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*Dedicated to Professor Eugen Segal*
*on the occasion of his 80th anniversary*

# SIMULTANEOUS DETERMINATION OF PARACETAMOL, PROPYPHENAZONE AND CAFFEINE FROM PHARMACEUTICAL PREPARATIONS IN THE PRESENCE OF RELATED SUBSTANCES USING A VALIDATED HPLC-DAD METHOD

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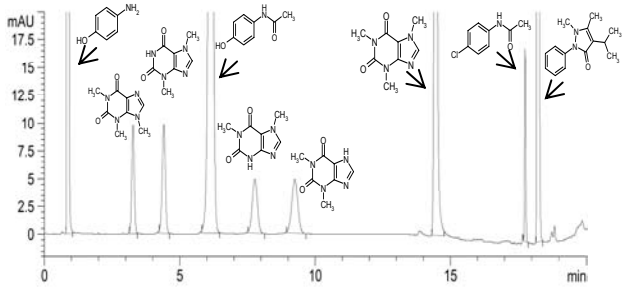
A new HPLC method was developed and validated for simultaneous determination of paracetamol, propyphenazone and caffeine from commercial pharmaceutical preparations in the presence of six related impurities. The HPLC separation of the studied compounds was achieved using a Luna 5u C18 column (100 mm x 4.6 mm, 5 µm particle) and a gradient elution of 0.05 M aqueous $NaH_2PO_4$ (containing 1% tetrahydrofuran and adjusted to pH 3.0 by addition of $H_3PO_4$) and acetonitrile. The injection volume of the standard and sample solutions was 20 µL, column temperature was set up at 25°C and the mobile phase flow rate was 1.5 mL/min. For UV detection of the active ingredients two wavelengths were used, corresponding to maximum absorbance: 245 nm for paracetamol and 275 nm for propyphenazone and caffeine. The proposed method was fully validated through linearity, limit of detection, limit of quantification, accuracy and precision over the concentration range of 42.8-127.6 µg/mL for paracetamol, 24.8-75.0 µg/mL for propyphenazone and 9.4-25.0 µg/mL for caffeine. The proposed method has been applied with good results on two real drug products for the determination of the active compounds.




## INTRODUCTION

New drug formulations on the market are designated to cover more than one pharmaceutical compound. Thus, formulations containing three active substances are designated to enhance the pharmaceutical effects of each substance and to cover a larger medical treatment. Tablets containing paracetamol, propyphenazone and caffeine are emerging as the most widely prescribed combination

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for pain relief. Paracetamol (PAR) is a widely used over-the-counter (OTC) analgesic and antipyretic agent. It is commonly used for the relief of headaches and other minor aches and pains and is a major ingredient in numerous cold and flu remedies.[1-2] Propyphenazone (PPH) is a non-steroidal anti-inflammatory drug (NSAID) derived from pyrazolone incorporated into many over-the-counter analgesic combinations in many countries. It is an analgesic, antipyretic and anti-inflammatory agent.[3-4] Caffeine (CAF) is a xanthine alkaloid substance used as a psychotropic stimulant drug[5-7] naturally found in coffee, tea, mate, guarana, cola and cocoa. This combination of active pharmaceutical ingredients (APIs) causes reduction in the amount of prostaglandin, whereas caffeine is also known to increase the analgesic effect of paracetamol and propyphenazone,[8-10] synergistically, providing relief from symptoms like headache, muscular aches, neuralgia, backache, joint pain, rheumatic pain, migraine, general pain, toothache and menstrual pain. The combination is also found to be effective in controlling fever originating from bacterial or viral infection. It is usually available in tablet dosage form as a single unit dose with concentration in varying proportions such as 250 mg paracetamol, 150 mg propyphenazone and 50 mg caffeine.

Simultaneous determination of these three APIs in ternary mixtures was carried out by spectrophotometry,[11-15] normal and reversed-phased thin-layer chromatography,[16-18] pressurized planar chromatography,[18] gas chromatography,[19] and micellar electrokinetic capillary chromatography.[20,21] There have been reported HPLC methods for analysis of paracetamol, propyphenazone and caffeine from blood,[22] and from pharmaceutical dosage forms.[23-25] However, none of the reported HPLC methods deal with the determination of these three compounds in the presence of their related impurities. It is the aim of this work to develop and validate a suitable method for separation and quantitative determination of these active substances from other six related impurities that could appear from the raw material manufacturing or from degradation process in the finished product (with denomination and structures given in Fig. 1).

