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Determination of clandestine Corticosteroids in Cosmetics with LC/DAD/MS

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Introduction

Many people with skin disorders are confident that phytotherapeutic products contain only active ingredients from natural sources. Such products should therefore not provoke side effects which can occur when using products containing synthetic corticosteroids. There are however reports which show that corticosteroids are sometimes used as adulterants in natural herbal products (1–3). The aim of such adulterations is to offer a natural and effective "cosmeceutical" and at the same time to evade tedious registration procedures required for drugs. In this paper we would like to present our method which we used successfully for detecting triamcinolone acetonide and betamethasone di propionate in phytotherapeutic ointments (4).

Experimental

Materials and instruments

Analytical balance (AT 200, Mettler Toledo, Greifensee), ultrasonic bath (Branson 3510, Merck, Zürich), centrifuge (Heräus Biofuge Primo, BGB, Anwil), vortex (Genie, Bender & Hobein, Zürich).

Quaternary gradient HPLC system consisting of a low pressure mixing quaternary gradient pump (Thermo Separations P4000), an autosampler (Thermo Separations AS3000), a photo diode array detector (Thermo Separations UV6000LP fitted with 2 µl 10 mm flowcell), an ion trap MS/MS mass spectrometer (LCQ Duo) and a data station (Excalibur), all from Thermo Finnigan (Spectronex, Birsfelden).

Analytical column: Phenomenex Prodigy ODS-3, 3 µm, 100×2 mm (Brechbühler, Schlieren), PTFE-Syringe filters, 13 mm diameter, 0.45 µm pore size (Titan Filtration Systems, Schmidlin, Neuheim).

Chemicals

Methanol for LC/MS (RdH), demineralised water for HPLC (J.T. Baker), ethanol p.a. (Merck), acetone p.a. (Merck), N,N-dimethylformamide p.a. (Merck). All reference substances and materials are listed in table 1.

Table 1
Reference substances and materials

Nr.	*Analyte*	*CAS-Nr.*	*Assay*	*Product*
1	hydrocortisone	50-23-7	>98 %	Sigma H0888
1a	hydrocortisone	50-23-7	10 mg/g	Pfizer Terracortril Ointment
2	dexamethasone	50-02-2	>98 %	Sigma D1756
2a	dexamethasone	50-02-2	1 mg/g	Medinova Dexalocal Scalp
3	triamcinolone acetonide	76-25-5	>99 %	Sigma T6501
3a	triamcinolone acetonide	76-25-5	1 mg/g	Sanofi Mycolog Cream
4	fluocinolone acetonide	67-73-2	>98 %	Sigma F8880
4a	fluocinolone acetonide	67-73-2	0.25 mg/g	Grünenthal Synalar Ointment
5	prednisolone 21-acetate	52-21-1	2.5 mg/g	Spirig Premandol Ointment
6	hydrocortisone 21-acetate	50-03-3	>98 %	Sigma H4126
6a	hydrocortisone 21-acetate	50-03-3	2.5 mg/g	Spirig Alfacorton Cream
7	methylprednisolone aceponate	86401-95-8	1 mg/g	Lederle Advantan Cream
8	clobetasol 17-propionate	25122-46-7	0.5 mg/g	Glaxo Wellcome Dermovate Cream
9	prednicarbate	73771-04-7	2.5 mg/g	Knoll Prednitop Ointment
10	betamethasone 17-valerate	2152-44-5	1 mg/g	Essex Celestoderm V Cream
11	betamethasone 17,21-dipropionate	5593-20-4	0.64 mg/g	Essex Diprosalic Ointment
12	mometasone 17-furoate	83919-23-7	1 mg/g	Essex Elocom Cream
13	flumetasone 21-pivalate	2002-29-1	0.2 mg/g	Novartis Locasalen Ointment

Procedures

Calibration

20 ml solutions of 10 mg of corticosteroids in ethanol were prepared. These solutions are stable for at least one month if stored in the dark at 4 °C.

1 to 20 ng/µl calibration solutions were prepared by dilution with ethanol.

