Simultaneous determination of ruscogenin, neoruscogenin, trimebutin, and parabens in cream formulation by reverse phase high performance liquid chromatography (RP-HPLC)

Gürkan Özen (gurkanozen@baskent.edu.tr)
Baskent University
Emirhan Nemutlu
Hacettepe University

Research Article

Keywords: Ruscogenin, neoruscogenin, trimebutine, propylparaben, methylparaben, determination, ointments, HPLC, validation

Posted Date: June 6th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2986997/v1

Version of Record: A version of this preprint was published at Chromatographia on October 4th, 2023. See the published version at https://doi.org/10.1007/s10337-023-04282-z.

Abstract

A simple and rapid reverse phase high performance liquid chromatography (RP-HPLC) method for analysis of ruscogenin (RUS), neoruscogenin (NRUS), trimebutine (TB), methylparaben (MP) propylparaben (PP) in pharmaceutical preparations has been developed and validated. In this study, a RP-HPLC method was developed for the determination of RUS, NRUS, TB, MP and PP and applied for analysis of pharmaceutical cream formulations. ACE C18 Column 121-2546 (250x4.6 mm) was used at 25 °C and gradient elution was performed with mobile phase consisting of a mixture of ACN:Buffer (pH:3.9). RUS, NRUS, TB, MP and PP eluted within 17 minutes. Peak homogeneity data of RUS, NRUS, TB, MP and PP in the pharmaceutical cream samples peaks obtained using photodiode array detector, in the cream sample chromatograms, demonstrated the specificity of the method. The developed method was validated according to the ICH guidelines. Linear ranges were 1.00, 5.00, 10.0, 25.0, 50.0, 100, 150 µg/mL for MP, RUS and 5.00 – 10.0, 25.0, 50.0, 100, 150, 200 µg/mL for NRUS, PP and 10.0-25.0, 50.0, 100, 150, 200 µg/mL for TB. Validation parameters, such as stability, linearity, sensitivity, accuracy, precision, recovery, robustness and ruggedness were evaluated according to ICH guidelines and the method was simple, rapid, selective, sensitive, accurate, precise, robust and rugged. We developed a fast, low-cost RP-HPLC method with time of 17 minutes for simultaneous analysis of RUS, NRUS, TB and preservatives (MP and PP) in pharmaceutical preparations with semi-solid dosage form. The developed method was successfully applied for the quantification of RUS, NRUS, TB and preservatives (MP and PP) in pharmaceutical preparations with semi-solid dosage form. The detection limit of the method was 0.07, 0.28, 0.07, 0.45, 0.02 µg mL^{-1} respectively for MP, TB, PP, NRUS and RUS.

Highlights

- This study is the first method for the simultaneous analysis of ruscogenin, neoruscogenin, trimebutine, propylparaben, and methylparaben.
- The developed RP-HPLC method is a reliable and stability-indicating for active pharmaceutical ingredients and preservatives.
- Validation studies were performed according to ICH guide.
- The developed method is simple, selective, and sensitive.

1. INTRODUCTION

Hemorrhoids are formed by aggregation of vascular tissues, smooth muscles and connective tissues extending in three columns along the anal canal in the left lateral, right anterior and right posterior positions. [1]. This disease has significant effects on human quality of life. Hemorrhoids is one of the most common diseases with a prevalence of 44% in western societies. [2, 3]. Medical treatment, instrumental treatment and surgical treatment methods are recommended according to the condition of the disease. [4]. Medical treatment methods never eliminate the disease but cause relief in the patient's symptoms. Semi-solid dosage formulations with active substance combinations of Ruscogenin (RUS) for anti-inflammatory

and anti-thrombotic activities and Trimebutin (TB) for antimuscarinic and weak mu opioid agonist effects can be used in medical treatment methods of hemorrhoids.

RUS was first isolated from the plant *Ruscus aculeatus* (Liliaceae family), a perennial rhizomatous and evergreen shrub. It is the steroid saponins of the traditional Chinese herb, Ophiopogon japonicas [5]. Saponins are glycosides that can be divided into two. The rhizomes of *R. aculeatus L.* contain steroidal saponins having ruscogenin (25R-spirost-5-ene-1b,3b-diol) and neoruscogenin ([spirosta-5, 25(27)-diene-1b,3b-diol) (Fig. 1a and b) [6]. In addition to its use in the treatment of hemorrhoids, this substances has also been reported to have beneficial effects in the treatment of acute and chronic inflammation, prevention of blood-brain barrier dysfunction and alleviation of acute lung injury, and pulmonary arterial hypertension diseases [7]. In addition to these, it suppresses hepatocellular carcinoma metastasis, alleviates lipopolysaccharide-induced pulmonary endothelial cell apoptosis, improves Sjögren's syndrome, and alleviates particulate matter-induced acute lung injury [8–11].

TB (2-(Dimethylamino)-2-phenylbutyl 3,4,5-trimethoxybenzoate) is a unique and multifunctional compound in its properties. TB has been used in the treatment of functional gastrointestinal disorders, particularly irritable bowel syndrome, has positive effects on wound healing, functional dyspepsia, and significantly inhibits the viability and colony formation of glioma cells with fatal malignancy [12–16].

Both RUS and TB active ingredients are commercially available as pharmaceutical cream formulations in semi-solid dosage form. These pharmaceutical creams are frequently used in the treatment of painful and itchy swollen anal symptoms, internal and external hemorrhoids, inflammatory diseases and symptomatic treatment of fissures, especially during hemorrhoid crises in the anal (breech) region. These cream preparations contain emulsifying beeswax, cetostearyl alcohol, isopropyl myristate, propylene glycol, glycerol monostearate, methyl parahydroxybenzoate (MP), propyl parahydroxybenzoate (PP), purified water, etc. as general excipients.

