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DEVELOPMENT OF A REVERSED-PHASE HPLC METHOD FOR ANALYSIS OF FLUOCINOLONE ACETONIDE IN GEL AND OINTMENT

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SUMMARY

An accurate and precise HPLC assay has been established for simultaneous determination of fluocinolone acetonide and additives in gel. Drugs were chromatographed on a C_{18} reversed-phase column with 55:45 (*v*/*v*) methanol–water as mobile phase and detection at 238 nm. Solution concentrations were measured on a weight basis to avoid the use of an internal standard. The method was statistically validated for linearity, accuracy, precision, and selectivity. Linearity for assay of fluocinolone acetonide, methyl 4-hydroxybenzoate (nipagin M), and propyl 4-hydroxybenzoate (nipagin P) were confirmed over the ranges 0.5–30, 5–200, and 10–120 μg mL^{-1}, respectively. Because of the simplicity and accuracy of the method, it was used for routine analysis of fluocinolone acetonide in ointment. It does not require any specific sample preparation.

INTRODUCTION

Fluocinolone acetonide (6α,9α-difluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxy-1,4-diene, 3,20-dione) is a corticosteroid used topically for treatment of a variety of skin disorders and inflammatory eye, ear, and nose diseases. It has high anti-inflammatory activity and is usually used formulated as a cream, gel, lotion, or ointment.

Detection and quantitative determination of fluocinolone acetonide in routine analysis of biological matrices, including human plasma, serum, and urine, is accomplished by variety of methods, including gas chromatography–mass spectrometry (GC–MS) [1–3], high-performance liquid chromatography–mass spectrometry (HPLC–MS) [4,5], and high-performance liquid chromatography with fluorimetric [6] or UV (HPLC–UV) [7] detection. Other analytical methods widely used to determine corticosteroids

are based on radioimmunoassay (RIA) procedures; these are characterized by high sensitivity but also by an important lack of specificity, owing to the cross-reactivity of related compounds [8,9].

GC–MS methods, although highly sensitive and specific, require derivatization before injection, because of the low volatility of these compounds, which are also thermally unstable [1–3]. In addition to the long separation time, necessary derivatization of the analytes complicates sample preparation, and modifying the chemical structure of the molecules reduces the quality of information and the selectivity.

HPLC enables analysis of corticosteroids without derivatization and results in high sensitivity and selectivity with normal or reversed-phase packings and isocratic or gradient elution. Fluocinolone acetonide has been determined in several biological fluids for doping control [10] and for clinical and pharmacokinetic studies [11]. Before analysis of corticosteroids by HPLC, sample pretreatment is usually necessary; methods include enzymatic hydrolysis, usually with β-glucuronidase [12], and solid-phase extraction [4,5,7] or double liquid–liquid extraction [13]. Liquid chromatographic methods with minimum sample preparation, i.e. without derivatization and complex extraction, but of high sensitivity, have not been widely employed. Reversed phase (RP) HPLC methods used for analysis of pharmaceutical creams and ointments usually require elaborate sample pretreatment, including removal of interferences and extraction of the analytes; this is problematical and frequently leads to low and variable recovery. Fluocinolone in cream has been analysed by high-performance liquid chromatography with fluorimetric detection after derivatization [14].

Fluocinolone acetonide in cosmetic products has also been determined by thin-layer chromatography combined with high-performance liquid chromatography (TLC–HPLC) [15], but the method is time-consuming and expensive solid-phase extraction was used.

Although a few micellar electrokinetic chromatographic (MEKC) and micellar liquid chromatographic (MLC) methods for determination of fluocinolone acetonide in very complex mixtures [16] and pharmaceutical products, for example cream and ointment [17,18], have been reported in the literature, no papers report concurrent identification and quantification of the active substance and other substances in gel.

The purpose of the work reported here was to develop an HPLC procedure for determination of fluocinolone acetonide in different drug formulations. Because methyl and propyl 4-hydroxybenzoate are used in many drugs, including gels and creams, in the work discussed in this pa-

per conditions for concurrent identification and quantitative determination of active substance and additives, if any, were established for HPLC with reversed-phase partition. This paper reports a simple, sensitive, and rapid method with minimum sample preparation, that is sufficiently sensitive for simultaneous determination of fluocinolone acetonide and additives in gel by HPLC with UV absorbance detection. The method requires limited pretreatment and is sufficiently sensitive to be an alternative to that proposed in Farmakopea Polska VI for determination of this active substance in two formulations (gel and ointment) [19].