## EXPERIMENTAL

Paracetamol, 4-aminophenol, 4'-chloroacetanilide and tetrahydrofuran were obtained from Merck (Darmstadt, Germany). Propyphenazone was supplied from Shandong Xinhua Pharmaceutical (Zibo, China), caffeine was purchased from Sigma-Aldrich (Buchs, Switzerland) and the impurity standards of caffeine (defined as "caffeine for system suitability standard" by European Pharmacopoeia, 7th edition): 1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (impurity A), 1,3,9-trimethyl-3,9-dihydro-1*H*-purine-2,6-dione (impurity C), 3,7-dimethyl-3,7-dihydro-1H-purine-2,6-dione (impurity D) and 1,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (impurity F) were obtained from European Directorate for the Quality of Medicines & HealtCare (EDQM). Caffeine for system suitability standard is a mixture of the four chemical related substances mentioned above.

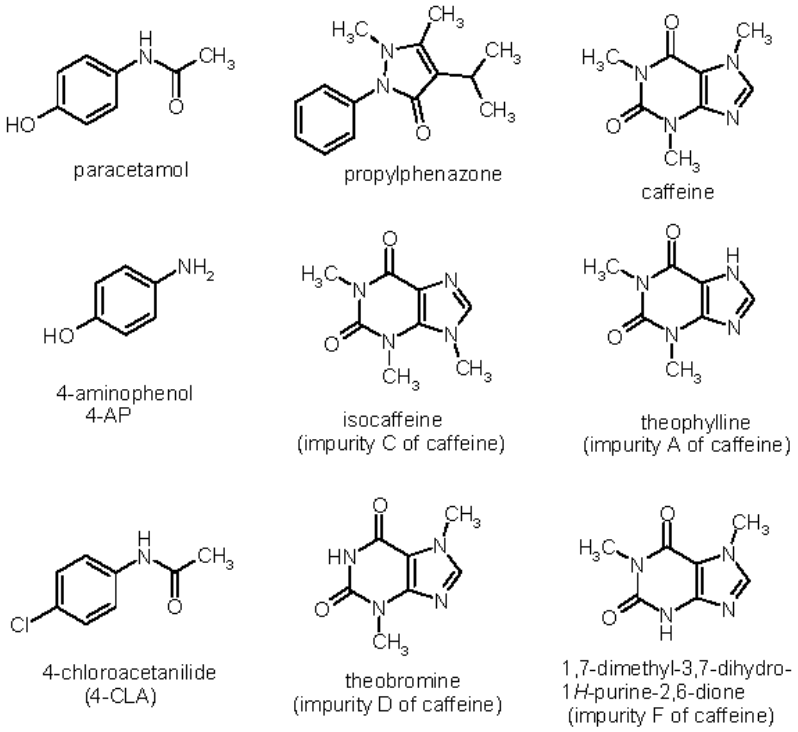


Fig. 1 – Chemical structures of the pharmaceutical compounds and related impurities studied in this work.

Colloidal silicon dioxide, talcum, isomalt, stearic acid and croscarmellose sodium were obtained as gift from Polipharma Industries SRL (Sibiu, Romania). HPLC gradient grade methanol and acetonitrile were procured from LGC Standards (Wesel, Germany). $H_3PO_4$ (85% conc.) was obtained from Lach-Ner (Neratovice, Czech Republic) and ultra-purified water with specific resistivity of 18.2 MΩ/cm obtained from a Milli-Q Direct 8 System (Millipore, Bedford, MA, USA) was used. All the solvents and samples were filtered through 0.45 µm nylon filter membranes (Phenomenex, Torrance, CA, USA) prior to their injection into HPLC system.

The chromatographic analysis was performed on an Agilent 1200 series (Waldbronn, Germany) equipped with an on-line vacuum degaser, a quaternary pump, a vial autosampler, a thermostated column compartment and a diode array detector. Data acquisition and peak integration was done with ChemStation software (revision B.04.02). The pH measurements were made using inoLab 740 pH meter from WTW (Weilheim, Germany) and for ultrasonication of the sample solutions Sonorex Digitec DT 1028H (Bandelin, Berlin, Germany) was employed.

