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High-performance liquid chromatography–diode array and electrospray-mass spectrometry analysis of non-allowed substances in cosmetic products for preventing hair loss and other hormone-dependent skin diseases

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ABSTRACT

A simple high-performance liquid chromatography (HPLC) method with ultraviolet diode array (UV-DAD) and electrospray ionisation mass spectrometry (ESI-MS) detection has been developed for the determination of minoxidil, progesterone, estrone, spironolactone, canrenone, hydrocortisone and triamcinolone acetonide in cosmetic products. The presence of these substances in commercial cosmetic samples is prohibited. The compounds were separated by reversed phase chromatography with water (0.1% trifluoroacetic acid) and acetonitrile gradient elution and detected by UV-DAD at 230, 254 and 280 nm and by ESI-MS positive ionisation mode. Benzoic acid was used as internal standard. Linearity was studied with UV-DAD detection from 1.50 to 1000 μg/ml or μg/g range, depending on the different compounds and type of cosmetic preparation and with ESI-MS in the 50–1000 ng/ml or ng/g range. Good determination coefficients ($r^2 \geq 0.99$) were found in both UV and ESI-MS. At three concentrations spanning the linear dynamic ranges of both UV-DAD and ESI-MS assay, mean recoveries were always higher than 90% for the different analytes. This method was successfully applied to the analysis of substances under investigations illegally added in cosmetic cream and lotions, sold on internet web sites to prevent hair loss and other hormone-dependent skin diseases, like acne and hirsutism.

1. Introduction

Baldness, alopecia, hirsutism and acne are skin and hair diseases complicating social relationship of the affected people [1–4]. The most common topically active anti-alopecia and baldness, anti-hirsutism and anti-acne drugs are: minoxidil, glucocorticoids (e.g. hydrocortisone or triamcinolone acetonide), spironolactone and its primary metabolite canrenone used alone or in combination to prevent hair loss but also effective in the treatment of hirsutism and acne [5–15] and oral contraceptives such as progesterone and estrone used to fight biochemical signs of hyperandrogenism (hirsutism, acne, male-pattern hair loss) (Fig. 1) [16]. At present, in approved marketed Italian topic products, only a combination of minoxidil and hydrocortisone exists in two different lotions containing the first compound in a range of 12–15% (v/v) and the second in 0.1% (v/v). Conversely, one component cream and two cutaneous solutions for topic use are present in the market and contain 0.5% hydrocortisone and 2–5% minoxidil, respectively. The other reported substances (triamcinolone acetonide, spironolactone, canrenone, estrone and progesterone) are only present in pharmaceutical preparations (e.g. tablets, gels or vaginal ovules) for systemic use.

Recently, the Italian anti-adulteration and safety bureau (NAS) seized several illegal cosmetic preparations sold through internet web sites or illegal circuits (e.g. private doctors, fitness centers, odd stores) as promising remedies for these hormone-dependent pathologies. Although in the majority of cases substances contained in the products are not listed, the high suspicion that the above-reported pharmacologically active substances could be illegally employed in these formulations prompted the bureau to request for specific analysis of seized products.

Article I from CEE Cosmetic Directive 76/768/EEC, aiming at regulating the production of cosmetic products, defined exactly what can be considered a cosmetic: "For the purposes of this law, a "cosmetic product" shall mean any substance or preparation other than medicines, to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view, exclusively or mainly to cleaning them, perfuming them, protecting them, keeping them in a good conditions, changing their appearance and/or correcting body odors [17]. Cosmetic products have no therapeutic purposes and shall

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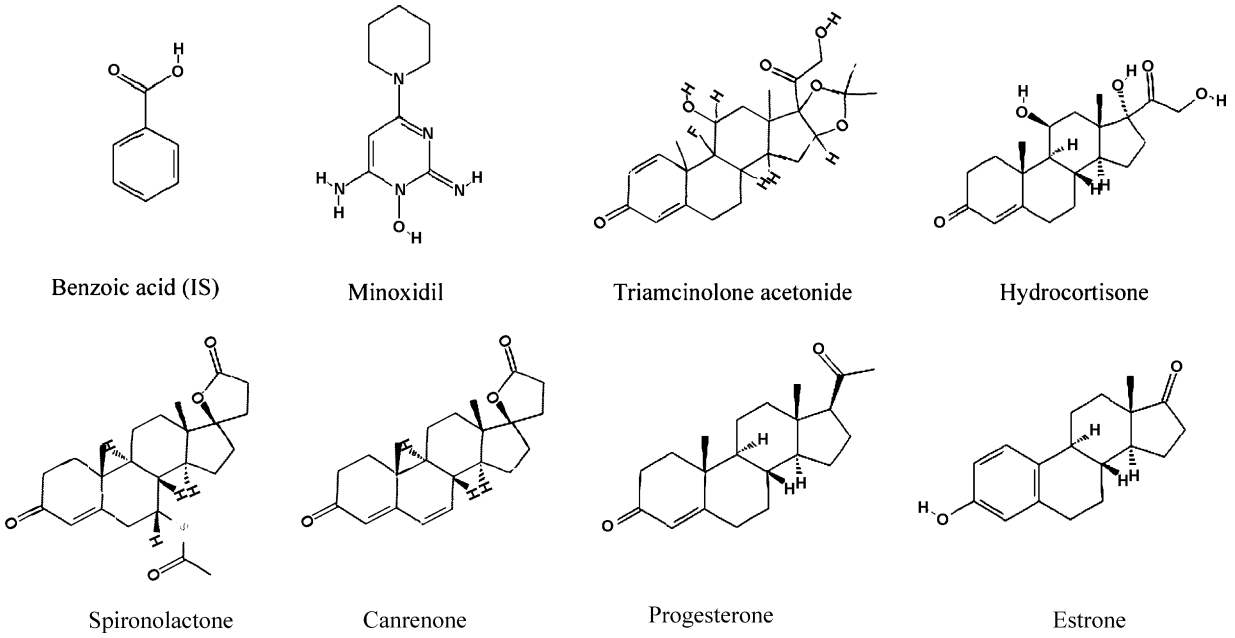

Fig. 1. Chemical structure of the compounds of interest and internal standard.

not claim any therapeutic action. In particular, the products listed in Annex II to this law are cosmetic products pursuant to the preceding paragraphs".