Parabens are widely used as preservatives in pharmaceutical and cosmetic products. Parabens can prolong the shelf life of products for months or even years by preventing the formation of bacteria and mold in the product. Parabens are often used in combination with other preservatives to protect the product from a wide variety of bacteria and microbes. Parabens have toxic and toxic properties. Reduced glutathione (GSH), an intracellular thiol plant, is known to provide protection against radical species. Consuming them by parabens can cause DNA damage[17]. An overall positive correlation was demonstrated between parabens and 8-hydroxy-2'-deoxyguanose, a marker of intracellular oxidative stress. Methylparaben is one of the parabens that increases the 8-hydroxy-2'-deoxyguanose substance the most. [18]. Due to the widespread use of parabens in cosmetic and pharmaceutical preparations, the determination of the amounts of these chemicals is of great importance.

There are many methods described in the literature for the determination of either RUS or TB alone from plants, biological materials, and pharmaceutical formulations. But, none of them simultaneously analyzed RUS and TB. Quantitative determination of RUS from plants and biological materials were performed using different analytical methods including HPLC [19–21], enzyme-linked immunosorbent (ELISA) [22], liquid

chromatography-tandem mass spectrometry (LC-MS/MS) [23, 24] and capillary electrophoresis [25]. None of these methods were developed for the analysis of RUS from pharmaceutical preparations. Reported methods for the determination of TB of from different matrices include HPLC [26–33], LC-MS/MS [34–36], spectrophotometric [28, 37–41], and electrochemical [42–48]. There was only one method developed and validated for TB determination from the pharmaceutical formulations [42]. A method is needed for the simultaneous determination of RUS, NRUS, TB active substances in pharmaceutical preparations as well as preservatives (MP and PP). We developed for the first-time simultaneous analysis of RUS, NRUS, TB and preservatives. However, in recent years, there has been an increasing interest in the simultaneous analysis of more than one active substance in a single pharmaceutical preparation with a single analytical method in a shorter and cheaper way. [49]. We developed a fast, low-cost RP-HPLC method with time of 17 minutes for simultaneous analysis of Rus, NRUS, TB and preservatives (MP and PP) in pharmaceutical preparations with semi-solid dosage form.

2. MATERIALS AND METHOD

2.1 Chemicals and reagents

RUS, NRUS and TB reference standard substances were kindly supplied from Abdi İbrahim Pharmaceutical Company Turkey. Pharmaceutical cream samples (Proctolog) were commercially obtained from a local pharmacy. All inactive placebo substances (emulsifying wax, cetostearyl alcohol, isopropyl myristate, propylene glycol, glycerol monostearate, methyl parahydroxybenzoate, propyl parahydroxybenzoate) were of analytical purity. MP, PP and HPLC-grade acetonitrile (ACN) and methanol were purchased from Merck-Sigma Aldrich (Darmstadt, Germany). Milli-Q water was obtained from a Barnstead Nanopure™ system from Thermo and all the solutions were prepared in Milli-Q water.

2.2 Apparatus and Chromatographic conditions

HPLC analyses were performed on a Shimadzu HPLC system. The liquid chromatographic system was comprised of a solvent delivery system (Shimadzu LC-20AB) and a diode array detector (Shimadzu SPD-M20A). The autosampler (Shimadzu SIL-20AC) was used for sample injection and the column was kept in the oven (Shimadzu CTO-20AC). Chromatographic separations were carried on a C18 column (ACE-121-2546). The mobile phase comprised sodium dihydrogen phosphate buffer (pH = 3.9) (A) and ACN (B) in a gradient mode as follows: 0–8. min, 80% B; 8–17. min, 95–0% A; 7.75–8.5 min, 0% A; 8.5–8.51 min, 0–95% A; 8.51–10 min, 95% A. The flow-rate was 0.225 mL min − 1(Table 1). The column temperature was set at 25°C. The injection volume was 10 µL and 200 nm was selected as the detection wavelength for the diode array detector. Peak identity was confirmed by retention time compariso*n.*

Table 1
Gradient elution program for the developed method.

Tıme (min.)	Flow Rate (mL/min)	Buffer pH 3.9 (A)	Acn (B)
0.0	1.0	80	20
1.0	1.0	80	20
8	1.0	20	80
12	2.0	20	80
14	2.0	20	80
17	1.0	80	20

2.3 Data processing

Data acquisition and processing were done with the LabSolutions software (version 1.25, Shimadzu). The quantification of RUS, NRUS, TB, MP and PP in the samples was performed using calibration curves. Therefore, least squares regression was used to create the calibration curves by plotting RUS, NRUS TB, MP and PP concentrations to the respected peak areas. The statistical calculations were carried out using Microsoft Excel software.

2.4 Preparation of Calibration Standards Solutions

The 3 µg/mL standard stock solutions of RUS, NRUS, TB, MP and PP were separately prepared in methanol. The calibration standard solutions were daily prepared from the standard stock solutions by diluting with the mobile phase (ACN:buffer 80:20 v/v). Linear ranges were 1.0-5.0-10-25-50-100-150 for MP, RUS and 5.0-10-25-50-100-150-200 µg/mL for NRUS, TB and PP.

2.5 Sample preparation

For the analysis,10 g of the cream (Proctolog®) was accurately weighed and transferred into 25 mL flask. Then 10 mL of methanol was added and sonicated for 30 min in an ultrasonic water bath at 50°C with occasional stirring. The sample was cooled to room temperature and filtered through a syringe strainer (Millex-LG, filter, 0.20 µm, Hydrophilic, PTFE, 25 mm). Then the volume was made up to 25 mL with methanol. Appropriate volumes were taken from final solution, diluted with ACN:buffer (pH:3.9) mixture (80:20 v/v), and analyzed with developed RP- HPLC method.

2.6 Placebo preparation

A placebo cream (all excipients excluding the active ingredient) was prepared according to the manufacturing protocol of the formulation. The placebo cream consisted of 3% cetostearyl alcohol, 5% propylene glycol, 1% glycerol monostearate, 0.07% methyl parahydroxybenzoate, 0.03%propyl parahydroxybenzoate and total 10% emulsifying beeswax, 5% ısopropyl myristate. Accurately weighed amounts of the placebo cream were analyzed at the concentration levels mentioned.

2.7 Analytical Method Validation

The method was validated according to the International council for harmonization of technical requirements for pharmaceuticals for human use (ICH) [50]. The following validation characteristics were addressed to selectivity, linearity, sensitivity, precision, accuracy, robustness, and ruggedness.