The separation of the studied compounds was carried out on a Luna 5u C18 100A column (100 mm x 4.6 mm, 5 µm particle size, Phenomenex, Torrance, CA, USA). The mobile phase consisted of solvent A (0.05 M $NaH_2PO_4$ aqueous buffer with 1% THF, adjusted to pH 3.0 with $H_3PO_4$) and solvent B (acetonitrile). The starting composition was 100% solvent A and the binary gradient elution was as follows: 0% solvent B over 0-12 min, 0-80% solvent B in linear gradient over 12-20 min, 80% solvent B over 20-25 min, returned from 80% to 0% solvent B in 0.1 min and re-echilibration from 25.1-30 min. The flow rate was 1.5 mL/min, column temperature was set to 25°C and the injection volume was 20 µL. The chromatograms were recorded simultaneously at 245 nm and 275 nm and whenever necessary, UV spectra were recorded within the range of 200-400 nm.

A stock standard solution of accurately weighed APIs having the concentration of 0.84 mg/mL for PAR, 0.50 mg/mL for PPH and 0.168 mg/mL for CAF was prepared in a 100 mL volumetric flask using solvent A as sample diluents, and then diluted (1:10) to the final concentrations of 84 µg/mL for PAR, 50 µg/mL for PPH and 16.8 µg/mL for CAF.

For real sample preparation 20 tablets from each pharmaceutical product to be tested were weighed and homogenized into powder using a grinding mortar. A precise quantity of powder from each sample was then dissolved in a mixture of $CH_3OH$ : solvent A (1:1) by ultrasonication (15 min) and magnetic stirring (10 min). The sample solutions were filtered next through a medium pore-size filter paper in order to remove the insoluble excipients ($SiO_2$, talcum, etc.). Finally, the filtered solutions were diluted using solvent A so that the final labeled concentration was in the range of the working standards.

## RESULTS AND DISCUSSION

### Detection parameters

UV-detection ensures the sensitivity of the chromatographic analysis for the aim of this study. The detection wavelengths of 245 nm and 275 nm were selected considering the UV absorption spectra of the three APIs. As it can be seen from Fig. 2, the wavelength of 245 nm is optimal for paracetamol, while 275 nm can be used for both caffeine and propyphenazone.

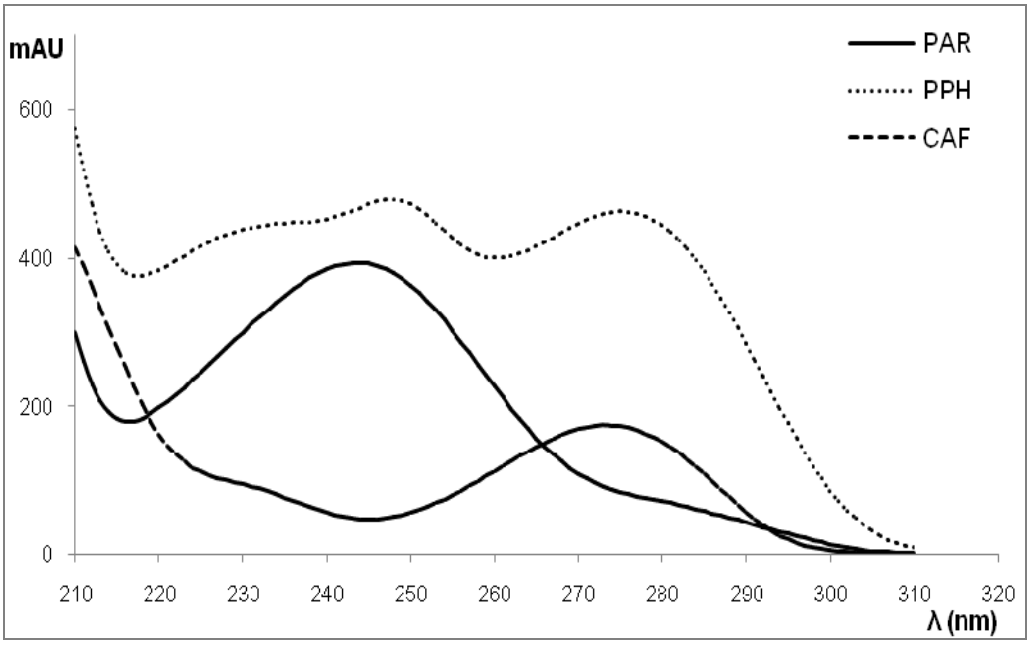


Fig. 2 – UV spectra of paracetamol, propyphenazone and caffeine.