The majority of the compounds considered in the present study are included in the Annex II of the above-mentioned Directive and are expressly forbidden (minoxidil, spironolactone, progesterone, estrone, hydrocortisone, triamcinolone). Even though not mentioned in the above reported Annex II, canrenone is also a pharmacologically active compound and therefore it should not be added to a cosmetic product.

There is no literature on the simultaneous separation by high-performance liquid chromatography (HPLC) and detection by ultraviolet-diode array (UV-DAD) and electrospray ionisation mass spectrometry (ESI-MS) of the seven active substances that we sought to investigate. Numerous methodologies have been published about the analysis of substances under investigation in a variety of matrices with different chromatographic techniques. Among the most recent, ion-pair HPLC for minoxidil in plasma [18], tandem thin-layer chromatography and HPLC for hydrocortisone and other steroids in cosmetic products [19], HPLC–atmospheric pressure chemical ionization-MS for spironolactone and canrenone in plasma [20] and finally HPLC for progesterone and estrone and HPLC–ESI-MS for triamcinolone in cosmetics [21,22].

In this study a simple HPLC separation method with both UV-DAD and ESI-MS detection to investigate the illegal presence of analytes under investigation in cosmetic preparations was developed. It was hypothesized that, in order to produce a pharmaceutical effect, an illegal product should contain an amount of these substances comparable to the minimum of these active substances usually administered in pharmaceutical preparations. If an illegal product contains the investigated substances at this level of concentration, UV-DAD detection is usually adequate. Nonetheless, to detect these substances in an illegal product at lower concentrations and in order to have a high degree of specificity and additional information about the structure of the analytes, the use of ESI-MS is more suitable. For this purpose, the developed method has been applied to some cosmetic preparations sold on internet web sites with the purpose of preventing and fighting hair loss and other hormone-dependent skin diseases, like acne and hirsutism.

2. Experimental

2.1. Chemicals and reagents

The pharmaceutical standards (purity >99%) of minoxidil, hydrocortisone, triamcinolone acetonide, spironolactone, progesterone, estrone, benzoic acid (used as internal standard, IS), methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate and butyl 4-hydroxybenzoate were purchased from Sigma and canrenone was supplied by Sanofi-Aventis.

All reagents were of analytical-reagent grade and were used without further purification. Methanol and trifluoroacetic acid (TFA) were purchased from Aldrich, acetonitrile and HPLC grade methanol were supplied by Merck (Darmstadt, Germany). Ultrapure water and all other reagents of analytical grade were obtained from Carlo Erba (Milan, Italy). An oil-in-water blank cream containing about 30% fatty substances (water, isopropyl palmitate, octyl octanoate, sucrose cocoate, glycerin, glyceryl stearate, stearyl alcohol, alcohol) and a hydro-alcoholic blank lotion (water, alcohol, glycerine) free from the analytes under investigation were prepared in our laboratory according to Italian Pharmacopoeia and used to prepare calibration standards and quality control samples.

All solvents and solutions for HPLC analysis were filtered through a Millipore filter (pore size: 0.45 μm) and vacuum degassed, by an ultrasound treatment, before use.

2.2. Cosmetic products

Four different cosmetic products (one cream and three lotions) were obtained from two internet web sites, where they were sold as promising remedies for hyperandrogenism-dependent pathologies (hair loss, baldness, acne, hirsutism). The products presented a label with generic indications such as:

- Cream 1: better skin for woman, 3 oz., apply before getting to bed, made in India;
- Cream 2:, 3 oz., apply once a day, made in UK;
- Lotion 1: regain hair, 100 ml, apply once a day, made in UK;

- Lotion 2: solution for man, 100 ml, apply twice a day, made in UK;
- Lotion 3: natural lotion for woman, 100 ml, apply once a day, made in UK;
- Lotion 4: natural lotion for men, 100 ml, apply once a day, made in UK;

No ingredients were reported on the label and the manufacturer was not indicated.

2.3. Instrumentations and conditions

2.3.1. HPLC-UV-DAD

HPLC-UV-DAD analyses were performed using an Agilent 1100 series HPLC system consisting of a G 1312A binary pump, a G1322A degasser and an ALS G1329A autosampler (Agilent Technologies, Palo Alto, CA) interfaced to an Agilent1100 UV-DAD detector. Data acquisition and analysis were performed using standard software supplied by the manufacturer.

Chromatographic separation was achieved using Zorbax SB-CN (250 mm × 4.6 mm; 5 μm) (CPS analitica, Milan, Italy). The mobile phase used in the separation, at a flow rate of 1.2 ml/min, consisted of (A) water (0.1% TFA) and (B) acetonitrile programmed as follows: 90% A for 1 min, decreased to 10% in 40 min, then increased again to 90% A in 10 min. The injection volume was 10 μl and the column temperature was set at 30 °C. The DAD detector allowed the evaluation of the peak purity factors, very useful in the analysis of real samples to confirm the absence of interfering co-eluting compounds and the selection of appropriate wavelength to obtain the best sensitivity for all the investigated compounds. On the basis of preliminary analyses on methanolic working solution of analytes under investigation, three different detection wavelengths (230, 254 and 280 nm) were selected.