Sample preparation

500 mg of sample were weighed into two 50 ml erlenmeyer flasks. 25 ml of ethanol were added to one and 25 ml of acetone to the other flask. Each flask was vortexed thoroughly for one minute and then sonicated for 15 minutes in an ultrasonic bath at 40 °C. If the sample either could not be dissolved or did not give a homogenous suspension, N,N-dimethylformamide or a mixture of 70 % ethanol with 30 % water were used as alternative solvents. This may especially be favorable for the polar corticosteroids 1 to 6. Raising the extraction temperature to 50 °C can be a another powerful tool to improve extraction, but stability of the analytes has to be taken into account. Sample solutions were filtered through a 0.45 µm PTFE HPLC filter.

HPLC parameters

HPLC analysis was performed with a gradient elution as described in table 2. Flow rate was 0.25 ml/min, run time was 27 minutes and column temperature 30 °C. The usual injection volume was 1 µl.

Table 2
Gradient time table

time/min	*acetonitrile*	*methanol*	*water*
0	10 %	45 %	45 %
5	10 %	45 %	45 %
12	20 %	50 %	30 %
16	20 %	50 %	30 %
19	35 %	65 %	0 %
23	35 %	65 %	0 %
23.1	10 %	45 %	45 %
27.1	10 %	45 %	45 %

Spectra were recorded in the scan range of 220 nm to 360 nm, with a bandwith and a resolution of 1 nm at a rate of 1 Hz. One discrete channel at 240 nm was recorded with a scan rate of 10 Hz and a bandwith of 5 nm.

MS parameters

Quantitative measurements were made in the atmospheric pressure chemical ionisation (APCI) positive mode. For screening or verification purposes negative and positive mode were used in the same run. Vaporizer temperature was set at 400 °C, heated transfer capillary temperature at 170 °C. Sheath gas flow was held at a rate of 38 arbitrary units, no auxillary gas was used. Source voltage was set at 6 kV, source current was set at 5 µA and capillary voltage was set at 37 V.

Results and discussion

Extraction and chromatography

Many papers deal with the determination of corticosteroids in drugs. Most of them describe the determination of one single corticosteroid in a drug where dosages are high (per mil or even per cent range) and a very high precision and accuracy is required by law. Other published methods deal with the determination of one specific corticosteroid in biological fluids. In all these cases the identity of the analyte and the matrix is known in advance. Detecting stealth corticosteroids in phytocosmetics however presupposes a method capable of screening for up to 40 corticosteroids (5) in several matrices such as creams, ointments, gels or lotions. Several HPLC methods (6–9) were available but none of them comprised coupling to an MS or dealing with a wide range of analytes. Accurate quantification of towards 40 corticosteroids can only be done with an ad hoc validation in the case of positive samples. The method should also be capable of detecting recently introduced (respectively previously unknown) corticosteroids. This is possible with a group specific detection method. As corticosteroids contain the same chromophore (Fig. 1) and therefore almost identical UV spectra, diode array detection is the method of choice. Reliable identification of individual components however can only be done by MS.

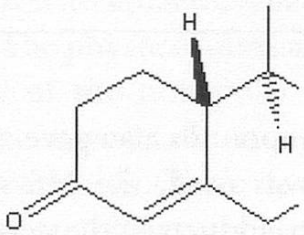

Figure 1 **Chromophore of corticosteroids**

Extraction and chromatography were optimised with thirteen often used corticosteroids of different polarity containing different functional groups (Table 1). The best results were achieved using a ternary gradient system with water, methanol and acetonitrile on a Prodigy ODS-3 column. Acetonitrile used as a modifier for LC often leads to a loss of sensitivity in APCI-MS detection. Sensitivity was also decreased in this case using 10 % to 35 % acetonitrile but was still acceptable without using acid. This eluent allowed to reliably record UV spectra down to an analyte amount of about 1 ng on column (Fig. 2). As a further benefit, changing MS conditions from APCI positive to APCI negative mode could be done without modifying the chromatographic system. For a qualitative analysis, switching between negative and positive mode was even possible in the same run. This further meant a higher

