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Development and Validation of an HPLC Method for Determining Prednisolone in Dietary Supplements

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Abstract :

The widespread use of dietary supplements for enhancing physical performance and general well-being has raised significant safety concerns due to the adulterating for some products with undeclared pharmaceutical substances. Prednisolone, a potent synthetic glucocorticoid, is among the most frequently detected illicit additives. This study aimed to develop and validate a high-performance liquid chromatography (HPLC) method for simultaneously quantitatively determining prednisolone and other pharmacologically active substances in commercial dietary supplements.

International validation criteria validated the analytical method, including linearity, accuracy, and precision. Calibration curve for prednisolone demonstrated excellent linearity ($R^2 > 0.99$) over the tested concentration ranges. Accuracy, expressed as recovery percentage, ranged from 95.2% to 103.4%, and precision (RSD%) was below 1.1% for all analytes.

Application of the validated method to seventeen randomly selected dietary supplement products revealed the presence of prednisolone in nine samples, with concentrations ranging from 3.66 to 378.69 µg/ml. These findings indicate a potentially serious public health risk due to the unauthorized presence of corticosteroids and stimulants in non-compliant dietary supplements.

This study underscores the urgent need for stricter regulatory oversight and routine analytical screening of dietary supplements. The validated HPLC method provides a reliable and reproducible approach for detecting such adulteration.

Key words: HPLC, Prednisolone, dietary supplements, pharmaceutical adulteration, corticosteroids, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), regulatory oversight, quantitative analysis, qualitative analysis, public health.

تطوير والتحقق من طريقة HPLC لتحديد البريدنيزولون في المكملات الغذائية

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مستخلص:

الاستخدام الواسع للمكملات الغذائية بهدف تعزيز الأداء البدني والرفاهية العامة قد أثار مخاوف كبيرة تتعلق بالسلامة، بسبب غش بعض المنتجات بمواد دوائية غير معلنة. يُعد البريدنيزولون، وهو غلوكوكورتيكويد صناعي قوي، من أكثر المواد المضافة غير القانونية التي يتم اكتشافها بشكل متكرر. هدفَت هذه الدراسة إلى تطوير طريقة كروماتوغرافيا السائل عالية الأداء (HPLC) والتحقق من صحتها من أجل التحديد الكمي المتزامن للبريدنيزولون وغيره من المواد الفعالة دوائيًا في المكملات الغذائية التجارية.

تم التحقق من صحة الطريقة التحليلية وفقاً للمعايير الدولية للتحقق، بما في ذلك الخطية، والدقة، والتكرارية. أظهرت منحنيات المعايرة للبريدنيزولون خطية ممتازة ($R^2 > 0.99$) ضمن نطاقات التركيز المختبرة. تراوحت الدقة، والمُعبر عنها بنسبة الاسترداد، بين ٪95.2 و٪103.4، وكانت التكرارية (RSD%) أقل من ٪1.1 لجميع المواد المحللة.

أظهر تطبيق الطريقة المُتحقق منها على سبعة عشر منتجًا من المكملات الغذائية تم اختيارها عشوائيًا وجود البريدنيزولون في تسعة عينات، بتراكيز تراوحت بين 3.66 إلى 378.69 ميكروغرام/ مل. تشير هذه النتائج إلى خطر صحي عام محتمل وخطير ناتج عن وجود الكورتيكوستيرويدات والمنشطات غير المصرح بها في المكملات الغذائية غير المتوافقة.

تبرز هذه الدراسة الحاجة الملحة إلى رقابة تنظيمية أكثر صرامة وفحوصات تحليلية دورية للمكملات الغذائية. توفر طريقة HPLC المُتحقق منها نهجًا موثوقًا وقابلاً لإعادة التكرار للكشف عن هذا النوع من الغش.

الكلمات المفتاحية: كروماتوغرافيا السائل عالية الأداء (HPLC)، بريدنيزولون، المكملات الغذائية، الغش الدوائي، الكورتيكوستيرويدات، الدقة، التكرارية، حد الكشف (LOD)، حد التقدير (LOQ)، الرقابة التنظيمية، التحليل الكمي، التحليل النوعي، الصحة العامة.

1- Introduction

Nutritional supplements are extensively used for their recognized benefits in supporting physical and cognitive performance. However, the increasing incidence of adulteration in these products through the addition of banned synthetic pharmaceutical substances such as corticosteroids has become a growing concern for both regulatory and health authorities. Among these compounds, prednisolone, a potent synthetic glucocorticoid, is one of the most frequently reported adulterants in nutritional supplements(1).

