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PAPER

# Development and validation of a rapid HPLC method for the determination of ascorbic acid, phenylephrine, paracetamol and caffeine using a monolithic column†

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This article reports a fast and simple liquid chromatographic method for determination of ascorbic acid, phenylephrine, paracetamol and caffeine. Salicylic acid was used as internal standard. The analytes were successfully separated in less than 5 min by isocratic elution using monolithic column, Onyx Monolithic C18 (100 × 4.6 mm), with mobile phase composed of acetonitrile and phosphate buffer (pH 6.50) (10 : 90, v/v) at a flow rate of 1.0 mL min$^{-1}$ and 25 °C, sample volume was 10 μL. Detection was observed at two wavelengths 210 nm (phenylephrine, paracetamol and salicylic acid) and 235 nm (ascorbic acid and caffeine). The optimized method was applied for the determination of the analytes in pharmaceutical formulation Coldrex tablets (Smithkline Beecham Consumer Healthcare, United Kingdom) commonly used in virosis treatment.

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## Introduction

Ascorbic acid, phenylephrine, paracetamol and caffeine are widely used in diseases accompanied by pain and fever such as the common cold and other viral infections such as an analgesic, antipyretic and decongestant active substances.

A variety of methods for the determination of some of these compounds is found in literature[1–22] but only one of them[1] described the determination of all four compounds simultaneously. Analysis was performed with a particle column LiChrospher 100 RP 18 (250 × 4 mm, 5 μm); the mobile phase consisted of a mixture of methanol and phosphate buffer (pH 3) in ratio of 35 : 65 (v/v) and the analysis time was 30 min. Theophyline was chosen as internal standard. Developed method was used for analysis of tested compounds in pharmaceutical formulations (Panadol Extra, Vitaminum C and Efferalgan Vitamin C).

Most of the above mentioned methods were used for the determination of paracetamol combinations like paracetamol–ascorbic acid,[8,14] paracetamol–phenylephrine hydrochloride[5,10,11,13,20,21] and paracetamol–caffeine.[2,4,9,12,16,17,22]

The aim of this study was to develop and validate a rapid HPLC method for the determination of ascorbic acid, phenylephrine, paracetamol and caffeine using a monolithic column. The optimized method was applied for the determination of tested analytes in the pharmaceutical formulation Coldrex tablets (GlaxoSmithKline Dungarvan Ltd., Co. Waterford, Ireland) commonly used in virosis treatment.

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## Experimental

### Chemicals

Working standards of ascorbic acid (ASC), phenylephrine (PHE), paracetamol (PAR), caffeine (CAF) and salicylic acid (SAL) were provided by Sigma-Aldrich (Prague, Czech Republic). Monopotassium phosphate and disodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile and methanol were purchased from Sigma-Aldrich (Prague, Czech Republic). The deionised water was purified by a Milli-Q system (MilliporeTM, Czech Republic). All other used chemicals were of analytical grade quality. The pharmaceutical formulation used for method validation was Coldrex tablets (GlaxoSmithKline Dungarvan Ltd., Co. Waterford, Ireland). 1 tablet contains: Paracetamol (500 mg), caffeine (25 mg), phenylephrine hydrochloride (5 mg), terpine hydrate (20 mg), ascorbic acid (30 mg) and excipients including cornstarch, pregelatinized starch, talc, stearic acid, povidone, potassium sorbate, sodium lauryl sulphate, sunset yellow (E 110).

### Chromatographic system

Analyses were performed using the Breeze HPLC System (Waters Corp., USA), consisting of a binary pump (Waters 1525 Binary HPLC Pump), an autosampler (Waters 717 plus), a UV detector (Waters 2487 Dual λ Absorbance Detector) and a PC

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for data processing. The data were collected and analyzed by the Breeze software.

The analytes were successfully separated in less than 5 min by isocratic elution on the monolithic column (Onyx Monolithic C18, 100 × 4.6 mm, Phenomenex), with a mobile phase composed of acetonitrile/phosphate buffer (pH 6.50) (10 : 90, v/v) at a flow rate of 1.0 mL $min^{-1}$ at 25 °C, sample volume was 10 μL. Detection was observed at two wavelengths 210 nm (PHE, PAR and SAL) and 235 nm (ASC and CAF) to keep sufficient precision for all active substances.

