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High performance liquid chromatography-diode array and electrospray-mass spectrometry analysis of vardenafil, sildenafil, tadalafil, testosterone and local anesthetics in cosmetic creams sold on the Internet web sites

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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 vardenafil, sildenafil, tadalafil, testosterone, procaine, lidocaine, prilocaine, and benzocaine in cosmetic creams sold as promising remedies for male erectile dysfunction and female genitals stimulation. The presence of these substances in commercial cosmetic samples is prohibited. Aliquots (1 g) of the cosmetic creams under investigation were diluted 1:100 in methanol, subjected to ultrasonic treatment, added with benzoic acid as internal standard, and analyzed by HPLC–DAD and HPLC–ESI-MS after a further 1:1000 dilution. The compounds were separated by reversed phase chromatography with water (0.02% trifluoroacetic acid) and acetonitrile gradient elution and detected by UV-DAD at 228, 255 and 290 nm and by ESI-MS positive ionisation mode. Benzoic acid was used as internal standard. Linearity was studied with UV-DAD detection from 2.5–7.8 to 250 μg/g range, depending on the different compounds and with ESI-MS in the 3.3–8.9 to 250 ng/g range. Good determination coefficients ($r^2 \geq 0.99$) were found in both UV-DAD and ESI-MS. Limits of quantifications ranged between 2.5 and 7.8 μg/g for HPLC–UV-DAD assay and between 3.3 and 8.9 ng/g for HPLC–ESI-MS assay depending on different analyzed substances. 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 and intra-assay and inter-assay precision always better than 15% and 12%. This method was successfully applied to the analysis of substances under investigations present in cosmetic creams, freely sold on the Internet web-sites.

1. Introduction

Erectile dysfunction (ED) is a common medical condition that affects the sexual life of millions of men worldwide. Numerous physical and psychological factors are involved in normal erectile function, including neurological, vascular, hormonal and cavernous functions [1].

The physiological mechanism for penile erection during sexual stimulation involves release of nitric oxide (NO) from cavernous nerves and vascular endothelial cells in the corpus cavernosum, the activation of cyclic guanosine monophosphate (cGMP) and of cGMP-dependent protein kinases, responsible of free cytoplasmic calcium and smooth muscle relaxation lead to increased corporal blood flow and tumescence (erection). [2]. Phosphodiesterase type-5 (PDE-5) is responsible for degradation of cGMP [3]. Inhibition of PDE-5 slows cGMP degradation, leading to increased levels of cGMP and greater blood flow through the corpus cavernosum when NO is released during sexual stimulation [4].

Oral PDE-5 inhibitors are the current first-line treatment for ED [5]. After the first PDE-5 inhibitor, sildenafil approved by FDA in 1998, two other drugs vardenafil and tadalafil have been approved during 2003.

All three drugs have similar efficacy and toxicity profiles following oral administration. Sildenafil and vardenafil, with similar molecular structure, have half-lives of approximately 4 h, the half-life of structurally different tadalafil is 17.5 h (Fig. 1) [6]. Regarding the side effects, headache, facial flushing, nasal congestion, dyspepsia and back pain have been observed as the most common adverse events [7–9]. Visual events including nonarteritic anterior ischaemic optic neuropathy have been also reported and caution is