The unauthorized inclusion of prednisolone poses a serious health risk to consumers, as its uncontrolled use is associated with potentially serious side effects, including suppression of the immune system, increased risk of osteoporosis, and metabolic disorders. *These concerns highlight the urgent need* to develop accurate and reliable analytical mechanisms to monitor its presence in products available on the market(2).

High-performance liquid chromatography (HPLC) is a widely recognized pharmaceutical and food control technique thanks to its high resolution, excellent sensitivity, and ability to separate and identify trace amounts of compounds within complex matrices(3). Several methods have been documented using HPLC to determine prednisolone in various samples, such as pharmaceuticals and biological fluids. Still, the analysis of dietary supplements remains a challenge due to their multiple components and varying composition, which may lead to analytical interferences that affect the accuracy of the results(4).

This study aims to develop a quantitative analytical method using HPLC to determine prednisolone in a selection of commercially available dietary supplements, *evaluated based on international validation criteria* of linearity, precision, repeatability, limits of detection, and quantification(5). The study also seeks to evaluate the extent of prednisolone adulteration in the market, contributing to strengthening control systems and ensuring the safety of consumer products(6). The results of this work represent an important addition to ongoing monitoring and surveil-

lance efforts aimed at protecting public health and promoting transparency in the dietary supplement market(7).

2- Materials and Instruments

This section describes the chemical reagents, analytical instruments, and sample materials employed for detecting and quantifying prednisolone and related compounds in dietary supplements using high-performance liquid chromatography (HPLC).

2-1 Chemical Materials

All reagents used in this study were of analytical grade. These included methanol and deionized water (DI-H_2O). Standard solutions of prednisolone prepared at a concentration of 1000 µg/ml. Details are provided in Table 1.

Table 1: Chemical Materials and Suppliers

Item	Material Name	Chemical Formula	Purity or concentration	Supplier
1	Deionized Water	DI-H2O	-	Sigma
2	Methanol	CH3OH	99.9%	Sigma

3- Instruments

Analytical measurements were conducted using high-precision laboratory instruments listed in Table 2, including a high-performance liquid chromatograph (Agilent 1260 Infinity II, USA) and an atomic absorption spectrophotometer (PerkinElmer AAnalyst 400, USA). Analytical procedures were conducted using a High-Performance Liquid Chromatography (HPLC) system (Agilent 1260 Infinity II, USA) with a UV absorbance detector.

The following HPLC columns were employed: For corticosteroids and caffeine: Agilent SB-CN column (250 mm × 4.6 mm, 5 µm). For ephedrine: Thermo Scientific C18 column (250 mm × 4.6 mm, 5 µm).

Table 2: Instruments Used

Item	Instrument Name	Model and Origin
1	Electronic Balance	Sartorius Entris 224i, Germany
2	Magnetic Stirrer	IKA C-MAG HS 7, Germany
3	Drying Oven	Memmert UN55, Germany
4	High-Performance Liquid Chromatograph	Agilent 1260 Infinity II, USA

4- Samples

prednisolone was procured from certified sources with purity levels ranging from 99% to 100%. These are summarized in Table 3.

Table 3: Organic Materials for Determination

Item	Material Name	Chemical Formula	Purity
1	Prednisolone	$C_{21}H_{28}O_5$	99.7%

4-1 Sample Collection

Seventeen dietary supplement products were randomly purchased from local markets and fitness centres. The samples varied in form (protein powders, capsules, mass gainers), origin (USA, UK, Poland, Iraq), and packaging (plastic containers and nylon bags). Detailed information was recorded, including product name, manufacturer, country of origin, and packaging type.

5- Preparation of Tools and Glassware

All glassware and laboratory tools were thoroughly cleaned with tap water, rinsed with distilled and deionized water, and dried in a laboratory oven before use.

