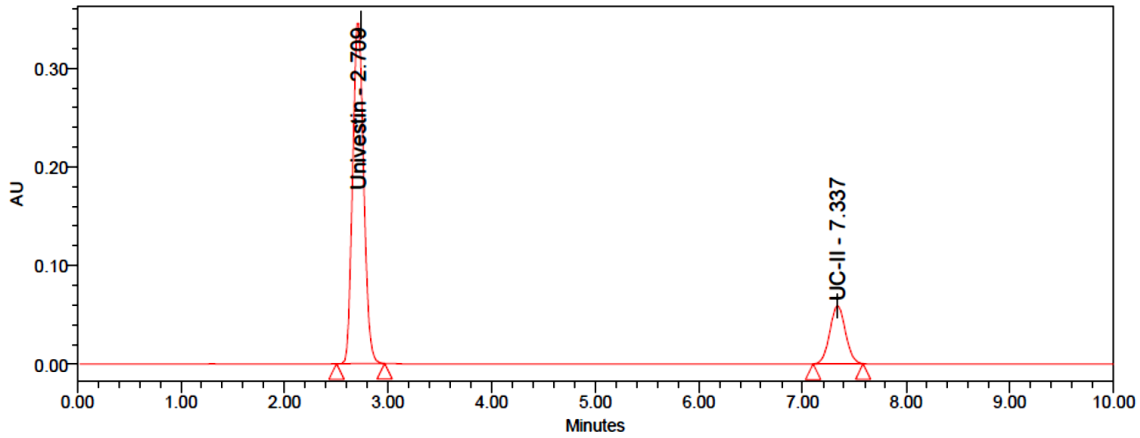

Fig. 1: Chromatogram of standard

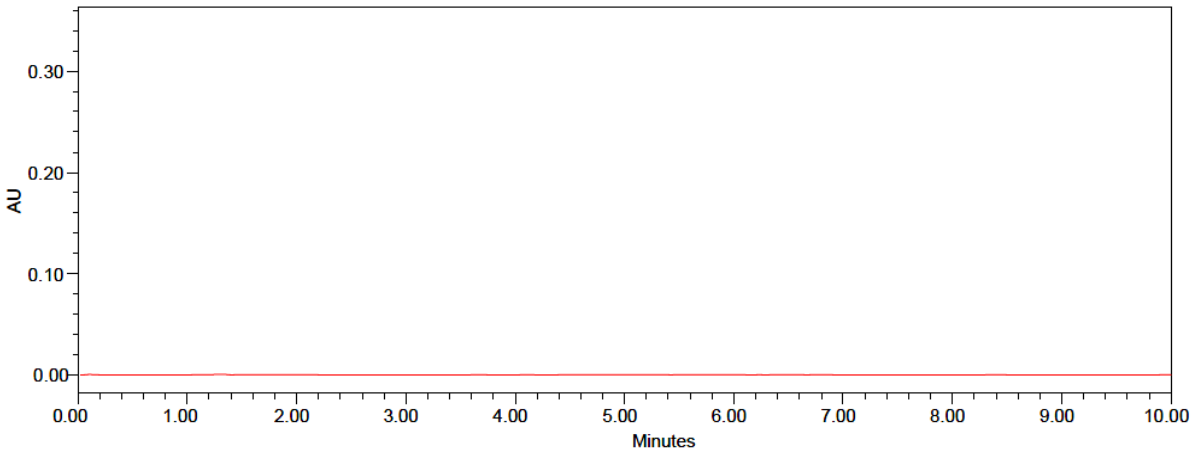

Fig. 2: Chromatogram of blank

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