2.7.1. Selectivity

The selectivity of the RP-HPLC method was investigated by peak purity index (PPI) values of analytes and comparing chromatograms obtained from placebo and MP, TB, PP, RUS and NRUS spiked placebo. The samples were analyzed under the optimum analytical conditions.

2.7.2. Linearity

The linearity studies were carried out by analyzing calibration solutions containing different concentrations of MP, RUS (1.00, 5.00, 10.0, 25.0, 50.0, 100, 150 µg/mL), PP, NRUS (5.00, 10.0, 25.0, 50.0, 100, 150, 200 µg/mL) and TB (10.0–25.0, 50.0, 100, 150, 200 µg/mL) using the proposed RP-HPLC method. The calibration plots were created by plotting the peak area of MP, TB, PP, RUS and NRUS against the concentrations with least-squares linear regression analysis.

2.7.3. Sensitivity

The sensitivity of the developed RP-HPLC method was evaluated with limit of detection (LOD) and limit of quantitation (LOQ) values based on signal-to-noise ratio at 3:1 and 10:1, respectively for each item analyzed.

2.7.4. Precision and Accuracy

Intra-day and inter-day precision and accuracy were estimated by analyzing three replicates containing MP, TB, PP, RUS and NRUS at four different concentration levels (0.15, 5.0, 25.0, 100.0 µg/mL for MP, 0.84, 5.0, 50.0, 150.0 µg/mL for TB, 0.2, 5.0, 50.0, 150.0 µg/mL for PP, 1.35, 5.0, 50, 100 µg/mL for NRUS and 0.06, 5.0, 25.0, 100 µg/mL for RUS) on the same day and three consecutive days, respectively. Relative standard deviation (RSD %) and relative error (RE) for precision and accuracy, respectively were calculated at each concentration level. In addition, the accuracy of the developed RP-HPLC method was also examined with recovery studies.

2.7.5. Robustness

The robustness of the method was tested using experimental design approach. Small changes were made in ACN percentage of the mobile phase (19–21%), flow rate (0.9–1.1 mL/min) and column temperature (24–26°C), pH (3.8-4.0), buffer concentration (19–21 mM), and gradient program (ACN:buffer pH:3.9 ratio at 8th minute 79–81%). The results obtained were evaluated statistically.

3. Results and Discussion

3.2. Method optimization

During the method development, analysis conditions were optimized considering the physical and chemical properties of RUS, NRUS, TB, MP, PP and analysis time. The aim of the study, simultaneous analysis of the RUS and TB in the presence of preservatives. Therefore, we start with the coherent detection wavelength for each substance, and 200 nm was chosen as the detection wavelength. The optimization of the mobile phase was first performed in isocratic elution mode using different mixture of water and ACN. As a result of isocratic elution, while satisfactory peak solubility values were obtained for the active substances, it was observed that the parabens peaks were not completely separated. It also extended the isocritical elution mode analysis times up to 30 minutes. It was found that it is not possible to separate all compounds by isocratic elution. Therefore, a gradient elution mode had to be used. Different pH values (3-3.5-4-4.5-5-5.5-6-6.5) of 20 mM sodium dihydrogen phosphate (NaH_2PO_4) buffer were tested. The obtained values were evaluated in terms of peak resolution, symmetry, retention factor and capacity factor. As a result, the optimum pH value was chosen as 4. Then, to obtain better chromatographic conditions, mobile buffer phases adjusted to pH 3.8-3.9-4.1 and 4.2 were tested and it was decided to work at pH 3.9. Since the substances to be separated have different polarity values, a wide gradient elution program was used (from the 1st minute to the 8th minute, the ACN ratio was increased from 20–80% by volume). The retention times of the RUS and NRUS substances were considerably greater than the other substances. In order to reduce the retention times of these two substances and to achieve shorter analysis times, the flow rate was 2 ml/min from the 8th minute to the 12 th minute (Table 1). Various sizes of C18 columns were investigated in order to optimize the chromatographic parameters. Firstly, Agilent Eclipse (4.6x150mm) C18 column was tried. Low values of Rs occurred for the MP and TB substances. To eliminate this problem, a longer column, ACE-121-2546 (C18, 4.6x 250mm) was used. In the developed method, all standards and parabens were eluted in 14 minutes (Fig. 2). The system suitability of the developed RP-HPLC method under optimum analysis conditions by injection of 50 µg/mL standards (n = 6) was evaluated in terms of retention time (Rt), column efficiency (theoretical plate number, N), capacity factor (k'), resolution (R), peak purity index (PPI) and tailing factor parameters (Tf) (Table 2).

Table 2
System suitability parameters for the developed RP- HPLC method.

	Rt	k'	N	Rs*	Tf	PPI
MP	6.7	1.239	18052	5.32	1.459	0.9999
TB	7.9	1.636	16396	2.86	1.265	0.9904
PP	8.5	1.844	32263	16.23	1.387	0.9998
NRUS	12.4	3.142	29375	4.88	1.457	0.9972
RUS	13.5	3.500	129600	-	1.33	0.9963

** RS values were calculated according to the next peak.*

3.3. Analytical Method Validation

The RP-HPLC method was validated for selectivity, linearity, sensitivity, precision, accuracy, robustness, and ruggedness following the ICH guidelines [50].

3.3.1. Selectivity

For selectivity studies, a solution of placebo and standards (50 µg/mL) was prepared and analyzed (Fig. 2). No distracting peaks detected in the retention times of the analytes. In addition, the selectivity of the method was evaluated with the PPI (Table 2). Evaluation of peak purity was performed to ensure that no interfering substance contributed to the response of the peaks. The results showed that the developed RP-HPLC method is selective.

3.2.2 Linearity

The calibration curves of RUS, NRUS, TP, MP, and PP were determination and summarized in Table 3. The RP-HPLC method showed an acceptable linearity range in the concentration ranges shown in for MP, TB, PP, RUS and NRUS.

Table 3
Linearity parameters for MP, TB, PP, RUS and NRUS.