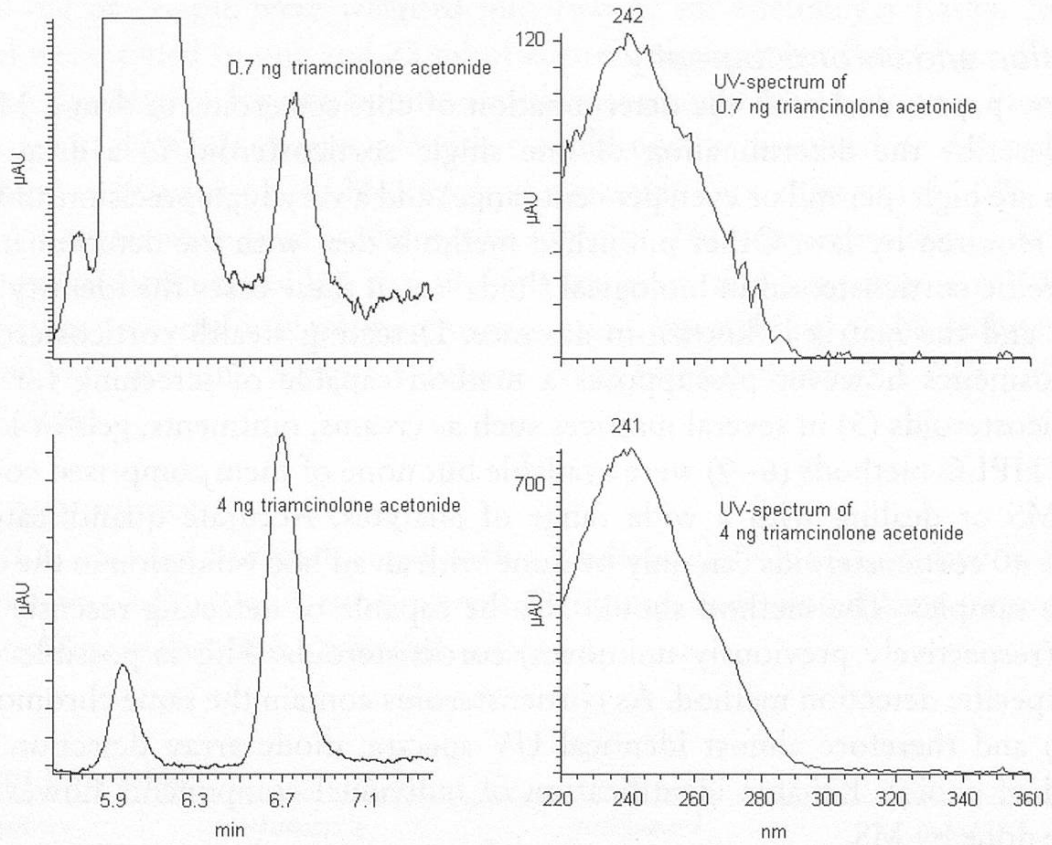

Figure 2 **Chromatograms and UV-spectra of a phytocosmetic sample containing 40 mg/kg (above) and a drug containing 1000 mg/kg (below) triamcinolone acetonide**

identification reliability as most compounds also gave a signal in the APCI negative mode. Although no formic acid was used, the MS system obviously contained enough formic acid to form formiate adducts in the negative mode. All analytes that gave signals in the negative mode had masses of detection of M+44 corresponding to the mass of the molecule plus formiate.

Surprisingly MS/MS detection was often more sensitive in the negative mode whereas full scan MS showed better sensitivity and reproducibility in the positive mode. Detection limits (LOD's) of all thirteen compounds were below 20 mg/kg which is only a fraction of the concentrations applied in drugs (per mil or low per cent range). Determination of corticosteroids by LC/MS is usually performed using electrospray ionisation (ESI). The ion trap mass spectrometer used in this study however proved to be more sensitive in the APCI mode. In table 3 the retention times and masses of detection of 13 corticosteroids are given. No interferences were observed when analysing drugs. This was also the case for the three illegal phytocosmetic samples we analysed. However, due to the many ingredients which can be encountered in cosmetics, interfering mass peaks can not be ruled out in general.