5-1 Preparation of Standard Solutions

The target analytes ' stock standard solutions (1000 µg/mL) were prepared by accurately weighing 1.0 g of each pure compound and dissolving it in

Table 4: Dietary Supplement Samples Analyzed

Code	Product Name	Manufacturing Company	Country of Origin	Packaging Type
A	Test + GH	BioTech	USA	Plastic Container
B	MASS Protein	Applied Nutrition	UK	Plastic Container
C	ISO Protein	MBN	Poland	Plastic Container
D	ISO Protein	Bar Mass	USA	Plastic Container
E	Gold lean Mass	Kevin Levrone	USA	Nylon Bag
F	ISO Protein	Black	USA	Plastic Container
G	MASS Protein	ANS	USA	Nylon Bag
H	ISO Protein	Kevin Levrone	USA	Plastic Container
I	ISO + Whey + hydro Protein	Fitness Authority (FA)	Poland	Plastic Container
J	Whey Protein	Fitness Authority (FA)	Poland	Plastic Container
K	REDOCN1	Muscle Pharm	USA	Plastic Container
L	Whey Protein	Kevin Levrone	USA	Plastic Container
M	Pure Whey ISO	YAVA LABS	UK	Plastic Container
N	BCAA	Applied Nutrition	UK	Plastic Container
O	Weight gain supplement COLOMBIA	COLOMBIA	Iraq	Capsules in Plastic Container
P	Weight gain supplement Filler	Abu Zahraa	Iraq	Capsules in Plastic Container
R	Weight gain supplement PUMA	PUMA	Iraq	Capsules in Plastic Container

methanol in 100 mL volumetric flasks. These were further diluted to the required calibration levels for analysis.

5-2 Sample Preparation for HPLC Analysis

Approximately 1 gram of each dietary supplement sample was weighed and dissolved in 40 mL of methanol using a mechanical shaker for 1 hour. The mixture was left to stand for 5 minutes, shaken again, then subjected to gentle heating (not exceeding 50°C). The solution was filtered and evaporated to dryness at room temperature. The residue was reconstituted in 3 mL of methanol and filtered before injection into the HPLC system.

5-3 Chromatographic conditions(8–10)

For Prednisolone and Related Com-

pounds:

Column: Agilent SB-CN (250 mm × 4.6 mm, 5 µm)

Mobile phase: Methanol: Water (1:1, v/v)

Flow rate: 1.0 mL/min

Injection volume: 24 µL

Detection wavelength: 250 nm

Run time: 15 minutes

All chromatographic analyses were performed at ambient room temperature.

6- Results

The analysis of dietary supplements for the presence and quantification of prednisolone and other related substances was performed using High-Performance Liquid Chromatography (HPLC) under optimized conditions. The method's performance was evaluated by constructing calibration curves and determining linearity, accuracy, and precision.

7- Calibration Curves and Linearity

Calibration curves were constructed for prednisolone by plotting the corresponding standard concentrations against the peak areas. The method's linearity was assessed based on the correlation coefficient (R^2) values obtained from the linear regression analysis of the calibration curves(11). The results, including the linear equations and R^2 values for each compound, are summarized in Table 5.

The high R^2 indicate excellent linearity of the method within the tested concentration ranges for all analyzed compounds, demonstrating a strong correlation between peak area and concentration.