### Mobile phase composition

According to the octanol/water partition coefficient log $D_{ow}$ estimated from Marvin program[26] the critical pair for separation is theophylline/theobromine, which have the same value of log $D_{ow}$ at pH = 3 of mobile phase used during the separation. Meanwhile, these target analytes are the less hydrophobic compounds and under usual mobile phase compositions (aqueous component and methanol or acetonitrile) they elute from chromatographic column close to the dead time of separation. Very hydrophilic analytes are also caffeine (log $D_{ow}$ = -0.55), and isocaffeine (log $D_{ow}$ = -0.66) and are expected to elute at small values of retention time. For these reasons, a choice of mobile phase composition based only on two components is not able to separate analytes with close values of log $D_{ow}$. A third organic additive in the mobile phase composition, such as tetrahydrofuran, may influence the balance of interactions of target analytes between mobile phase and stationary phase, a fact that was observed during method development and used for final validation. On the other hand, three of the target analytes are rather hydrophobic such that their interaction with stationary phase is rather strong, and consequently they elute at high retention times.[27] Thus, PAR has a log $D_{ow}$ = 1.72; propyphenazone – log $D_{ow}$ = 2.86, and 4-CLA – log $D_{ow}$ = 1.82 (at the same pH = 3). Therefore, a linear gradient with the increase content of the strong component (acetonitrile) in mobile phase up to 80% will decrease the retention time of these analytes. Under these eluting conditions all nine analytes are well separated and the final running time of the chromatogram is rather convenient for the purpose of this study (less than 20 minutes).

### Specificity

In order to be specific, an assay method should demonstrate that it can separate and quantify the active pharmaceutical ingredient from the excipients, chemical related substances and from other APIs. The proposed method was investigated in order to demonstrate that the studied compounds can be discriminated from their related substances and excipients. To observe any interference from excipients the original formulation (without the active ingredients) of one of the commercial drugs analyzed in this study (Pararemin®) was prepared having the following excipients: colloidal silicon dioxide, talcum, isomalt, stearic acid and croscarmellose sodium. This matrix served as a placebo that was next treated as in sample preparation, but the final concentration of the placebo solution was 5 times more concentrated than the sample solution. This was done to ensure that any possible excipient interference could be easily noticed. To evaluate the interference of the related substances a synthetic sample was prepared having all the APIs and the selected impurities (at a concentration above their normal acceptable limit in order to better illustrate the separation).

Standard solutions were prepared separately for each impurity with the following working concentrations: 0.1 mg/mL for 4-AP, 8 μg/mL for 4-CLA and 1.0 mg/mL as "caffeine for system suitability standard".

Fig. 3 presents a chromatogram of the synthetic sample that contains all three pharmaceutical compounds together with the chemical related substances. The excipients from pharmaceutical formulation do not generate any chromatographic peak that could interfere with the active components or the chemical related impurities. The separation parameters are given in Table 1. Thus, the ability of the this method to separate all the compounds is demonstrated by assessing the resolution between the peaks corresponding to various compounds, particularly for the compounds with the closest elution relative to paracetamol and propyphenazone.

The performance of this method is proved by its ability to separate the target compounds found in a synthetic mixture, which corresponds to the conditions imposed by European Pharmacopoeia. As can be seen from Fig. 4 even the critical pair (propylphenazone and 4'-chloroacetanilide) is separated with acceptable resolution.

### Linearity and detection parameters

Linearity was determined experimentally by analyzing a series of standard solutions at five different concentrations that cover 50-150 % of the expected working range of the assay. For each concentration level three replicates were prepared from the stock solution. The concentration range was 42.8-127.6 μg/mL for PAR, 24.8-75.0 μg/mL for PPH and 9.4-25.0 μg/mL for CAF.

