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**Original Article**

# Core shell column in high-performance liquid chromatography for the determination of polar compounds; troxerutin and carbazochrome

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**ABSTRACT**

**Background and Aims:** The combination of troxerutin and carbazochrome has been found efficacious for non-surgical patients with acute uncomplicated hemorrhoids. Therefore it is essential to develop a simultaneous analysis method of those drug substances. This study explains a simple and selective HPLC method to analyze troxerutin and carbazohrome in tablets.
**Methods:** The analysis was carried out using a core-shell pentafluorophenyl propyl polar column with a mobile phase consisting of (MeOH; % 5 HAc), (99.5:0.5 - V/V) at a flow rate of 0.25 mL/min. UV detection was set to 350 nm.
**Results:** The standard calibration curve was established between 1.00-20.00 µg/mL for troxerutin; 0.05-1.00 µg/mL for carbazochrome. LOD and LOQ were found to be 0.65 µg/mL and 1.00 µg/mL for troxerutin and 0.01 µg/mL and 0.05 µg/mL for carbazochrome, respectively.
**Conclusion:** The proposed method was validated for specificity, linearity, accuracy, precision, LOQ and LOD and then successfully applied for the analysis of these substances in tablets.

**Keywords:** Troxerutin, carbazochrome, high performance liquid chromatography, core-shell column, tablet

## INTRODUCTION

Hemorrhoids, a pathologic dilatation of the hemorrhoidal venous plexus, is a common clinical problem world-wide. Venous insufficiency, of which hemorrhoids is one type, is an underestimated public health problem and can have a serious impact on patients' quality of life. The underlying cause of hemorrhoids can be treated with venotonic agents. It has been found that flavonoids, such as troxerutin, are not only effective but also safe agents for the treatment of chronic venous insufficiency (Basile et al., 2001). Troxerutin (TROX) is a trihydroxyethylrutin (Figure 1). It has antithrombotic, antierythrocytic, fibrinolytic, oedema-protective, andrheological activity and has been used therapeutically to treat chronic venous insufficiency, varicose veins and haemorrhoids. Troxerutin significantly inhibits platelet adhesion to the extracellular matrix, yields an anti-erythrocyte aggregation effect and exerts a favorable action on the blood fibrinolytic system. This is the reason for its positive effect on capillary perfusion, stasis and trophic complications of chronic venous insufficiency (Basile et al., 2001; Cui et al., 2011). Chemical formula of Carbazochrome (CARBO) is 5,6-dioxo-3-hydroxy-1-methyl-2,3,5,6-tetrahydro-1H-indole 5-semicarbazone (Figure 1). It is an adrenochrome derivative and has been widely preferred for treatment of hemorrhage due to the fragility of capillaries. It has been shown that carbazochrome can decrease the pulmonary dysfunction and vascular hyper-permeability (Song et al., 2010). In patients with chronic venous insuf-

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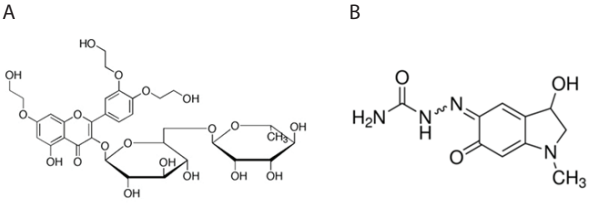


**Figure 1.** Structures of troxerutin (A) and carbazochrome (B).

ficiency, this combination of drugs has been proved to have a good efficacy and well tolerated (Squadrito et al., 2000).

Several methods were reported on each drug individually. In the literature, high-performance liquid chromatography (HPLC) methods (Joseph & Deepthi 2015; Hepsebah, Nihitha & Kumar, 2014; Yang et al., 2007), spectrophotometric method (Chen et al., 2015) and capillary electrophoresis method (Guo, Ron, Bı, & Sun, 2004) have been reported for analysis of troxerutin. Carbazochrom was examined using spectrophotometry (Gan et al., 2012; Wu & Liu, 2008), HPLC (Hu et al., 2014) methods, and the chemiluminescence method (Wang et al., 2011). Khattab et al. (2015) and Abdelrahman et al. (2016) determined that carbazochrome and troxerutin were from injectable pharmaceutical forms simultaneously.

The ability to retain and separate polar, hydrophilic molecules in reverse phase chromatography can be challenging and problematic. Separation generally requires the use of ion pair reagents, mobile phase and pH modification. C18 column has been used as a stainonary phase for HPLC methods in the literature (Joseph & Deepthi, 2015; Hepsebah et al., 2014; Yang et al., 2007; Hu et al., 2014; Abdelrahman, Abdelaleem, Ali & Emam, 2016). However we chose the core-shell pentafluorophenyl propyl polar column (PFP) to separete substances efficiently. Nowadays, the use of core shell technology has become increasingly popular to provide efficient separation and good peak shape in chromatography. Due to the core-shell particles, rapid mass transfer occurred and broadening from longitudinal diffusion, known as B term in the Van Deemter equation, was reduced (Hayes et al., 2014). The core shell particles column technology has gained a position of primary importance in HPLC due to advantages of resolution power.