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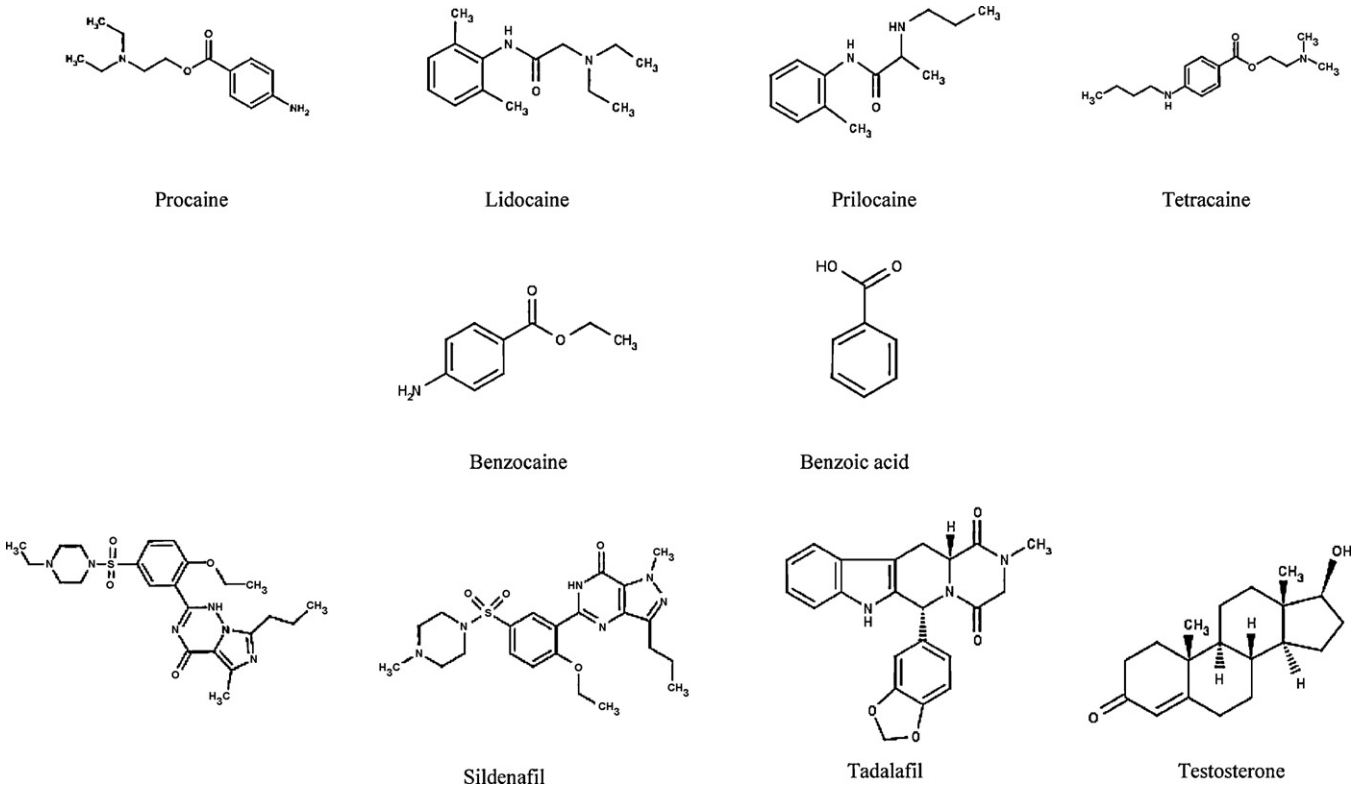

Fig. 1. Chemical structure of the compounds of interest and internal standard.

advised in prescribing PDE-5 inhibitors to patients who have had a myocardial infarction or stroke, or who have resting hypotension or hypertension, unstable angina or cardiac failure, as there are no controlled clinical data in these patient types [4,9].

In recent years, cosmetic preparations for topical use (e.g. creams) containing sildenafil, vardenafil or tadalafil) appeared in the Internet web sites market or in illegal circuits (e.g. private doctors, fitness centers) as promising remedies for male erectile dysfunction, premature ejaculation and female orgasmic dysfunction. Being preparations for topical use, they are seen as "safer" by the consumers than capsules and tablets with respect to eventual acute and chronic toxicity.

Although in the majority of cases all the substances contained in the products are not listed, naive information from consumers and web sites themselves let suspect the simultaneous presence of PDE-5 inhibitors with topical testosterone, proved to be effective in improving erectile dysfunction in aged men [10] and local anaesthetics, used in pharmacological treatment of premature ejaculation [11].

The presence of two latter type of compounds (testosterone and local anaesthetics) in cosmetic creams is expressly forbidden (Annex II) by the Article I from CEE Cosmetic Directive 76/768/EEC, aiming at regulating the production of cosmetic products [12]. In addition, even though not mentioned in the above reported Annex II, PDE-5 inhibitors should not be added to a cosmetic product, since they are also pharmacologically active compounds requiring medical prescription even when present in topical preparations.

Recently, the Italian anti-adulteration and safety bureau (NAS) seized several illegal cosmetic creams sold through Internet web sites or illegal circuits and 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.

Although analytical methodologies exist to measure PDE-5 inhibitors and testosterone in dietary supplements, 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 substances in cosmetics and more specifically in oily preparations, while only one assay has been reported on the analysis of some local anesthetics in after-sun lotions [13–16]. In this study a simple HPLC separation method with both UV-DAD and ESI-MS detection to investigate the illegal presence of vardenafil, sildenafil, tadalafil, testosterone, procaine, lidocaine, prilocaine, tetracaine, benzocaine in cosmetic creams was developed. The developed method has been applied to some creams for topical use sold on Internet web sites.