	Slope	P Value (Slope)	Intercept	P Value (Intercep)	R^2	LOD	LOQ
MP	48899	$1.79\ 10^{-11}$	10460	0.48	0.9998	0.05	0.15
TB	21644	$1.81\ 10^{-06}$	80142	0.23	0.9998	0.28	0.84
PP	37914	$1.63\ 10^{-06}$	-36610	0.82	0.9925	0.07	0.20
NRUS	36087	$2.42\ 10^{-06}$	168341	0.33	0.9913	0.45	1.35
RUS	21381	$1.74\ 10^{-10}$	168341	0,322	0.9998	0.02	0.06

3.2.3 Sensitivity

LOD and LOQ values in this study was selected as shown in Table 3. The developed method was highly sensitive for estimating MP, TB, PP, RUS and NRUS in samples.

3.2.4 Precision and Accuracy

Precision is the degree of repeatability of an analytical method under normal operational conditions. For intraday and interday precision and accuracy studies, three replicates of standard solutions of RUS, NRUS, TB, MP and PP (in four different concentrations covering the linear range) were prepared and analyzed

using the proposed method. Accuracy is the percent of analyte recovered by assay from a known added amount. The accuracy of the method was determined by recovery studies. The recovery study was carried out by performing analyzes on three solutions of different concentrations. In this study, 80%, 100% and 120% of the standard MP, TB, PP, RUS and NRUS within linear ranges were added to the placebo.

Results of intraday and interday precision and accuracy studies are summarized in Table 4. Low RSD and RE values indicate that the method is precise and accurate.

The recovery data of MP, TB, PP, NRUS and RUS are shown in Table 5. Recovery values for all analyzed compounds were between 98 and 102% and the relative standard deviation values (RSD) within the limit or < 2%.

Table 4
Intra- and inter-day accuracy and precision of the developed method (RE, relative error, RSD, relative standard deviation)

Concentration (µg/mL)	Intraday (n = 3)		Interday (n = 3)	
	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)
MP				
0.15	0.63	1.04	0.85	0.21
5.00	1.83	0.22	0.26	0.43
25.00	-0.37	1.52	0.04	0.60
100.00	0.87	0.70	-0.42	0.89
TB				
0.84	0.31	0.98	-0.24	1.17
5.00	-0.26	1.49	0.05	0.51
50.00	1.29	0.39	-0.17	0.59
150.00	0.34	0.80	0.27	0.68
PP				
0.20	-0.22	1.76	0.42	1.64
5.00	0.44	0.53	-0.39	0.44
50.00	0.99	0.73	-0.13	0.82
150.00	0.82	1.70	0.09	0.78
NRUS				
1.35	1.17	0.72	0.53	1.39
5.00	0.80	0.36	-0.47	0.68
50.00	-0.61	1.19	0.04	0.79
150.00	-0.98	0.42	0.66	0.53
RUS				
0.06	1.12	2.09	-1.11	1.12
5.00	1.22	1.28	-2.09	0.37
25.00	0.76	1.99	0.02	0.71
100.00	-0.88	1.39	0.93	0.25

Table 5

Results of recovery studies for the developed method. (n: number of repetitions, SE: standard error, RSD: relative standard deviation)

Initial Amount (µg/mL)	Amount Added (%), n = 3	Average Amount Found (µg/ml) ± SE	Average Recovery (%)	RSD (%)
MP				
25	80	20.01 ± 0.01	100.05	0.52
	100	24.92 ± 0.05	99.68	1.63
	120	30.04 ± 0.04	100.13	1.22
TB				
50	80	39.94 ± 0.04	99.85	0.79
	100	51.24 ± 0.07	102.48	1.19
	120	59.79 ± 0.12	98.65	1.79
PP				
50	80	40.35 ± 0.89	100.88	7.55
	100	50.64 ± 0.43	101.28	2.91
	120	59.44 ± 0.09	99.06	0.53
NRUS				
50	80	40.06 ± 1.04	100.15	4.51
	100	50.77 ± 0.96	101.54	3.33
	120	61.01 ± 0.51	101.68	1.44
RUS				
25	80	20.06 ± 1.04	100.3	2.41
	100	24.77 ± 0.96	99.08	1.33
	120	30.56 ± 0.51	101.86	1.19

3.2.5 Robustness

Robustness tests measure the effects of experimental indicators on analysis results. It can also be defined as the ability of the method to detect acceptable accuracy and precision of analytical results under various conditions. Robustness enables a decision to be made whether the developed method needs revalidation when one or more of its indicators are changed. ICH documents; recommends considering the robustness improvement of the method at the method development stage[51, 52]. For robustness tests of the

developed method, a nine-stage fractional factor design including seven experiments was applied under optimized conditions Table 6. The results of the analysis were statistically compared with ANOVA test and p values of regression coefficient and regression equation were calculated Table 7. When the results were evaluated statistically, there was no significant difference between them ($p \geq 0.05$). The lack of significant effects of small changes on peak area, retention time and peak symmetry indicates the robustness of the developed method.

Table 6
The parameters and their levels for robustness study.

Parameters	**Level**		
	-1	0	+ 1
ACN (%)	19	20	21
pH	3..8	3.9	4
Flow rate (mL/min)	0.9	1	1.1
Column Temp. (°C)	24	25	26
Buffer conc. (mM)	19	20	21
Detection wavelength (nm)	199	200	201
Gradient program (ACN rate at 8 min.)	70	80	81

Table 7
The experimental design and results for robustness study. (RT: retention time, PS: peak symmetry)