EXPERIMENTAL

Chemicals and Materials

Fluocinolone acetonide substance and preparations (Flucinar gel and ointment) were purchased from Jelfa (Jelenia Góra, Poland). Other compounds (methyl 4-hydroxybenzoate and propyl 4-hydroxybenzoate) and internal standard (ethyl 4-hydroxybenzoate) were obtained from Fluka Chemie (Switzerland). Ethanol (UV grade) used for sample preparation was supplied by POCh (Gliwice, Poland). Methanol (HPLC grade) for preparing the mobile phase was from Merck (Darmstadt, Germany). Water was redistilled.

Apparatus and Chromatographic Conditions

High-performance liquid chromatography was performed with a Knauer (Berlin, Germany) system comprising a Mini-Star K-500 pump, a K-2500 UV detector, operating at 238 nm, and a Knauer interface box. Compounds were separated on a 125 mm × 4 mm i.d., 5 μm particle, Nucleosil 100 RP-18 analytical column from Knauer. Methanol–water, 55:45 (*v*/*v*), at 1 mL min^{-1} was used as mobile phase. The same volumes of standard and sample solutions were introduced to the column by means of a 20-μL loop (Rheodyne) and the corresponding chromatographic data were collected and processed with a computer system for data acquisition (Eurochrom 2000). Individual peaks were identified from retention time and concentrations were derived from the peak area for appropriate standard and sample solutions. Analysis was performed at room temperature.

Other laboratory equipment used was a temperature-controlled centrifuge and an instrument for mechanical shaking of samples.

Extraction Procedure

Ethanol (96%; 4 mL) and internal standard solution in ethanol (1 mg mL^{-1}, 30 μL) were added to 0.1 g gel. The tubes were capped and shaken vigorously in a water bath for 0.5 h at 50ºC. The samples were then removed from the bath and frozen at −20ºC. The organic layer was decanted and the extraction procedure was repeated. The solidified aqueous layer was then discarded. The ethanol extracts were combined and evaporated to dryness on a water bath at 80ºC under a stream of air. The residue was dissolved in 1 mL mobile phase. After centrifugation 20 μL of this mixture was injected into the chromatograph. Gel calibration standards were prepared containing 5, 10, 15, 20, 25, and 30 μg mL^{-1} fluocinolone acetonide and 30 μg mL^{-1} internal standard; replicate analysis was then performed to measure the recovery, precision, and linearity of the method.

RESULTS AND DISCUSSION

The method described required systematic optimisation of methanol–water mixtures. The internal standard, nipagin A, was chosen because it is structurally similar to additives nipagin M and nipagin P.

To verify its suitability for determination of fluocinolone acetonide and other additives in gel, the method was subjected to preliminary validation. Chromatogram peaks were identified for appropriate solutions of the constituents under investigation and the effect of vehicle (gel or ointment base, also termed matrix) was examined. (The term “matrix” denotes all components of the ointment except for the active substance or all the ingredients of the gel except active substance and preservatives.) With 55:45 (*v*/*v*) methanol–water as mobile phase retention times obtained for the gel were approximately 6.97 min for fluocinolone acetonide and 2.4 and 5.95 min for nipagin M and nipagin P, respectively. Nipagin A, retention time 3.6 min, was used as internal standard. Examples of the chromatograms obtained are presented in Figs 1A and 1B. It is clearly apparent that fluocinolone acetonide was well separated from additives under the conditions mentioned above.