2.3.2. HPLC–ESI-MS

HPLC–ESI-MS analyses were also performed the same HPLC system (Agilent 1100 series system) consisting of a G 1312A binary pump, a G1322A degasser, and an ALS G1329A autosampler (Agilent Technologies, Palo Alto, CA) interfaced to an Agilent 1100 series G1946D single quadrupole mass spectrometer equipped with an ESI interface. Data acquisition and analysis were performed using standard software supplied by the manufacturer

The conditions used for chromatographic separation were the same as the one used for HPLC-UV-DAD assay apart from injection volume that, in this case, was 20 μl.

The mass spectrometer was operated in positive ESI mode with selected ion monitoring (SIM) acquisition. The following ESI parameters were applied: drying gas (nitrogen) heated at 350 °C at a flow rate of 10.0 l/min; nebulizer gas (nitrogen) at a pressure of 40 psi; capillary voltage at 4000 V.

MS characterization (purity and identity) of compounds under investigation was achieved using flow injection analysis (FIA). The substances, dissolved in mobile phase, were infused through an integrated syringe pump into the ESI probe at rate of 1 ml/min. In FIA experiments, full scan acquisitions were made over the (50–550 *m*/*z*) range using both negative and positive ionisation. On the basis of these experiments, the best acquisition parameters were selected.

Two different fragmentation voltages (130 and 170 V) were applied in order to obtain a quantifying ion (the protonated molecular ion in the majority of the cases) and two significant qualifying ions for the different analytes under investigation. Dwell time was set at 43 ms, and mass peak width at 0.10 min.

More specifically, at 130 V ions at *m*/*z* 210, 193 and 164 were selected for minoxidil; *m*/*z* 271, 253 and 179 for estrone; *m*/*z* 315, 250 and 109 for progesterone; *m*/*z* 123, 105 and 77 for IS were selected. At 170 V ions at *m*/*z* 341, 363 and 267 for canrenone; *m*/*z* 341, 363 and 249 for spironolactone; *m*/*z* 363, 287 and 270 for hydrocortisone; *m*/*z* 435, 474 and 415 for triamcinolone acetonide. The underlined ions were used for quantification. The acceptance criterion for selected ion intensity ratios was a deviation ≤20% of the average of the ion intensity ratios of all the calibrators.

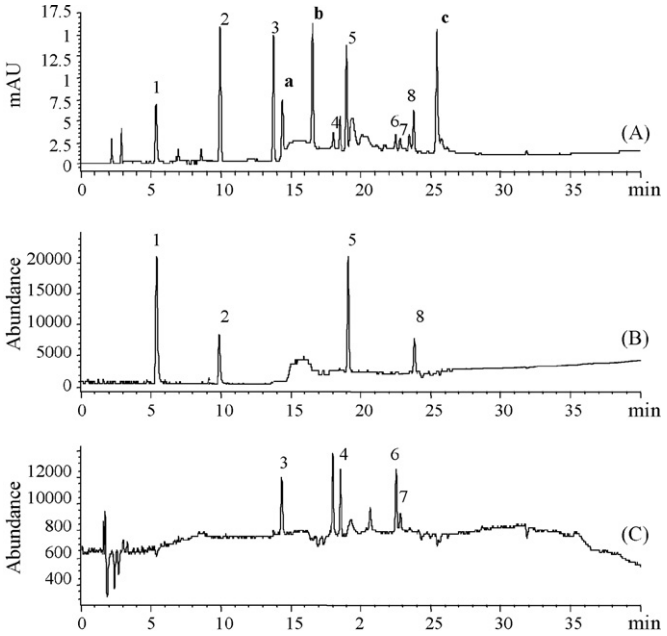

Fig. 2. Overlay of (A) HPLC-UV-DAD chromatogram at 254 nm of a blank cosmetic cream added with 60 μg/g minoxidil (1), hydrocortisone (3), triamcinolone acetonide (4), estrone (5), canrenone (6), spironolactone (7), progesterone (8) and 300 μg/g IS (2), (a) ethyl 4-hydroxybenzoate, (b) propyl 4-hydroxybenzoate and (c) butyl 4-hydroxybenzoate. (B and C) HPLC–ESI-MS-TIC chromatograms at 130 and 170 fragmentation voltages, respectively of a blank cosmetic lotion added with 60 ng/ml minoxidil (1), hydrocortisone (3), triamcinolone acetonide (4), estrone (5), canrenone (6), spironolactone (7), progesterone (8) and 300 ng/ml IS (2). Benzoates are not seen at those fragmentation voltages.

2.4. Calibration standards and quality control samples

2.4.1. Calibration standards and quality control samples for HPLC-UV-DAD

Standard stock solutions (5 mg/ml) of analytes were prepared in methanol and stored at +4 °C. The internal standard (IS) working solution was prepared at a concentration of 3 mg/ml. Diluted dispersions of blank lotion and blank cream were prepared transferring 1 ml lotion and 1 g cream to a 100 ml volumetric flask and taken to volume with methanol. The dispersions were subjected to ultrasonic treatment for 10 min at 40 °C. After centrifugation, the clear supernatant was collected (solutions A blank).

Calibration standards containing 300 μg IS and different μg amounts (LOQ, 1000 μg/ml or μg/g) of analytes under investigation were prepared for each analytical batch by adding suitable amounts of standard stock solutions to 1 ml blank lotion and blank cream solutions A. Calibration samples were treated and processed as unknown samples. Several aliquots of quality control samples at 40, 300 and 600 μg/ml or μg/g concentration were prepared in blank cosmetic products to be used for the calculation of validation parameters. Blank solutions containing 2000 and 5000 μg analytes under the investigation per ml or per g products were prepared as over-curve samples, to be tested for accuracy and precision once diluted 5 and 10 times, respectively.

