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Short communication

HPLC determination of calcium pantothenate and two preservatives in topical cream

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Abstract

A RP-HPLC method for simultaneous determination of calcium pantothenate and two preservatives methylparaben and propylparaben present in topical cream was developed. Different analytical columns with various stationary phases were tested. During method development, Supelco Discovery C18 column (125 mm × 4.0 mm, 5 μm) and Zorbax SB-CN column (150 mm × 4.6 mm, 5 μm) were tested. Both were not convenient for analytical separation because of the co-elution of calcium pantothenate with dead volume, and problems with the peak-shape of all components. Good separation was achieved using Zorbax TSM (250 mm × 4.6 mm, 5 μm) and Hypersil ODS column (250 mm × 4.6 mm, 5 μm), the latter was finally used for the analysis. The analysis time was 12 min, at flow rate 0.7 ml min^{-1}. Chromatography was performed using binary mobile phase composed of methanol and phosphoric acid, pH 2.5, 65:35 (v/v). UV detection was accomplished at 214 nm. The method was validated according to ICH guideline recommendations.

The method is suitable for practical routine analysis of commercially produced topical pharmaceutical preparations.

Keywords: Calcium pantothenate; HPLC; Pharmaceuticals; Parabens

1. Introduction

Calcium pantothenate (Fig. 1) is a calcium salt of pantothenic acid, chemically *N*-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-β-alanine [1].

Pantothenic acid is traditionally considered to be a Vitamin B_5. It is a component of coenzyme A which is essential in the metabolism of carbohydrates, fats and proteins. Pantothenic acid has no accepted therapeutic use in human medicine, though it has been often administered in form of calcium salt by mouth as a nutrition supplement, and usually with other vitamins of the B group. Calcium pantothenate is besides other B vitamins used as a supplement to treat, e.g. acne, osteoarthritis, rheumatoid arthritis, wounds, and to support the immune system [2].

Calcium pantothenate is used in many cosmetic and pharmaceutical topical preparations for its skin emollient, regeneration and hair conditioning properties. Calcium pantothenate has a positive effect on migration, proliferation and protein synthesis of human dermal fibroblasts. It is suitable for dry skin problems or as a supplement in treatment of dermatitis, eczema, herpes simplex and herpes zoster diseases [3].

Methylparaben (MP) and propylparaben (PP) are commonly used as preservatives in foods, beverages, cosmetics and pharmaceuticals because of their antibacterial and antifungal effect. MP and PP are used together since they have synergistic effect [4].

The European Pharmacopoeia (Ph. Eur. 4) used titration method for determination of calcium pantothenate. The substance is dissolved in 50 ml of anhydrous acetic acid R and titrated with 0.1 M perchloric acid determining the end-point potentiometrically [5].

USP 28 recommended titration method for the determination of calcium pantothenate using 0.05 edetate disodium VS until the solution was a distinct blue in color (hydroxy naphthol blue as indicator). Each milliliter of 0.05 M edetate disodium is equivalent to 2.004 mg of Ca [1].

Recently, there has been found a number of reports dealing with determination of calcium pantothenate and other vitamins in multivitamin preparations using high performance liquid chromatography [6–11].

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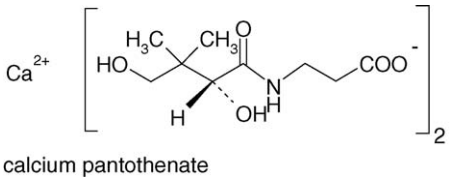

Fig. 1. Chemical structure of major component calcium pantothenate, a calcium salt.

A reverse phase liquid chromatographic method for determination of calcium pantothenate and dexpanthenol in pharmaceutical preparations using Nucleosil 5C18 column (150 mm × 4.6 mm) was developed, operated at 40 °C, with the detection at 208 nm. Elution was performed with 10 mM phosphate buffer, pH 3.0, and acetonitrile [12].

A chiral purity of calcium pantothenate was also determined, enantiomers were separated only partially using analytical column containing chiral stationary phase (CSP) MCI gel CRS 10W (50 mm × 4.6 mm, 3 μm) and mobile phase consisting of 2 mM $CuSO_4$ [13].

Another method used to study drug purity and pharmacokinetics of calcium pantothenate, D-pantheine, dexpanthenol, homopantothenic acid, pantolactone and pantheine was developed. All substances were separated on a Bondapack C18 column (50 mm × 4.1 mm) with 20 mM $KHPO_4$ (pH 5.0) and methanol as mobile phase, detection at 210 nm [14].

