Simultaneous Determination of Piroxicam and Codeine Phosphate Hemihydrate in a Pharmaceutical Dosage Form Using Validated HPLC Method

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Abstract
An easy, eclectic, precise high-Performance Liquid Chromatographic (HPLC) procedure was evolved and validated to estimate of Piroxicam and Codeine phosphate. Chromatographic demarcation was accomplished on a C18 column [Use BDS Hypersil C18, 5µ, 150 x 4.6 mm] using a mobile phase of methanol:phosphate buffer (60:40, v/v, pH=2.3), the flow rate was 1.1 mL/min, UV detection was at 214 nm. System Suitability tests (SSTs) are typically performed to assess the suitability and effectiveness of the entire chromatography system. The retention time for Piroxicam was found to be 3.95 minutes and 1.46 minutes for Codeine phosphate. The evolved method was validated through precision, limit of quantitation, specificity, limit of detection linearity and accuracy. (LOD) was 1.92 µg/mL and (LOQ) was 6.336 µg/mL for Piroxicam, whereas (LOD) for Codeine phosphate was 0.29 µg/mL and (LOQ) was 0.95 µg/mL. Piroxicam and Codeine phosphate showed a linear signal in the domain of 5-50 µg/mL for each compound. This research presided to evolve and validate an HPLC method, and the proposed procedure can be used to estimate these drugs in their combined dosage forms.

Keywords: Codeine Phosphate, HPLC, Piroxicam, System Suitability Test, Validation.

Introduction:
Piroxicam (Fig. 1) is a drug classified as a non-steroidal anti-inflammatory drug (NSAID) having an antipyretic and analgesic characteristic. It is used to treat rheumatic diseases such as inflammation, pain from injury, menstrual cramps, arthritis, musculoskeletal disorders, and non-rheumatic diseases such as biliary and ureteral colic, dysmenorrhea inflammation and fever 1. Piroxicam has an oxime group and N-heterocyclic carboxamide. Piroxicam has an IUPAC name (4-Hydroxy-2-methyl-N-(2-pyridinyl)-2H,1,2-benzothiazine-3-carboxamide 1,1-dioxide), it is white to light yellow, crystalline powder with a molecular weight of 331.35 g/mol and practically insoluble in water 2,3. Codeine Phosphate (Fig. 2) is an opioid derived from the immature poppy seed plant (Papaver somniferum). It has a phenolic hydroxyl group with a methyl substitution and has a morphine-like structure. The chemical description of the phosphate salt form is 7,8-Didehydro-4,5 alpha-epoxy-3-methoxy-17-methylmorphinan-6 alpha-ol phosphate hemihydrate and the molecular form is C18H20NO5·P·½H2O. Codeine has one chiral center. Codeine has a molecular weight of 406.4 g/mol 4.

Figure 1. Structure of Piroxicam

Figure 2. Structure of Codeine phosphate
Literature reviews do not reveal any analytical techniques for the determination of Piroxicam and Codeine Phosphate as a combination in Pharmaceutical Dosage Forms, but they reported some analytical methods to estimate Piroxicam that include: spectrophotometric method \(^5\text{–}\)\(^8\), high-performance liquid chromatographic method \(^5\text{–}\)\(^12\) and capillary electrophoresis \(^13\). On the other hand, literature reviews reported several analytical methods to estimate Codeine Phosphate\(^14\) individually or in combination with other active pharmaceutical ingredients in different pharmaceutical dosage forms. The fixed-dose presence of piroxicam and Codeine Phosphate is not indexed in any of the official pharmacopoeias. So, in the current research, we have approached a novel validated easy HPLC method and credible for the sake of simultaneous estimation of Piroxicam and Codeine Phosphate in their formulated tablets.