Exp. No	ACN (%)	pH	Flow rate (mL/min)	Column temp. (°C)	Buffer conc. (mM)	Det. wav. (nm)	Gradient prog. (ACN rate at 8 min.)	RT	Peak Area	PS
MP										
1	1	1	1	1	1	1	1	7.2	2690883	1.5
2	1	1	-1	1	-1	-1	-1	6.9	2566881	1.5
3	1	-1	1	-1	-1	1	-1	6.5	2259609	1.4
4	1	-1	-1	-1	1	-1	1	7.1	2509976	1.7
5	-1	1	1	-1	1	-1	-1	7.2	2549970	1.6
6	-1	1	-1	-1	-1	1	1	7.3	2410722	1.6
7	-1	-1	1	1	-1	-1	1	6.7	2455491	1.4
8	-1	-1	-1	1	1	1	-1	7.2	2440898	1.7
9	0	0	0	0	0	0	0	6.7	2628684	1.5
P values	0.58	0.77	0.20	0.51	0.24	0.72	0.65			
TB										
1	1	1	1	1	1	1	1	7.2	2430853	1.5
2	1	1	-1	1	-1	-1	-1	8.0	2187169	1.2
3	1	-1	1	-1	-1	1	-1	7.5	2364066	1.6
4	1	-1	-1	-1	1	-1	1	8.0	2234324	1.4
5	-1	1	1	-1	1	-1	-1	8.2	2157532	1.3
6	-1	1	-1	-1	-1	1	1	8.2	2227608	1.2
7	-1	-1	1	1	-1	-1	1	7.8	2169118	1.4
8	-1	-1	-1	1	1	1	-1	8.1	2281175	1.3
9	0	0	0	0	0	0	0	7.9	2473621	1.3
P Values	0.39	0.27	0.21	0.87	0.63	0.41	0.53			
PP										
1	1	1	1	1	1	1	1	8.8	2277676	1.5

Exp. No	ACN (%)	pH	Flow rate (mL/min)	Column temp. (°C)	Buffer conc. (mM)	Det. wav. (nm)	Gradient prog. (ACN rate at 8 min.)	RT	Peak Area	PS
MP										
2	1	1	-1	1	-1	-1	-1	8.6	2824055	1.6
3	1	-1	1	-1	-1	1	-1	8.4	2352782	1.4
4	1	-1	-1	-1	1	-1	1	8.7	2584573	1.5
5	-1	1	1	-1	1	-1	-1	9.1	2489970	1.4
6	-1	1	-1	-1	-1	1	1	8.8	2184157	1.6
7	-1	-1	1	1	-1	-1	1	8.6	2421267	1.5
8	-1	-1	-1	1	1	1	-1	8.7	2233926	1.5
9	0	0	0	0	0	0	0	8.5	2784888	1.4
P Values	0.84	0.59	0.33	0.67	0.51	0.93	0.64			
NRUS										
1	1	1	1	1	1	1	1	12.5	424755	1.5
2	1	1	-1	1	-1	-1	-1	12.2	660802	1.4
3	1	-1	1	-1	-1	1	-1	12.2	669229	1.4
4	1	-1	-1	-1	1	-1	1	12.3	685827	1.4
5	-1	1	1	-1	1	-1	-1	12.5	746453	1.4
6	-1	1	-1	-1	-1	1	1	12.4	638089	1.5
7	-1	-1	1	1	-1	-1	1	12.3	626078	1.5
8	-1	-1	-1	1	1	1	-1	12.5	692630	1.5
9	0	0	0	0	0	0	0	12.4	721360	1.5
P Values	0.02	0.14	0.35	0.06	0.11	0.06	0.07			
RUS										
1	1	1	1	1	1	1	1	13.1	38227	1.4
2	1	1	-1	1	-1	-1	-1	13.1	56168	1.6
3	1	-1	1	-1	-1	1	-1	13.1	73615	1.4

Exp. No	ACN (%)	pH	Flow rate (mL/min)	Column temp. (°C)	Buffer conc. (mM)	Det. wav. (nm)	Gradient prog. (ACN rate at 8 min.)	RT	Peak Area	PS
MP										
4	1	-1	-1	-1	1	-1	1	13.2	61724	1.4
5	-1	1	1	-1	1	-1	-1	13.4	89574	1.4
6	-1	1	-1	-1	-1	1	1	13.3	63808	1.6
7	-1	-1	1	1	-1	-1	1	13.2	64486	1.6
8	-1	-1	-1	1	1	1	-1	13.4	67877	1.4
9	0	0	0	0	0	0	0	13.5	81513	1.6
P Values	0.07	0.88	0.15	0.31	0.10	0.29	0.33			

3.2.6 Determination in pharmaceutical product

The chromatogram in Fig. 3 was obtained using the described RP-HPLC method with a topical cream (1%) sample. All compounds presented in the sample (MP, TB, PP, RUS and NRUS) are clearly separated. Since the active ingredients in the analyzed cream samples were stated as ruscogenins (RUS + NRUS), the sum of the RUS and NRUS peak areas was proportional to the total concentration in the calculation of ruscogenins. The average determined amounts of the MP, TB, PP, RUS and NRUS in pharmaceutical cream 1% (n = 3) is summarized in Table 8. Under the described chromatographic conditions, a linear relationship between peak areas and analyte concentrations were found for pharmaceutical cream.

Table 8
Analysis findings of pharmaceutical preparations by RP- HPLC method.

Cream (100 g)	MP (0.070 g)	TB (5.800 g)	PP (0.030 g)	RUS + NRUS (0.500 g)
Found values	0.071	5.800	0.031	0.510
	0.072	5.922	0.030	0.501
	0.071	5.701	0.031	0.499
	(n = 3)	(n = 3)	(n = 3)	(n = 3)
Mean ± standard error	0.071 ± 0.0003	5.808 ± 0.064	0.031 ± 0.0003	0.503 ± 0.003
SD	0.001	0.111	0.001	0.006
% RSD	0.809	1.906	1.883	1.164
Confidence interval (α = 0.05)	0.071–0.072	5.682–5.933	0.030–0.031	0.497–0.510

SD: Standard Deviation; % RSD: Relative Standard Deviation; Confidence Interval

4. Conclusion

In this study, an accurate and reliable RP-HPLC method with diode-array has been developed for the determination of MP, TB, PP, RUS and NRUS. Through the developed method, the five analytes were eluted and determined in a total time lower than 17 min. The method has been validated and the results obtained satisfactorily precise, simple, linear, specific, sensitive, and accurate. It was shown that the presence of cream placebo ingredients did not affect the MP, TB, PP, RUS and NRUS determination. The method can be used for routine analysis of compounds in pharmaceutical products containing active TB, RUS, preservatives MP and PP. this new method was successfully applied to the analysis of all compounds in a topical commercial rectal cream.