Table 3
Retention times and detection masses of selected corticosteroids

Substance	*min*	*M/z (MH+)*
hydrocortisone	4.1	363.1
dexamethasone	6.3	393
triamcinolone acetonide	6.7	435
fluocinolone acetonide	7	453
prednisolone 21-acetate	7.3	403
hydrocortisone acetate	7.4	405.2
methylprednisolone aceponate	16.7	473
clobetasol 17-propionate	17.1	467
mometasone furoate	17.7	520.9
prednicarbate	17.5	489
betamethasone 17-valerate	18.3	477
flumethasone 21-pivalate	19.3	495
betamethasone 17,21-dipropionate	19.8	504.9

The precision of the method was evaluated with two phytocosmetics. One contained between 15 and 40 mg/kg triamcinolone acetonide depending on the lot number. The maximum difference found between double determinations of four lots was 4 %. Analysis precision in the other product, which contained about 600 mg/kg of beta-methasone dipropionate was 7 % (relative standard deviation; n=4).

Analysis of a phytocosmetical formulation containing 1000 mg/kg triamcinolone acetonide gave good recovery rates with several detection modes (Table 4). The extraction was performed with ethanol, which works well with the polar corticosteroids 1 to 6 (of table 1). The phytocosmetical product having an in situ concentration of about 600 mg/kg of the non polar betamethasone dipropionate was extracted with three different solvents. In comparison to acetone being the best solvent for non-polar corticosteroids (Table 1, analytes 7 to 13), N,N-dimethylformamide (DMF) only gave a relative recovery rate of 17% whereas ethanol yielded 41 %. An efficient extraction requires the sample to be either dissolved or to be transformed to a slurry. In some cases adding small portions of water may help break up clumps resulting in better recovery rates. Extraction temperature was limited to 40 °C as we did not have data on all analytes concerning their thermostability, but raising the temperature can improve recovery rates if the compounds of interest are thermally stable.

Table 4
Method accuracy

Detection mode	*Trace*	*Content**	*Recovery**
UV	at 240 nm	0.100 %	100 %
APCI(+): Full Scan 230–600	at 435 (MH+)	0.102 %	102 %
APCI(–): Full Scan 230–600	at 479 (MFormiat(–))	0.112 %	112 %

*using a 0.1 % triamcinolone acetonide containing phytocosmetical ointment

Linearity for triamcinolone acetonide and betamethasone di propionate in the APCI(+) mode is established at least between 0.25 and 5 ng on column and similar results can be expected for the other substances if the necessary sensitivity can be achieved.

In samples with analyte concentrations above 100 mg/kg and with no observed interferences, quantification with the UV detector should be prefered because of its higher precision and stability. Nevertheless MS detection also proved to be reliable for quantitation when compared to data obtained by UV detection (Table 4).

Analysis of samples

The development of our method was triggered by the results of a clinical double blind study (4). In this study, a well sold phytocosmetical product, which was marketed in an European country for treating skin disorders, proved to have a therapeutic effect in patients with atopic dermatitis. The screening of the product for clandestine corticosteroids revealed, that it contained between 15 and 40 mg/kg triamcinolone acetonide, depending on the lot number. Parabens, sunscreen filters as well as over 20 other ingredients in the ointment did not interfere with UV detection of triamcinolone acetonide (Fig. 3) but parabens could interfere if earlier elut-