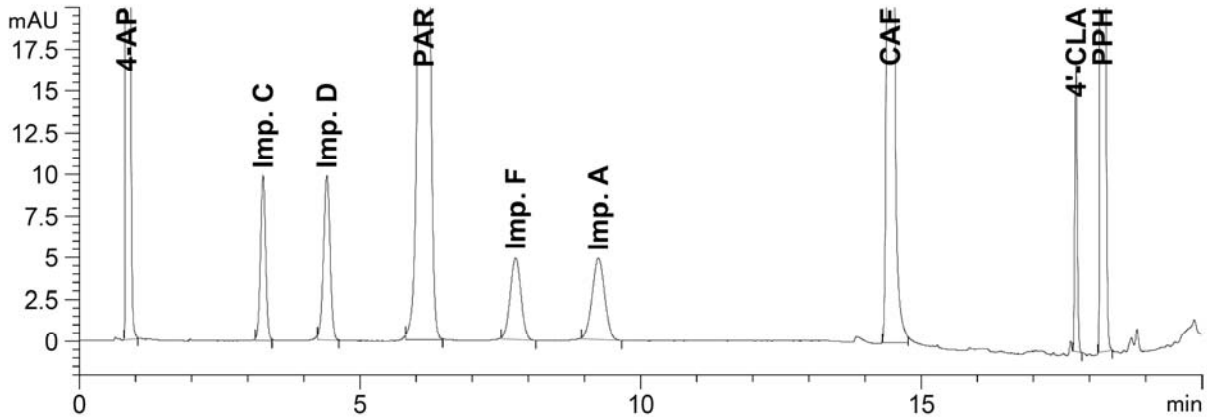


Fig. 3 – Typical chromatogram of the synthetic sample recorded at 275nm.

*Table 1*

Separation parameters of the synthetic sample

| Compound | Retention time (min) | Relative retention time* | Symmetry factor | USP tailing factor | Resolution |
|---|---|---|---|---|---|
| 4-Aminophenol | 0.846 | 0.138 | 0.371 | 2.075 | - |
| Impurity C | 3.277 | 0.534 | 0.968 | 1.006 | 19.33 |
| Impurity D | 4.413 | 0.719 | 0.996 | 0.976 | 6.12 |
| Paracetamol | 6.140 | 1.000 | 0.978 | 1.000 | 7.00 |
| Impurity F | 7.775 | 1.266 | 0.994 | 0.975 | 5.19 |
| Impurity A | 9.249 | 1.506 | 0.981 | 0.979 | 3.95 |
| Caffeine | 14.446 | 2.353 | 0.882 | 1.076 | 21.19 |
| 4'-Chloroacetanilide | 17.753 | 2.891 | 0.838 | 1.158 | 39.65 |
| Propyphenazone | 18.226 | 2.968 | 0.828 | 1.174 | 5.99 |

*with respect to paracetamol, the major active pharmaceutical ingredient.