Declarations

Ethical Approval

Ethical approval is not required as we did not use animals, humans or blood cells in our study.

Competing interests

This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

Authors' contributions

Gürkan ÖZEN: Conceptualization, Methodology, Formal Analysis, Writing - original draft, Visualization, Writing - review & editing.

Emirhan Nemutlu: Conceptualization, Supervision, Methodology, Visualization, Writing - review & editing.

Funding

No financial support was received from any institution in this study.

Availability of data and materials

There are no restrictions for researchers to access the datasets used in the article.

References

1. Shafik A (2009) Surgical anatomy of hemorrhoids. Surgical Treatment of Hemorrhoids. Springer, London
2. Dennison A, Paraskevopoulos J, Kerrigan D, Shorthouse A (1996) Minerva chirurgica 51:209-216.
3. Lohsiriwat V (2012) World journal of gastroenterology: WJG 18:2009.
4. Higuero T, Abramowitz L, Castinel A, Fathallah N, Hemery P, Duhoux CL, Pigot F, Pillant-Le Moult H, Senéjoux A, Siproudhis L (2016) Journal of visceral surgery 153:213-218.
5. Ercan G, Tartar RI, Solmaz A, Gulcicek OB, Karagulle OO, Meric S, Cayoren H, Kusaslan R, Kemik A, Kayali DG (2020) Asian Journal of Surgery 43:405-416.
6. Nazemiyeh H, Zengin G, Mehrad H, Farhoudi M, Bahadori MB (2020) European Journal of Integrative Medicine 40:101245.
7. Fu F, Lai Q, Hu J, Zhang L, Zhu X, Kou J, Yu B, Li F (2022) Antioxidants 11:583.
8. Hua H, Zhu Y, Song Y-H (2018) Biomedicine & Pharmacotherapy 101:115-122.
9. Wu Y, Wang Y, Gong S, Tang J, Zhang J, Li F, Yu B, Zhang Y, Kou J (2020) Biomedicine & Pharmacotherapy 125:109868.
10. He J, Wang Y, Xu L, Xu C, Zhu Y, Xu M, Chen Y, Guo L, Hu W, Xu D (2022) Evidence-Based Complementary and Alternative Medicine 2022.
11. Wang Y-w, Wu Y-h, Zhang J-z, Tang J-h, Fan R-p, Li F, Yu B-y, Kou J-p, Zhang Y-y (2021) Acta Pharmacologica Sinica 42:726-734.
12. Salvioli B (2019) Minerva Gastroenterologica e Dietologica 65:229-238.
13. Fan Y-p, Liu P, Xue W-k, Zhao W-j, Pan H-c (2018) Frontiers in Pharmacology 9:664.
14. Kountouras J, Gavalas E, Papaefthymiou A, Tsechelidis I, Polyzos SA, Bor S, Diculescu M, Jadallah K, Tadeusz M, Karakan T (2020) Medicina 56:339.

15. Nakajima S, Ogawa N, Yokoue N, Tachibana H, Tamada K, Okazawa M, Sato A, Oyama T, Abe H, Kamiya T (2020) Biochemical and Biophysical Research Communications 533:1155-1161.
16. Lee H, Kwon O-B, Lee J-E, Jeon Y-H, Lee D-S, Min S-H, Kim J-W (2021) Cells 10:918.
17. Martín JMP, Peropadre A, Herrero Ó, Freire PF, Labrador V, Hazen MJ (2010) Mutation Research/Genetic Toxicology and Environmental Mutagenesis 702:86-91.
18. Liao Q, Huang H, Zhang X, Ma X, Peng J, Zhang Z, Chen C, Lv Y, Zhu X, Zheng J (2022) Chemosphere 308:136394.
19. Güvenç A, Şatır E, Coşkun M (2007) Chromatographia 66:141-145.
20. Nikolov S, Joneidi M, Panova D (1976) Die Pharmazie 31:611-612.
21. DING R, DI T-y, WANG G-l, LIN R-c, LIU L-f (2011) Chinese Journal of Pharmaceutical Analysis 31:127-130.
22. Liu N, Wen X, Liu J, Liang M, Zeng H, Lin Y, Yu B (2006) Analytical and bioanalytical chemistry 386:1727-1733.
23. Ji P-y, Li Z-w, Yang Q, Wu R (2015) Journal of Chromatography B 985:71-74.
24. Zhang H, Xu T, Gao L, Liu X, Liu J, Yu B (2017) Molecules 22:1250.
25. Huang B, Yao C, Bian Q, Wang Z, Mo J (2011) Yao xue xue bao= Acta Pharmaceutica Sinica 46:443-446.
26. Lavit M, Saivin S, Boudra H, Michel F, Martin A, Cahiez G, Labaune JP, Chomard JM, Houin G (2000) Arzneimittelforschung 50:640-644.
27. Joo E-H, Chang W-l, Oh I, Shin S-C, Na H-K, Lee Y-B (1999) Journal of Chromatography B: Biomedical Sciences and Applications 723:239-246.
28. El-Gindy A, Emara S, Hadad GM (2003) Journal of pharmaceutical and biomedical analysis 33:231-241.
29. Zaitsev VN, Syrotchuk OA, Didukh IR (2015) Journal of Chemical and Pharmaceutical Research 7:609-615.
30. LIN Z-h, SONG M (2001) Chinese Journal of Pharmaceutical Analysis 21:25-27.
31. WANG W, ZUO W-j (2003) Chinese Journal of Pharmaceutical Analysis 23:111-113.
32. Larabi IA, Duverneuil-Mayer C, Abe E, Baud F, Alvarez J-C (2015) Journal of Analytical Toxicology 39:720-725.
33. Mostafa AM S, Ahmad AB (1999).
34. Wang H, Zhou H, Horimoto S, Jiang J, Mayumi T, Hu P (2002) Journal of Chromatography B 779:173-187.
35. Qin Y, Zhao H, Zhang W, Zhao N, Fan P, Zhang L, Zhang H (2013) Life Sci. J 10:2840-2849.
36. YANG Z-d, ZHANG Q, MA B, HU Y-y, ZHENG Y-y, WANG Y-l (2012) Chinese Journal of Pharmaceutical Analysis 32:1351-1356.
37. Lee S-H, Lee J-H, Cho S, Do S-H, Woo Y-A (2012) Archives of pharmacal research 35:1599-1607.
38. Ayad M, El-Balkiny M, Hosny M, Metias Y (2016) Indian J. of Advances in Chem. Sci 4:85-97.