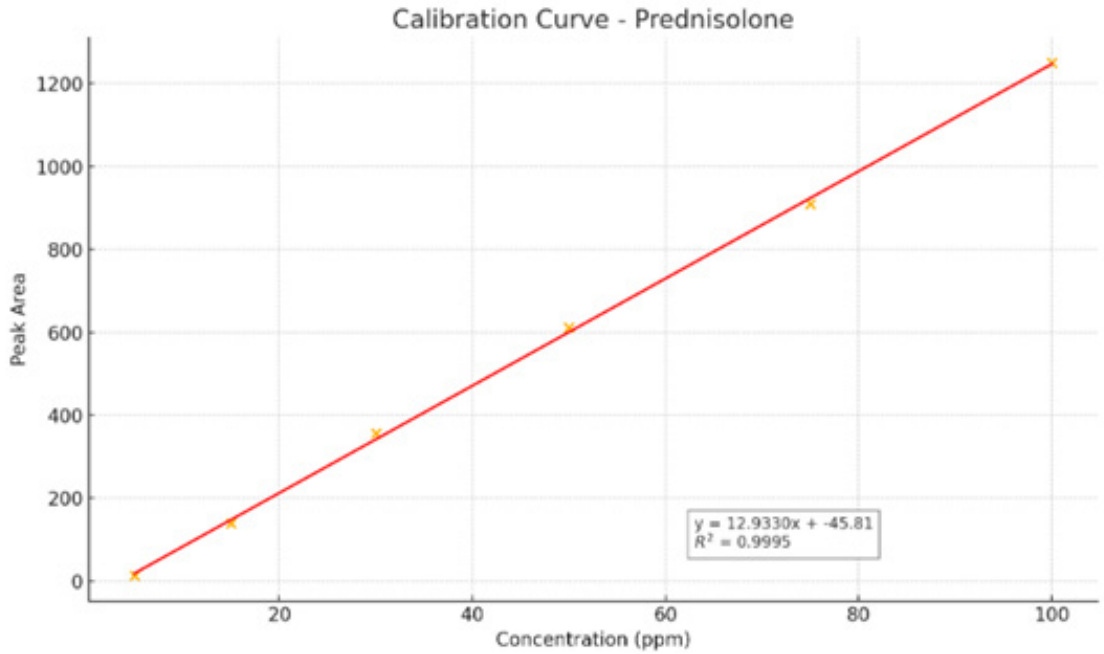


Fig.1 Calibration Curves of Prednisolone

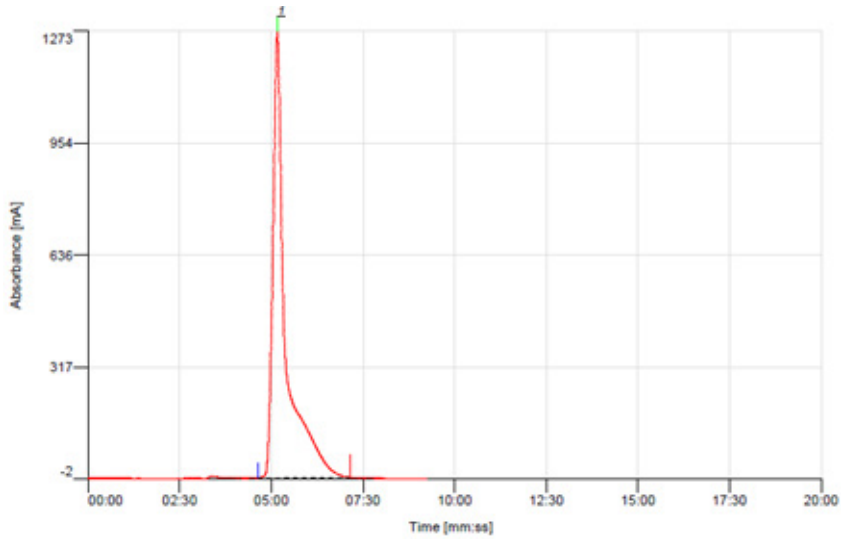
Table 5: Summary of Calibration Curve Parameters for Analyzed Compounds

Compound	Linear Equation	R^2 Value	Slope	Intercept	Linearity range µg/mL
Prednisolone	Y=12.9330x - 45.81	0.9995	12.9330	45.81	5-100

Calibration results for prednisolone demonstrated excellent linearity over the tested range ($R^2 = 0.9995$), as summarized in Table 5.

To visually confirm the retention time and chromatographic performance of the method, a standard solution of prednisolone (5 µg/mL) was analyzed using HPLC under the specified conditions.

The chromatogram is shown in Figure 2



No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]	Area %
001	***	05:10.0	04:37.6	07:09.3	30847.5	1272.1	100.000

Fig.2 HPLC chromatogram of standard prednisolone solution

8- Accuracy and Precision

Method accuracy and precision were assessed by analyzing a spiked dietary supplement sample (Sample M) containing each analyte's known concentration (5 µg/mL). Accuracy was determined by calculating the recovery percentage (%REC), while precision was evaluated based on the relative standard deviation (%RSD) of ten replicate injections(12).

Prednisolone exhibited recovery values within the generally accepted 96.8% range, indicating high analytical accuracy. Moreover, the %RSD values were consistently below 2%, confirming the method's precision and reproducibility. Detailed results are summarized in Table 6 .

Table 6: Accuracy and Precision of the HPLC Method

Compound	Mean Measured concentration (µg/mL)	Known concentration (µg/mL)	RE%	REC (%)	RSD (%)
Prednisolone	4.84	5	-0.2	96.8	1.03

The recovery percentages for Prednisolone were within acceptable limits (typically 96.8% for analytical methods), indicating good method accuracy. The low RSD% values (all below 2%) demonstrate high precision and reproducibility of the measurements.

(Note: Figures illustrating the accuracy and relative error and SD and RSD% values would typically be included here. These figures would visually represent the method's performance in terms of accuracy and precision.)

9- Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined using the standard deviation (SD) of the response and the slope (S) of the calibration curve according to the following formulas:(13)

$$LOD = (3 \times SD) / S$$

$$LOQ = (10 \times SD) / S$$

Table 7 Representative results for selected analytes

Compound	Slope (S)	SD	LOD (µg/mL)	LOQ (µg/mL)
Prednisolone	0.0773	0.06	0.02	0.0066

These values confirm that the developed HPLC method is sufficiently sensitive to detect trace amounts of the target analytes in complex matrices such as dietary supplements.

10- Determination of Prednisolone and Other Substances in Dietary Supplements

The developed and validated HPLC method was applied to determine the presence and concentration of prednisolone in various dietary supplement samples. The concentrations of the detected compounds were calculated using the respective calibration curve equations and the peak areas obtained from the analysis of each sample(14).

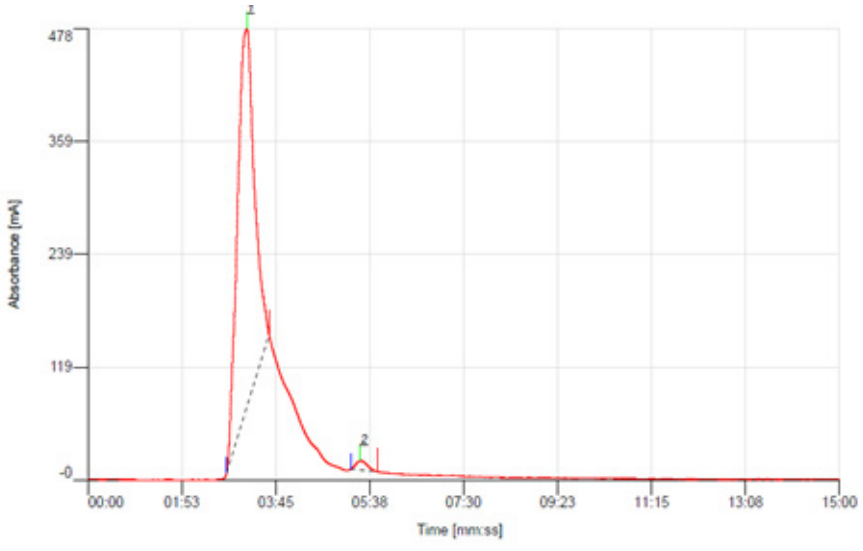
The calculated concentrations for prednisolone in the analyzed samples are presented in Table 8.

Table 8: Calculated Prednisolone concentrations in Dietary Supplement Samples

Sample Code	Sample Name	Peak Area (mAs)	Calculated concentration (µg/mL)
A	Test + GH	157.1	15.70903
C	ISO Protein	184.6	17.83478
D	ISO Protein	6.5	4.06765
F	ISO Protein	70.0	8.9762
H	ISO Protein	30.6	5.9305
J	Whey Protein	45.2	7.05916
N	BCAA	1.3	3.66569
O	Weight gain supplement COLOMBIA	4852.9	378.69437
P	Weight gain supplement Filler	360.0	31.3932
R	Weight gain supplement PUMA	406.2	34.96446

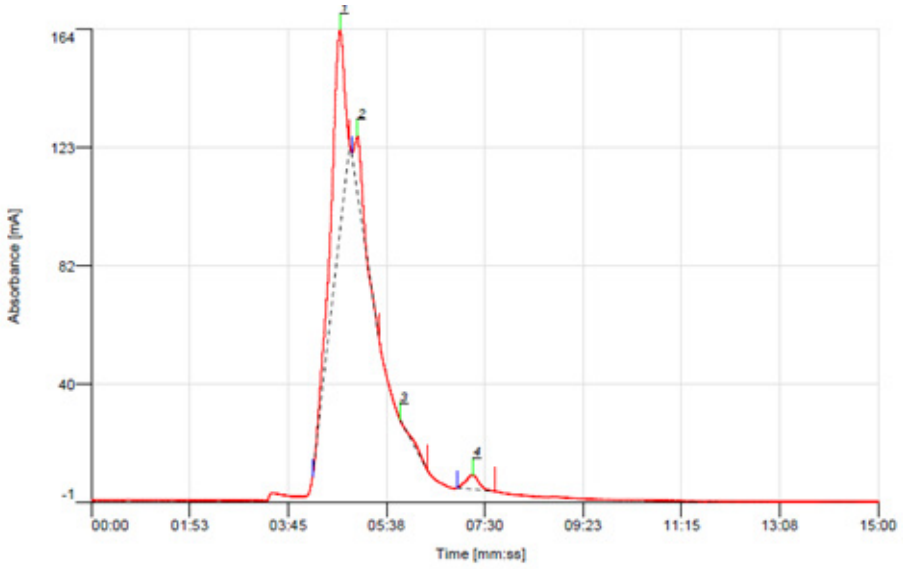
The validated HPLC method was applied to seventeen dietary supplement samples. Prednisolone was detected in multiple samples with varying concentrations, as shown in Table 8.

