New Approach for Simultaneous Analysis of Commonly Used Antigout Drugs by HPLC/UV Detection; Application in Pharmaceutical and Biological Analysis

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Abstract

An innovative, simple, fast, specific and sensitive high performance liquid chromatographic method with Ultraviolet detection (HPLC/UV) was established and validated for simultaneous determination of a combination of three commonly prescribed antigout drugs namely; Colchicine (CLN), Probenecid (PRD) and Febuxostat (FBX) in dosage forms and in human urine samples. GraceSmart RP C_{18} column was utilized for separation of the studied mixture using isocratic mode of mobile system consisting of acetonitrile: water (55: 45, v/v; containing 0.5% v/v formic acid). Flow rate and detection wavelengths programming were designed to obtain rapid and efficient separation. The cited drugs were separated within less than 9 minutes. The retention times (t_R) were 3.597, 5.357 and 8.250 for CLN, PRD and FBX; respectively. The linearity range for all the investigated drugs was 0.040 – 50.0 µg/mL with detection limits of 1.94 - 6.48 ng/mL. Additionally, the developed method was validated according to ICH and US-FDA guidelines and was successfully applied for simultaneous estimation of the studied drugs in their pure form, and in laboratory-made tablets mixture. The proposed method was also used for analysis of CLN and PRD in their combined tablets and in human urine samples obtained from healthy volunteers with good recoveries (98.39- 101.87%). Furthermore, the stability of CLN-PRD mixture in urine samples was studied. This method is suitable for quality control purposes for simultaneous analysis of these co-administered antigout agents in their binary and ternary mixtures.

Keywords

Colchicine; Probenecid; Febuxostat; High Performance Liquid Chromatography; Tablets; Urine samples.

1. Introduction

Gout is a common rheumatic disease. During the last four decades, global studies have found an increase in mean serum uric acid which is the final product of the metabolism of endogenous and exogenous purine in man [1]. So, treatment of gout aims to alleviate the acute attack, prevent future attacks, and lower plasma-urate concentration. This can be achieved by using urate-lowering drugs. These drugs are classified into three main groups; xanthine oxidase inhibitors (XOIs), uricosurics, and recombinant uricases [2]. Febuxosat (FBX) is a non-purine selective XOI which was approved by the U.S. Food and Drug Administration (FDA) on 2009. FBX reduces uric acid production by blocking the molybdenum pterin center on the oxidized and reduced form of xanthine oxidase which is responsible for conversion of hypoxanthine and xanthine to uric acid [3]. Furthermore, Probenecid (PRD) is one of uricosuric agents that are considered the second-line therapy for management of gout which act by increasing renal urate excretion [4].

On the other hand, an attack of acute inflammatory gouty arthritis is best treated as soon as possible with a Non-Steroidal Anti-inflammatory Drug (NSAID). Aspirin or other salicylates are not suitable since they may increase plasma-urate concentrations. Colchicine (CLN) is an effective alternative for those unable to tolerate NSAIDs. It decreases swelling and lowers the uric acid crystals build up that cause pain in the affected joint [2].

All recent guidelines support the combination of XOIs, uricosurics and/or CLN when monotherapy is not effective [3-7]. Case *et al.* (2018) illustrated the synergistic effect of PRD that was added to FBX regimen for patients with refractory hyperuricaemia. It was found that the PRD-FBX combination successfully reduced uric acid to the target level especially in patients with chronic kidney disease [5].

Also, Yamanaka *et al.* (2018) recommended a stepwise dose increase in FBX plus low-dose CLN to give effective reduction of gout flares compared with FBX alone [6]. In addition, the combination of CLN and PRD is widely prescribed to prevent gouty arthritis in people who have frequent severe gout attacks [7].

The chemical structures of the studied drugs are given in (Figure 1).

Regarding literature, many methods have been reported for analysis of CLN, PRD and FBX alone or combined with other drugs; including liquid chromatography (HPLC) [8-18], gas chromatography [19-24], thin layer chromatography (TLC) [25-31], fluorimetric and spectrophotometric methods [32-39].

Until now, two methods (HPLC and HPTLC) were reported for simultaneous estimation of CLN-PRD combination in dosage forms [17, 28]. Both methods suffer from low sensitivity, complex mobile phases, and tedious extraction procedures with limited application. To the best of our knowledge, no method has been reported for analysis of CLN-PRD in biological fluids. Also, there is no reported method for quantitation of CLN-PRD-FBX mixture.

In the present study and for the first time, a rapid, specific and sensitive HPLC/UV method was established for simultaneous estimation of three commonly prescribed antigout drugs namely; Colchicine (CLN), Probenecid (PRD) and Febuxostat (FBX) in dosage forms and in human urine samples. The proposed method depends on using GraceSmart RP C_{18} column (250 x 4.6 mm, 5µm) and acetonitrile: water (55: 45, v/v; containing 0.5% v/v formic acid) as a mobile phase for separation of the studied mixture. Modeling flow- and detection wavelength- programming was created to ensure rapid and efficient separation of the cited drugs.

In this work, the detection (LOD) and quantitation (LOQ) limits (15.70, 5.88, 19.65 and 5.18, 1.94, 6.48 ng/mL for CLN, PRD and FBX; respectively) are lower than

those of other reported methods [8-16]. This sensitivity is convenient for the assessment of the targeted drugs in real urine samples with no need of tedious extraction steps. As shown in **Table 1**, the method's simplicity and sensitivity are superior to other reported HPLC methods where complex mobile phases were used with complicated pretreatment procedures.

2. Experimental

2.1. Apparatus

PerkinElmer Series 200 HPLC System (Model LCTURBO, Serial # ZAAA0646, PerkinElmer® Inc., USA) connected to series 200 degasser, pump and UV/VIS detector was used. A rheodyne injection valve (Model 7725i, USA) with a 20 µL loop and 100 µL Hamilton – Bonaduz, Schweiz (Switzerland) sample syringe were used for sample application. The HPLC system was operated with TotalChrom Workstation version 6.3.4 software. The separation was performed on GraceSmart RP C_{18} encapped column (250 mm length x 4.6 mm inner diameter, 5µm particle size, 120 Å pore size).

Thermo Scientific-Evolution 220 UV-VIS spectrophotometer with 1 cm quartz cuvettes (Waltham, Massachusetts, USA) was utilized for determination of corresponding maximum wavelengths of the studied drugs. Also, Analytical series Fisher-Scientific balance-PAS214C (China), ultrasonic cleaner- Kerry PUL 125 (England), laboratory centrifuge (Bremsen ECCO, Germany) and FALC vortex-MIX (FALC Instruments, Italy) were used. Jenway 350 pH meter (China) was utilized to check pH of the mobile phase. Furthermore, Nylon 66 membranes (0.45µm pore size, 47.0 mm diameter) and Whatman™ Nylon syringe filters (0.2 µm) were obtained from SUPELCO, USA and Fisher Scientific Co., UK; respectively.