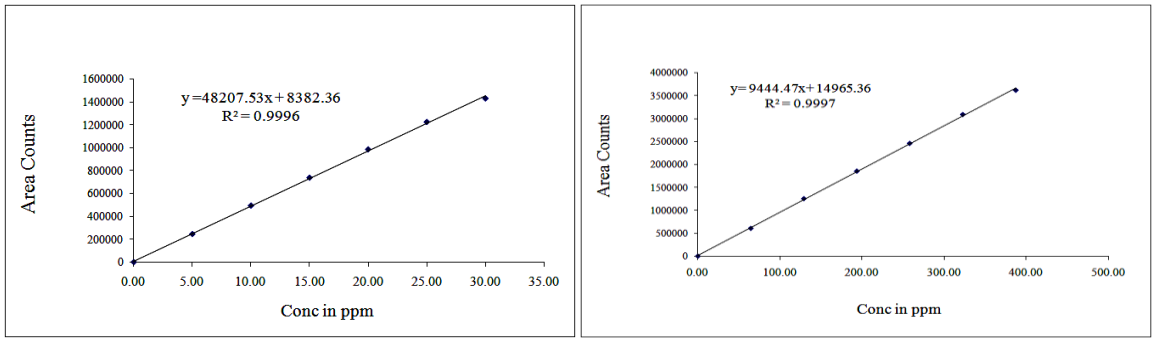


UC-II Univestin

Fig. 3: Calibration plots of (A) UC-II (B) Univestin

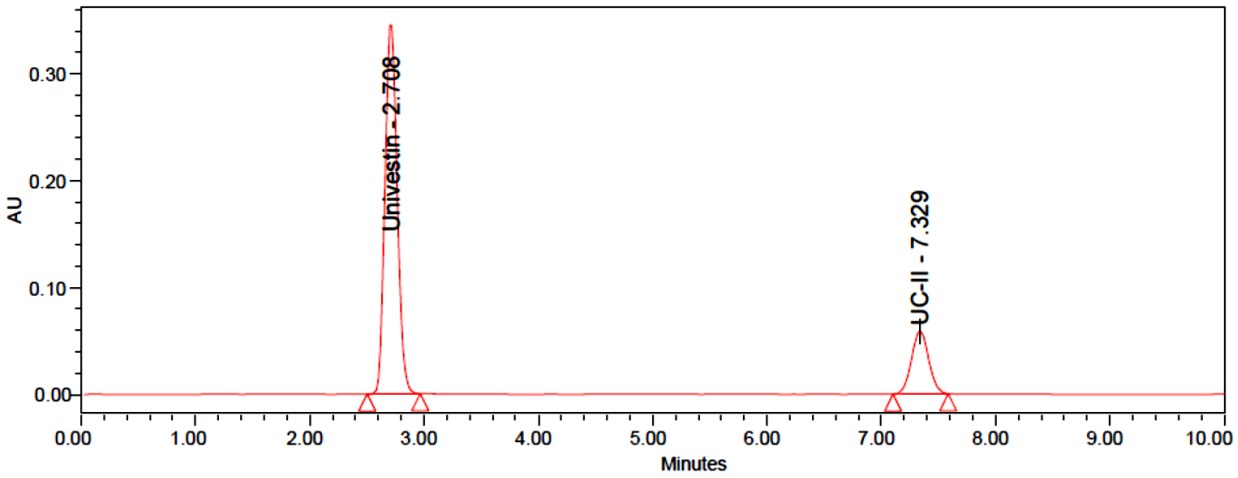
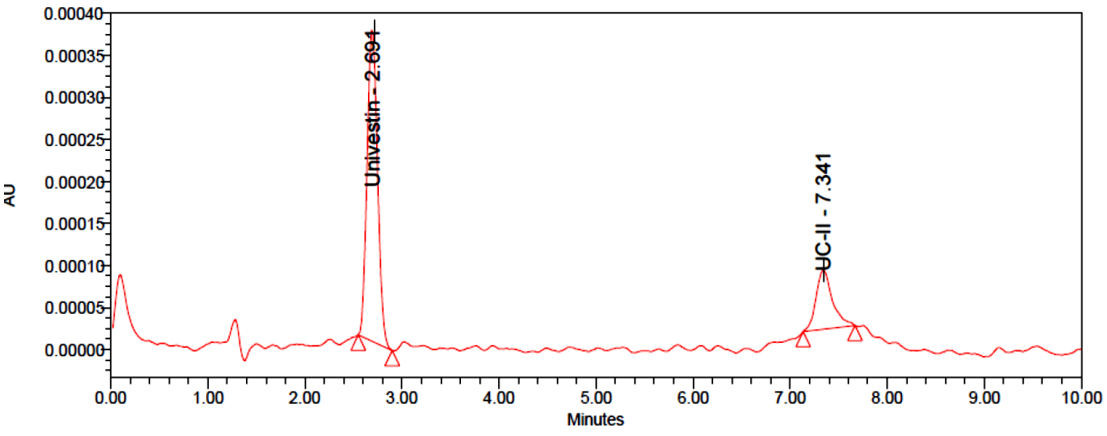