2. Experimental

2.1. Chemicals and reagents

The pharmaceutical standards (purity > 99%) of procaine hydrochloride, lidocaine hydrochloride, prilocaine hydrochloride, tetracaine hydrochloride, benzocaine, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, testosterone, butyl-4 hydroxybenzoate and benzoic acid (used as internal standard, IS) were purchased from Sigma (Milan, Italy). Vardenafil was purchased from Bayer Italia (Milan, Italy), Sildenafil from Pfizer Italia S.r.l. (Milan, Italy) and Tadalafil from Lilly (Sesto Fiorentino, Florence, Italy). Trifluoroacetic acid (TFA) was purchased from Aldrich (Milan, Italy), HPLC grade acetonitrile and 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 water, glycerine, butylene glycol dicaprylate/dicaprate, denaturated alcohol, C18–36 acid triglyceride, Ceteareth-20 and an water-in-oil cream containing PEG-8-caprylate/Caprate, water, Polyglyceryl-10 diisostearate, cyclomethicone, Propylene glycol, tetrasodium EDTA 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

Five different cosmetic creams were obtained from Internet web sites suggested by consumers, where these products were sold as promising remedies for male erectile dysfunction, male and female genitals stimulation and sexual arousal. The creams (200 mg each product) were contained in white plastic tubes with no label and no indication of the manufacturer.

2.3. Instrumentations and conditions

2.3.1. HPLC–UV-DAD

HPLC–DAD analyses were performed using a Varian Prostar HPLC system consisting of a 210 binary pump, a 410 autosampler (Varian Italia SpA, Turin, Italy) interfaced to a Varian 330 UV-DAD detector. Data acquisition and analysis were performed using standard software Star Varian 5.5 (Varian).

Chromatographic separation was achieved using Waters Sunfire (150 × 4.6 mm; 5 μm; Waters Italy SpA, Milan, Italy). The mobile phase used in the separation, at a flow rate of 1.0 mL/min, consisted of (A) water (0.02% TFA) and (B) acetonitrile (0.02% TFA) programmed as follows: 90% A for 1 min, decreased to 75% A in 5 min and then decreased to 10% A in 25 min, with 10 min to re-establish the initial conditions. The injection volume was 20 μL and the column temperature was set at 30 °C. The DAD detector allowed the evaluation of the peak purity factors 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, 255 nm wavelength was selected as the best compromise for all the compounds under investigation.

2.3.2. HPLC–ESI-MS

HPLC–ESI-MS analyses were performed using Alliance HPLC system (Waters, Etten-Leur, The Netherlands) interfaced to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters) equipped with an electrospray and chemical ionization (ESCi) interface.

The conditions used for chromatographic separation (e.g. column, injection volume, column temperature, mobile phase) were the same as the ones used for HPLC–DAD assay apart from flow rate which was splitted at 1:4 ratio. All chromatographic solvents were degassed with helium before use.

MS characterizations (purity and identity) of compounds under investigation were acquired as follows. The substances, dissolved in mobile phase, were infused through an integrated syringe pump into the ESCi interface in ESI (+), ESI (−), atmospheric pressure chemical ionization (APCI) (+) and APCI (−) in single quadrupole mode at rate of 1 mL/min. On the basis of these experiments, the following optimized conditions were used: capillary voltage at 3.0 kV, cone voltage at 15 V, source temperature and desolvation temperature at 350 °C. The cone and desolvation gas flows were set at 50 and 400 L/h, respectively.

The mass spectrometer was finally operated in positive ESI mode selected full scan acquisition. Qualifying ions were: *m*/*z* 237, 164 and 120 for procaine, *m*/*z* 235, 86 and 58 for lidocaine, *m*/*z* 221, 144 and 86 for prilocaine, *m*/*z* 489, 312 and 151 vardenafil, *m*/*z* 265, 209 and 176 for tetracaine, *m*/*z* 475, 311 and 283 for sildenafil, *m*/*z* 166, 138 and 94 for benzocaine, *m*/*z* 390, 268 and 168 for tadalafil, *m*/*z* 289, 109 and 97 for testosterone and *m*/*z* 123, 105 and 77 for IS. The underlined ions were used for quantification. The presence of 4-hydroxybenzoate esters, present as cosmetics preservatives, was also checked using *m*/*z* 167, 139 and 121 for ethyl 4-hydroxybenzoate, *m*/*z* 181, 139 and 121 for propyl 4-hydroxybenzoate and *m*/*z* 195, 139 and 121 for butyl 4-hydroxybenzoate. The acceptance criterion for selected ion intensity ratios was a deviation ≤ 20% of the average of the ion intensity ratios of all the calibrators.