2.4.2. Calibration standards and quality control samples for HPLC–ESI-MS

Standard stock solutions (50 μg/ml) of analytes were prepared in methanol and stored at +4 °C. The IS working solution was prepared at a concentration of 30 μg/ml. Diluted dispersions of blank lotion and blank cream were prepared by transferring 1 ml lotion and 1 ml cream to a 100 ml volumetric flask and taken to volume with methanol. One ml of each methanolic dispersion was further diluted 1:1000 with methanol. The dispersions were submitted to ultrasonic treatment for 10 min at 40 °C. After centrifugation, the clear supernatant was collected (solutions B blank).

Calibration standards containing 300 ng IS and different ng amounts (LOQ, 1000 ng/ml or ng/g) of analytes under investigation were prepared for each analytical batch by adding suitable amounts of standard stock solutions to 1 ml blank lotion and blank cream solutions B. Calibration samples were treated and processed as unknown samples. Several aliquots of quality control samples at 60, 400 and 800 ng/ml or ng/g concentration were prepared in blank cosmetic products to be used for the calculation of validation parameters. Blank solutions containing 2000 and 5000 ng analytes under the investigation per ml or per g products were prepared as over-curve samples, to be tested for accuracy and precision once diluted 5 and 10 times, respectively.

2.5. Samples preparation and extraction

Aliquots (1 ml or 1 g) of the cosmetic sample (lotions and cream), were diluted and treated as above reported for calibration standards and quality control samples. An amount of 10 μl solutions B were injected, after filtration on Millipore Filter, into the HPLC-UV-DAD. For HPLC–ESI-MS analysis, 1 ml solutions B were diluted 1:1000 with methanol, added with IS and 20 μl were injected into chromatographic column.

2.6. Validation procedures

Prior to application to real samples, both HPLC-UV-DAD and HPLC–ESI-MS were tested in a validation protocol following the accepted criteria for bioanalytical method validation [23,24]. Selectivity, matrix effect, recovery, linearity, limit of detection (LOD) and quantification (LOQ), precision, accuracy and stability were assayed as previously reported [25,26].

In brief, blank products were extracted and analyzed for assessment of potential interferences due to endogenous substances. The apparent responses at the retention times of the analytes under investigation and IS were compared to the response of analytes at the LOQ and IS at its lowest quantifiable concentration. The potential or carryover was investigated by injecting extracted blank products, with added IS, immediately after analysis of the highest concentration point of the calibration curve on each of the days of the validation protocol and measuring the area of eventual peaks, present at the retention times of analytes under investigation.

Analytical recoveries were calculated by comparing the peak areas obtained when calibration samples were analyzed by adding the quality control samples and the IS in the extract of blank products prior to and after the extraction procedure. The recoveries were assessed by quality control samples using four replicates at each level.

For an evaluation of matrix effects, the peak areas of extracted blank products spiked with quality control samples after the extraction procedure were compared to the peak areas of pure diluted substances.

Linearity was studied with in the calibration curves range, depending on the different compounds and type of cosmetic preparation and on UV-DAD and ESI-MS detection. Six points calibration curves were tested in triplicate using peak area ratios between compounds and IS for calculations. A weighted (1/concentration) least-squares regression analysis was used for slopes and intercepts (SPSS, version 9.0.2 for Windows). Standard deviation (S.D.) of the mean noise level over the retention time window of each analyte was used to determine detection limit (LOD = 3S.D.) and the quantification limit (LOQ = 10S.D.). To be accepted, the calculated LOQ had to show precision and accuracy within the 20% relative S.D. (R.S.D.) and relative error, respectively.

A total of five replicates at each of quality control samples added to blank products and over-curve samples, diluted to optimal levels, extracted as reported above were analyzed for the determination of intra-assay precision and accuracy. Precision was expressed as the R.S.D. of concentrations calculated for quality control samples and accuracy as the relative error of the calculated concentrations. Both parameters had to be within 20% of R.S.D. or error for both qauilty control and over-the-curve samples.

The effects of three freeze–thaw cycles (storage at −20 °C) on the stability of compounds in lotion and cream were evaluated by repeated analysis ($n = 3$) of quality control samples. In addition, mid-term stability test was performed for quality control and real samples (Lotion 1 and Cream) stored at −20 °C. Three replicates of both quality control and real samples were analyzed once a month during a 4-month period. The stability was expressed as a percentage of the initial concentration (first analyzed batch) of the analytes both in quality control and real samples.

3. Results and discussion

3.1. Chromatography and validation results

The linear gradient with a simple binary mixture with TFA in the aqueous phase produced well-shaped chromatographic peaks and permitted an excellent separation of all the tested compounds, which differ in lipophilicity. Fig. 2 presents an overlay of UV DAD at 254 nm and ESI-MS total ion current (TIC) chromatograms indicating coincidence of the signals of interest and Fig. 3 shows the ESI-MS SIM responses presented by the analytes under investigation extracted by a cream, respectively.

Samples following the ones exceeding the linear range in the chromatographic run were re-injected to check eventual contamination by carryover. Nonetheless, no carryover was observed in this case, nor when extracted blank cosmetic were injected after the highest point of the calibration curve, using both UV-DAD and ESI-MS detection. The additional peaks present in UV-DAD chromatograms, in any case at retention times different from those of analytes under investigation, were identified as 4-hydroxybenzoate esters (methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate and butyl 4-hydroxybenzoate) used as preservatives in cosmetic products (Fig. 1).

With respect to the matrix effect, the comparison between peak areas of analytes spiked in extracted blank products versus those for pure diluted standards showed less than 10% analytical signal suppression due to co-eluting endogenous substances.

Absolute analytical recoveries (mean ± S.D.) obtained after extraction procedure for the three different quality control samples using the two different detection modes, were always around 90% (Table 1).