Fig. 4: Chromatogram of method precision



A

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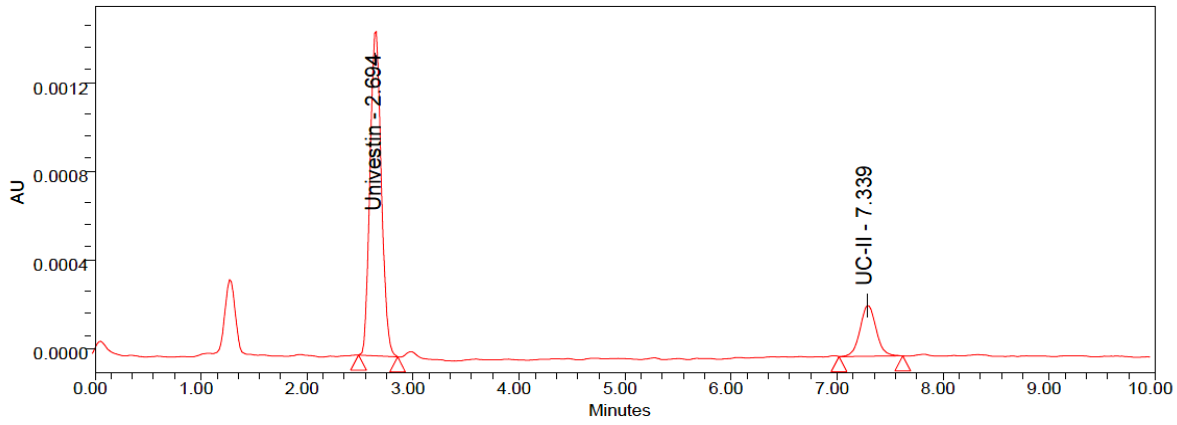

B

Fig. 5: Chromatogram of (A) LOD and (B) LOQ

ChemometricAnalysis:

In thischemometrics assisted HPLC study ,PCA,PLS calibrations were used to analysethe drugs of Univestin and collagen type II at 308 nm by using PDA detector. The data obtained from analysed drugs were stored in computer having required software to performchemometric analysis.

Acquisitionsoftware: Inpresentstudyweareusingfollowingchemometrictechniques.

- Principalcomponentanalysis(PCA)
- Partialleastsquarestechnique(PLS)

We are download the unscrambler (camo software),it facilitates the PCA,PLS analysis morerobust,accessible.

PLSApproach:

PLScalibrationusingtheorthogonalizedPLSalgorithminvolves,simultaneously,independentanddependentvariablesonthedatacompressionanddecompositionoperations.IntheHPLCdataanalysis,HPLC-PLScalibrationwasobtainedbydecomposition of both the drugs of concentration , peak area matrix into latent variables.PLS calibration was obtained using therelationship between the decomposed peak area .

Table : 8 : PLS Accuracynumericaldata ofUnivestin and Collagen Type-II

	YREFERENCE	YPREDICTED	YPREDICTED
		UNIVESTIN	CT-II
	1	2	2
1	50.000	101.9937	49.9435
2	50.000	101.9986	50.1832
3	50.000	97.4663	50.1293
4	100.000	92.5418	99.8576
5	100.000	101.4895	99.4355
6	100.000	101.5013	100.2942
7	150.000	101.0049	148.5840
8	150.000	101.0150	151.5758
9	150.000	100.9889	149.9968