2.2. Drugs and Solvents

CLN (≥95%), PRD (≥98%) and FBX (≥98%) were purchased from Sigma-Aldrich Co., UK. HPLC grade acetonitrile, methanol (99.9%) and 90 % formic acid were supplied by Fisher Scientific Co., UK. All other used chemicals and solvents were of analytical grade.

2.3. Dosage forms

Colchicine and Probecid® tablets (containing 0.5 mg CLN and 500 mg PRD per tablet; respectively) were purchased from the British local drug stores. Goutifade film-coated tablets (80 mg FBX per tablet, manufactured by Global Napi Pharmaceutical Company, Egypt) and Goutyless tablets (0.5 mg CLN + 500 mg PRD per tablet, October Pharma co., Egypt) were bought from the Egyptian local market.

2.4. Standard Solutions preparation.

Stock standard solutions of CLN, PRD and FBX (1.0 mg/mL) were prepared by dissolving 25.0 mg of each drug in 25.0 mL methanol. These stock solutions were kept in a refrigerator away from light.

To obtain the working standard solutions (0.040-50.0 μg/mL), the stock solutions were appropriately diluted with the mobile phase (acetonitrile: double distilled water, 55:45, v/v, containing 0.5% formic acid).

2.5. Analytical procedures.

2.5.1. General Chromatographic Procedure.

Twenty microliter of the working standard or sample solution of the investigated mixture was injected on PerkinElmer Series 200 HPLC System. The separation was performed on GraceSmart RP C_{18} column (250 mm length x 4.6 mm inner diameter, 5μm particle size, 120 Å pore size). Then, isocratic elution was carried out by mobile phase consisting of acetonitrile-double distilled water, 55:45, v/v, containing 0.5% formic acid (filtered by Nylon 66 membranes and degassed by sonication before use).

The total run time was 10 minutes. Flow rate and wavelengths were programmed via TotalChrom Workstation version 6.3.4 software.

Flow rate was firstly set at 1.0 mL/min for 7 min followed by 1.2 mL/min for the last 3 min. The UV/VIS detector was also programmed in parallel with the run time at three wavelengths corresponding to the studied drugs. The detector programming was set at 350 nm from 0- 4.5 min, 249 nm form 4.5 – 7.0 min and 320 nm from 7.0 – 10.0 min for CLN, PRD, and FBX; respectively.

2.5.2 Construction of Calibration Curves.

Working standard solutions of the CLN-PRD-FBX mixture equivalent to (0.040, 0.10, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/mL of each drug) were prepared in the mobile phase. Then, the assay was performed as mentioned under the general chromatographic procedure (section 2.5.1).

The peak area values (µV*Sec) of each drug were plotted against the drug final concentrations (µg/mL) to get the calibration graph. Thereafter, the corresponding regression equations were calculated.

2.5.3. Procedure for Tablets.

a) **For CLN-PRD-FBX ternary mixture;** Colchicine, Probecid® and Goutifade film-coated tablets were used to prepare laboratory-made mixture containing the three studied antigout drugs. These tablets were separately weighed, grinded and mixed. An accurate weight of the mixed powder of each pharmaceutical formulation, equivalent to 50.0 mg of each drug was transferred into a 50-mL calibrated flask. 25.0 mL of methanol was added and sonication was done for 15 min. Subsequently, completion to the mark with methanol and filtration through SUPELCO nylon membranes were performed to obtain 1.0 mg/mL of stock sample solution. Appropriate dilutions of the

filtrate solutions were made with the mobile system to obtain the working sample solutions. Then, the assay was completed as mentioned under the general procedure (section 2.5.1).

b) **For CLN-PRD binary mixture;** Ten Goutyless tablets (containing, 0.5 mg CLN plus 500 mg PRD per tablet) were weighed, grinded then mixed. An accurate weight of the mixed powder was dissolved in methanol, sonicated and filtered to prepare stock sample solution containing 1.0 and 1000.0 mg/mL of CLN and PRD; respectively. Thereafter, CLN-PRD stock sample solution was appropriately diluted by mobile phase to obtain working sample solutions of CLN and PRD in the ratio of 1:1000. After that, the general procedure (section 2.5.1) was followed.

2.5.4. Procedure for Spiked Urine Samples.

It was reported that in patients with normal kidney function, renal excretion accounts 10-20% and 77-88% of total CLN and PRD clearance; respectively [41-46]. So, the developed method was utilized for analysis of CLN-PRD binary mixture in urine samples in presence of FBX as an internal standard (IS).

In dry clean glass test tube, drug- free urine samples were collected from healthy volunteers and freshly used on the day of analysis. Into a series of tapered bottom centrifuge tubes, an aliquot of 2.0 mL blank human urine was spiked with 150 µL of 1.0 mg/mL of FBX which was used as an IS (IS final concentration = 30.0 µg/mL). 1.0 mL of the working standard solution of the studied CLN-PRD mixture was also added and followed by dilution to 5.0 mL with acetonitrile (to obtain final concentrations ranging from 0.040 to 50.0 µg/mL). The prepared spiked samples were vortexed and centrifuged at 4000 c/s for 30 sec and 5 min; respectively. After that, filtration of the spiked urine samples was carried out by the aid of (0.2 µm)

Whatman™ Nylon syringe filters. The obtained filtrates were directly injected on the HPLC system as mentioned before (section 2.5.1). A blank experiment of the drug-free urine sample was performed in the same manner excluding the drugs.

To construct calibration curves of CLN and PRD, peak area ratios of the studied drug to IS against different drug concentrations in urine samples were processed by linear least square regression analysis.

2.5.5. Procedure for Real Urine Samples.

Due to the developed method's sensitivity, the analysis of CLN-PRD combination in urine samples was achieved. Many terminal half-lives in healthy volunteers were mentioned (1-10.5 and 2-12 hour) for CLN and PRD; respectively. Furthermore, 40 – 65% of the total absorbed dose of CLN was recovered unchanged in urine after oral administration of 1.0 mg [42]. While, PRD was excreted in urine mainly as monoacyl glucuronide and intact drug [44].

Goutyless tablets (labeled to contain 0.5 mg CLN plus 500 mg PRD per tablet) were orally administered to three healthy human volunteers (32- years' old men, their average weight is 80 Kg). They did not take any medication for two week before the experiment. Urine samples were collected in glass bottles after 6.0 h posterior to receiving the medication. 2.0 mL of the collected urine sample was transferred into a tapered bottom centrifuge tube and spiked with 120.0 µL of 1.0 mg/mL of FBX (IS final concentration = 30.0 µg/mL) then completed to 2.0 mL with acetonitrile. This prepared sample was vortexed for 30 sec and centrifuged at 4000 c/s for 5 mins. Then, filtration step of the urine sample was done using (0.2 µm) Whatman™ Nylon syringe filters. 30 µL of the obtained clear solution was directly loaded on injection valve and 20 µL was injected on the HPLC system as aforementioned (section 2.5.1).

A blank experiment was established in the same way without the drugs.