**Material and Methods:**

**Instrumentation and Chemicals**

A Shimadzu Prominence SPD-20A HPLC PDA, Japan and an auto sampler (Shimadzu, Japan, model SIL-10AD) and a model SPD-20AV UV-VIS detector were used for chromatographic measurements. Working standards Piroxicam (99.5\% w/w) and Codeine Phosphate (99.3\% w/w) were kindly gifted by UNIPHARMA and ULTAMEDICA respectively (a Syrian pharmaceutical companies in the Damascus countryside, Syria). Fixed-dose combination tablets that contain 10 mg of Piroxicam and 10 mg of Codeine Phosphate Hemihydrate were formulated in the Industrial Pharmacy Department, Faculty of Pharmacy, Damascus University, Syria. Disodium hydrogen phosphate, monosodium dihydrogen phosphate, orthophosphoric acid, potassium dihydrogen phosphate and triethylamine (analytical grades) were gifted by MEDICO labs (a Syrian pharmaceutical company in the Homs countryside, Syria). Methanol (HPLC grade) were purchased from J.T. Baker (USA).

**Preparation of Standard Stock and Working Solutions**

Preparation of methanolic hydrochloric acid 0.01 N: It is prepared by diluting 0.9 mL of hydrochloric acid with methanol to a volume of 1 L.\(^15\).

Preparation of phosphate buffer solution pH=6.8: Dissolving 8.722 g of disodium hydrogen phosphate (Na\(_2\)HPO\(_4\).2H\(_2\)O) and 7.038 g of monosodium dihydrogen phosphate (NaH\(_2\)PO\(_4\).2H\(_2\)O) in sufficient water to produce 1000 mL pH adjusted to 6.8 with ortho-phosphoric acid.

Preparation of potassium phosphate buffer solution pH=2.3: Dissolving 2.04 g of potassium dihydrogen phosphate (KH\(_2\)PO\(_4\)) in sufficient water to produce 1000 mL, then add 2 mL of triethylamine (TEA). pH adjusted to 2.3 with ortho-phosphoric acid.\(^16\)

Preparation of Standard Solutions: Piroxicam stock solution (0.1 mg/mL) and Codeine phosphate stock solution (0.1 mg/mL) were prepared in methanolic hydrochloric acid 0.01 N. Mixed standard stock solution containing 50 µg/mL of Piroxicam and 50 µg/mL of Codeine phosphate was prepared by diluting the standard stock solution in methanolic hydrochloric acid 0.01 N.

**Analytical Conditions of HPLC Method**

The HPLC system (Shimadzu Prominence HPLC, Japan) consisted of a Pump that was set to inject 20 µl per injection at once. The detector (SPD-20A PDA) consisted of UV/ VIS in the one hundred ninety to seven-hundred nm range which can be used for all UV analyses. BDS Hypersil C\(_18\) analytical column (150 × 4.6 mm, 5µ) (stationary phase) was utilized for LC separations. The mobile phase contained methanol: phosphate buffer pH=2.3 (6:4, v/v), and ortho-phosphoric acid was used to adjust pH to 2.3. Degassing then filtering procedures were performed on the mobile phase before using it. The pump was set at a flowing rate of 1.1mL/min. All measurements were accomplished at ambient temperature with a detection wavelength of 214 nm.

**Validation of the HPLC Method**

System Suitability test should be performed before starting validation of the HPLC method where the standard solutions of the two drugs are injected sundry times and the relative standard deviation is calculated, which should be less than \(\%2\) at all.

**System Suitability Test (SST)**

SST is particularly fulfilled to decide the column efficiency, resolution, and repeatability of a specific chromatographic system to affirm its ability for a described evaluation. SST is an important characteristic of all HPLC analytical systems. When the system suitability for the evaluation method uses the high-performance liquid chromatography, the subsequent parameters are had to be in the appropriate outlines\(^17\):

**Retention Time (Rt):** It is the interval time between the injection of the sample into the tool and the advent of the maximal peak at the detector.\(^18\text{,}\)\(^19\)
Capacity Factor: It expresses the overlap of the injected sample material with the column filling and the mobile phase and has to be more than 2.\textsuperscript{18,19}

Resolution: It demonstrates the dissociation power of the whole chromatographic system to the specific compounds of the commixture. It is expressed as the proportion of the range between peaks to the peak width value. If R is identical to or greater than one then components are absolutely separated. If R is much less than one, then components are overlapped.\textsuperscript{18}