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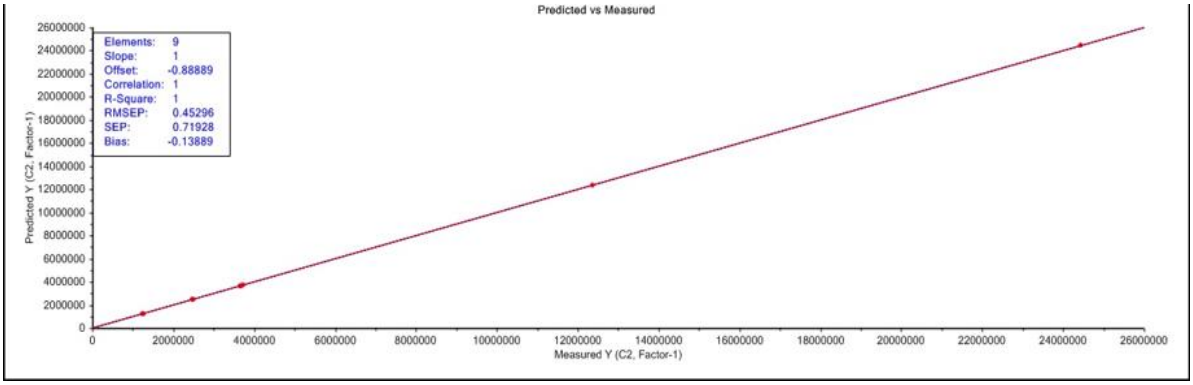

Fig 6: PCA Accuracy SpectralData ofUnivestin of Univestin

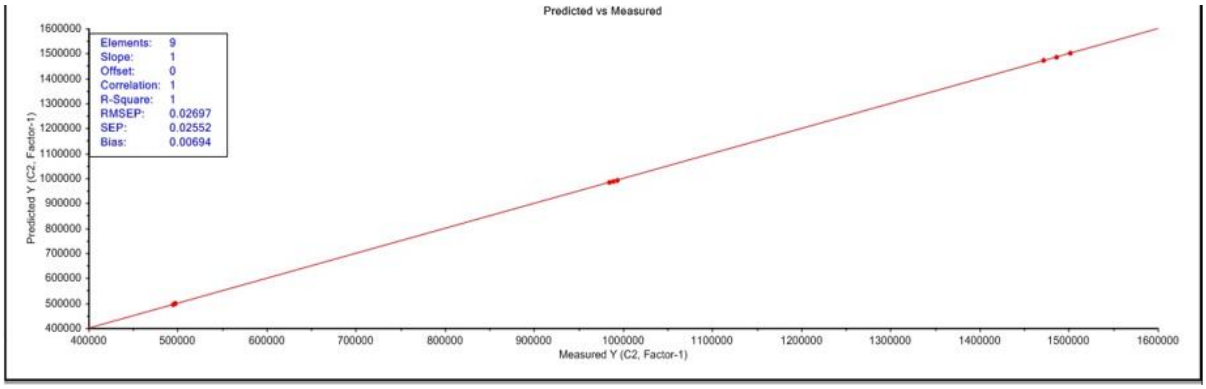

Fig 7 : PCA Accuracy SpectralData ofCollagen type II

Table 9: PLS Accuracynumericaldata ofUnivestin

	YREFERENCE	YPREDICTED
		Univestin
	1	2
1	0.000	-1.5002
2	64.50	61.4955
3	129.00	130.5756
4	193.50	194.7843
5	258.00	259.0260
6	322.50	2326.0309
7	387.00	382.5877

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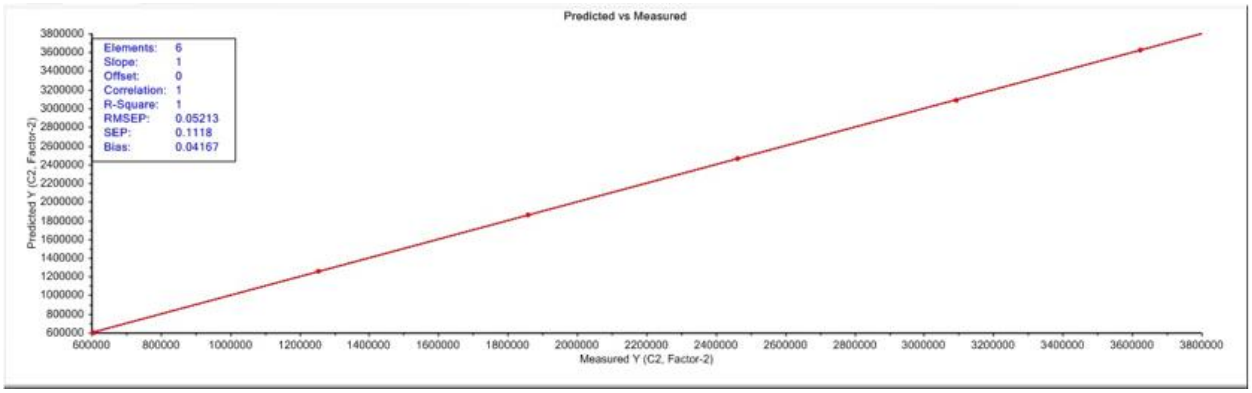