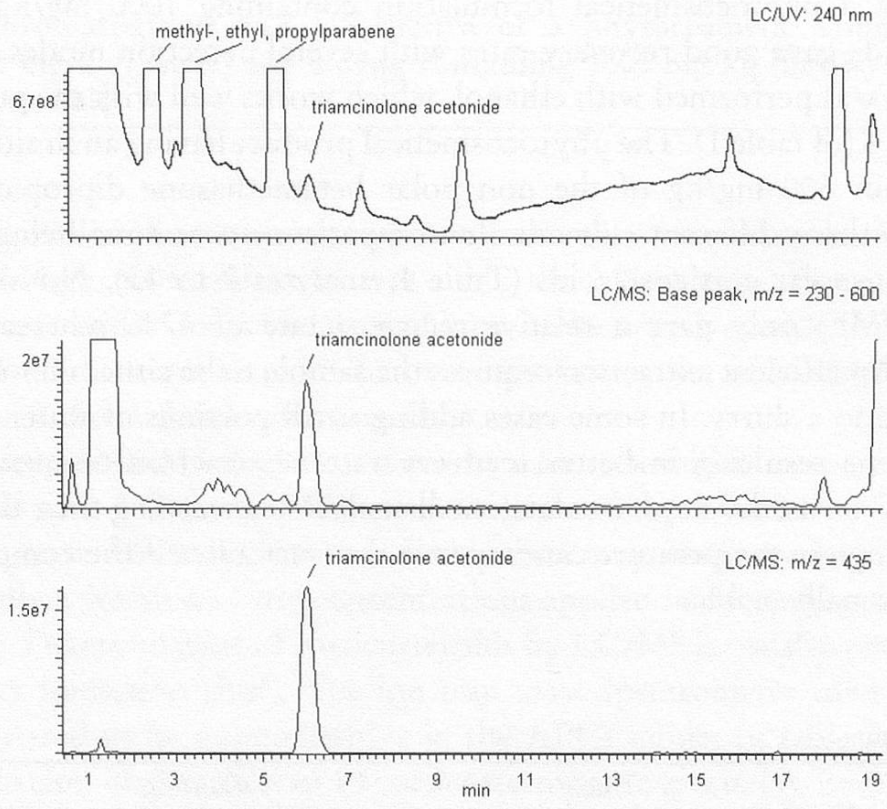

Figure 3 **Comparison of HPLC/DAD with HPLC/APCI(+)– MS (base peak of full scan and m/z=435) of a phytocosmetic sample containing about 40 mg/kg triamcinolone acetonide**

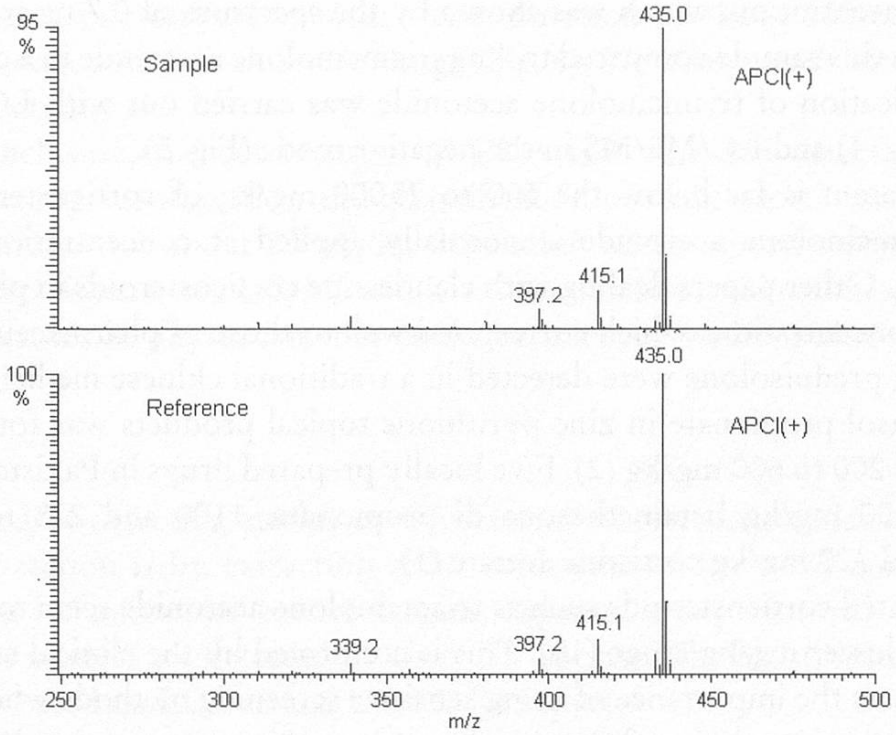

Figure 4 **APCI(+) MS spectra of triamcinolone acetonide in a phytocosmetic sample and a reference solution**