### Standards preparation

Stock and standard solutions of ASC, PHE, PAR, CAF and SAL were prepared by dissolution in mobile phase. Mixed standard solution for separation experiments contained 300 μg $mL^{-1}$ of ASC, 50 μg $mL^{-1}$ of PHE, 100 μg $mL^{-1}$ of PAR, 250 μg $mL^{-1}$ of CAF and 50 μg $mL^{-1}$ of SAL in mobile phase.

All stock and standard solutions were stored at 4 °C and mixed standard solution was prepared weekly.

### Sample preparation

A total amount of 10 tablets (Coldrex tablets) was accurately weighed and powdered in a mortar. Quantity equivalent to one quarter of tablet was accurately weighed and dissolved in internal standard solution (50 μg $mL^{-1}$ of SAL) in mobile phase in 25.00 mL volumetric flask. After 15 min in an ultrasonic bath, the solution was transferred to centrifuge tube and centrifuged at 3904 × $g$ (6000 rpm) for 15 min. Supernatant was filtered through membrane filter (Nylon, pore size 0.45 μm) (Solution A—expected concentrations corresponded to mixed standard solution) and then diluted 50 times with internal standard solution to a final concentration (Solution B—for PAR determination at 100 μg $mL^{-1}$ level). Solution A was used for quantitation of ASC, PHE, CAF and solution B for evaluation of PAR concentration.

## Results and discussion

### Optimization of chromatographic parameters

Separation efficiency of monolithic columns (C18; 25–100 mm long) with various internal diameters (2–4.6 mm) were compared, and 100 mm long monolithic column with internal diameter 4.6 mm was chosen for the next optimization due to sufficient resolution of all analyzed substances.

During our preliminary experiments, several combinations of the mobile phase composition (acetonitrile, methanol and mixture of acetonitrile, methanol and water or buffers) and pH were tested to obtain the optimum separation conditions.

Acetonitrile percentages (5–15%) and mobile phase pH (2.5–7.5) were examined in detail. Prolonged retention time with decreased acetonitrile content was more significant for CAF (as the last peak). Retention times of CAF ranged from 2.60 min (15% of acetonitrile) to 12.20 min (5% of acetonitrile). Among analyzed substances ASC ($pK_a$ = 4.2) represented acidic compounds, PHE, PAR and CAF were basic compounds with $pK_a$ in the range 8.9–10.4. Separation of such mixture in the short analysis time needed to keep pH of the mobile phase close to neutral conditions. Small changes of pH did not affect elution of ASC and CAF because they were analyzed in ionized form. pH lower than 5 caused movement of PHE peak close to the peak of ASC. pH higher than 7.5 caused overlapping of PHE and PAR peaks.

The best separation was achieved with mobile phase composed of acetonitrile and phosphate buffer (pH 6.50) (10 : 90, v/v). The method of internal standard was used for precise data evaluation and decreasing effect of inaccurate injections, pumping and UV lamp lifetime together with sample preparation differences and several compounds were tested throughout the method development (methylparaben, diclophenac, ibuprofen, salicylic acid, sodium benzoate). SAL was chosen as internal standard because its retention time was in between other analyzed substances, did not prolong the analysis time and was not close to other analytes. Problems associated with other tested compounds were found mainly in elution together with other analyzed substances or prolonged separation.

Extraction procedure for tablets containing ASC, PHE, PAR and CAF was optimized as well. The isolation procedure was based on previous experience with analysis of tablets routinely carried out in our laboratory. Two extraction media—methanol and mobile phase were tested and mobile phase was chosen because of lower recovery of ASC (86.51%) and PHE (85.49%) using methanol as extraction agent.

### Analytical parameters and validation

All compounds were successfully separated using the proposed procedures (Onyx Monolithic C18, 100 × 4.6 mm, Phenomenex, 10 μL sample volume, a mobile phase composed of acetonitrile and phosphate buffer pH 6.50, 10 : 90 at a flow rate of 1.0 mL $min^{-1}$, ambient temperature, detection wavelengths of 210 and 235 nm) and several chromatographic parameters, such as peak asymmetry, peak resolution, number of theoretical plates, height equivalent to a theoretical plate, repeatability of peak area and retention time, were calculated from experimental data. Results are given in Table 1 and obtained values are in good agreement with limits set by validation authorities (ICH; European Pharmacopoeia).[23–25] The repeatability (Table 1) of the method was checked by six injections of standard solution (ASC, PHE, PAR, CAF and SAL) and was expressed as the relative standard deviation values (RSD%).