Representative chromatograms of selected positive samples are presented below to illustrate the presence of prednisolone at a retention time of approximately 5.10 minutes



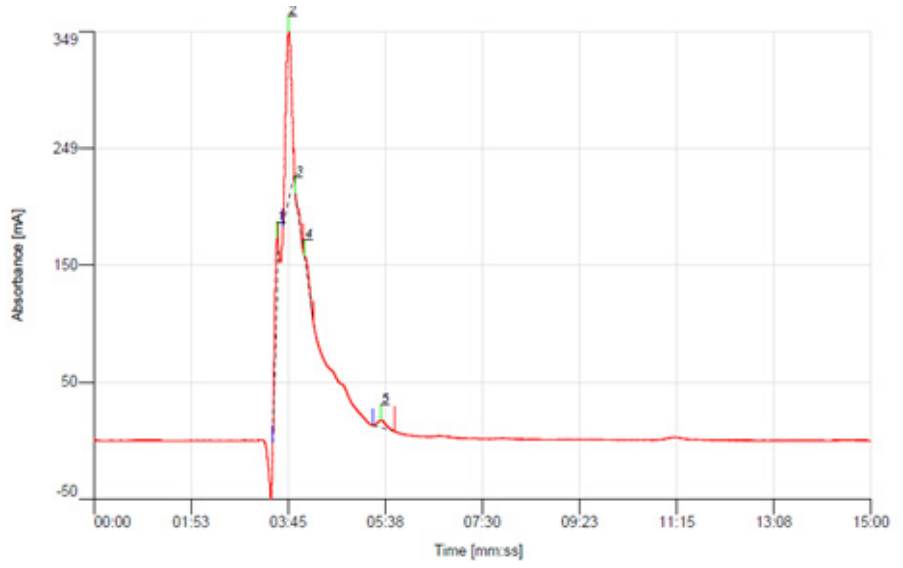
No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]	Area %
001	* * *	03:10.7	02:46.1	03:37.1	9576.3	400.4	98.386
002	prednisolone	05:26.4	05:14.8	05:47.4	157.1	10.0	1.614

Fig.3 HPLC chromatogram of sample A



No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]	Area %
001	* * *	04:44.6	04:12.9	04:55.3	1196.1	69.6	80.552
002	prednisolone	05:04.3	04:57.4	05:28.9	184.6	20.0	12.432
003	Dexamethazon	05:52.8	05:52.8	06:24.3	19.9	0.0	1.338
004	Triamcinolone	07:16.4	06:58.0	07:41.4	84.3	5.0	5.678

Fig.4 HPLC chromatogram of sample C



No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]	Area %
001	* * *	03:32.3	03:27.4	03:33.9	236.5	47.9	16.229
002	Caffein	03:45.8	03:39.3	03:52.3	1096.3	143.8	75.229
003	* * *	03:53.4	03:53.4	04:02.1	16.5	0.0	1.130
004	* * *	04:04.3	04:04.3	04:15.1	38.0	0.0	2.609
005	prednisolone	05:33.2	05:23.5	05:48.5	70.0	6.2	4.804

Fig.5 HPLC chromatogram of sample F

11- Discussion

The results obtained from the HPLC analysis provide valuable insights into the presence and concentration of prednisolone and several other pharmacologically active substances in the analyzed dietary supplement samples. The developed and validated HPLC method demonstrated good linearity, accuracy, and precision for the simultaneous determination of these compounds, as evidenced by the high correlation coefficients (R^2) of the calibration curves, acceptable recovery percentages, and low relative standard deviations (RSD%) from the method validation study.