2.6 CLN-PRD Mixture Stability.

2.6.1. Quality control (QC) samples preparation.

In accordance to US-FDA requirements for bioanalytical method validation [45], two QC samples at low and high concentrations (0.50 and 50.0 µg/mL) were prepared for study of CLN-PRD combination stability in urine.

Firstly, freshly prepared QC samples were analyzed immediately at zero time (baseline) to determine the accuracy and precision of the developed method. Furthermore, Short- and long-term stabilities were carried out on two QC concentrations (0.50 and 50.0 µg/mL).

For short-term stability study, standing on the bench-top for 4 hours at ambient temperature was required or storing the QC samples at −80º C for 7 weeks before their analysis for long-term studies. In addition, freeze-thaw stability was investigated by melting at room temperature subsequent to freezing (at −80º C for 24 hours) for three cycles.

All the above-mentioned samples were analyzed in triplicates, and the results were compared with those gained from the freshly prepared samples.

2.6.2. Stock solutions.

The two working standard solutions of CLN and PRD (0.5 and 50.0 µg/mL) were analyzed at baseline, subsequent to storage for 48 hours at room temperature, and after storage for seven weeks in deep freezer (-80º C).

2.7. Validation

As stated in ICH and US-FDA guidelines on the validation of analytical/bioanalytical methods, linearity, detection (LOD) and quantification (LOQ) limits, precision, accuracy, robustness, selectivity, specificity, stability and system suitability were evaluated [45,46].

2.7.1. Linearity.

Linearity was conducted by plotting either peak area of the cited drugs (CLN, PRD and FBX) or peak area ratio of CLN or PRD to IS in urine versus drug concentration (µg/mL). This study was performed on seven concentration levels and six calibration curves were constructed. After that, slopes, intercepts and correlation coefficients were calculated.

2.7.2. Detection and quantification limits (LOD and LOQ).

3 σ/S and 10 σ/S were used for calculation of LOD and LOQ of the studied drugs; respectively. Where, σ is the standard deviation of y-intercept of the regression equation and S is the slope of the calibration curve.

2.7.3. Accuracy.

Regarding evaluation of the developed method's accuracy, 0.5, 30.0, and 50.0 µg/mL were selected to cover the low, medium, and high concentration ranges of the calibration curves of the investigated drugs. By injecting each concentration in six replicates on the HPLC system, recovery percentages were derived.

2.7.4. Precision.

Triplicate analysis of low, medium and higher concentration solutions (0.5, 30.0, and 50.0 µg/mL) were used for investigation of intra-day precision of this method. This study was repeated for three successive days to determine the inter-day precision (n=9). Recoveries, standard deviation (SD) and Relative standard deviation (RSD) were computed.

2.7.5. Robustness.

The influence of minor changes of the optimal method parameters (including, flow rate, mobile system composition, analytical wavelengths, and formic acid concentration) on the method's performance was determined.

2.7.6. Selectivity.

The selectivity was tested by the analysis of the dosage forms, blank urine (free from the studied drugs), the spiked and real urine samples to exclude any interference from tablet excipients or endogenous urine components.

2.7.7. Stability.

Low and high concentrations QC samples (0.5 and 50.0 µg/mL) were utilized for evaluation of CLN-PRD mixture stability in human urine. Short- and long-term stabilities in addition to freeze-thaw studies were done. CLN-PRD stock solution stability was also investigated (at baseline, after storage for 48 hours at room temperature, and following seven weeks-storage in deep freezer).

Triplicates analysis was performed for each QC sample, and the obtained results were compared with those of the freshly prepared samples.

2. Results and Discussion

The main objective of this work is to establish and validate a rapid, highly sensitive and specific HPLC/UV for simultaneous estimation of CLN, PRD and FBX combination in dosage forms and in human urine samples.

3.1. Chromatographic Conditions Optimization.

For achievement of good resolution within short analysis time and symmetric peaks, detection wavelengths, mobile phase composition, flow rate and the system suitability were tested.

3.1.1. Analytical wavelengths

Thermo Scientific-Evolution 220 UV-VIS spectrophotometer with 1 cm quartz cuvettes was utilized for determination of maximum absorption wavelengths of the cited drugs. As shown in Figure 2, CLN has two maxima at 246 and 350 nm, 230 and 249 nm for PRD and 320 nm for FBX. As a result of the observed difference in these

measured wavelengths, wavelength programming was designed to achieve a higher sensitivity and specificity for quantitation of the studied drugs by the proposed method. Three wavelengths (350, 249 and 320 nm) were selected for the analysis of CLN, PRD and FBX; respectively. The detection was performed by using PerkinElmer Series 200 UV/VIS detector and programmed with TotalChrom Workstation version 6.3.4 software. The detector programming was set at 350 nm from 0- 4.5 min, 249 nm for 2.5 min and 320 nm for the last 3 min of the total run time (10 min).

3.1.2. Mobile phase

Several trials were performed to select the optimum mobile system for the separation of CLN-PRD-FBX ternary mixture. Acetonitrile, ethanol, and methanol were tested as organic solvents. It was found that good resolution, high peak areas and sharp peaks obtained when acetonitrile used as organic modifier. While, methanol and ethanol gave asymmetric peaks with CLN (broad) and FBX (forked). Also, the percentage of the selected organic modifier was varied (from 10% to 90%). Upon increasing the acetonitrile content ≥70%, the cited drugs eluted faster with improper resolution. When the acetonitrile content was decreased ≤40%, good separation was observed within longer run time (>11 min). So, 55% acetonitrile to 45% water was chosen as the optimum mobile phase ratio.

To improve the peak shape and prevent the tailing, formic, acetic, ortho-phosphoric acids as well triethylamine were tested as mobile phase additives. The results revealed that Acidic additives gave better separation of the studied drugs than triethylamine. Bad Peaks of PRD (pKa=3.53) and FBX (pKa=3.08) were observed by using trietylamine, this might due to the ionization of these drug in alkaline medium.

Formic acid (0.5%) was the selected acid that gave minimal baseline noise, symmetric peaks with higher peak areas.

Finally, formic acid (0.5 %v/v) dissolved in acetonitrile and water mixture (in ratio 55: 45, v/v; pH= 2.31 ± 0.02) was found to be the optimum eluent that gave the best resolution and sharp peaks of the investigated drugs separated within reasonable time. The retention times (t_R) were 3.597, 5.357 and 8.250 for CLN, PRD and FBX; respectively. The HPLC chromatogram for the cited mixture was shown in Figure 3.

3.1.3. Flow rate

The influence of flow rate on the separation of the targeted drugs was studied in the range of 0.3-2 mL/min. In the preliminary studies, flow rate in isocratic mode was tested. At higher flow rates ≥1.2, overlapping and poor resolution was observed between CLN and PRD peaks. But at lower flow rates ≤0.8, good resolution was achieved with broad asymmetric peaks and longer run time (more than 15 min) for the entire elution of the cited drugs. So, we tried to model a gradient flow program to enhance the method's resolution and decrease the required separation time (to less than 9 min). Flow rate was programmed via TotalChrom Workstation version 6.3.4 software.