The chromatogram in Fig. 1 was obtained using the HPLC method for separation of mentioned substances in the standard solution. Fig. 2 shows separation of tested analytes in Coldrex tablets (including sample solutions A and B).

The validation parameters included linearity, precision and accuracy and results are described in Table 2. Values of mentioned parameters were, in all cases, calculated as a ratio of the respective value for individual compound and the value for internal standard obtained in one analysis.

Linearity (Table 2) was established with a series of working solutions prepared by dilution of the stock solution in mobile phase to the final concentrations corresponded to 50, 70, 90, 100, 120, and 150% of the labelled content of each active substance. The calibration graphs thus involved six experimental points for each compound and solutions were injected in triplicate. The

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**Table 1** Evaluation of separation process[a]

| Parameter | Criteria | Ascorbic acid | Phenylephrine | Paracetamol | Salicylic acid | Caffeine |
|---|---|---|---|---|---|---|
| Peak resolution | $R > 1.5$ | — | 2.03 | 4.02 | 2.99 | 3.52 |
| N. of theor. plates | — | 1760 | 2346 | 3704 | 3636 | 3007 |
| HETP (μm) | — | 56.81 | 42.62 | 27.00 | 27.50 | 33.26 |
| Peak asymmetry | $0.8 < T < 2$ | 1.30 | 1.48 | 1.40 | 1.50 | 1.66 |
| Repeatability of $t_R$ (%)[b] | RSD < 1 | 0.46 | 0.32 | 0.43 | 0.43 | 0.48 |
| Repeatability of A (%)[b] | RSD < 1 | 0.28 | 0.39 | 0.20 | — | 0.16 |

[a] HETP—height equivalent of the theoretical plate; N. of theor. plates—number of theoretical plates; A—peak area. [b] RSD for repeated injections of standard solution ($n = 6$).

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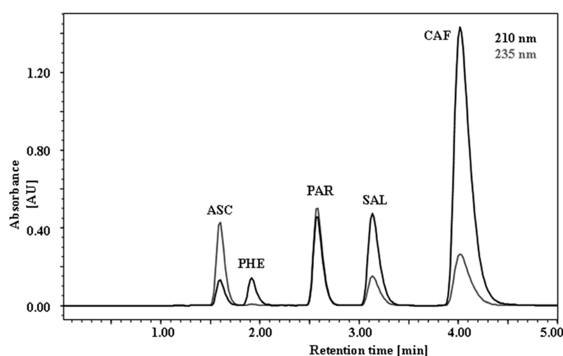


**Fig. 1** HPLC chromatogram of standard solution using Onyx™ Monolithic C18 column (100 × 4.6 mm), mobile phase acetonitrile : phosphate buffer (pH 6.50) 10 : 90 (v/v), flow rate 1.0 mL min$^{-1}$. ASC—ascorbic acid, PHE—phenylephrine, PAR—paracetamol, SAL—salicylic acid, CAF—caffeine.

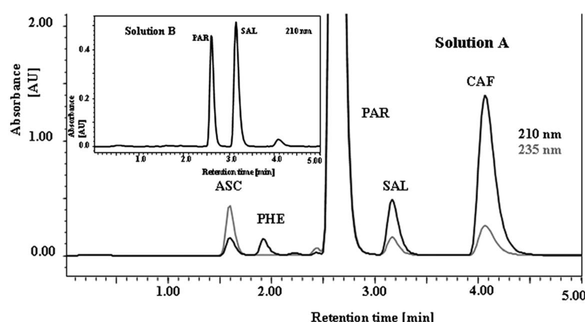


**Fig. 2** HPLC chromatogram of sample solution A and B (Coldrex tablets) using Onyx™ Monolithic C18 column (100 × 4.6 mm), mobile phase acetonitrile : phosphate buffer (pH 6.50) 10 : 90 (v/v), flow rate 1.0 mL min$^{-1}$. Main figure—solution A, inserted figure—solution B; ASC—ascorbic acid, PHE—phenylephrine, PAR—paracetamol, SAL—salicylic acid, CAF—caffeine.

correlation coefficient values were 0.9992 for ASC, 0.9994 for PHE, 0.9999 for PAR and 0.9992 for CAF, respectively.