The aim of this study is to develop a simple, safe method for the determination of the polar compounds based on UV- absorption detection with high performance liquid chromatography technique using core-shell PFP column and application of this method on tablet pharmaceutical preparations.

## MATERIALS AND METHODS

### Apparatus and reagents

The HPLC system of Agilent Infinity 1260 Series with UV detector were used for analysis of troxerutin and carbazochrome. The HPLC system consisted of 1260 Quat pump, 1260 ALS, 1290 Thermostat, 1260 TCC, 1260 DAD. Chemstation was used as instrument soft-ware.

Troxerutin and carbazochrome were obtained from World Medicine, Turkey with their certificates. Methanol and acetonitrile, which are HPLC grade, were purchased from Merck. Labels of acetic acid (100%) and analysis grade dimethyl sulfoxide were also obtained from Merck.

### Chromatographic conditions

Chromatographic separation was carried out at a temperature of 25°C using a Phenomenex Kinetex Core-shell Pentafluorophenyl Propyl, 2,6 µm, 4,6x150 mm column. The mobile phase consisted of (MeOH:%5 HAc)(99.5:0.5-V/V) (v/v). The flow rate of the mobile phase was 0.25 mL/min. The detector was set at 350 nm. The injection volume of 5 µL was chosen.

### Preparation of standard solutions and quality control samples

The stock solutions of troxerutin were prepared in ultrapure water (0.5 mg/mL) and diluted with methanol (100 µg/mL). The stock solutions of carbazochrome were prepared in dimethyl sulfoxide (0.5 mg/mL) and also diluted with methanol (5 µg/mL). All solutions were stored at 4°C until the end of the study. The five calibration standard containing different ratios of TROX (1-20 µg/mL) and CARBO (0.05-1.00 µg/mL) were prepared with methanol. The concentrations of quality control (QC) samples were at 1.00; 3.00; 15.00; 20.00 µg/mL for troxerutin and 0.05; 0.30; 0.70; 1.00 µg/mL for carbazochrome. All calibration standards and QC samples were stored at 4°C until the end of the study.

### Preparation of tablet samples

Ten tablets containing 300 mg TROX and 3 mg CARBO were powdered and ¼ tablet weight was diluted in a 50 ml flask to volume with dimethyl sulfoxide, 6 replicates were prepared in this way. Recovery samples were also prepared by spiking drug substances in to plecoba at the same concentration. Further dilution was carried out by transferring the appropriate amount in methanol for 15.00 ug/mL for TROX and 0.15 ug/mL for CARBO final concentration.

### Evaluation of the analytical method

The method development process is influenced by the nature of the analytes. The main part of this process is the selection of column and mobile phase composition. In this study, different column types and mobile phase compositions were used to separate CARBO and TROX. C18 and C8 columns with different length and particule sizes were tried to separate both subtances. The best separation was gained by using core-shell pentafluorophenyl propyl column. Firstly a system suitability test was performed to check the sufficiency of the system. Then validation parameters were performed. The limits were determined based on commonly recommended ranges according to EMA Regulations (EMA, 1995). Linearity specificity sensitivity, accuracy, precision parameters were evaluted. Back calculated concentrations of the calibration standards and quality control samples were within ± 15% of the nominal value, except for the LLOQ for which it was within ± 20%. LOD values were also calculated with the following expression:

LOD= 3.33 x SD of the regression line /slope

## RESULTS

Different column types and mobile phases were used to separate CARBO and TROX. C18 stationary phase with different

**Table 1. System suitability parameters of troxerutin and carbazochrome**

| Parameter | Value (Troxerutin) | Value (carbazochrome) | Limit (FDA guideline) |
|---|---|---|---|
| Retention Time | 5.67 | 6.07837 | - |
| Peak Width (W) | 0.1192 | 0.1617 | - |
| Tailing (T) | 1.32400 | 1.13889 | T ≤ 2 |
| Theoretical Plates (N) | 10974 | 6902 | N > 2000 |
| Injection Precision (RSD) | 0.424 | 0.189 | RSD% < 1% , n ≥ 5 |
| Resolution (R) | 2.9213 | 2.9213 | Rs > 2 |
| Capacity Factor (k) | 6.87 | 7.43 | k' > 2 |

FDA: Center for Drug Evaluation and Research

particle sizes and shapes was tried in order to separate two molecules but the resolution of the chromatograms was not satisfactory. The use of core-shell pentaflorophenyl propyl column provided relevant resolution.