Chromatographic conditions proposed by European Pharmacopoeia 5.0 for determination of the substance results in a longer sample-analysis time (retention time for fluocinolone acetonide was 10 min) [20] compared with our method (retention time 6.97 min, and complete analysis time for

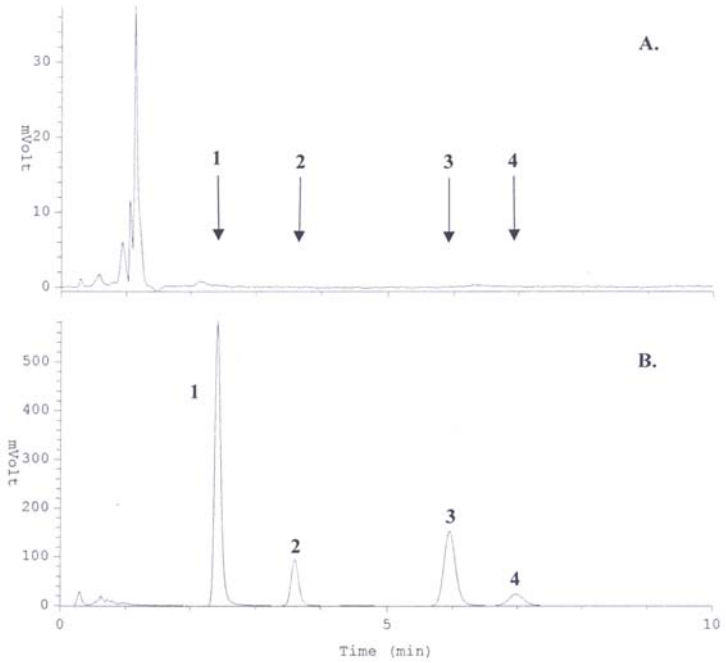

Fig. 1

Chromatograms obtained from vehicle gel extract (A) and Flucinar gel extract (B) containing of nipagin M (1), nipagin A (2; internal standard), nipagin P (3) and fluocinolone acetonide (4) at concentrations 155, 30, 86, and 23.25 μg mL^{-1}, respectively

a single sample was 10 min). Short assay time is very important in analysis of series of many samples.

Further investigations were focused on the effect of the matrix used for the drug under examination. Solutions were prepared containing the gel base at concentrations corresponding to those in the drug. Although individual signals in the form of peaks were recorded, their retention times were different from those of nipagin A (internal standard), nipagin M, nipagin P, or fluocinolone acetonide, thus indicating no effect on the determination of these constituents (Fig. 1B). It was found that chromatogram peaks of retention time below 2 min originated from the vehicle constituents and mobile phase solvents.

The limit of detection (LOD) was derived from the concentrations corresponding to the mean value obtained from blank test plus three standard deviations. The limit of quantitation (LOQ) corresponds to the limit

of detection plus ten standard deviations for the blank test. The results are presented in Table I. Response for fluocinolone acetonide, nipagin M, and nipagin P were linearly dependent on concentration in the ranges 0.5–30, 5–200, and 10–120 μg mL^{-1}, respectively; the regression equations and correlation coefficients, *r*, are also listed in Table I.

Table I

Limits of detection and quantitation, regression equations, and correlation coefficients, *r*

Compound	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Regression equation	*r*
Fluocinolone acetonide	250	500	S/S_{IS} = 0.025 (±0.0005)*C* + +0.015 (±0.009)	0.9993
Nipagin M	100	200	S/S_{IS} = 0.03 (±0.0007)*C* + +0.45 (±0.089)	0.9990
Nipagin P	150	300	S/S_{IS} = 0.032 (±0.0007)*C* + +0.14 (±0.059)	0.9989

C, calculated concentration of fluocinolone acetonide; *S*, peak-area of fluocinolone acetonide, S_{IS}, peak-area of nipagin A (internal standard); *r*, correlation coefficient
Statistical evaluation was performed on results from determination of six concentrations in six independent series of samples (i.e. *n* = 6).

A reference sample containing fluocinolone acetonide at a concentration of 250 μg L^{-1} and additives (nipagin M and nipagin P) at given concentrations recalculated on the basis of the active substance were used for evaluation of the precision of analysis of the gel. The chromatogram obtained under the conditions described above revealed the presence of a peak assigned to the active substance, fluocinolone acetonide, with peaks from nipagin M, nipagin A (internal standard), nipagin P, and other peaks originating from the matrix (Figs 1A and 1B). The peaks of the analysed components were very well resolved and there was no interference from peaks arising from the vehicle gel (Figs 1A and 1B), as is apparent from both chromatograms. The accuracy of the method was expressed as percentage recovery obtained when analysing reference drug. The recovery was 97.8% for fluocinolone acetonide and 99.0 and 98.1% for nipagin M and P, respectively.