2.4. Calibration standards and quality control samples

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

Standard stock solutions of 1 mg/mL were prepared in methanol and stored at +4 °C. Diluted dispersion of blank creams were prepared transferring 1 g water-in oil and oil-in water creams to a 100 ml volumetric flask and taken to volume with methanol. The dispersions, added with 60 (g IS, were subjected to ultrasonic treatment for 10 min at 40 °C. After centrifugation, the clear supernatant was collected (solutions A blank).

Calibration standards containing different (g amounts (LOQ-250 (g/g) of analytes under investigation were prepared for each analytical batch by adding suitable amounts of standard stock solutions to 1 ml solutions A. Calibration samples were treated and processed as unknown samples. Several aliquots of quality control (QC) samples (low, medium and high, respectively) at 10, 80 and 200 μg/g were prepared in solutions A to be used for calculation of validation parameters. Blank solutions containing 500 and 1000 (g analytes under the investigation were prepared as over-curve samples, to be tested for accuracy and precision once diluted 5 and 10 times, respectively.

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

Standard stock solutions of 1 μg/mL were prepared in methanol and stored at +4 °C. Diluted dispersions of blank water-in-oil and oil-in-water creams were prepared transferring 1 g 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, added with 60 ng IS, were submitted to ultrasonic treatment for 10 min at 40 °C. After centrifugation, the clear supernatant was collected (solutions B blank).

Calibration standards containing IS and different ng amounts (LOQ-250 ng/g) of analytes under investigation were prepared for each analytical batch by adding suitable amounts of standard stock solutions to 1 ml solutions B. Calibration samples were treated and processed as unknown samples. Several aliquots of quality control (QC) samples (low, medium and high, respectively) at Calibration samples were treated and processed as unknown samples. Several aliquots of quality control (QC) samples (low, medium and high, respectively) at 10, 80 and 200 μg/g were prepared in solutions B to be used for calculation of validation parameters. Blank solutions containing 500 and 1000 ng analytes under the investigation per g products were prepared as over-curve samples, to be tested for accuracy and precision once diluted 5 and 1 0 times, respectively.

2.6. Samples preparation

2.6.1. Samples preparation for HPLC–UV-DAD

Aliquots (1 g) of the cosmetic creams were put into a 100 ml volumetric flask and taken to volume with methanol. The dispersions, added with 60 (g IS, were subjected to ultrasonic treatment for 10 min at 40 °C. A 20 (l amount of samples and calibration standards solutions A were injected, after filtration on Millipore Filter, into the HPLC-UV-DAD. Analyses were executed in triplicate.