A thermospray LC–MS method was used for analysis of water-soluble vitamins. The analysis was performed on a Shim-pack CLC-ODS (M) column (150 mm × 4.6 mm, 5 μm) at 50 °C. Acetonitrile–H_2O containing 0.1 M formic acid and 0.1 M ammonium formate (7:93, v/v; pH 3.5) served as mobile phase. Detection was performed using TSI-MS [15].

None of these methods were developed and validated for the determination of calcium pantothenate with two preservatives in topical cream.

2. Experimental

2.1. Chemicals and reagents

Working standards of calcium pantothenate, methylparaben, propylparaben and ketoprofen were used for the purpose of this study. Calcium pantothenate active substance (*N*-(2,4-dihydroxy-3,3-dimethylbutyryl)-β-alanine calcium salt) was provided by Herbacos (Pardubice, Czech Republic). The reference standards of methylparaben and propylparaben were bought from Sigma–Aldrich GmbH, Germany. Ketoprofen (internal standard) was obtained from Herbacos (Pardubice, Czech Republic).

Chloroform for HPLC, Acetonitrile Chromasolv for HPLC gradient grade, and Methanol Chromasolv for HPLC were provided by Sigma–Aldrich (Prague, Czech Republic). Phosphoric acid 85% p.a. was obtained from Merck (Darmstadt, Germany). Natrium hydroxide was bought from Lachema (Brno, Czech Republic). HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and it met all European Pharmacopoeia requirements.

2.2. Chromatographic system

A Shimadzu LC-2010 C system (Shimadzu Corp., Kyoto, Japan) was applied to perform all the analyses. Detection was accomplished using built-in UV–VIS detector. The built-in auto sampler was conditioned at 25 °C. Chromatographic software Class VP 6.12 was used for data collection and processing.

Different analytical columns with various stationary phases were tested: the conventional octadecylsilica sorbent Supelco Discovery C18 (125 mm × 4.0 mm, 5 μm) Sigma–Aldrich (Prague, Czech Republic), Zorbax SB-CN (150 mm × 4.6 mm, 5 μm) and Zorbax TMS (250 mm × 4.6 mm, 5 μm) column. Zorbax columns were bought from Agilent Technologies (Prague, Czech Republic). The last analytical column tested was Hypersil ODS (250 mm × 4.6 mm, 5 μm) purchased by Hypersil, UK.

2.3. Reference standard preparation

The concentration of each compound in the reference standard was selected so as to correspond to concentrations in a real sample of topical cream.

First, the stock solution of an internal standard was prepared by dissolving of 100.0 mg of ketoprofen working standard in 100.0 ml of a mixture of methanol and phosphate buffer, pH 3.1 [16], 80:20 (v/v). Reference standard solution for calcium pantothenate cream analysis was prepared in 100 ml volumetric flask by dissolving 125.0 mg calcium pantothenate, 5.0 mg methylparaben and 1.25 mg propylparaben in a mixture of methanol and phosphate buffer, pH 3.1, 80:20 (v/v). Thereafter, 5.0 ml of internal standard ketoprofen stock solution were added and the flask was topped up to the volume with a mixture of methanol and phosphate buffer, pH 3.1, 80:20 (v/v).

Working solution of internal standard was prepared by diluting 5.0 ml of the internal standard stock solution by a mixture of methanol and phosphate buffer, pH 3.1, 80:20 (v/v) to a volume of 100 ml. The final concentration of the internal standard was always approximately 50 mg l^{-1}.

2.4. Sample preparation

0.5 g of the cream (corresponding to 25.0 mg of the active substance calcium pantothenate) was accurately weighted and transferred into a centrifuge flask. 2.0 ml of chloroform were added and the mixture was placed in a hot water bath at 75 °C for 20 min. 20.0 ml of working solution of internal standard ketoprofen were added. The mixture was placed in an ultrasonic bath for 20 min and centrifuged at 6000 rpm for 15 min. The supernatant was filtered trough the 0.45 μm filter and injected directly into the chromatographic system.

3. Results and discussion

3.1. Chromatographic conditions

HPLC with UV detection as a fast and simple separation analytical technique was chosen for determination of calcium pantothenate active substance and two preservatives present in

topical cream. Final chromatographic conditions and composition of mobile phase were optimized with the regards to the peak resolution, co-elution with dead volume, peak interference with placebo, and analysis time as well.