The accuracy and precision results further support the suitability of the developed HPLC method for the intended purpose. The recovery percentages, generally within the acceptable range of 90-110%, suggest that the method can accurately quantify the target compounds in the matrix of dietary supplements. The low RSD% values indicate that the method is reproducible and reliable, providing consistent results upon repeated analysis.

The application of the validated method to the dietary supplement samples revealed the presence of several of the targeted compounds in varying concentrations. Specifically, prednisolone was detected in multiple samples. The presence of prednisolone, a potent corticosteroid, in dietary supplements is a significant finding, as it is a prescription medication with potential side effects, and its undeclared presence poses serious health risks to consumers, especially those with underlying health conditions or those who may be taking other medications.

Detecting other pharmacologically active substances like caffeine and ephedrine in some samples is also noteworthy. While caffeine is a common ingredient in some supplements, its presence should be declared, and the concentration should be within safe limits. Ephedrine, a stimulant, has been associated with significant health risks and is banned in many countries for use in dietary supplements. Such undeclared or potentially harmful substances underscore the importance of stringent quality control and regulatory oversight of the dietary supplement

industry.

Comparing these findings with existing literature on the analysis of dietary supplements for undeclared substances is crucial. Numerous studies have reported the adulteration of dietary supplements with various pharmaceutical ingredients, including corticosteroids, stimulants, and weight-loss drugs. These studies often highlight the challenges in detecting such adulterants due to variations in concentration, masking agents, and the complexity of supplement matrices. The HPLC method developed in this study demonstrates its capability to detect and quantify some of these substances, contributing to the analytical tools available for monitoring supplement quality and safety.

The presence of high concentrations of prednisolone in some samples, as calculated in this study, is particularly concerning. Prednisolone is typically prescribed at much lower doses, and its unsupervised use can lead to severe side effects, including immune system suppression, adrenal insufficiency, osteoporosis, and psychological disturbances. The levels detected in some samples could lead to significant health issues for consumers. This finding emphasizes the critical need for regulatory bodies to actively monitor dietary supplements for the presence of potent pharmaceuticals and take action against products found to be adulterated.

Furthermore, the study's findings highlight the importance of robust analytical methods for accurately determining active compounds and potential adulterants in dietary supplements. With its versatility and sensitivity, HPLC remains a cornerstone technique for such analysis. The method described in this research, with its validated parameters, provides a reliable approach for simultaneously determining prednisolone and other related substances.

Future research could expand the analysis scope to include a wider range of potential adulterants commonly found in dietary supplements. Additionally, investigating the source of contamination or adulteration in the supply chain of these supplements would be valuable in preventing their distribution.

12- Conclusion

This study successfully developed and validated an HPLC method for determining prednisolone and other related substances in dietary supplements. The method demonstrated excellent linearity, accuracy, and precision, making it suitable for quantitatively analyzing these compounds in complex matrices. The application of this method to a selection of dietary supplement samples revealed the presence of prednisolone in several products. This finding is of significant concern due to the potential health risks associated with the undeclared consumption of corticosteroids. The study also detected other pharmacologically active substances like caffeine and ephedrine in some samples, further highlighting the issue of adulteration in the dietary supplement market. The results underscore the critical need for rigorous quality control and regulatory monitoring of dietary supplements to ensure consumer safety and prevent the distribution of products containing undeclared and potentially harmful pharmaceutical ingredients. Future research should broaden the scope of analysis to detect a wider range of adulterants and investigate the sources of contamination within the supply chain.

The primary objective of this study is to develop and validate a reliable and sensitive High-Performance Liquid Chromatography (HPLC) method for detecting and quantifying prednisolone and other pharmacologically active compounds in commercially available dietary supplements. This analytical approach is designed to meet international validation criteria, including linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ), to ensure its applicability in real-world regulatory and quality control settings.

This study is significant because it contributes to public health protection by addressing the growing concern of dietary supplement adulteration with undeclared corticosteroids and stimulants. Prednisolone, a potent synthetic glucocorticoid, is frequently added illicitly to enhance the physiological effects of these products, posing serious health risks to unsuspecting consumers. By providing a validated and re-

producible method for detecting such adulterants, this study supports regulatory agencies and quality assurance laboratories in their efforts to monitor, evaluate, and enforce safety standards in the dietary supplement industry.

Moreover, this research adds to the scientific literature by offering quantitative data on the prevalence and concentration of prednisolone in locally and internationally marketed supplements, highlighting the urgent need for routine screening and stricter regulatory oversight.

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