Theoretical plates: The quantity of theoretical plates is a degree of the overall performance of the column and has to be more than 2000.\textsuperscript{17}

The HPLC procedure was established with appreciation to the subsequent Parameters according to the ICH guidelines.\textsuperscript{20}

Linearity: The capacity of the analytical method to offer measurement outcomes immediately proportional to the concentration of the analyzed material within the sample in the given range, either immediately or after performing precise mathematical transformations. The linearity of a standard series preparation within the range of 5-50 \( \mu \text{g/mL} \) has been determined. Linear standardization curves had been generated with the aid of using plotting the peak area in opposition to the concentration of the drug.\textsuperscript{21,22}

Accuracy: The HPLC procedure accuracy was carried out inside the procedure range, as follows: three weights of every drug was prepared to give concentration (10, 25 and 45) \( \mu \text{g/mL} \). The percentage for every sample and the relative standard (RSD) was Calculated. The relative standard value should not be greater than 2\%\textsuperscript{21}

Precision: Studies of repeatability and intermediate precision were carried out to analyze the precision of the method. Repeatability studies had been done by evaluation of three different concentrations of (10, 25 and 45) \( \mu \text{g/mL} \) for every drug by HPLC. Procedure repeatability was performed from RSD\% values gained by duplicating the measure several times around the same time for intra-day accuracy. The intermediate (inter-day) precision of the procedure was tested by a seeming similar framework on various days under similar trial conditions.

Specificity: It was prescribed by injecting solution of the excipients which has the selfsame concentration of the tablet solution.\textsuperscript{21,22}

Limit of Detection (LOD): It means the lowest quantity of the two drugs that detected at 214 nm. It was calculated from the subsequent equation: LOD= 3.3 SD/m.

Limit of Quantification (LOQ): It is expressed by the lowest quantity of the two drugs that may be quantitatively marked with appropriate precision and accuracy.

LOQ and LOD had been calculated by the subsequent equations: LOD= 3.3 SD/m, LOQ= 10 SD/m, in which SD is the standard deviation of the intercept of the regression line, and m is the slope of the regression line.\textsuperscript{20,24}

Analysis of the Two Drugs in the Tablets (Assay): Five tablets of the prepared tablets (containing 10 mg Piroxicam and 10 mg Codeine phosphate) were weighted and crushed. A precise weightiness of the powder equals to 10 mg of Piroxicam and 10 mg of Codeine phosphate was transmitted into a 25 mL volumetric flask which contains 15 mL of methanol, ultrasonicated for 45 minutes and 25 mL of methanol were added to the mark. The proposed solution was passed through a 0.45 \( \mu \text{m} \) membrane filter. Suitable dilutions were made utilizing the mobile phase to make the final tablet solution with a concentration of 10 \( \mu \text{g/mL} \) for Piroxicam and 10 \( \mu \text{g/mL} \) for Codeine phosphate. Tablet solutions consequently was filtered and analyzed according to the proposed analytical method.

Results and Discussions

Validation of HPLC Method

System Suitability Test (SST)

The System suitability test was conducted by injecting six injections of resolution solution. The dissociation was carried out by setting the pump at a flowing rate of 1.1 mL/min. The retention times for Piroxicam and Codeine phosphate were 3.95 and 1.460 minutes, respectively (Fig.3). Acceptable retention time (Rt), theoretical plates, tailing factor and good resolution for Piroxicam and Codeine phosphate were obtained as shown in Table 1.
Figure 3. Chromatogram of the two drugs with Concentration 10 µg/mL of Piroxicam (Rt 3.955) and 10 µg/mL of Codeine phosphate (Rt 1.462)

Table 1. System Suitability studies

|                  | Theoretical plates | Resolution on Retention Time (min) | Tailing Factor | Drugs          |
|------------------|--------------------|------------------------------------|----------------|----------------|
| Piroxicam        | 2881974.65         | 8.649                              | 1.220          | 3.955          |
| Codeine phosphate| 2857275            | -                                  | 1.417          | 1.462          |