39. Abdel-Wadood HM (2002) Bulletin of Pharmaceutical Sciences. Assiut 25:137-144.
40. Shaban M (2002) Scientia Pharmaceutica 70:341-351.
41. Walash M, El Abass SA, Fathy M (2018) Anal. Methods 10:4511-4517.
42. Adhoum N, Monser L (2005) Journal of pharmaceutical and biomedical analysis 38:619-623.
43. Li F, Yu L (2001) Biomed. Chromatogr. 15:248-251.
44. Elqudaby H, Mohamed GG, El Din GM (2014) International Journal of Electrochemical Science 9:856-869.
45. Elqudaby HM, Mohamed GG, El Din GM (2013) journal of pharmacy research 7:686-691.
46. Ayad M, El-Balkiny M, Hosny M, Metias Y (2016) Indian J Adv Chem Sci 4:149-159.
47. Yu L, Li F (2001) Yao xue xue bao= Acta Pharmaceutica Sinica 36:131-133.
48. Ali TA, Mohamed GG, El-Sonbati AZ, Diab MA, Elkfass AM (2020) Iranian Journal of Pharmaceutical Research: IJPR 19:533.
49. Rasool N, Kanwal Q, Waseem M, Khan MI (2021) Biomed. Chromatogr. 35:e4997.
50. Guideline IHT (2005) Q2 (R1) 1:05.
51. Q8 I (2005) International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Topic Q9: Quality Risk Management Geneva
52. ICH-Q2B (1995) International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use: Validation of analytical procedures: Methodology.

Figures

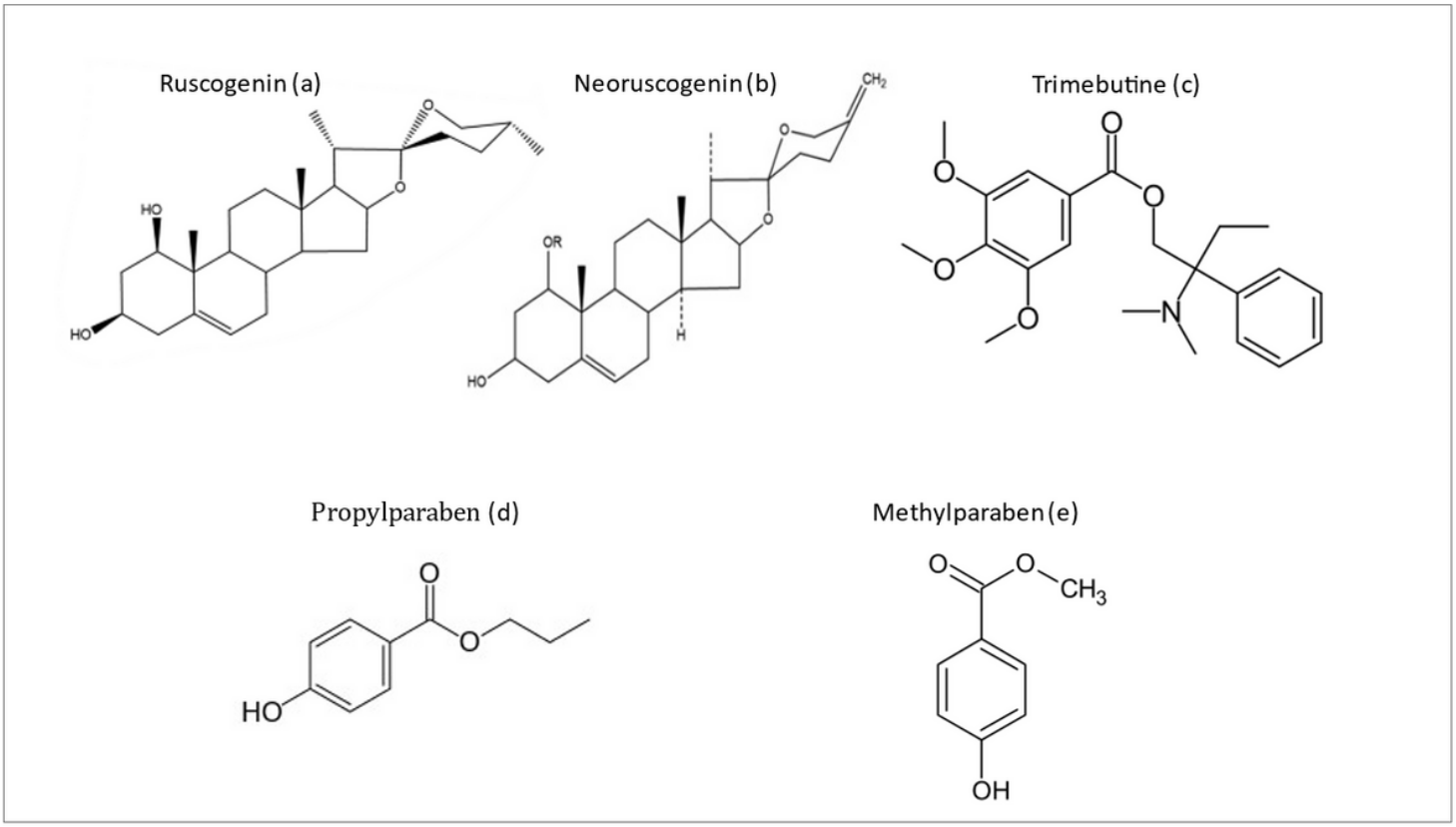

Figure 1

Chemical structures of ruscogenin (a), neoruscogenin (b), trimebutynin (c), propylparaben (d) and methylparaben (e)