To validate the precision (Table 2) of the method six sample solutions prepared from the same pharmaceutical formulation were analyzed consecutively; three injections of each preparation were used for evaluation. The results were expressed as RSD values and obtained results were 2.24% for ASC, 0.30% for PHE, 1.29% for PAR and 2.17% for CAF, respectively.

The accuracy of the method was carried out measuring the pharmaceutical samples fortified with a known quantity of the analytes (addition of 100% amount of the standard to sample solution) using only one concentration level following the recommendations of the local drug control authority. Spiked and un-spiked sample solution results were compared for recovery evaluation. Obtained values of the recoveries (and RSD) were 101.28% (2.51%) for ASC, 103.30% (1.81%) for PHE, 98.67% (1.80%) for PAR and 101.87% (3.64%) for CAF. Accuracy proved that the method allows direct determination of ASC, PHE, PAR and CAF in tablets of Coldrex formulation in the presence of other excipients.

The developed method was then applied for the determination of tested substances in the commercially available tablet, Coldrex (Smithkline Beecham Consumer Healthcare, United Kingdom), commonly used in virosis treatment. Active substance content was evaluated as percentage of labelled amount and obtained results were: 98.52% for ASC, 97.45% for PHE, 96.76% for PAR and 97.04% for CAF. These values were in set limits for content of the active ingredient in tablets with amount lower than 0.05 g (ASC, PHE, CAF; limit 5%) or higher than 0.05 g (PAR; limit 10%).

## Conclusions

The HPLC method with the monolithic column and UV spectrophotometric detection for the determination of ASC, PHE, PAR and CAF in the pharmaceutical formulation Coldrex tablets (GlaxoSmithKline Dungarvan Ltd., Co. Waterford, Ireland) using SAL as internal standard was developed. Complete separation of all mentioned compounds was carried out in 5 min with the 100 × 4.6 mm monolithic column. The mobile phase consumption was 5 mL (0.5 mL of acetonitrile) per single analysis. In the field of drug control, the modern trend of monolithic columns offers quick analysis compared to particle based columns and in the described determination analysis time was shortened 6-times.

As many pharmaceutical formulations used in virosis treatment (with the same active substances as Coldrex tablets) are produced, the proposed method can be used for the separation and simultaneous determination of tested compounds in different formulations after re-validation to the respective formulation.

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**Table 2** Method validation results

| Parameter | Criteria | Ascorbic acid | Phenylephrine | Paracetamol | Caffeine |
|---|---|---|---|---|---|
| Calibration range (μg mL$^{-1}$)[a] | | 150–450 | 25–75 | 50–150 | 125–375 |
| $A$ coefficient (%)[d] | | $0.0063 \pm 9 \times 10^{-5}$ | $0.0022 \pm 3 \times 10^{-5}$ | $0.0078 \pm 5 \times 10^{-5}$ | $0.0080 \pm 1 \times 10^{-4}$ |
| $B$ coefficient[d] | | $0.0190 \pm 0.0092$ | $8 \times 10^{-4} \pm 0.0029$ | $0.0160 \pm 0.0049$ | $0.0080 \pm 0.0120$ |
| Correlation coefficient | $R > 0.9900$ | 0.9992 | 0.9994 | 0.9999 | 0.9992 |
| Method precision (%)[b] | RSD < 5 | 2.24 | 0.30 | 1.29 | 2.17 |
| Accuracy (% RSD)[b] | RSD < 5 | 2.51 | 1.81 | 1.80 | 3.64 |
| Accuracy (% recovery) | 100 ± 5 | 101.28 | 103.30 | 98.67 | 101.87 |
| Determination of analytes (%)[c] | | 98.52 | 97.45 | 96.76 | 97.04 |

[a] Each concentration was measured in triplicate. [b] R.S.D. for repeated injections of sample preparations ($n = 6$), three injections of each preparation. [c] Percentage of labeled amount. [d] Linear regression $y = Ac + B$; $y$—ratio of peak areas of analyte and SAL; $c$—concentration in % of declared amount.

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