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Fig 7 :PLS LinearityspectraldataofUnivestin

Table 10 : PLS Accuracynumericaldata ofCollagen type II

	YREFERENCE	YPREDICTED
		Collagen type II
	1	2
1	0.000	-0.1643
2	5.000	4.7922
3	10.00	10.0110
4	15.00	15.0806
5	20.00	20.2789
6	25.00	25.2738
7	30.00	29.5635

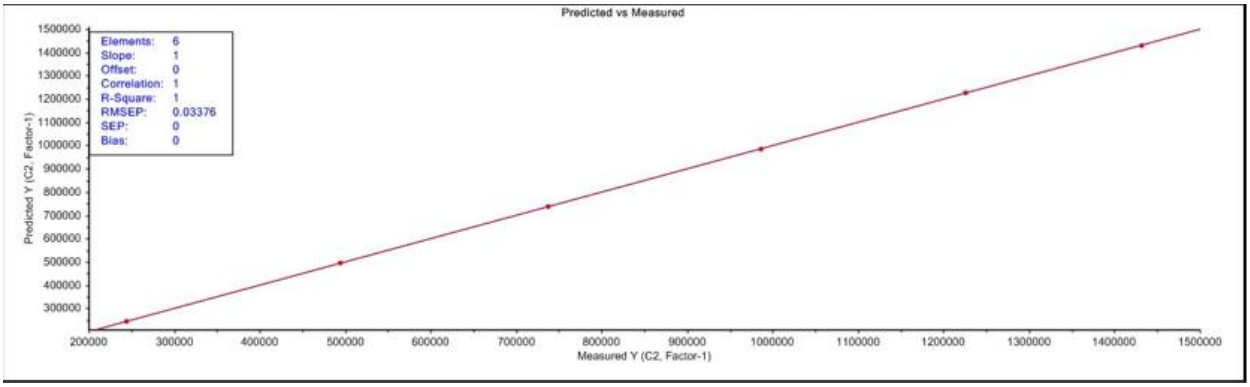

Fig 8 : PLS Accuracynumericaldata ofCollagen type II

PCA approach:

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In PCA technique it gives relevant information from data set, and it can be used express thedata on the basis of their similarity and differences. It is used to develop correlationstructure between variables, and examine the changes. In PCA data transferred to describethe amount of same variability. In these HPLC data analysis the data of both drugs ofUnivestin andCollagen Type-II peakarea we getthe Bio-plot.

Table 11 :PLS Accuracynumericaldata ofUnivestin and Collagen type II

PC-1	PC-2
1252983.2500	5068.8364
1243588.2500	5073.9165
1238031.1250	5076.9102
24452669.7500	4731.0049
2486091.0000	4731.0127
24644037.5000	4730.9097
3676590.0000	5068.3188
3665655.0000	5062.4526
3715108.0000	5039.3485