With both UV-DAD and ESI-MS, linear calibration curves showed determination coefficients (r^2) higher than 0.99 in all cases. LODs and LOQs values were adequate for the purposes of the present study (Table 1). Particularly, the calculated LOQ tested for precision

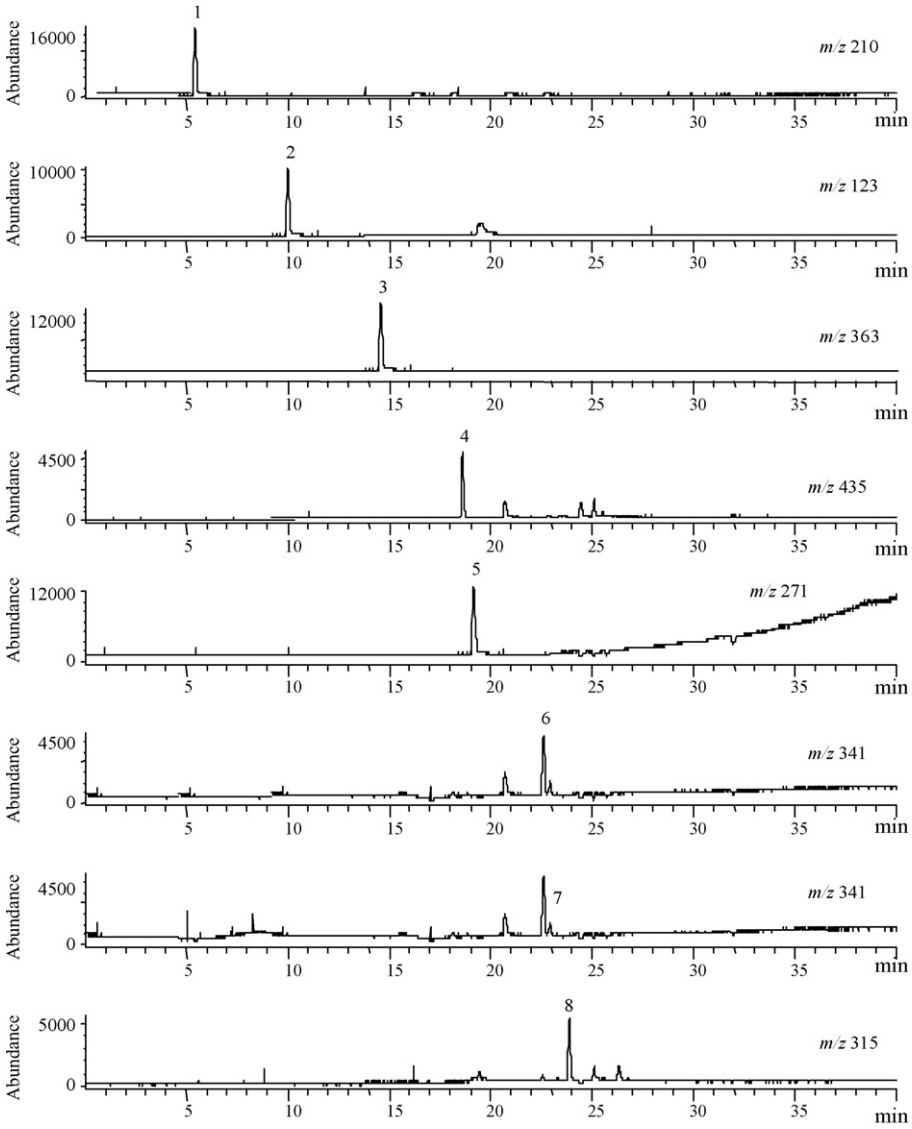

Fig. 3. HPLC–ESI-MS SIM chromatogram of an extract of a blank cosmetic cream added with 60 ng/g minoxidil (1), hydrocortisone (3), triamcinolone acetonide (4), estrone (5), canrenone (6), spironolactone (7), progesterone (8) and 300 ng/g IS (2).

and accuracy presented coefficient of variations always better that 20%.

The results obtained for intra-assay and inter-assay precision and accuracy satisfactorily met the internationally established acceptance criteria [23,24] (Table 2). Over-curve samples, tested for accuracy and precision after diluting 10 and 50 times, gave values always better than 10% R.S.D. and error %.

No relevant degradation was observed after any of the three freeze–thaw cycles, with differences in the initial concentration less than 10%. Similar results (differences to the initial concentration always lower than 10%) were obtained in the case of the mid-term stability test for both QC and real samples assuring the feasibility of stored samples analysis.

3.2. Analysis of cosmetic products

The validated HPLC-UV-DAD and HPLC–ESI-MS assays have been applied to the analysis of some cosmetic preparations sold on internet web sites with the purpose of preventing and fighting hair loss and other hormone-dependent skin diseases, like acne and hirsutism.

Figs. 4 and 5 and Table 3 show that, in all the examined products, more than one forbidden compound was present. In details, minoxidil was always present in the lotions sold for preventing hair loss and hirsutism together with canrenone and progesterone. Spirono-

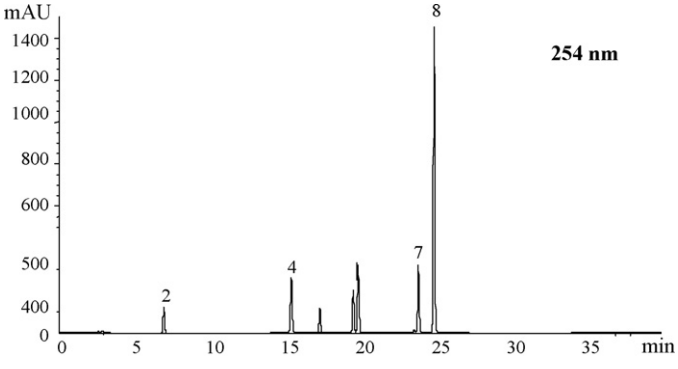

Fig. 4. HPLC-UV-DAD chromatogram at 254 nm of an extract of commercial cosmetic cream (diluted 1:100) containing 12.1 mg/g triamcinolone acetonide (4), 17.3 mg/g spironolactone (7) and 49.0 mg/g progesterone (8) and IS (2).