It was found that 1.0 mL/min for the first 7 min followed by 1.2 mL/min for the last 3 min of the total run time (10 min) was the optimum flow programming for CLN, PRD and FBX elution within less than 9 min. Thus, the developed method can successfully applied for rapid assay of these antigout agents in tablets and in biological fluids.

3.1.4. System suitability

The system suitability parameters such as peak area, height, retention time, theoretical plates' number, resolution, tailing, capacity, and separation factors were studied for evaluation of the system performance and the method repeatability. CLN-PRD-FBX

combination (30.0 µg/mL of each drug) was injected in six replicates and analyzed under the optimal chromatographic conditions. The results presented in **Table 2** refer to acceptable ranges of repeatability and peak symmetry.

3.2. Method Validation.

3.2.1. Linearity and sensitivity parameters.

According to the optimal chromatographic conditions, six calibration curves for standard solutions of the CLN-PRD-FBX combination were constructed by plotting peak area against drug concentration (µg/mL). The results in **Table 3** indicate the good linearity over a range of 0.040 – 50.0 µg/mL for all drugs with acceptable correlation coefficients (0.99914-0.99955).

The method's sensitivity was assured by the lower LOD and LOQ values (5.18, 1.94, 6.48 and 15.70, 5.88, 19.65 ng/mL for CLN, PRD and FBX respectively). This sensitivity permits the application of the developed method in biological analysis. Figure 4 represents three dimensional diagram of HPLC - chromatograms of the studied ternary mixture in seven concentration levels (0.040 – 50.0 µg/mL).

3.2.2. Accuracy and precision

After six replicates analysis of low, medium, and high concentration ranges (0.5, 30.0, and 50.0 µg/mL) of the studied mixture by the proposed method (Figure 5), the recoveries were in the range from 98.53 to 101.04% with RSD ≤1.99 indicating good accuracy of the method for quantitation of CLN-PRD-FBX combination (**Table 4**). Moreover, the three above-mentioned concentrations were analyzed in triplicates for intra-day precision and gave recoveries of (98.63-100.44%) and (98.93-102.16%) for inter-day precision (n=9). RSD values were found to be ≤1.12 and ≤ 2.0 for intra- and inter-day precision; respectively (**Table 5**). These results prove that the acceptable

repeatability and accuracy of the proposed method for estimation of the cited drugs either in pharmaceutical or in biological samples.

3.2.3. Robustness.

Minor changes of the optimal chromatographic parameters were tested. It was found that the slight alteration in flow rate, mobile system composition, analytical wavelengths, and formic acid concentration had no significant influence on the method's performance. The results shown in **Table 6** indicate the reliability of our method during normal usage.

3.2.4. Specificity and selectivity.

The proposed method is able to extract the studied drugs completely from tablets or from urine samples. After injection of the obtained extracts of the tablets and urine samples, clean chromatograms were observed without any interfering peaks from excipients or endogenous urine components especially at the target retention times (Figure 6, 7). Additionally, the method's specificity was enhanced by using detection wavelength programming at the drugs' maxima wavelengths.

3.2.5. Stability.

Low and high concentrations QC samples (0.50 and 50.0 µg/mL) were utilized for evaluation of CLN-PRD mixture stability in human urine. In **Table 7,** the good recoveries of short- and long-term as well freeze-thaw studies (97.91- 100.77 %) confirm the absence of significant degradation of the studied drugs. Moreover, the stock solution recoveries (after storage for 48 hours at room temperature, and for 7 weeks in deep freezer) were ranged from 98.04 to 101.68%. CLN and PRD showed their stability in pure form and in urine.

This guarantees the handling of the samples containing CLN-PRD mixture under normal laboratory conditions without significant loss or alteration of these drugs.

3.3. Applications

3.3.1. Pharmaceutical analysis

Laboratory-made solutions of Colchicine, Probecid® and Goutifade tablets were analyzed successfully by the developed method (Figure 6). In **Table 8**, the calculated t- and F- values assure that there is no significant difference between the obtained results by our method and the reported methods [17, 28]. Also, standard addition method was performed for the cited drugs in their respective tablets with excellent recoveries (99.43- 101.02%) as shown in **Table 9**. Furthermore, the proposed method was applied for quantitation of CLN and PRD present in Goutyless tablets (Figure 8) with agreeable recovery and RSD (99.05- 101.53% ± 0.86-1.58) as presented in **Table 10**. These results confirm the absence of interference from either frequently encountered additives or excipients. So, the proposed method is convenient, sensitive, accurate and suitable for the attainment of the cited drugs in their dosage forms and for application in QC laboratories.

3.3.1. Urine analysis

Due to the reasonable sensitivity of the developed method, the assessment of CLN-PRD combination in both spiked and real human urine samples was achieved. CLN and PRD are mainly excreted unchanged in urine whereas FBX is excreted as its metabolites. Thus, the established method was employed for CLN-PRD mixture analysis using FBX as an IS. Simple extraction procedure was followed using acetonitrile for deproteinization of the urine. Additionally, a blank experiment of the drug-free urine sample was treated in the same manner omitting the drugs. Linear regression equations of CLN and PRD were obtained by plotting peak area ratios of CLN or PRD to IS versus different drug concentrations in urine samples (y= 0.0191x- 0.003, r=0.9986 for CLN and y= 0.0133x-0.0004, r=0.9992 for PRD). Results represented in **Table 11** indicate good

recoveries and accepted RSD values for analysis of CLN-PRD mixture in spiked urine samples (98.39-101.87% ± 0.21-0.96).

As presented in Figure 7, it was observed that no extra peaks appeared with insignificant changes in peak areas and retention times of the targeted drugs. This proves that absence of urine components interference during the analysis.

On the other hand, the proposed method was used efficiently to estimate CLN-PRD mixture in real urine samples collected from healthy volunteers received Goutyless tablets (**Table 12**). The HPLC chromatogram of human urine sample obtained from healthy volunteers receiving Goutyless® tablets shows the excreted intact drugs as well as metabolites (Figure 9). An extra peak was observed at 4.9 min. It is likely to be the metabolite of PRD (monoacyl glucuronide) that is excreted mainly in urine [43]. As a result, our method is characterized to be convenient for specific quantitation of the target drugs even in presence of their metabolites.

3. Conclusion.

For the first time, a rapid, specific and sensitive HPLC/UV method was established for simultaneous estimation of three commonly prescribed antigout drugs namely; Colchicine, Probenecid and Febuxostat in tablets and in human urine samples. Modeling of flow rate with detection wavelengths programming was innovated to obtain fast and efficient separation. The targeted drugs were separated within less than 9 minutes using GraceSmart RP C_{18} column with mobile system consisting of acetonitrile: water (55: 45, v/v; containing 0.5% v/v formic acid). The linearity range for all the investigated drugs was 0.040 – 50.0 µg/mL with limits of detection of 1.94 - 6.48 ng/mL. Additionally, the developed method was validated according to ICH and US-FDA requirements and was successfully applied for simultaneous estimation of the above-mentioned drugs in their pure form, and in laboratory-made tablets

mixture. Also, the proposed method was utilized for analysis of CLN and PRD in their combined tablets and in human urine samples obtained from healthy volunteers with good recoveries. Our method was proved to be suitable for quantitation of the target drugs even in the presence of excipients, urine components and metabolites. Furthermore, the stability of CLN-PRD mixture in urine samples was studied. Lastly, this method is suitable for quality control measurements for simultaneous analysis of these co-administered antigout agents in their binary and ternary mixtures.