Chromatographic separation of compounds was optimized to provide acceptable resolution, good peak shape and intensity of the response. Mobile phase composition was changed systematically to establish chromatographic conditions giving an acceptable resolution.

**System suitability test**

System suitability parameters summarized in Table 1 were within acceptable limits (Reviewer Guidance, FDA, 1994). There are also extra data in the table (retention times and peak widths of analytes). Data and calculations were produced by Agilent Chemstation Software.

**Specificity**

The specificity of method was assessed by analyzing the inactive ingredients of pharmaceutical preparations. Chromatograms of blank samples (sample without drug) and chromatograms of samples were compared for method selectivity (Figure 2). A clear baseline was seen and there was no interference at retention times of analytes.

**Limit of quantitation (LOQ) and limit of detection (LOD)**

The lower standard 1.00 µg/mL for TROX and 0.05 µg/mL for CARBO on the calibration curve were identified as the lower limit of quantification (LOQ) with a precision of less than or equal to 20%. LOD values were also calculated and found to be 0.65 µg/mL and 0.01 µg/mL for TROX and CARBO respectively.

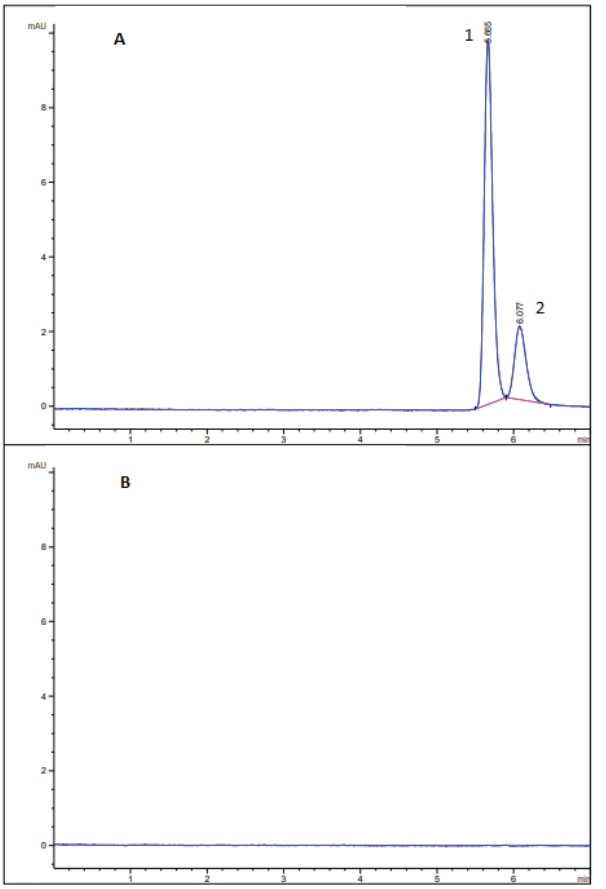


**Figure 2.** (A) Chromatograms of sample, (B) Placebo. 1: Troxerutin, 2: Carbazochrome.

**Linearity**

The calibration curves were prepared from five calibration samples within the range of 1.00-20.00 µg/mL including LOQ for TROX and 0.05-1.00 µg/mL including LOQ for CARBO. The standard calibration curves were linear over the concentration with mean r2= 0.9998 for TROX and r2= 0.9999 for CARBO. Regression equation of TROX was (y= 23.37416.x-1.24667) and regression equation of CARBO was (y= 72.58107.x -0.520126).

**Accuracy and precision**

Repeatability - intermediate precision and accuracy of TROX and CARBO were carried out within the range of calibration curves using six individual quality control samples at four concentrations including low, medium and high concentration QC samples according to EMA guidlines (EMA, 1995). The precision was expressed as coefficient of variation (CV%), accuracy was expressed as relative error (Table 2, Table 3). Analysis of these QC samples were carried out on three separate days.

The values of RD and CV% were within ±15% for the QC samples, except for the LLOQ which was within ±20%. The methods are quite accurate and precise.

**Table 2. Results of accuracy and intermediate precision for troxerutin**

| Sample | Concentration (µg/mL) | Average (µg/mL) | RD | SD | CV% | n |
|---|---|---|---|---|---|---|
| QC1 | 1.00 | 1.13 | 12.58 | 0.06 | 5.62 | 18 |
| QC2 | 3.00 | 3.10 | 3.42 | 0.08 | 2.48 | 18 |
| QC3 | 15.00 | 14.82 | -1.17 | 0.27 | 1.80 | 14 |
| QC4 | 20.00 | 19.90 | -0.52 | 0.46 | 2.29 | 18 |