Results obtained from determination of precision and accuracy for fluocinolone acetonide, methyl 4-hydroxybenzoate, and propyl 4-hydroxybenzoate are listed in Tables II, III, and IV, respectively; the results are

consistent with the declared constituent concentrations and statistical analysis shows the results are characterised by high repeatability and low scatter.

Table II

Within-day precision and accuracy for analysis of fluocinolone acetonide

Average nominal concentration (AVG) (μg mL^{-1})	Average concentration found (AVG) (μg mL^{-1})	SD	RSD (%)
0.5	0.51	0.0468	9.18
1.0	1.09	0.0927	8.50
5.0	5.20	0.4195	8.08
10.0	10.13	0.5888	5.81
15.0	14.80	0.7483	5.06
20.0	19.73	0.9266	4.70
25.0	24.93	0.8548	3.43
30.0	29.80	0.8390	2.82

AVG, SD, and RSD denote mean value, standard deviation, and relative standard deviation, respectively (n = 6)

Table III

Within-day precision and accuracy for analysis of methyl 4-hydroxybenzoate (nipagin M)

Average nominal concentration (AVG) (μg mL^{-1})	Average concentration found (AVG) (μg mL^{-1})	SD	RSD (%)
5	5.17	0.4592	8.88
15	14.72	0.9291	6.31
30	30.56	1.7606	5.76
60	58.67	1.5766	2.69
100	102.11	2.5880	2.53
140	139.00	2.5899	1.86
200	199.56	2.5698	1.29

AVG, SD, and RSD denote mean value, standard deviation, and relative standard deviation, respectively (n = 6)

The suitability of the method under the conditions described above was checked by simultaneous determination of active substance and additives in Flucinar-gel. As in analysis of the gel discussed above, chromatograms recorded for Flucinar gel revealed no interference between the peaks

Table IV

Within-day precision and accuracy for analysis of propyl 4-hydroxybenzoate (nipagin P)

Average nominal concentration (AVG) (μg mL^{-1})	Average concentration found (AVG) (μg mL^{-1})	SD	RSD (%)
10	10.05	0.8710	8.66
20	19.79	1.2587	6.36
40	39.90	1.9713	4.94
60	60.21	2.7657	4.59
100	99.59	3.4491	3.46
120	120.37	2.3815	1.98

AVG, SD, and RSD denote mean value, standard deviation, and relative standard deviation, respectively (n = 6)

of fluocinolone acetonide and nipagins M and P, and the peaks assigned to the matrix (Fig. 1B). Results from determination of fluocinolone acetonide and additives were characterised by high repeatability and low scatter around the mean value. The results were within accepted tolerances for such drugs. The concentration of nipagin M (0.14%) was lower than that of nipagin P (0.52%).

The good performance of the method was further confirmed in quantitative analysis (Table V), because the results obtained were comparable with the amounts of components used to prepare the gel. The same method was used for determination of fluocinolone acetonide in ointment from

Table V

Results from quantitative analysis of fluocinolone acetonide in Flucinar gel

Declared concentration (μg g^{-1})	Concentration found (μg g^{-1})
250	227.5
	232.5
	227.5
	232.5
	222.5
	237.5
Mean	230.0
SD	5.244
RSD (%)	2.28
n	6

which nipagin M and nipagin P were absent. The quantitative results obtained are shown in Table VI. The quantitative results listed in Tables V and VI were calculated after analysis of 1 g gel or ointment and are in accordance with the amounts declared by the producer.

Table VI

Results from quantitative analysis of fluocinolone acetonide in Flucinar ointment

Declared concentration (μg g^{-1})	Concentration found (μg g^{-1})
250	222.5
	227.5
	222.5
	217.5
	227.5
	222.5
Mean	223.33
SD	3.7639
RSD (%)	1.685
n	6

Chromatograms obtained from ointment matrix using the procedure presented here do not show the presence of interfering peaks (Figs 2A and 2B). Good separation is achieved for fluocinolone acetonide and the internal standard peaks, as described above. The only differences were for quantification of individual components. Although different results were obtained for gel and ointment, the differences were within ranges accepted by validation rules.