References

[1] Sweetman S.C., Martindale: The Complete Drug Reference, 38th ed., Pharmaceutical Press, London, 2016.

[2] Bernal JA, Quilis N, Andres M, Sivera F, Pascual E. Gout: optimizing treatment to achieve a disease cure. Ther. Adv. Chronic Dis. 2016; 7(2):135–144.

[3] Chinchilla SP, Urionaguena I, Perez-Ruiz F. Febuxostat for the chronic management of hyperuricemia in patients with gout. Expert Rev. Clin. Pharmacol. 2016; 9(5): 665–73.

[4] Sattui SE, Gaffo AL. Treatment of hyperuricemia in gout: current therapeutic options, latest developments and clinical implications. Ther. Adv. Musculoskeletal Dis. 2016; 8(4):145–159.

[5] Case, R., Wentworth, B., & Jester, G. (2018). Effective uric acid reduction with probenecid and febuxostat in a patient with chronic kidney disease. BMJ case reports, 2018. doi: 10.1136/bcr-2017-222845.

[6] Yamanaka H, Tamaki S, Ide Y, Kim H[4], Inoue K[5], Sugimoto M[6], Hidaka Y[7], Taniguchi A[1], Fujimori S[8], Yamamoto T[9]., Stepwise dose increase of febuxostat is comparable with colchicine prophylaxis for the prevention of gout flares during the initial phase of urate-lowering therapy: results from FORTUNE-1, a prospective,

multicentre randomised study. Ann. Rheum. Dis. 2018 Feb; 77(2):270-276. doi: 10.1136/annrheumdis-2017-211574. Epub 2017 Nov 4.

[7] https://www.webmd.com/drugs/2/drug-2234/probenecid-colchicine-oral/details

[8] Joshi, S. A., Jalalpure, S. S., Kempwade, A. A., & Peram, M. R. (2018). Development and Validation of HPLC Method to Determine Colchicine in Pharmaceutical Formulations and its Application for Analysis of Solid Lipid Nanoparticles. Current Pharmaceutical Analysis, 14(1), 76-83.

[9] Çankaya, N., Bulduk, İ., & Çolak, A. M. (2018). Extraction, development and validation of HPLC-UV method for rapid and sensitive determination of colchicine from Colchicum autumnale L. Bulbs. Saudi Journal of Biological Sciences. https://doi.org/10.1016/j.sjbs.2018.10.003

[10] Samanidou, V. F., Sarantis, G. A., & Papadoyannis, I. N. (2006). Development and validation of a rapid HPLC method for the direct determination of colchicine in pharmaceuticals and biological fluids. Journal of liquid chromatography & related technologies, 29(1), 1-13.

[11] Aparna, T., Kistayya, C., Vageesh, N. M., & Reddy, T. K. (2018). Analytical Method Development and Validation for Simultaneous Assessment of Cefadroxil and Probenecid in Bulk Pharmaceutical Formulation. Innovat International Journal of Medical & Pharmaceutical Sciences, 3(7).

[12] Hansen-Møller J & Schmit U. (1991). Rapid high-performance liquid chromatographic assay for the simultaneous determination of probenecid and its glucuronide in urine. Irreversible binding of probenecid to serum albumin. J Pharm Biomed Anal. 9(1):65-73.

[13] Derasari JM, Patel VB. Development and Validation of Stability Indicating LC-PDA method for simultaneous assessment of febuxostat and ketorolac tromethamine in tablet dosage form. Asian J. Chem.; Ghaziabad 2017;29(1): 69–75.

[14] Lukram O, Parmar S, Hande A. Determination of febuxostat in human plasma using ultra-performance liquid chromatography tandem mass spectrometry. Drug Test. Anal. 2013; 5(6): 492–499.

[15] Panigrahy UP, Reddy ASK. Development and validation of stability indicating method for simultaneous estimation of febuxostat and diclofenac potassium in bulk and tablet dosage form using RP-HPLC. Int. J. Pharm. Sci. Res. 2015; 6(2): 798–807.

[16] Tandel D, Shah P, Patel K, Thakkar V, Patel K, Gandhi T. Salting-out assisted liquid–liquid extraction for quantification of febuxostat in plasma using RP-HPLC and its pharmacokinetic application. J. Chromatogr. Sci. 2016; 54(10): 1827-1833.

[17] Lo, W. Y., & Krause, G. M. (1987). Simultaneous Determination of Probenecid and Colchicine in Solid Dosage Form by Reversed Phase High Performance Liquid Chromatography. Drug Development and Industrial Pharmacy, 13(1), 57-66.

[18] Reddy, B. S., & Rao, N. V. B. (2013). A stability indicating RP-HPLC method development for determination of febuxostat in tablet dosage form. Caribbean J Sci Technol, 1, 228-37.

[19] Brunelli, C., Bicchi, C., Di Stilo, A., Salomone, A., & Vincenti, M. (2006). High-speed gas chromatography in doping control: Fast-GC and fast-GC/MS determination of β-adrenoceptor ligands and diuretics. Journal of separation science, 29(18), 2765-2771.

[20] Sabih K, Klaassen CD & Sabih K. (1971). Combined GC and high resolution mass spectrometric determination of probenecid. J Pharm Sci. 60(5):745-748.

[21] Olga Zaporozhets, Iuna Tsyrulneva & Mykola Ischenko (2012). Determination of 8 Diuretics and Probenecid in Human Urine by Gas Chromatography-Mass Spectrometry. American Journal of Analytical Chemistry, 3(4), DOI:10.4236/ajac.2012.34044

[22] Gao Rui-yin, Wei Fu-rong, Nie Li-yun, Wu Lin, Chen Jin SM. Determination of residual organic solvents in febuxostat by GC. Qilu. Pharm. Affairs 2011; 1. Retrieved from http://en.cnki.com.cn/Article_en/CJFDTOTALSDYG201101017.htm

[23] Zhang Jing, Zhou Wen-yan WQ. Determination of the residual organic volatile solvents in febuxostat by gas chromatography. Strait Pharm. J. 2011; 2. Retrieved from http://en.cnki.com.cn/Article_en/CJFDTotal-HAIX201102026.htm

[24] Zhu Sheng-liang, Li Yu-ru SX. Simultaneous determination of 3 kinds of trace organic acids in febuxostat by headspace GC with pre-column derivatization. China Pharmacy 2012; 25. Retrieved from http://en.cnki.com.cn/Article_en/CJFDTOTAL-ZGYA201225028.htm

[25] Rahmawati, S. I., Susilowati, A., Yunus, A., & Widyastuti, Y. (2018, March). Determination colchicine content in aquadest-extracted Gloriosa superba seed from Sukoharjo and Gunung Kidul. In IOP Conference Series: Earth and Environmental Science (Vol. 142, No. 1, p. 012053). IOP Publishing.