The aim of our study was firstly to find the optimal chromatographic conditions for separation of compounds in the sample and secondly the comparison of analyses performed on different stationary phases. The method should be robust enough for routine use in pharmaceutical control laboratory.

3.1.1. Optimal stationary and mobile phase

Firstly, Supelco Discovery C18 (125 mm × 4.0 mm, 5 μm) column packed with stationary phase based on octadecylsilica sorbent was examined for analysis. Mobile phases consisting of acetonitrile and water, or acetonitrile and phosphate buffer in pH range from 2.5 to 6.0 and in different volume ratios were tested. The peaks of methylparaben and propylparaben were separated well from calcium pantothenate. The main problem was the retention time of calcium pantothenate. Calcium pantothenate active substance co-eluted with dead volume under all tested conditions. This problem was not solved by using combinations of methanol and phosphoric acid, pH 2.5, as mobile phase as well.

Analytical column Zorbax SB-CN (150 mm × 4.6 mm, 5 μm) was tested thereafter. Mobile phases of similar compositions were tested, but the results obtained were still not satisfactory. The main problem was peak fronting and tailing of all separated components under chosen conditions and co-elution of calcium pantothenate with the dead volume.

Good results were obtained using Zorbax TMS (250 mm × 4.6 mm, 5 μm analytical column). Mobile phases consisting of acetonitrile and phosphate buffer in pH range from 2.5 to 6.0 and in different volume ratios were tested. This column enabled elution of calcium pantothenate separately from the dead volume (tested by using of a solution of potassium iodide) and also good separation of all tested components in 16 min was observed. Chromatography with a reference standard solution was performed using binary mobile phase composed of acetonitrile and phosphate buffer, pH 5.0 (27:73, v/v). The problem was placebo interferences (after isolation procedure) at the same retention times of tested compounds.

3.2. Comparison of two TMS columns of various series

The next step was to compare analyses results achieved with two Zorbax TMS columns (Agilent Technologies, 250 mm × 4.6 mm, 5 μm) manufactured in different batches (different serial number). Zorbax TMS columns were labeled as Number 1 and Number 2. The optimal conditions for separation with Zorbax TMS Nr. 1 column were: acetonitrile and phosphate buffer, pH 5.0 (27:73, v/v), as mobile phase at flow rate 0.6 ml min^{-1}. Ethylparaben 50 mg l^{-1} was used as internal standard.

Analysis with Zorbax TMS Nr. 2 under same conditions was completely different—the retention time of calcium pantothenate increased only about 30 s, but retention time of methylparaben increased from 8.8 min (with column Nr. 1) to 18.8 min (with column Nr. 2). Fig. 2 demonstrates changes in retention time.

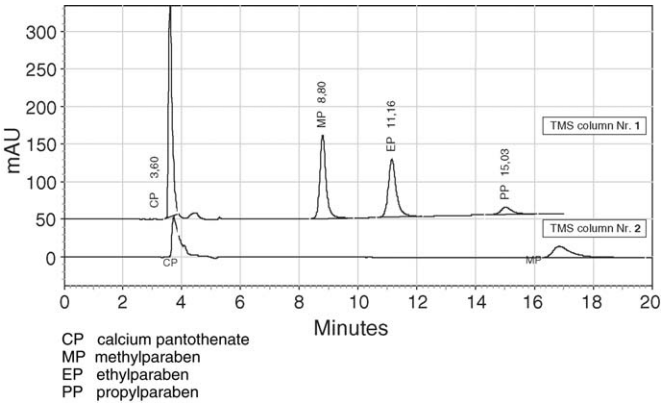

Fig. 2. Chromatogram 1: chromatogram at 214 nm, analysis of compounds in standard solution, demonstrates changing of retention time of preservative methylparaben using two identical columns of various batches.

A quicker analysis was achieved with acetonitrile and phosphate buffer, pH 5.0 (50:50, v/v), as mobile phase at flow rate 0.8 ml min^{-1}. The main problem was tailing of all peaks in the chromatogram. The mixture of acetonitrile and phosphoric acid, pH 3.1 (50:50, v/v), was also tested. A double peak of the active substance calcium pantothenate was observed. In the next step mixtures of methanol and phosphoric acid, pH 2.5, in different volume ratios were tested as binary mobile phases. Satisfactory results for analysis of reference standard solution were achieved with a binary mobile phase consisting of methanol and phosphoric acid, pH 2.5 (55:45, v/v), using flurbiprofen as internal standard (concentration of flurbiprofen 50 mg l^{-1}). The analysis time was 10 min. This method however was not convenient for our purposes, because it was not robust and not sufficiently reproducible. The decrease of retention times of propylparaben and flurbiprofen (IS) was 1 min in 28 analyses. The R.S.D. (%) of flurbiprofen IS retention time reproducibility was higher than 1% (nine replicates made).