Twofold extraction with ethanol was found to be satisfactory, because this solvent resulted in reliable recoveries without extracting interfering compounds and it can be evaporated more rapidly than some other organic solvents. The liquid–solid extraction technique previously employed [13] was more time-consuming than twofold extraction with ethanol.

The high precision and accuracy of the method are apparent from the results presented in Tables II–IV. Linearity is maintained over a wide range of concentrations. Limits of detection for fluocinolone acetonide, nipagin M, and nipagin P were 250, 100, and 150 ng mL^{-1}, respectively, indicating the suitability of the method for determination of low concentrations of substances in the presence of additives which are present at high concentrations compared with the active compound. In our opinion this is

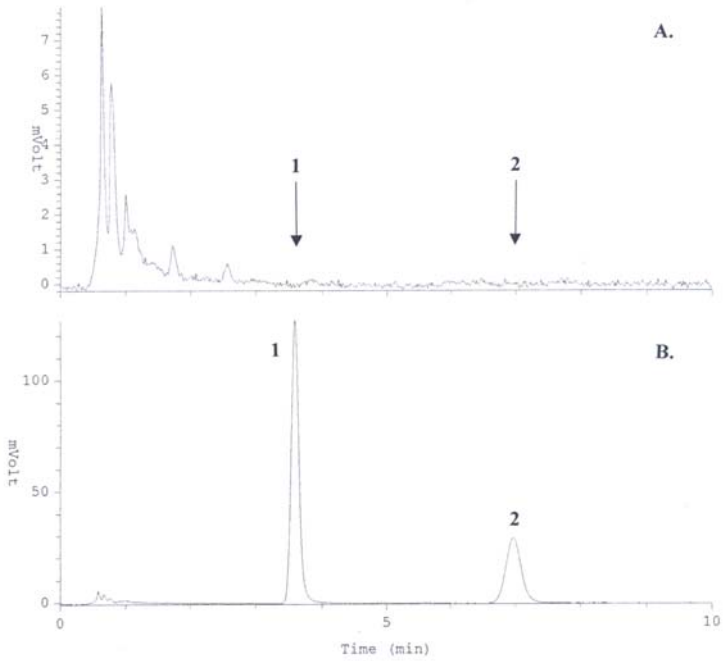

Fig. 2

Chromatograms obtained from vehicle ointment extract (A) and Flucinar ointment extract (B) containing 22.75 μg mL^{-1} fluocinolone acetonide (1) and 30 μg mL^{-1} nipagin A (2; internal standard).

the method of choice for the determination of fluocinolone acetonide in gel and ointment, because it does not require derivatization and complex instrumentation such as in GC–MS, HPLC–MS, micellar electrokinetic chromatography (MEKC), and micellar liquid chromatography (MLC). Extraction is also rapid and simple.

The substance analysed was received with a quality-control certificate; this was checked using the method recommended in the pharmacopoeia [20] and by use of the method normally applied by the company. The method described could be alternative tool to pharmacopoeia [19] method for determination of the active substance. The method proposed also enables quality-control analysis of additives in addition to the main active substance. We recommend our method for quality-control analysis not only of fluocinolone acetonide but also preservatives (nipagin M and P) added

by the pharmaceutical company in significant quantities. It should be emphasized that these additional substances are very important for protecting the preparation against microbiological agents. Determination upper limit of additives such as derivatives of 4-hydroxybenzoate acid is very essential because their presence at concentrations which are too high can cause allergic effects.

CONCLUSIONS

An HPLC method with UV detection has been developed for determination of fluocinolone acetonide in gel. Results obtained in the validation process and in drug analysis are encouraging and indicate suitability for routine tests. Under the conditions established there are no interference from vehicle. In addition, good separation is achieved for the constituents of interest. The method is characterised by good precision and accuracy.

The method enabled successful separation and quantitative determination of fluocinolone acetonide and additives in gel and of fluocinolone acetonide in ointment.

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