[26] Ali, A., Qadir, A., Khan, M. A., Al-Otaibi, F. O., Khan, S., & Abdin, M. Z. (2017). A Simple Validated HPTLC Method for Simultaneous Analysis of Colchicine and Gallic Acid in Polyherbal Formulation: Habb-E-Irqun-Nisha. Food Analytical Methods, 10(9), 2934-2942.

[27] Sireesha, K. R., Mhaske, D. V., Kadam, S. S., & Dhaneshwar, S. R. (2004). Development and validation of a HPTLC method for the simultaneous estimation of

cefuroxime axetil and probenecid. Indian journal of pharmaceutical sciences, 66(3), 278.

[28] Mohamed, A. M. I., Omar, M. A., Derayea, S. M., Hammad, M. A., & Mohamed, A. A. (2018) Validated thin-layer chromatographic method for alternative and simultaneous determination of two anti-gout agents in their fixed dose combinations. Open Chemistry, 16(1), 496-510.**DOI:** https://doi.org/10.1515/chem-2018-0050

[29] Mojeeb Gulzar Khan M. & Arun Shirkhedkar A. (2016). Stability-indicating TLC/densitometric method for simultaneous analysis of febuxostat and diclofenac potassium in bulk and tablets. Curr. Pharm. Anal. 12(3): 266–273.

[30] El-Gizawy SM, Atia NN. & Hosny NM. (2017). Dual-wavelength thin-layer chromatographic–densitometric determination of febuxostat in combination with acetaminophen in synthetic mixture and in pharmaceutical formulations. J. Planar Chromatogr. - Mod. TLC; 30(6): 488-494.

[31] Hosny NM , Atia NN , El-Gizawy SM , Badary DM & Hareedy MS (2018). Innovative HPTLC method with fluorescence detection for assessment of febuxostat-montelukast combination and study of their protective effects against gouty arthritis. Analyst; 143(18):4366-4378. doi: 10.1039/c8an00772a.

[32] Yongming, L., & Guizhi, L. (2000). Determination of colchicine in Tablet by Fluorimetry. Chinese Journal of Analytical Chemistry, 28(3), 330-332.

[33] Bourdon, R., & Galliot, M. (1976). Determination of colchicine in biological fluids. Annales de biologie Clinique, 34(6), 393-401.

[34] Arslan, M., & Duymus, H. (2007). Spectroscopic studies of charge transfer complexes between colchicine and some π acceptors. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 67(3-4), 573-577.

[35] Cunningham, R. F., Israili, Z. H., & Dayton, P. G. (1978). New spectrophotofluorometric assay for probenecid. Journal of pharmaceutical sciences, 67(3), 434-436.

[36] Amareshwar, S., Swathi, A., Prashanthi, Y., Vishwanadham, Y., Sreekanth, D., & Vivekananda, B. (2017). Development and In vitro Characterization of Probencid Rapidly Disintegrating Tablets by Using Different Co-Processed Super Disintegrants, World Journal of Pharmacy and Pharmaceutical Sciences. 6(12), 1660-1672.

[37] Kurian, T., & Kurien, J. (2011). Simultaneous multicomponent spectrophotometric analysis of ampicillin and probenecid in pharmaceutical formulation by derivative spectroscopy. J. Drugs Med, 3, 57-61.

[38] El-Gizawy SM, Atia NN, Hosny NM. Fluorimetric determination of febuxostat in dosage forms and in real human plasma via Förster resonance energy transfer. Luminescence. 2018 Apr 24. doi: 10.1002/bio.3485.

[39] Derasari JM, Patel VB. Spectrophotometric determination of febuxostat and diclofenac potassium in their combined dosage form by simultaneous equation and first order derivative methods. Asian J. Res. Chem. 2013; 6(10): 968–972.

[40] Niel E, & Scherrmann JM. Joint Bone Spine. Colchicine today.2006 ;73(6):672-8. Epub 2006 Sep 1.

[41] Solak Y., Siriopol D., Yildiz A., Yilmaz M.I., Ortiz A., Covic A., & Kanbay M. Colchicine in Renal Medicine: New Virtues of an Ancient Friend. Blood Purif**.** 2017; 43:125-135**,** https://doi.org/10.1159/000454669

[42] https://www.drugbank.ca/drugs/DB01394

[43] https://en.wikipedia.org/wiki/Probenecid

[44] Cunningham RF, Israili ZH, & Dayton PG. Clinical pharmacokinetics of probenecid. Clin Pharmacokinet., 1981; 6(2):135-151.

[45] Food and Drug Administration, Guidance for Industry: Bioanalytical Method Validation, 2018. https://www.gmp-compliance.org/gmp-news/revised-fda-guidance-on-the-validation-of-analytical-methods

[46] ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology, Q2 (R1) Geneva, 2005, http://www.ich.org.

Figure captions

Figure 1. Chemical structures of the investigated drugs.

Figure 2. Absorption spectra of standard solutions of the studied drugs (20.0 μg/mL of each drug prepared in the mobile phase).

Figure 3. HPLC - Chromatogram of CLN– PRD –FBX synthetic mixture (Final drug conc. = 30.0 μg/mL of each drug).

Figure 4. Three dimensional diagram of HPLC – Chromatograms of laboratory-made synthetic mixture in seven concentration levels (0.040 – 50.0 μg/mL); three replicates for each concentration.

Figure 5. Accuracy expressed by three dimensional diagram of HPLC – Chromatograms of laboratory-made synthetic mixture in three concentration levels (0.50, 30.0 and 50.0 μg/mL); six replicates for each concentration.

Figure 6. HPLC - chromatograms of (**A**) standard solution of Colchicine-Probenecid-Febuxostat mixture and (**B**) extract obtained from Colchicine®, Probenecid® and Goutifade film-coated tablets (30.0 μg/mL of each drug**).**

Figure 7. Three dimensional diagram of HPLC – Chromatograms of blank human urine **(1)**, human urine sample spiked with 30.0 μg/mL of FBX **(2)**, human urine samples spiked with CLN, PRD and 30.0 μg/mL of FBX **(3-8)** in six concentration levels (0.040 – 50.0 μg/mL for CLN and PRD).

Figure 8. HPLC - Chromatogram of extract obtained from Goutyless® tablets (0.05 and 50.0 μg/mL for CLN and PRD; respectively).

Figure 9. Three dimensional diagram of HPLC – Chromatograms of blank human urine sample **(1)**, human urine sample obtained from healthy volunteers receiving Goutyless® tablets **(2)**, human urine sample spiked with CLN, PRD and FBX (30.0 μg/mL for each drug) **(3)**, and standard solution of CLN-PRD-FBX mixture (30.0 μg/mL for each drug) **(4)**.