Table 1
Method calibration data and recovery of analytes under investigation

Analytes	Determination coefficient (r^2)[a]	LOD (μg/ml or μg/g)	LOQ (μg/ml or μg/g)	Mean recovery (%)[b] (μg/ml or μg/g)		
				40	300	600
HPLC-UV-DAD						
Minoxidil	0.9991 ± 0.004	0.6	1.8	92.5 ± 2.3	94.7 ± 2.5	94.2 ± 2.5
Triamcinolone acetonide	0.9938 ± 0.006	0.5	1.5	91.7 ± 2.1	94.7 ± 2.2	94.8 ± 3.2
Hydrocortisone	0.9991 ± 0.007	0.7	2.1	92.0 ± 3.4	96.0 ± 2.4	97.0 ± 2.2
Spironolactone	0.9989 ± 0.006	0.5	1.6	93.5 ± 3.7	95.5 ± 2.8	94.5 ± 2.3
Canrenone	0.9947 ± 0.005	0.4	1.3	92.5 ± 2.9	94.5 ± 2.4	94.7 ± 2.6
Progesterone	0.9993 ± 0.006	0.6	1.9	91.1 ± 1.8	97.1 ± 2.5	96.1 ± 3.1
Estrone	0.9947 ± 0.005	0.5	1.5	95.2 ± 3.7	95.7 ± 2.7	95.6 ± 1.8
Analytes	Determination coefficient (r^2)[a]	LOD (ng/ml or ng/g)	LOQ (ng/ml or ng/g)	Mean recovery (%)[b] (ng/ml or ng/g)		
				60	400	800
LC-ESI-MS						
Minoxidil	0.9902 ± 0.006	14	46	92.7 ± 3.5	96.7 ± 2.1	96.0 ± 2.0
Triamcinolone acetonide	0.9923 ± 0.007	15	50	94.0 ± 2.8	96.0 ± 2.5	96.8 ± 2.4
Hydrocortisone	0.9992 ± 0.003	18	54	91.5 ± 3.4	95.8 ± 2.9	94.8 ± 2.5
Spironolactone	0.9941 ± 0.005	16	48	92.5 ± 3.8	93.5 ± 2.1	93.3 ± 2.3
Canrenone	0.9992 ± 0.003	15	50	92.5 ± 2.9	97.5 ± 2.7	96.5 ± 1.7
Progesterone	0.9902 ± 0.006	17	52	92.1 ± 3.7	96.5 ± 2.3	96.0 ± 2.6
Estrone	0.9941 ± 0.004	15	50	93.7 ± 2.7	95.5 ± 2.8	95.7 ± 1.8

[a] Mean ± S.D. of three replicates.
[b] Mean ± S.D. of four replicates.

Table 2
Intra- ($n = 5$) and inter-assay ($n = 15$) precision and accuracy for the analytes under investigation in quality control samples

Analytes	Intra-assay						Inter-assay					
	Precision (R.S.D.)			Accuracy (% error)			Precision (R.S.D.)			Accuracy (% error)		
	40[a]	300[a]	600[a]	40[a]	300[a]	600[a]	40[a]	300[a]	600[a]	40[a]	300[a]	600[a]
HPLC-UV-DAD												
Minoxidil	11.2	9.8	7.8	2.2	1.8	1.2	4.8	3.3	3.7	8.9	8.8	7.6
Triamcinolone acetonide	8.9	8.7	6.2	6.2	9.9	7.9	6	5.7	5.8	11.6	9.7	9.8
Hydrocortisone	12.8	7.7	6.1	8.4	4.3	3	7.9	3.9	3.8	8.8	7.8	6.4
Spironolactone	8.2	3	1.9	4.8	3	1.9	5	2.9	2.8	11.7	7.9	6.3
Canrenone	13.8	11.7	9.9	6.1	3.1	3.2	7.3	6	3.1	12.9	10.7	8.2
Progesterone	12.1	8.9	4.8	4.9	5.1	3.8	7.6	3.9	3.7	11.4	8.9	6.4
Estrone	9.8	8.7	3.9	8.9	9	7.3	7.8	8.4	3.9	9.9	11.1	8.7
Analytes	Intra-assay						Inter-assay					
	Precision (R.S.D.)			Accuracy (% error)			Precision (R.S.D.)			Accuracy (% error)		
	60[b]	400[b]	800[b]	60[b]	400[b]	800[b]	60[b]	400[b]	800[b]	60[b]	400[b]	800[b]
HPLC–ESI-MS												
Minoxidil	7.4	7.4	6.4	9.4	11.3	8.7	3.4	2.5	6.7	2.5	8.8	12.2
Triamcinolone acetonide	5.7	9.8	12.1	1.7	1.2	0.7	3.7	5.7	7.3	2.5	2.2	1.9
Hydrocortisone	5.6	5.3	9.9	5.6	9.9	9.9	8.5	6.6	9.9	1.4	7.7	9.9
Spironolactone	2.4	4.7	7.2	10	8.5	6.9	1.2	2.8	4.4	10.8	8.5	6.3
Canrenone	14.8	7.9	1	12.5	7.3	2.1	4.8	7.2	0.6	13.3	7.6	1.8
Progesterone	10.6	7.8	5.4	9.2	6.5	3.7	6.1	4.6	3.3	10	7.1	4.2
Estrone	14.4	8.4	2.3	7.5	9.6	11.8	8.7	9.5	3.3	6.6	9	11.5

[a] Expressed in μg/ml lotion and μg/g cream.
[b] Expressed in ng/ml lotion and ng/g cream.

lactone was also present in two out of the three lotions. Cream, sold as anti-acne and anti-hirsutism product contained triamcinolone acetonide together with spironolactone, canrenone and progesterone. Neither hydrocortisone, nor estrone was present in the examined preparations.