Linearity: The plotting drug concentrations against peak areas for each compound showed linear relationships. The curve equation was $y = bx + m$ with linear regression method to estimate drugs concentration. Piroxicam and Codeine phosphate showed a linear signal in the domain of 5-50 µg/mL for each compound. The corresponding linear regression equations were $y = 57128x + 18809$ and $(R^2)$ of 0.9999 for Piroxicam, $y = 54397x + 33635$ and $(R^2)$ of 0.9991 for Codeine phosphate (Fig. 4). A prime correlation existed between the peak areas and concentrations of Piroxicam and Codeine phosphate was presented in Table 2.

Figure 4. Calibration curve of Piroxicam and Codeine phosphate.
Table 2. Linear regression data for calibration curves.

| Parameters          | Codeine Phosphate | Piroxicam |
|---------------------|-------------------|-----------|
| Linearity range     | 5-50 µg/mL        | 5-50 µg/mL|
| Regression equation | y = 54397x + 18809| y = 57128x + 18809|
| Correlation coefficient $R^2$ | 0.9991 | $R^2 = 0.9999$ |
| Accuracy RSD%       | 0.48              | 0.29      |
| LOD ($µg/mL$)       | 6.336             |           |
| LOQ ($µg/mL$)       | 0.95              |           |

**Accuracy**: The accuracy test affirmed appropriate retrievals % with small (RSD%) against concentrations. The outcomes signify that the procedure is highly precise for simultaneous estimation of the mentioned drugs, as shown from the data in Table 3.

Table 3. Accuracy studies.

| Concentration added ($µg/mL$) | Concentration found ($µg/mL$) | Recovery % | Concentration added ($µg/mL$) | Concentration found ($µg/mL$) | Recovery % |
|------------------------------|------------------------------|------------|------------------------------|------------------------------|------------|
| 10                           | 9.93                         | 99.3%      | 10                           | 9.981                        | 99.8%      |
| 10                           | 9.92                         | 99.2%      | 10                           | 9.98                         | 99.8%      |
| 10                           | 9.91                         | 99.1%      | 10                           | 9.93                         | 99.3%      |
| 25                           | 24.54                        | 98.1%      | 25                           | 25.04                        | 100%       |
| 25                           | 24.78                        | 99.1%      | 25                           | 24.66                        | 98.6%      |
| 25                           | 24.85                        | 99.4%      | 25                           | 24.78                        | 99.0%      |
| 45                           | 44.7                         | 99.3%      | 45                           | 44.99                        | 99.9%      |
| 45                           | 44.66                        | 99.2%      | 45                           | 44.33                        | 98.5%      |
| 45                           | 24.99                        | 99.9%      | 45                           | 44.55                        | 99.9%      |
Precision: The results of the repeatability and intermediate precision experiments are shown in Tables 4 and 5. The proposed method was precise according to the RSD values which were less than 2%, respectively as advised by the international council for harmonization guidelines.

Table 4. Precision studies of Piroxicam.

| (n=5) | Intermediate precision | Between-day | Repeatability Within-day (n=5) |
|-------|------------------------|-------------|-------------------------------|
| SD    | R          | D | Me | Recovery % | Re | SD | R | D | Me | Recovery % |
| .419  | .416       | .35| 99. |  2 | .99. | | 0 | .72 | .710 | 27 | 2 | .99. |
| .79   | .78        | .88| 99. |  4 | .4443| | 0 | .443 | .76 | 24 | 2 | .99. |
| .3815 | .3817      | .05| 99. |  5 | .346 | | 0 | .345 | 768 | 35 | 2 | .99. |

Table 5. Precision studies of Codeine phosphate.

| (n=5) | Intermediate precision | Between-day | Repeatability Within-day (n=5) |
|-------|------------------------|-------------|-------------------------------|
| SD    | R          | D | Me | Recovery % | Re | SD | R | D | Me | Recovery % |
| .473  | .472       | .76| 99. | .4 | .99. | | 0 | .688 | .683 | 3 | 1 | .99. |
| .9    | 10         |    | 22 | 10 |    |
Specificity: The specificity was realized by completing the demarcation of Piroxicam and Codeine phosphate peaks in the existence of the tablet excipients (Fig. 6). No overlap was noticed due to any indeterminate excipients of the formulated tablet at the retention times of Piroxicam and Codeine phosphate. (The used excipients were sodium starch glycolate, Crosspovidone-XL and crosscarmellose sodium), (Fig. 5).