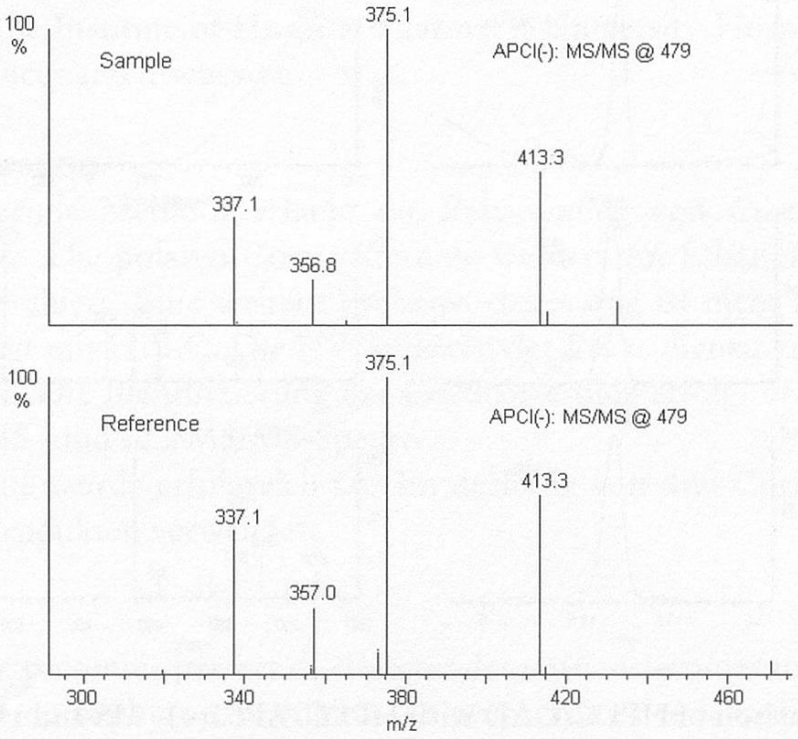

Figure 5 **APCI(–) MS/MS spectra of triamcinolone acetonide in a phytocosmetic sample and a reference solution**

ing steroids were present. Group specific detection with DAD was possible even at this low concentration, which was shown by the spectrum of 0.7 ng triamcinolone acetonide in this sample compared to 4 ng triamcinolone acetonide in a drug (Fig. 2). The identification of triamcinolone acetonide was carried out with LC/MS in the positive (Fig. 4) and LC/MS/MS in the negative mode (Fig. 5).

This content is far below the 500 to 25000 mg/kg of corticosteroid used in drugs. Triamcinolone acetonide is normally applied at concentrations of about 1000 mg/kg. Other papers dealing with clandestine corticosteroids in phytocosmetics found concentrations which correspond well to those of pharmaceuticals (1–3). 5000 mg/kg prednisolone were detected in a traditional chinese medicine ointment (3). Clobetasol propionate in zinc pyrithione topical products was found to be in the range of 200 to 600 mg/kg (2). Five locally prepared drugs in Pakistan contained 902 and 1400 mg/kg betamethasone di propionate, 1100 and 278 mg/kg prednisolone and 320 mg/kg cortisone acetate (1).

Fluorinated corticosteroids such as triamcinolone acetonide seem to be effective even in the lower mg/kg range (10). This is confirmed by the clinical study (4) and clearly stresses the importance of using sensitive screening methods when analysing suspicious phytocosmetics. Screening for about 40 corticosteroids in the mg/kg range calls for a method capable of zeroing in on the peaks of interest. UV detection proved to be a useful tool for sorting out peaks.