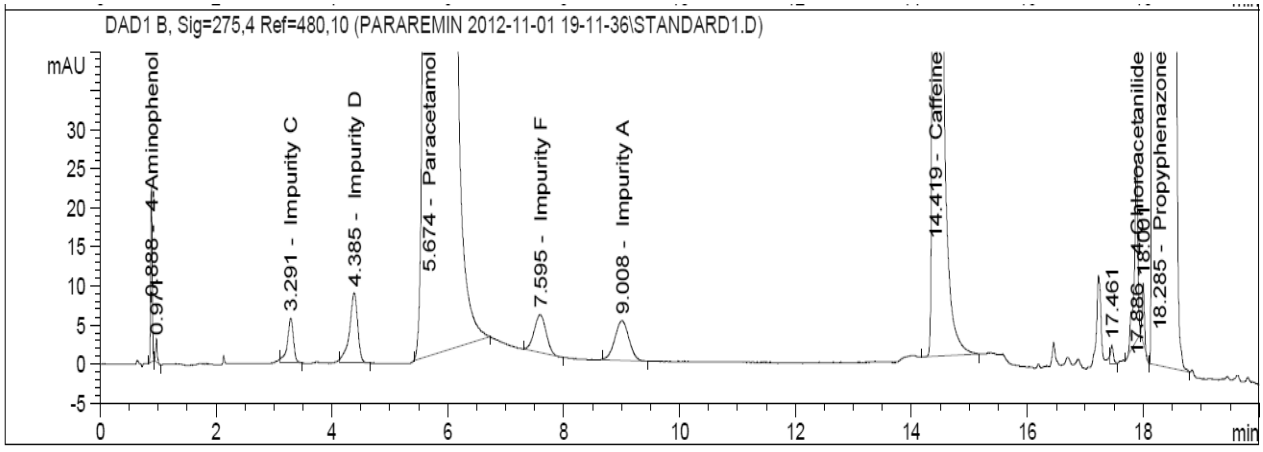


Fig. 4 – Chromatogram recorded for a standard solution containing 5000 ppm paracetamol; 5 ppm 4-aminophenol; 1.6 ppm impurity C; 3.5 ppm impurity D; 100 ppm caffeine; 2.3 ppm impurity F; 2.8 ppm impurity A; 3000 ppm propyphenazone, and 1 ppm 4-chloroacetanilide (20 μL injection volume in solvent A:acetonitrile 1:1 (v/v).

After the injection and chromatographic run of each standard solution, the calibration curve of the peak area (mAU·x s) versus the concentration was evaluated. The results of the linearity experiments, presented in Table 2, demonstrate that there is a linear relationship between the signal (peak area) of the studied APIs and the corresponding concentrations over the entire concentration range. The correlation coefficients obtained using linear regression model of calibration curve are greater than 0.9998. Further evaluation of residual plots was carried out to check the model's adequacy. The residuals did not exceed 2% of the peak area corresponding to the nominal working concentration (the middle of the range) for each of the studied compounds.

For estimation of LOD and LOQ, standard solutions of the studied compounds were prepared by successive dilutions of the stock solution used in the linearity experiments. The standard solutions were analyzed and the smallest concentration that gave a signal-to-noise ratio of 3 and 10 were considered as LOD and LOQ, respectively.[28] The values of LOD were estimated to 42 ng/mL for paracetamol, 194 ng/mL for propyphenazone and 74 ng/mL for caffeine, while LOQ values were 125 ng/mL for paracetamol, 776 ng/mL for propyphenazone and 295 ng/mL for caffeine.

## Accuracy and precision

The accuracy was investigated at three concentration levels by adding known amounts of standard to the sample matrix. Each concentration level was prepared and tested three times. The accuracy was evaluated by the percentage recovery and relative standard deviation (RSD) values.

Accuracy was determined over the entire range of the assay (50-150% of the nominal working concentration) by calculating the percentage ratio between the determined mean concentration and the theoretical (calculated) concentration of the spiked samples. The results of the accuracy are presented in Table 3 with recovery found to be within the targeted limit (100 ± 2%), with values ranging from 98.96 to 101.00% and an RSD value from 0.21 to 1.64%.

Precision has been estimated by repeatability and its intermediate estimate. For that, six consecutive measurements have been performed using a standard solution at the nominal concentration. Intermediate precision was checked by evaluating the repeatability of the entire analytical procedure in the same laboratory, but on two different days by two different investigators on two different chromatographic systems and on two different columns (different batch, same manufacturer). The values of relative standard deviation (RSD%) of peak areas corresponding to the repeatability study for the three APIs were following: 0.07% for paracetamol; 0.03% for caffeine, and 0.05% for propyphenazone. The results for intermediate precision study are given in Table 4. The RSD values are within the range of 0.03-0.07% for repeatability and are less than 2% for intermediate precision, which correspond to a good precision of the analytical method.

## Solution stability and analysis of commercial drugs

The chemical stability of the stock solutions containing the APIs in mobile phase (solvent A) has been studied for a period of 48 hours at room temperature (25°C). The solutions were found to be stable for 24 hours at 25°C. No other peaks in chromatograms were observed during the stability studies.