This is the first remarkable result of the study, which demonstrates the presence of a variety of forbidden substances (hormones, diuretics, minoxidil) in cosmetic products, as also demonstrated in case of anesthetics and antihistaminics illegally added to after-sun cosmetics [27].

The second important non expected result is that the percentages of non-allowed substances in examined products is extremely high, one order of magnitude more than those usually employed in cosmetic samples [27], and comparable to percentage of pharmacologically active substances in pharmaceutical preparations [28].

In conclusion, this paper reports the development of an HPLC method based on two detection methods:

- a diode array detection which can be used by all control laboratories still not equipped with an HPLC–MS instrument for the routine control of substances forbidden in cosmetic products, such as the ones detected in our study;
- an ESI-MS detection, which identifies with a high grade of certainty unknown substances which can be illegally added in cosmetics based on their structure and molecular weight.

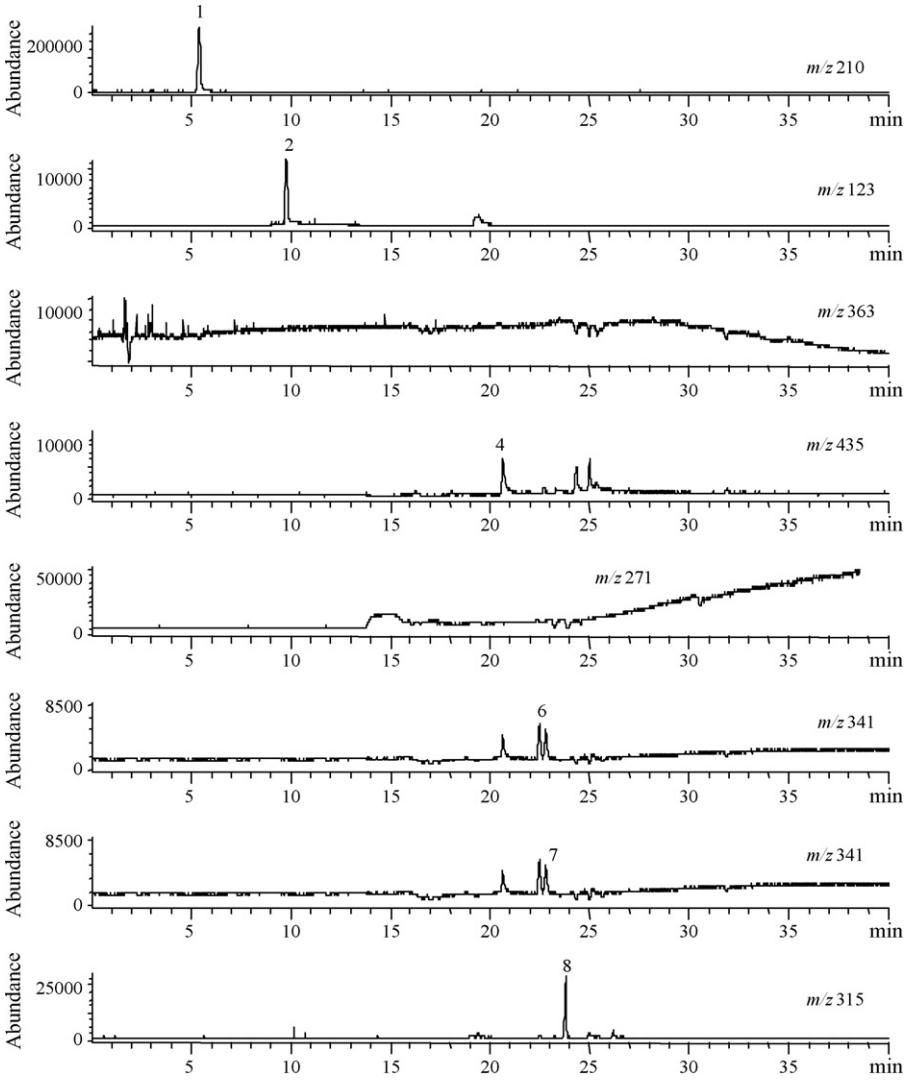

Fig. 5. HPLC–ESI-MS SIM chromatogram of an extract of commercial cosmetic lotion (diluted 1:100,000) containing 74.0 mg/ml minoxidil (1), 11.1 mg/ml canrenone (6), 25.8 mg/ml spironolactone (7) and 31.6 mg/ml progesterone and IS (2).

Table 3
Concentration of non-allowed substances in cosmetic products for preventing hair loss and other hormone-dependent skin diseases by HPLC-UV-DAD and HP LC-ESI-MS (in mg/ml lotion, mg/g cream and in % w/v or w/w substance in cosmetic product)