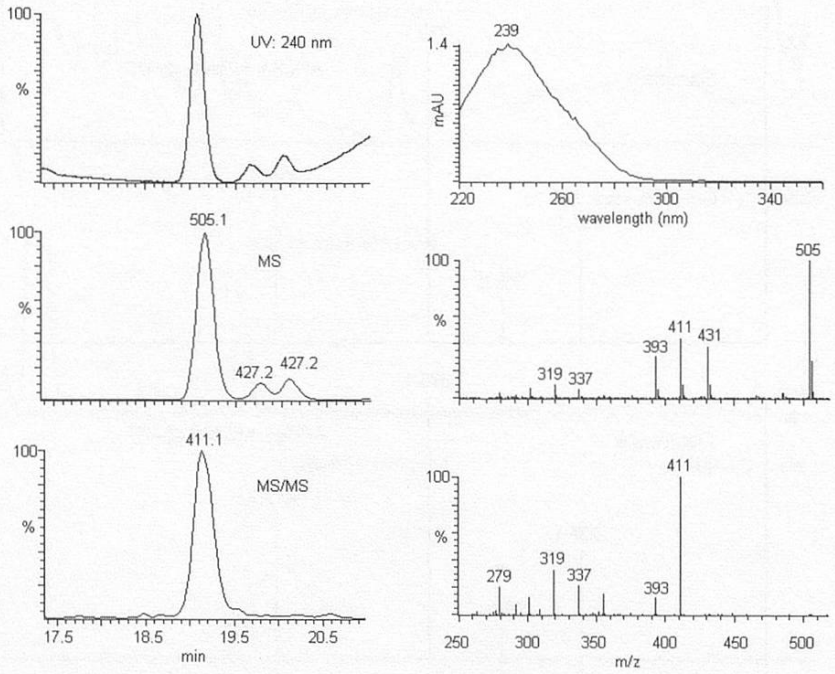

Figure 6 **Comparison of HPLC/DAD with HPLC/APCI(+)– MS and HPLC/APCI(+)–MS/MS of a phytocosmetic sample containing about 600 mg/kg Betamethasone di propionate; LC/DAD notation: spectrum maximum (nm); LC/MS notation: m/z of most intense ion (base peak)**

The analysis of two other suspicious phytocosmetic samples revealed in both cases betamethasone dipropionate in a concentration of about 600 mg/kg which equals the dosage found in skin care drugs (Fig. 6). No compounds interfered with the determination.

As a conclusion, the combination of PDA-UV with MS detection allows a reliable screening, identification and quantification of stealth corticosteroids down to a concentration of about 20 mg/kg depending on the specific compound and sample matrix. When using LC/MS and UV detection in combination, screening for 40 corticosteroids in phytocosmetics could be achieved with a rather easy sample preparation.

Summary

A method of detecting stealth corticosteroids in phytocosmetics is presented. Sample preparation is by extraction either with ethanol for polar compounds or with acetone for non-polar corticosteroids. Separation is performed with HPLC and peaks of interest are detected by their UV spectra. The identification of corticosteroids is based on LC/MS and LC/MS/MS spectra.

The method was suscessfully used in revealing the presence of corticosteroids in three suspect products.

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Zusammenfassung

Die vorliegende Methode erlaubt die Bestimmung von Corticosteroiden in Phytokosmetika. Die polaren Corticosteroide werden mit Ethanol, die unpolaren mit Aceton extrahiert. Eine weitere Probenvorbereitung ist nicht notwendig. Die Trennung erfolgt mit HPLC. Die UV-Spektren der Peaks dienen zur Selektion auf Corticosteroide. Die Identifizierung der Corticosteroide erfolgt über die Auswertung der LC/MS- und LC/MS/MS-Spektren.

Die Methode wurde erfolgreich zur Entdeckung von drei Corticosteroiden in verdächtigen Produkten verwendet.

Résumé

La méthode présentée permet de détecter des corticostéroïdes interdits clandestins dans les produits phytocosmétiques. La préparation des échantillons se fait par extraction avec de l'éthanol pour les substances polaires ou avec de l'acetone pour les composants non-polaires. La séparation est faite par HPLC et les pics importants

sont détectés par leur spectre UV. L'identification des corticostéroides se base sur les spectogrammes de masses par LC/MS et LC/MS/MS.

La méthode a été utilisée avec succès et a permis de révéler la présence de corticostéroides dans trois produits du marché.

Key words

Corticosteroids, Cosmetics, LC/MS

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