n: Number of replicate at same concentration

**Table 3. Results of accuracy and intermediate precision for carbazochrome**

| Sample | Concentration (µg/mL) | Average (µg/mL) | RD | SD | CV% | n |
|---|---|---|---|---|---|---|
| QC1 | 0.05 | 0.05 | -2.06 | 0.01 | 11.38 | 15 |
| QC2 | 0.30 | 0.32 | 6.81 | 0.02 | 5.40 | 18 |
| QC3 | 0.70 | 0.72 | 3.29 | 0.04 | 6.08 | 18 |
| QC4 | 1.00 | 1.02 | 1.65 | 0.07 | 7.41 | 18 |

n: Number of replicate at same concentration

**Recovery of the drug substances**

Calculation of the percent recovery was made using equation of calibration curve. They were found to be 101.18±1.13% and 103.43±2.75% for TROX and CARBO respectively.

## DISCUSSION

TROX and CARBO have been shown to be safe and effective agents for the treatment of chronic venous insufficiency. There are therefore many separate studies about quantitative analyses of troxerutin and carbazochrome and a few combined studies. Khattab et al. (2015) developed the derivative spectrophotometric method for simultaneous determination of troxerutin and carbazochrome from injectable preparation. The spectrophotometric method is cheap and easy but lack of analysis automation is a major drawback. Abdelrahman et al. (2016) determined carbazochrome and troxerutin from injectable pharmaceuticals using the HPLC and HPTLC-densitometric method. While they used C18 column in their methods to apply to injectable pharmaceuticals, in our study core-shell PFP column was used to separate molecules and the method was applied to tablet forms of combination of troxerutin and carbazochrome. Abdelrahman et al. (2016) provided much better resolution, however the theoretical plates (N) values did not meet the acceptance criteria. We achieved a much better theoretical plates value and a better capacity factor. They did not perform injection precision for system suitability. Tailing factors and linearity values were nearly the same. The developed method was almost five times more sensitive than Abdelrahman et al.'s study. In conclusion, the present method is different and has powerful aspects for the determination of troxerutin and carbazochrome. In this paper, the method based on UV- absorption detection with HPLC technique using core-shell PFP column was developed. The electronic transitions in organic compounds can be determined by UV. TROX and CARBO molecules have conjugated π bonds and free electron pairs which are donated as n. These two compounds show $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. The absorption maxima of these two compounds was found to be 348 nm for TROX and 355 nm for CARBO. 350 nm were chosen for determination both of TROX and CARBO.

Sufficient retention and separation of very polar TROX and CARBO were not provided with traditional C18 column. In order to maximize efficiency, chromatographic separations, sensitivity and improve peak capacity, core-shell pentafluorophenyl propyl column was used. Electronegative flourine groups affect polar functional groups of analytes. Also planar interactions of column improve resolution. Interaction between π- π electrons of the carbon ring and π- π electrons of analyte contribute to the increasein retention time in non-acetonitrile mobile phases.

This method exhibited excellent regression in the range of 1.00-20.00 µg/mL including LOQ for TROX including LOQ (r= 0.9998) and 0.05-1.00 µg/mL including LOQ for CARBO (r= 0.9999). The accuracy and precision of the method was given in Table 2 for troxerutin and Table 3 for carbazochrome. RD and CV% terms were used to express the accuracy and precision of the method. The CV% and RD for troxerutin is within the range 1.80 - 5.62 and (-0.52) - 12.58 respectively. The CV% and RD for carbazochrome is within the range 5.40-11.38 and (-2.06)- 6.81 respectively. Values found were evaluated according to EMA guidelines (EMA, 2011). It was seen that they met the acceptance criteria (CV should be maximally 15% (LLOQ: 20%)). Deviation of LOQ samples was higher than the others but they also met the acceptance criteria because of having a wider limit range. In addition, the literature survey shows that most studies with high RSD value have low sensitivity. Hu et al. (2014) developed a method to carbazochrome sodium sulfonate for injection and studied 0.08865 mg/mL-0.4432 mg/mL calibra-

tion range. Hepsebah et al. (2014) determined troxerutin and calcium dobesilate simultaneously and found the linearity of the method over the range 62.5-250 µg/mL for both the drugs.

The method developed is sensitive and selective enough for the determination of TROX and CARBO from pharmaceuticals and also this method can be applied to biological material analysis, for example pharmacokinetic and therapeutic drug monitoring studies.

## CONCLUSION

The developed method for the determination of TROX-CARBO from pharmaceutical preparation was found to be accurate, precise, selective, and suitable for the quality control analysis. In this study, we demonstrated the relevant separation with a core shell column technique. Separation with core shell technology column resulted in significant reduction in solvent consumption and time, combined with good resolution for compounds and better selectivity without the need of high cost instruments such as mass spectrometry.

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**Conflict of Interest:** The authors have no conflict of interest to declare.

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