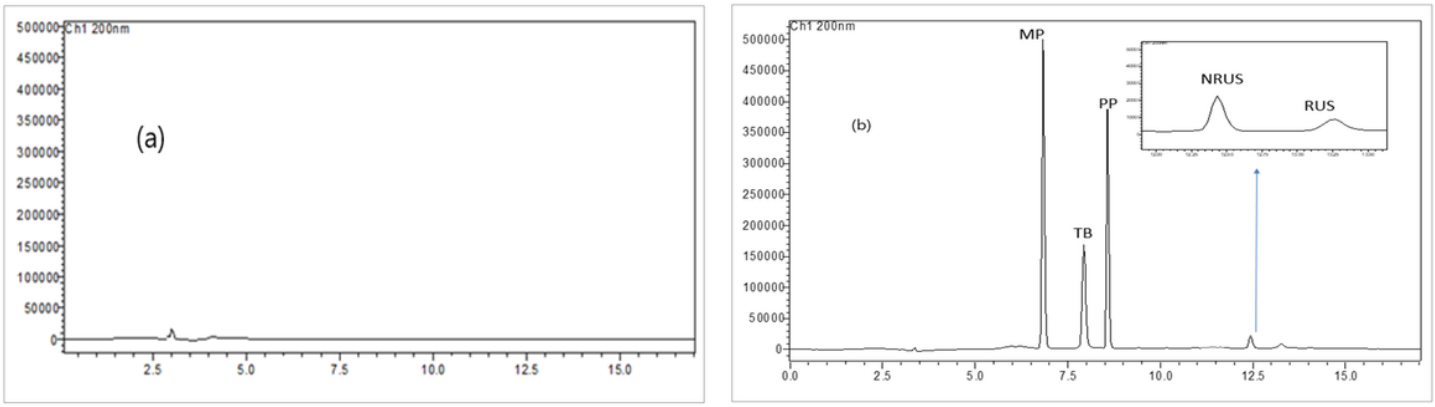

Figure 2

Chromatograms obtained under optimum chromatographic conditions; (a) Chromatogram of placebo of proctolog cream (b) MP, TB, PP, NRUS and RUS standard solution (50 µg/mL).

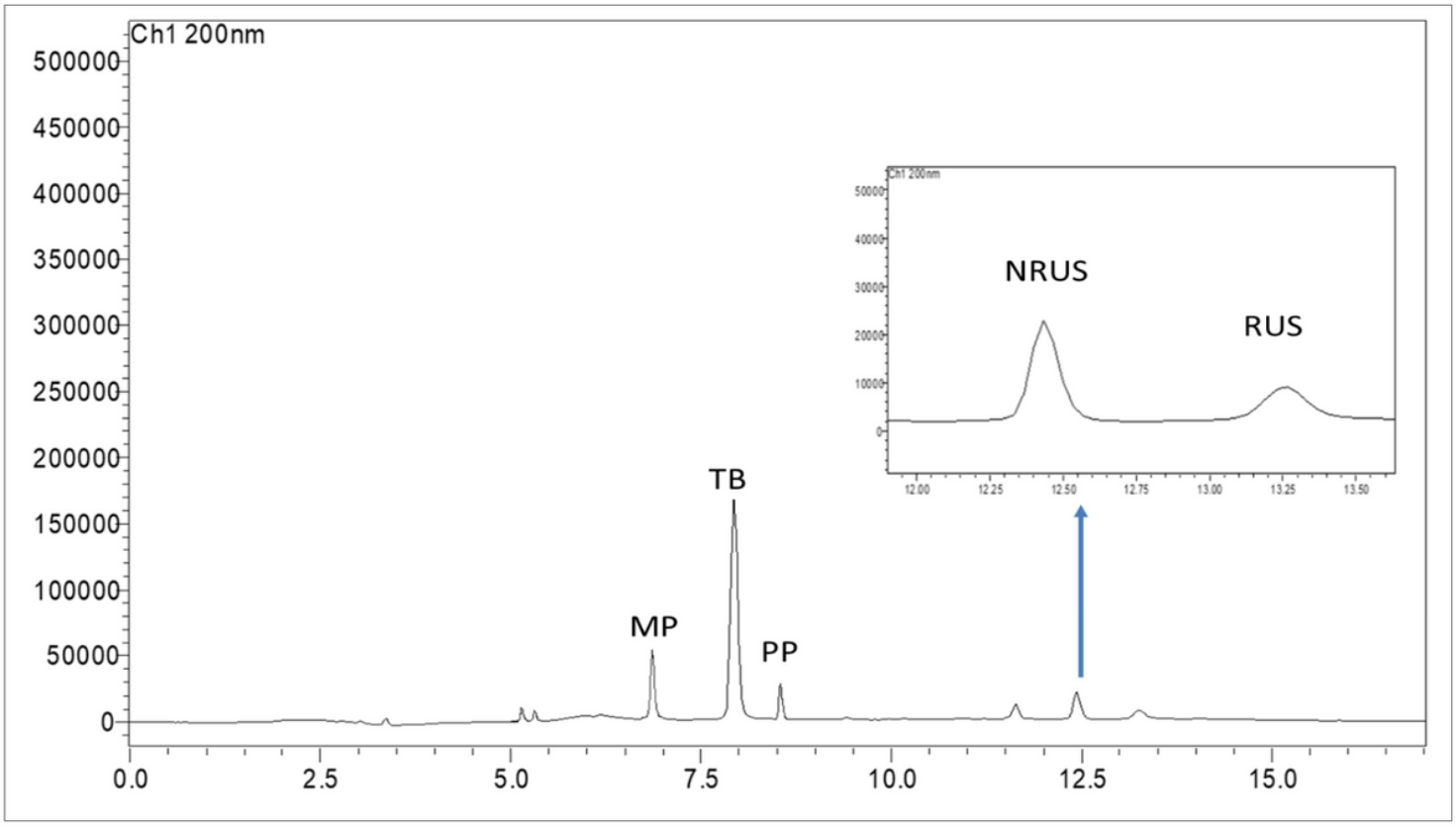

Figure 3

Chromatogram of 1% cream sample.