*Table 2*

The results of the linearity experiments; x – concentration; y – peak area (mAU·s)

| | **PAR** | **PPH** | **CAF** |
|---|---|---|---|
| Range (μg/mL) | 42.8 - 127.6 | 24.8 - 75.0 | 9.4 - 25.0 |
| Regression equation | y = 51.02·x – 23.46 | y = 31.28·x – 3.98 | y = 39.19·x – 7.33 |
| Correlation coefficient (r) | 0.99995 | 0.99990 | 0.99988 |
| Standard error of estimate (SEE) | 16.79 | 8.28 | 3.81 |
| F (Fischer test) | 122630.1 | 63496.2 | 54351.5 |

*Table 3*

Accuracy of the method (n = 3)

| Analyte | Concentration level | Theoretical concentration (μg/mL) | Assayed concentration* (μg/mL) | Recovery (%) | Mean recovery (%) | RSD (%) |
|---|---|---|---|---|---|---|
| Paracetamol | 50% | 42.57 | 42.10 ± 0.52 | 98.88 | 98.96 | 0.21 |
| | 100% | 84.16 | 83.48 ± 1.01 | 99.20 | | |
| | 150% | 128.13 | 126.61 ± 1.68 | 98.81 | | |
| Propyphenazone | 50% | 24.97 | 24.92 ± 0.12 | 99.77 | 99.64 | 0.30 |
| | 100% | 49.67 | 49.59 ± 0.18 | 99.84 | | |
| | 150% | 75.55 | 75.05 ± 1.00 | 99.30 | | |
| Caffeine | 50% | 8.62 | 8.87 ± 0.03 | 102.92 | 101.00 | 1.64 |
| | 100% | 17.24 | 17.23 ± 0.04 | 99.93 | | |
| | 150% | 25.86 | 25.90 ± 0.13 | 100.15 | | |

*mean ± standard deviation.

*Table 4*

Results for intermediate precision of the analytical process

| Compound | Retention time (min, n = 6) | | Assay (% of declared, n = 6) | | Mean (% of declared) | RSD (%) |
|---|---|---|---|---|---|---|
| | Investigator 1* | Investigator 2** | Investigator 1* | Investigator 2** | | |
| PAR | 6.279 | 6.284 | 100.83 | 99.55 | 100.19 | 0.91 |
| CAF | 14.556 | 14.551 | 97.82 | 97.97 | 97.90 | 0.11 |
| PPH | 18.250 | 18.247 | 102.48 | 100.49 | 101.49 | 1.39 |

*Results obtained in day 1.
**Results obtained in day 2.

*Table 5*

The results of the analysis on commercial pharmaceutical preparations by this method

| Active ingredient | Labeled content (mg/dose unit) | Pararemin® | | Saridon® | |
|---|---|---|---|---|---|
| | | Found* (mg/unit dose) | Recovery (%) | Found* (mg/unit dose) | Recovery (%) |
| Paracetamol | 250 | 244.7 ± 4.4 | 97.9 ± 1.8 | 255.8 ± 2.7 | 102.3 ± 1.1 |
| Propyphenazone | 150 | 151.6 ± 3.7 | 101.1 ± 2.5 | 147.3 ± 1.6 | 98.2 ± 1.1 |
| Caffeine | 50 | 49.2 ± 1.3 | 98.4 ± 2.6 | 45.7 ± 0.3 | 97.2 ± 0.5 |

*mean ± standard deviation

The proposed method has been applied for quantitative evaluation of paracetamol, propyphenazone and caffeine from PARAREMIN® (produced by Polipharma Industries S.R.L., Romania) and from SARIDON® (produced by Bayer Santé Familiale, France). Both pharmaceutical products have the same content of active ingredients. The obtained results (given in Table 5) are in agreement with the labeled content for both drug products, with recovery values situated within the limit of 100 ± 5%.

## CONCLUSIONS

A new HPLC method has been developed and validated for simultaneous quantitative evaluation

of paracetamol, propyphenazone and caffeine from commercial pharmaceutical preparations. The chromatographic conditions were adjusted in such a way that the chemical-related impurities or the excipients did not interfere with the APIs. The proposed method was validated and proven to have adequate selectivity, good linearity, accuracy, precision, low limits of detection and quantification. Furthermore, the assay was applied with good results for quality control of the APIs in two commercial drugs.

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