Sample	Minoxidil	Triamcinolone acetonide	Hydrocortisone	Spironolactone	Canrenone	Progesterone	Estrone
Cream 1							
HPLC-UV-DAD	n.d.	12.1 (1.2%)	n.d.	17.3 (1.7%)	n.d.	49.0 (4.9%)	n.d.
HPLC–ESI-MS	n.d.	10.2 (1.0%)	n.d.	15.1 (1.5%)	5.3 (0.5%)	47.4 (4.7%)	n.d.
Cream 2							
HPLC-UV-DAD	n.d.	n.d.	n.d.	n.d.	n.d.	39.0 (3.9%)	0.001 (0.1%)
HPLC–ESI-MS	n.d.	n.d.	n.d.	n.d.	n.d.	37.4 (3.7%)	0.009 (0.009%)
Lotion 1							
HPLC-UV-DAD	61.9 (6.1%)	n.d.	n.d.	13.4 (1.3%)	7.0 (0.7%)	28.7 (2.8%)	n.d.
HPLC–ESI-MS	82.3 (8.2%)	n.d.	n.d.	6.3 (0.6%)	5.9 (0.6%)	34.3 (3.4%)	n.d.
Lotion 2							
HPLC-UV-DAD	62.4 (6.2%)	n.d.	n.d.	n.d.	24.0 (2.4%)	26.8 (2.6%)	n.d.
HPLC–ESI-MS	68.1 (6.8%)	n.d.	n.d.	n.d.	22.3 (2.2%)	29.3 (2.9%)	n.d.
Lotion 3							
HPLC-UV-DAD	63.4 (6.3%)	n.d.	n.d.	27.0 (2.7%)	13.0 (1.3%)	30.7 (3.1%)	n.d.
HPLC–ESI-MS	74.0 (7.4%)	n.d.	n.d.	25.8 (2.5%)	11.1 (1.1%)	31.6 (3.2%)	n.d.
Lotion 4							
HPLC-UV-DAD	68.3 (6.8%)	n.d.	0.0022 (0.2%)	n.d.	n.d.	n.d.	n.d.
HPLC–ESI-MS	80.0 (8.0%)	n.d.	0.0033 (0.3%)	n.d.	n.d.	n.d.	n.d.

n.d. not detected.

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References

[1] M.E. Sawaya, J. Shapiro, Clin. Dermatol. 18 (2000) 177–186.
[2] V.M. Meidan, E. Touitou, Drugs 61 (2001) 53–69.
[3] A. Mofid, S.A. Seyyed Alinaghi, S. Zandieh, T. Yazdani, Int. J. Clin. Pract. 62 (2008) 433–443.
[4] J. Ayer, N. Burrows, Postgrad. Med. J. 82 (2006) 500–506.
[5] Q.Q. Dinh, R. Sinclair, Clin. Interv. Aging 2 (2007) 189–199.
[6] R.M. Trüeb, Clin. Interv. Aging 1 (2006) 121–129.
[7] N. Otberg, A.M. Finner, J. Shapiro, Endocrinol. Metab. Clin. North Am. 36 (2007) 379–398.
[8] D. Wasserman, D.A. Guzman-Sanchez, K. Scott, A. McMichael, Int. J. Dermatol. 46 (2007) 121–131.
[9] M.E. Sawaya, M.K. Hordinsky, J. Invest. Dermatol. 194 (1995) 30S.
[10] A. Tosti, F. Camacho-Martinez, R. Dawber, J. Eur. Acad. Dermatol. Venereol. 12 (1999) 205–214.
[11] P.G. Nielsen, G.A. Sobbrio, A. Granata, A. Panacea, F. Trimarchi, Minerva Endocrinol. 14 (1989) 105–108.
[12] B.A. Swiglo, M. Cosma, D.N. Flynn, D.M. Kurtz, M.L. Labella, R.J. Mullan, P.J. Erwin, V.M. Montori, J. Clin. Endocrinol. Metab. 93 (2008) 1153–1160.
[13] P.G. Nielsen, Dermatologica 166 (1983) 275–276.
[14] J.C. Shaw, L.E. White, J. Cutan. Med. Surg. 6 (2002) 541–545.
[15] M.H. Winston, A.R. Shalita, Pediatr. Clin. North Am. 38 (1991) 889–903.
[16] T.L. Setji, A.J. Brown, Minerva Med. 98 (2007) 175–189.
[17] http://www.ec.europa.eu/enterprise/cosmetics/html/consolidated_dir.htm (accessed March 2008).
[18] A. Zarghi, A. Shafaati, S.M. Foroutan, A. Khoddam, J. Pharm. Biomed. Anal. 36 (2004) 377–379.
[19] L. Gagliardi, D. De Orsi, M.R. Del Giudice, F. Gatta, R. Porrà, P. Chimenti, D. Tonelli, Anal. Chim. Acta 457 (2002) 187–198.
[20] H. Dong, F. Xu, Z. Zhang, Y. Tian, Y. Chen, J. Mass Spectrom. 41 (2006) 477–486.
[21] S. Zhao, D. Wu, P. Wang, Se Pu 22 (2004) 267–269.
[22] A. Panusa, M. Ottaviani, M. Picaro, E. Camera, L. Gagliardi, P. Cimenti, A. Granese, D. Tonelli, Analyst 129 (2004) 719–723.
[23] ICH Topic Q 2 B Validation of Analytical Procedures: Methodology, The European Agency for the evaluation of Medicinal Products. Available at: http://www.emea.eu.int/htms/human/ich/quality/ichfin.htm. November 1996, London: ICH Technical coordination.
[24] Guidance for Industry, Bioanalytical Method validation, US Department of Health and Human Services, Food and Drug Administration, May 2001. Available at: http://www.fda.gov/cder/guidance/4252fnl.htm.
[25] E. Marchei, M. Pellegrini, R. Pacifici, I. Palmi, S. Pichini, J. Pharm. Biomed. Anal. 37 (2005) 499–507.
[26] S. Pichini, M. Pujadas, E. Marchei, M. Pellegrini, J. Fiz, R. Pacifici, P. Zuccaro, M. Farré, R. de la Torre, J Pharm Biomed Anal. 47 (2008) 335–342.
[27] R. Porrà, S. Berri, L. Gagliardi, P. Chimenti, A. Granese, D. De Orsi, I. Carpani, D. Tonelli, Anal. Bioanal. Chem. 380 (2004) 767–772.
[28] C. Hoedemaker, S. van Egmond, R. Sinclair, Australas. J. Dermatol. 48 (2007) 43–45.