Table 1. Comparison between the proposed method and other reported chromatographic methods.

Drug	Method/ detection	Sample	Linearity range	LOD	LOQ	Stationary phase/mobile system	Ref.
CLN	HPLC/UV	Dosage forms	2-12 µg/mL	0.6121µg/ml	1.854 µg/ml	RP-C18, acetonitrile: 0.1% ortho-phosporic acid (35:65 v/v)	8
	HPLC/UV	*Colchicum autumnale L. Bulbs*	100–500 µg/ml	3.3731 µg/ml	7.7390 µg/ml	RP-C18, Phosphate buffer: methanol (45:55 v/v), pH 5.5; adjusted with dilute phosphoric acid.	9
	HPLC/UV	Serum, urine	0.05-2.5 µg/ml	0.025 µg/ml	0.0758 µg/ml	RP-C18, Ammonium acetate: methanol (48:52 v/v).	10
PRD	HPLC/ PDA*	Tablets	25-125 µg/ml	2.4 µg/ml	7.3 µg/ml	RP-C18, Triethylamine buffer: methanol (65:35 v/v), pH 4.5.	11
	LC/UV	Human urine	0.1-100 µg/ml	0.1 µg/ml at signal to noise=2	-----	RP-C8, Methanol: water: acetic acid (50:50: 1 v/v/v) and tetrabutylammonium bromide.	12
FBX	LC/PDA*	Tablets	50-150 µg/mL	0.246 µg/mL	0.741 µg/mL	RP-C18, Phosphate buffer: acetonitrile (60:40 v/v), pH 4; adjusted with ortho-phosphoric acid.	13
	LC/MS	Human plasma	0.075-12 µg/mL	0.025 µg/mL	0.075 µg/mL	RP-C18, 1% formic acid:acetonitrile (25:75v/v), FBX was dissolved in DMSO.	14
	LC/UV	Tablets	10-60 µg/mL	0.52 µg/mL	1.57 µg/mL	RP-C18, Phosphate buffer: acetonitrile (60:40 v/v), pH 5; adjusted with ortho-phosphoric acid.	15
	SALLE-HPLC/UV*	Rat plasma	0.3-20 µg/mL	0.0659 µg/mL	0.1995 µg/mL	RP-C18, 25 mM potassium dihydrogen orthophosphate buffer, adjusted to pH 6.8 with triethylamine: methanol (35:65 v/v)	16
CLN-PRD-FBX mixture	**Our method**	**Tablets, human urine**	**0.04 –50.0 µg/mL**	**1.94-6.48 ng/mL**	**5.88-19.65 ng/mL**	**Acetonitrile: Water (55: 45, v/v; containing 0.5% formic acid; pH= 2.31 ± 0.05)**	**This work**

***PDA** photodiode array; **SALLE** salting-out assisted liquid–liquid extraction.

Table 2. System suitability parameters.

Studied mixture	**CLN– PRD -FBX**		
Component drugs	**CLN**	**PRD**	**FBX**
Retention time, t_R (min)	3.597 **0.14**	5.357 **0.34**	8.250 **0.29**
Peak area (µV*Sec)	2214268 **0.56**	1537293 **0.45**	3657269 **0.57**
Peak height (µV)	222376 **1.60**	127131.8 **0.77**	240953.8 **0.44**
Number of theoretical plates (plate)	3412.333 **1.49**	4521.333 **1.49**	6174.333 **1.39**
Tailing factor at 5% of peak height	1.188 **1.58**	1.200 **1.59**	1.232 **1.59**
Capacity factor, K'	0.28 **0.73**	0.91 **0.76**	1.94 **0.61**
Separation factor, α	3.185 **0.50**		2.126 **0.09**
Resolution, Rs	6.020 **1.60**		8.066 **1.49**
Peak width at 0.05 (Sec)	21.92 **1.24**	25.95 **1.47**	33.68 **1.64**
Peak width at 0.10 (Sec)	18.33 **1.62**	21.73 **1.71**	27.69 **1.91**

- Relative standard deviation percentages are expressed in **bold digits**.
- Void time (t_o) = 2.80 min.

Table 3. Optimum chromatographic conditions and quantitative parameters of the proposed HPLC/UV method for determination of CLN– PRD -FBX synthetic mixture.

Studied mixture	**CLN– PRD -FBX**		
Mobile phase	**Acetonitrile: Water (55: 45, v/v; containing 0.5% formic acid; pH= 2.31 ± 0.02)**		
Component drugs	**CLN**	**PRD**	**FBX**
Analytical wavelength (nm)	**350**	**249**	**320**
Flow rate (mL/min)	**1.0**	**1.0**	**1.2**
Retention time; t_R (min)	3.597	5.357	8.250
Linearity range (µg/mL)	0.040 – 50	0.040 – 50.0	0.040 – 50.0
Correlation coefficient (r) ± SD*	0.99914 $\pm 1.6\times10^{-4}$	0.99955 $\pm 1.3\times10^{-4}$	0.99925 $\pm 1.3\times10^{-4}$
R^2 ± SD*	0.9983 $\pm 1.95\times10^{-3}$	0.9991 $\pm 2.63\times10^{-3}$	0.9985 $\pm 2.63\times10^{-3}$
Intercept (a) ± SD*	-12290.0 ± 105.36	1352.0 ± 31.113	45723.3 ± 215.95
Slope (b) ± SD*	6.71×10^4 ± 918.17	5.29×10^4 ± 3910.91	10.99×10^4 ± 1907.88
LOD[a]	5.18	1.94	6.48
LOQ[b]	15.70	5.88	19.65

*Average of six replicates.

[a]Limit of detection (ng/mL).

[b]Limit of quantitation (ng/mL).

Table 4. Accuracy of the proposed HPLC method for determination of CLN– PRD –FBX synthetic mixture.

Studied mixture	Authentic drug	Concentration (µg/mL)	% Recovery ± SD*	RSD%
CLN-PRD-FBX	CLN	0.5	100.39 ± 1.99	1.99
		30	99.90 ± 0.64	0.64
		50	101.04 ± 1.34	1.33
	PRD	0.5	98.53 ± 1.38	1.41
		30	100.85 ± 1.96	1.94
		50	98.92 ± 1.25	1.26
	FBX	0.5	100.16 ± 1.21	1.21
		30	100.20 ± 1.42	1.42
		50	100.19± 1.52	1.51

*Average of six replicates, results are calculated from standard curves.

Table 5. Intra-day and Inter-day precision of the proposed HPLC method for determination of CLN– PRD –FBX synthetic mixture.