Limit of Detection (LOD) and Limit of Quantification (LOQ): (LOD) was 1.92 µg/mL and (LOQ) was 6.336 µg/mL for Piroxicam, whereas (LOD) for Codeine phosphate was 0.29 µg/mL and (LOQ) was 0.95 µg/mL. Table 2.

Analysis of the Drugs in the Tablets (Assay):
Utilizing the proposed analytical procedure, assays of Piroxicam and Codeine phosphate in their tablets were accomplished. Satisfying outcomes were found for the two drugs in prime adjustment with the mentioned amounts proposing the appropriation of the procedure (Fig. 3). The retrieval % ± RSD of five duplicate outcomes were 99.41 ± 0.53 for Piroxicam, 100.3 ± 0.62 for Codeine phosphate. Table 6.

Figure 5. Chromatogram of the formulation that contains just excipients without drugs.
Table 6. Analysis of the formulation (Assay)

| Drugs          | Mentioned mg tablet⁻¹ | Drug content (n=5) ± SD | RSD (%) |
|----------------|-----------------------|-------------------------|---------|
| Piroxicam      | 10                    | 99.41 ± 0.66           | 0.53    |
| Codeine phosphate | 10               | 100.3 ± 0.71           | 0.62    |

Figure 6. Chromatogram of the formulation containing excipients and drugs

Conclusion
The proposed HPLC method demonstrates an easy, precise and reproductive method of quantitative analysis for concurrent estimation of Piroxicam and Codeine phosphate in bulk and tablet dosage forms. The HPLC method was validated according to ICH guidelines, and it was distinctive and there is no overlap from any of the sample compounds. It was deduced that the evolved procedure provided numerous profits such as speedy, slight-effective, easy mobile phase and making steps, innovated sensitivity and comparative low run time made it specific, dependable and effortlessly reproductive in any quality control set-up imparting all of the parameters are accompanied appropriately for its meant use.

Authors' Contributions Statement:
A M. AS contributed to the design and implementation of the research to the analysis of the results and to the writing of the manuscript. J A.H and AM.A L contributed to the revision and proofreading of the research.

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Authors' Declaration
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Besides, the Figures and images, which are not ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Damascus.
التعيين المتواطئ للبيروكسيكام والكودئين فوسفات ضمن شكل صيدلاني جرعي باستخدام كروماتوكرافيا العمود السائلة عالية الدقة

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الخلاصة:
تم تطوير وتوثيق طريقة تحليلية من أجل التعيين المتواطئ لمادة البيروكسيكام والكودئين فوسفات باستخدام كروماتوكرافيا العمود السائلة عالية الدقة (HPLC) حيث تحتوي هذه الطريقة على قدرة عالية في التدقيق والتأكيد. حيث قدرة سطوية تصل إلى 2.3 حمجة/جم ونسبة خسارة للالتحاص إلى 0.1%. بعد تطبيق القياس، وتم استخدام عينة صغيرة من المادة المعالجة في المحلول الدقيق للحصول على النتائج. وتم تحليل النتائج باستخدام برنامج HPLC وتم توثيق النتائج باستخدام نظام HPLC واللفتحات الدقيقة في الجراحة. وتم توضيح النتائج بناءً على النتائج المقدمة، حيث أن النتائج تظهر أن البترول والكودئين فوسفات كانتا 1.3 مكغ/مل و0.9 مكغ/مل، على التوالي. وتم تطبيق هذه الدراسة على تطوير طريقة تحليلية باستخدام كروماتوكرافيا، وتم استخدام عينة صغيرة مثل السائل إلى تركيزات ومقدرات هذه القائمة في الأشكال الجراحية التوزيعية الأبدام.