TMS column is designed for high stability at low pH (e.g. <5). The analyses were performed in acidic pH (aqueous part of mobile phase, pH 2.5). The temperature effect was tested (column oven control). Finally, it was concluded that TMS analytical column is not convenient for analysis of calcium pantothenate.

The Zorbax TMS column was changed for a Hypersil ODS column (250 mm × 4.6 mm, 5 μm). Mobile phases consisting of methanol and phosphoric acid, pH 2.5, or methanol and water in different volume ratios were tested. Calcium pantothenate was eluted after dead volume, separated enough and also, all other compounds were separated well. Finally, chromatography with a standard reference solution (Fig. 3) as well as with a real sample (after isolation procedure) was performed using binary mobile phase composed of methanol and phosphoric acid, pH 2.5 (65:35, v/v), at flow rate 0.7 ml min^{-1}.

3.2.1. Detection wavelength

The detection wavelength 214 nm was chosen with regards to the UV spectrum of calcium pantothenate and both preservatives.

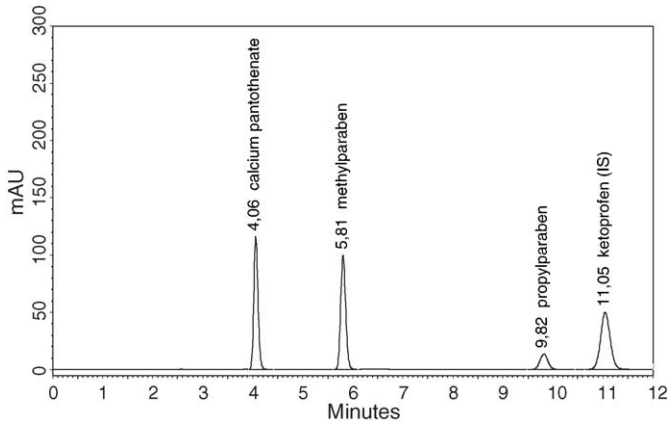

Fig. 3. Chromatogram 3: chromatogram at 214 nm, analysis of compounds in standard solution—calcium pantothenate (active substance), methylparaben, propylparaben and ketoprofen (IS).

3.2.2. Internal standard

Analysis of creams and gels requires using of the method of internal standard, mainly for the reason of recovery problems during sample preparation procedures. Ethylparaben, butylparaben, flurbiprofen and ketoprofen were tested as internal standards. Ketoprofen has been chosen as internal standard for analysis with Hypersil ODS column due to the convenient retention time. The analysis occurred without placebo interferences and any interference with a peak of solvent from isolation procedure (chloroform).

3.3. Isolation procedure

The isolation procedure was based on extraction procedures routinely applied for analysis of topical preparations in our laboratory, which include sonication and centrifugation. The isolation procedure commonly used in our laboratory includes extraction into organic solvent (or appropriate mixture of solvents), 20 min of sonication followed by centrifugation for 15 min at 3000 rpm. Using such a procedure the recovery was 45% for parabens and only 10% for calcium pantothenate.

Acetonitrile and methanol were tested as extraction media. Neither acetonitrile nor methanol gave satisfactory results related to the recovery. Mixtures of acetonitrile as well as of methanol with phosphate buffer in pH range from 2.5 to 6.0 in various volume ratios were tested. Extraction procedures by elevated as well as by reduced temperature were examined. Finally, a mixture of methanol and phosphate buffer, pH 3.1, 80:20 (v/v) was used as extraction medium. The optimal time of sonication and centrifugation was tested. Isolation procedure including 20 min in hot water bath at 75 °C, followed by 20 min of sonication and centrifugation for 15 min at 6000 rpm gave recovery about 85%.

It was necessary to dissolve the topical cream in 2 ml of chloroform before adding the extraction medium. Chloroform was evaporated under elevated temperature after dissolving and subsequently the chosen extraction medium was added. The above-described procedure meets the requirements of recovery in range of 95–105% for all tested compounds. The chromatogram shown in Fig. 4 illustrates the separation of all compounds tested after isolation from a pharmaceutical preparation. In placebo background there were no other substances, which might co-elute with tested analytes. This was verified during the method validation.