Studied mixture	Authentic drug	Concentration (µg/mL)	Intra-day precision		Inter-day precision	
			% Recovery ± SD*	RSD%	% Recovery ± SD**	RSD%
CLN-PRD-FBX	CLN	0.5	100.12 ± 0.61	0.61	98.93 ± 1.69	1.70
		30	99.64 ± 0.38	0.38	99.95 ± 0.68	0.68
		50	99.53 ± 1.11	1.12	99.13 ± 1.59	1.61
	PRD	0.5	99.55 ± 0.48	0.48	98.05 ± 1.78	1.82
		30	99.94± 0.88	0.88	102.16 ± 2.04	2.00
		50	98.63 ± 0.60	0.61	99.20 ± 1.25	1.26
	FBX	0.5	99.64 ± 0.74	0.74	100.76 ± 1.70	1.69
		30	100.44 ± 0.39	0.39	99.86 ± 1.59	1.59
		50	100.14± 0.92	0.92	99.44± 1.96	1.97

*Average of three replicates.

**Average of nine replicates.

Table 6. Robustness of the proposed HPLC method for determination of CLN– PRD –FBX synthetic mixture.

Studied mixture	**FXD - PSE**		
Mobile phase	Acetonitrile: Water (55: 45, v/v; containing 0.5% formic acid; pH= 2.31 ± 0.02)		
Component drugs	**CLN**	**PRD**	**FBX**
Analytical wavelength (nm)	**350**	**249**	**320**
Flow rate (mL/min)	**1.0**	**1.0**	**1.2**
Concentration (µg/mL)	**30**		
No variation	100.29 ± 0.34	101.24 ± 1.09	101.20 ± 0.73
ACN: Water ; 58:42, v/v	101.15 ± 0.42	100.53 ±0.54	103.96 ±1.35
52:48, v/v	101.29 ± 0.62	100.86 ±0.83	100.48 ±1.20
Wavelength; +5 nm	99.80 ±0.80	98.94 ±0.65	96.94 ±0.40
-5 nm	98.42 ±1.49	98.14 ±1.02	100.35 ±1.39
Flow rate; +0.1 mL/min	96.30 ±1.22	96.29 ±1.64	100.64 ±1.76
-0.1 mL/min	103.35 ±0.67	97.98 ±0.15	101.74 ±1.06
Formic acid concentration; +0.05 %	98.80±1.14	98.20 ±1.18	99.02 ±0.51
-0.05 %	97.85 ±0.53	98.16 ±0.85	98.62 ±0.20

*Average of three replicates, Results are expressed as % Recovery ± standard deviation.

Table 7. Stability of CLN-PRD mixture under various storage conditions.

Drug	Concentration (µg/mL)	Stability [a] (% Recovery ± SD)				
		Urine samples			Stock solutions [b]	
		Short-term (4 h)	Long-term (7 weeks)	Freeze-thaw cycle	48 h at room temperature	7 weeks at -80 °c
CLN	**0.50**	100.66 ± 1.27	99.74 ± 0.41	98.32 ± 1.00	99.91 ± 1.50	99.13 ± 1.27
	50.0	99.97 ± 0.88	99.72 ± 1.68	97.91± 0.56	101.68 ± 0.85	98.04 ± 0.62
PRD	**0.50**	99.15 ± 0.68	98.98 ± 1.10	98.90 ± 0.31	100.23 ± 0.54	99.35 ± 0.48
	50.0	99.67 ± 1.56	98.93 ± 0.54	100.77 ± 0.57	100.48 ± 0.30	98.63 ± 0.87

[a] Stability represents the recovery (%) and equals mean measured concentration (n = 3) at the indicated time divided by mean measured concentration (n = 3) at baseline x 100.

[b] Stock solutions of CLN-PRD mixture are prepared in methanol.

Table 8. Assay of tablets of the investigated drugs by the developed method and reported methods.

Pharmaceutical formulation	Component drug	%Recovery ± SD*		t-value	F-value
		Proposed method	Reported method		
Colchicine tablets	**CLN**	101.38 ± 1.83	100.25 ± 0.92[a]	1.35	3.93
Probecid® tablets	**PRD**	100.84 ± 0.98	99.58 ± 1.72[a]	1.55	3.04
Goutifade film-coated tablets	**FBX**	101.41 ± 1.02	100.05 ± 1.72[b]	1.66	2.86

*Average of six determination ± standard deviation.

** Theoretical values at 95% confidence limit; t = 2.228, F = 5.053.

[a] Reference [17] for CLN and PRD.

[b] Reference [18] for FBX.

Table 9. Analysis of the laboratory-made pharmaceutical formulation by the proposed HPLC method for determination of CLN– PRD –FBX mixture using standard addition method.

Pharmaceutical formulation	Component drug	Claimed taken (µg/mL)	Authentic added (µg/mL)	Total concentration found (µg/mL)	% Recovery ± SD*	RSD%
Colchicine tablets	**CLN**	**5.0**	0.0	5.001	100.02 ± 0.78	0.78
			5.0	9.943	99.43± 1.07	1.07
			25.0	30.123	100.41± 0.52	0.52
			45.0	49.99	99.98 ± 0.88	0.88
Probecid® **tablets**	**PRD**	**5.0**	0.0	5.0295	100.59 ± 0.45	0.45
			5.0	9.976	99.76 ± 1.49	1.49
			25.0	30.114	100.38± 0.39	0.39
			45.0	49.965	99.93 ± 1.50	1.50
Goutifade film-coated tablets	**FBX**	**5.0**	0.0	4.9815	99.63 ± 0.74	0.75
			5.0	9.998	99.98 ± 1.60	1.60
			25.0	30.306	101.02 ± 1.09	1.08
			45.0	49.91	99.82 ± 1.15	1.15

*Average of six replicates.

Table 10. Assay of tablets of investigated Colchicine – Probenecid mixture by the developed method.

Dosage form	Component drug	Content (mg/tab)	Claimed taken (µg/mL)	Found concentration (µg/mL)	% Recovery ± SD*	RSD%
Goutyless® tablets	**CLN**	**0.5**	0.04 0.05	0.04048 0.05076	101.21 ± 0.95 101.53 ± 1.60	0.94 1.58
	PRD	**500**	40.0 50.0	39.62 50.48	99.05 ± 0.87 100.96 ± 0.86	0.87 0.86

*Average of three replicates.

Table 11. Analysis of CLN-PRD mixture in spiked urine samples.

Targeted drug	Concentration (µg/mL)	% Recovery ± SD*	RSD %
CLN	**0.5**	101.87 ± 0.98	0.96
	30	100.44 ± 0.39	0.38
	50	99.38 ± 1.38	1.39
PRD	**0.5**	99.10 ± 0.31	0.31
	30	100.45 ± 0.35	0.35
	50	98.39 ± 0.21	0.21

* Average of three replicates.

Table 12. Analysis of CLN-PRD combination in real human urine samples.

Experiment No.	Found concentration* (µg/mL)	
	CLN	**PRD**
1	0.0159	6.088
2	0.0161	6.116
3	0.0158	6.109
Average	0.01593	6.1043
SD	1.53×10^{-4}	1.46×10^{-2}
RSD%	0.96	0.45

*Results are calculated from the linear regression equations of CLN and PRD in spiked human urine samples.