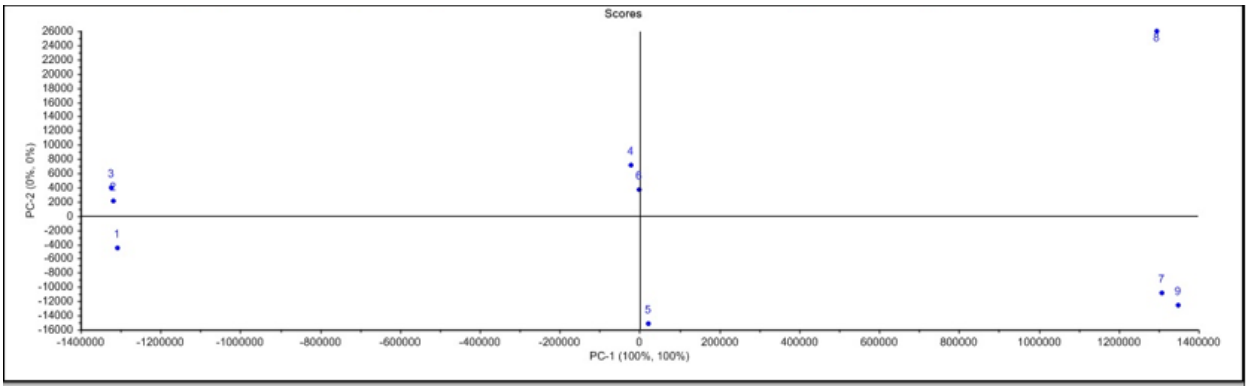

Fig 9:PLS Accuracyspectral data ofUnivestin and Collagen type II

Table12 :PCALinearity NumericalDataofUnivestin and Collagen Type-II

PC-1	PC -2
-4025.2326	-1635.128
605268.2500	20.63.0032
12552685.125	1891.0262
1858029.1250	1807.3346
2465749.2500	1809.1190
3092618.5000	1900.6732
3622946.0000	2040.5751

Figure83:PCAAccuracySpectralDataofUnivestin and Collagen type II

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PC-1	PC -2
-41.5002	-17.1643
605268.2500	2063.0032
1252685.1250	1891.0262
1858029.1250	1807.3346
2465749.2500	1809.1190
3092618.5000	1900.6732
3622946.0000	2040.5751

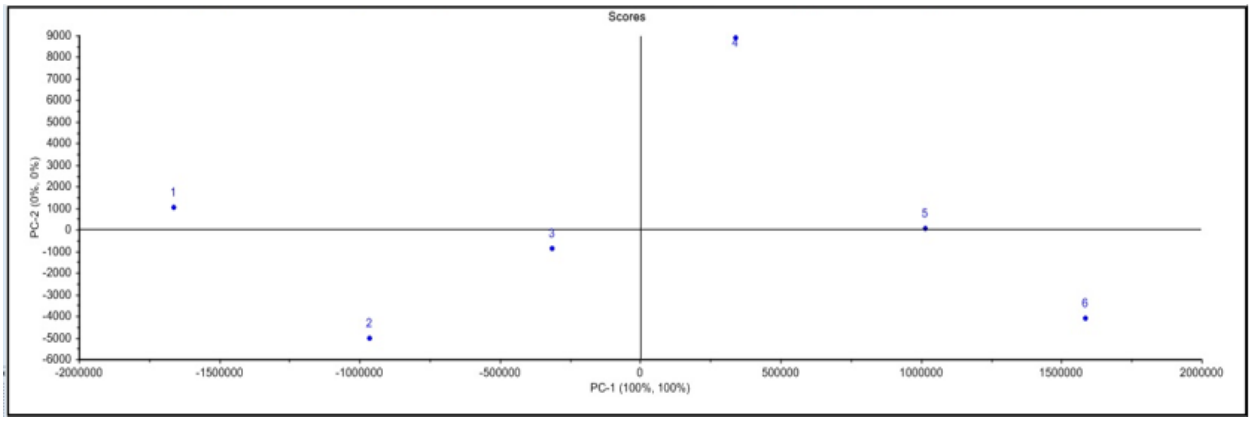

Fig 10 :PCALinearitySpectralData ofUnivestin and Collagen Type-II

PCA analysis produces several types of outputs which must be taken into account whendrawing conclusions. It gives some guidance on interpretation but it is not intended to be anexhaustive list (please note some of theoutputs mentioned will not be available in allsoftware packagesandthe terminologyalsovariesslightlyacrosspackages)

CONCLUSION

We present in this article simple, selective, validated and well-defined stability that shows isocratic RP-HPLC methodology for the quantitative determination of UC-II and Univestin. The evolved technique was observed to be accurate, precise, linear and reliable. The benefit comes from the ease with which the sample was prepared, as well as the use of less expensive reagents. The proposed HPLC conditions ensure adequate resolution and, as a result, accurate compound quantification. The precision and reproducibility data are satisfactory, according to the testing results. The developed chromatographic technique was widely used in drug testing for routine study.

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