3.4. Method validation

After developing and optimizing of chromatographic conditions it was necessary to carry out the method validation. SST and validation parameters were measured according to ICH guideline recommendations (Q2A and Q2B) [17] and in-house guidelines of our laboratory.

Method validations covers System Suitability Test (repeatability, number of theoretical plates, resolution and asymmetry) and the determination of validation parameters including

Table 1
Method validation results

Parameter	Calcium pantothenate	Methylparaben	Propylparaben	Criteria
SST				
Repeatibility—retention time[a] (R.S.D (%))	0.01	0.06	0.04	$X < 1\%$
Repeatibilty—area[a] (R.S.D. (%))	0.12	0.23	0.15	$X < 1\%$
Theoretical plates[b]	10042	14860	17068	$N > 1500$
Resolution[b]	9.94	10.07	16.15	$R_{ij} > 1.5$
Asymmetry[b]	1.14	0.95	1.06	$T < 2$
Validation				
Precision[c] (R.S.D. (%))	3.36	1.65	1.60	$X < 5\%$
Linearity[d] (correlation coefficient)	0.99981	0.99987	0.99985	$R > 0.9990$
Accuracy[c]				
Recovery (%)	100.25	100.69	101.92	$X = 100 \pm 5\%$
R.S.D. (%)	0.65	0.74	0.47	$X < 5\%$
Selectivity	No interference	No interference	No interference	

[a] Made in six replicates.
[b] Made in three replicates.
[c] Six samples injected three times each.
[d] At 40, 60, 80, 10, 120 and 140% levels, three replicates.

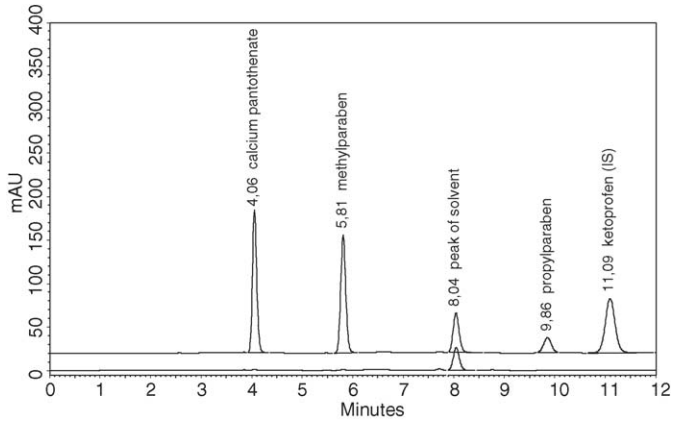

Fig. 4. Chromatogram 3: chromatogram at 214 nm, analysis of topical pharmaceutical preparation calcium pantothenicum cream and placebo chromatogram to demonstrate method selectivity.

accuracy, precision, selectivity, linearity (calibration curve with correlation coefficient) and short-term stability.

Relative standard deviation (R.S.D.) values were calculated for repeated standard solution injections to check the method repeatability as well as repeated injections of multiple sample preparations to check method precision. Accuracy was measured using placebo samples spiked with standard solution. Linearity was determined in 40–160% range of concentrations of substances present in topical cream. Selectivity was checked by comparison of chromatograms of standard solution and placebo solution analyses.

The short-term stability of all compounds was evaluated by comparison of response factors of freshly prepared and stored standards. The standard solutions were analyzed during storage at decreased temperature (4 °C).

The method validation results are summarized in Table 1. All tested parameters meet the requirements of regulative authorities.

4. Conclusion

HPLC method with UV detection at 214 nm using analytical column Hypersil ODS for the separation and determination of calcium pantothenate and two preservatives present in topical cream was developed and validated.

It was found out, that the results of analyses performed under the same conditions with two identical analytical columns produced by one manufacturer can be very different. To achieve satisfactory results it was necessary to change not only the volume ratios of aqueous components in the mobile phase but also the pH value of aqueous part of mobile phase and also the organic phase of binary mobile phase.

The analysis time of present HPLC method was less than 15 min. The method can be used in analytical drug control laboratory for routine quality control of pharmaceuticals containing calcium pantothenate and preservatives methylparaben and propylparaben.

Acknowledgements

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