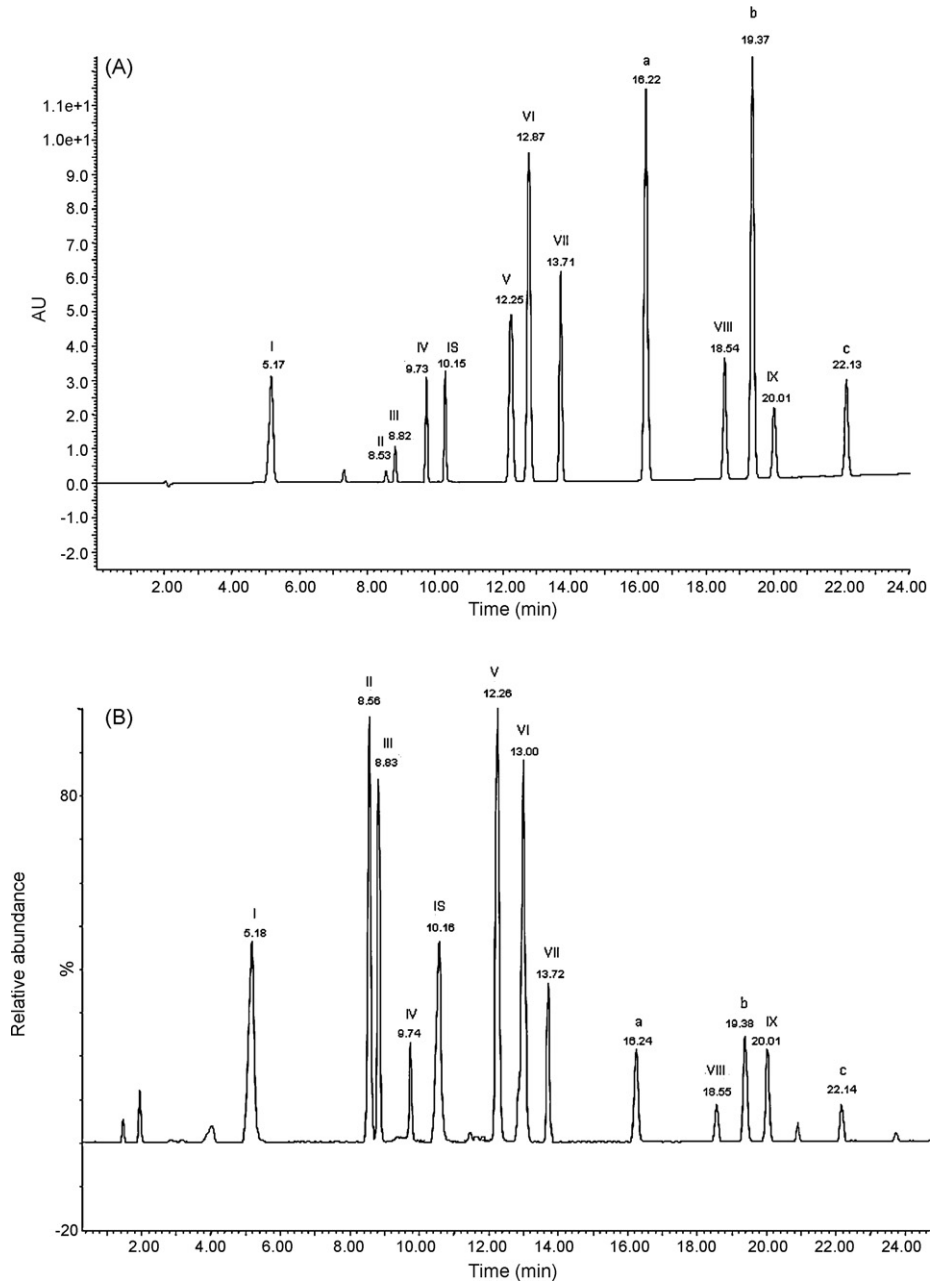

Fig. 2. Overlay of (A) HPLC–UV-DAD chromatogram at 255 nm of blank cosmetic cream added with 80 μg/g Procaine (I), Lidocaine (II), Prilocaine (III), Vardenafil (IV), Tetracaine (V), Sildenafil (VI), Benzocaine (VII), Tadalafil (VIII), Testosterone (IX) and 60 μg/g IS; ethyl 4-hydroxybenzoate (a), propyl 4-hydroxybenzoate (b) and butyl 4-hydroxybenzoate (c). (B) HPLC–ESI-MS chromatogram of blank cosmetic cream added with 80 ng/g procaine (I), lidocaine (II), prilocaine (III), vardenafil (IV), tetracaine (V), sildenafil (VI), benzocaine (VII), tadalafil (VIII), testosterone (IX) and 60 ng/g IS; ethyl 4-hydroxybenzoate (a), propyl 4-hydroxybenzoate (b) and butyl 4-hydroxybenzoate (c).

2.6.2. Samples preparation for HPLC–ESI-MS

Aliquots (1 g) of the cosmetic creams were put into a 100 ml volumetric flask and taken to volume with methanol. One ml of methanolic dispersion was further diluted 1:1000 with methanol. The dispersions, added with 60 ng IS, were submitted to ultrasonic treatment for 10 min at 40 °C. A 20 (l amount of samples and calibration standards solutions B were injected, after filtration on Millipore Filter, into the HPLC–ESI-MS. Analyses were executed in triplicate.

2.7. Validation procedures

Prior to application to real samples, both HPLC–DAD and LC–MS were tested in a validation protocol following the accepted criteria for bioanalytical method validation [17,18]. Selectivity, matrix effect, recovery, linearity, limit of detection (LOD) and quantification (LOQ), precision, accuracy and stability (freeze/thaw cycles and four-month mid-term stability) were assayed as we previously reported [19].

In brief, blank creams 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 creams, 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.

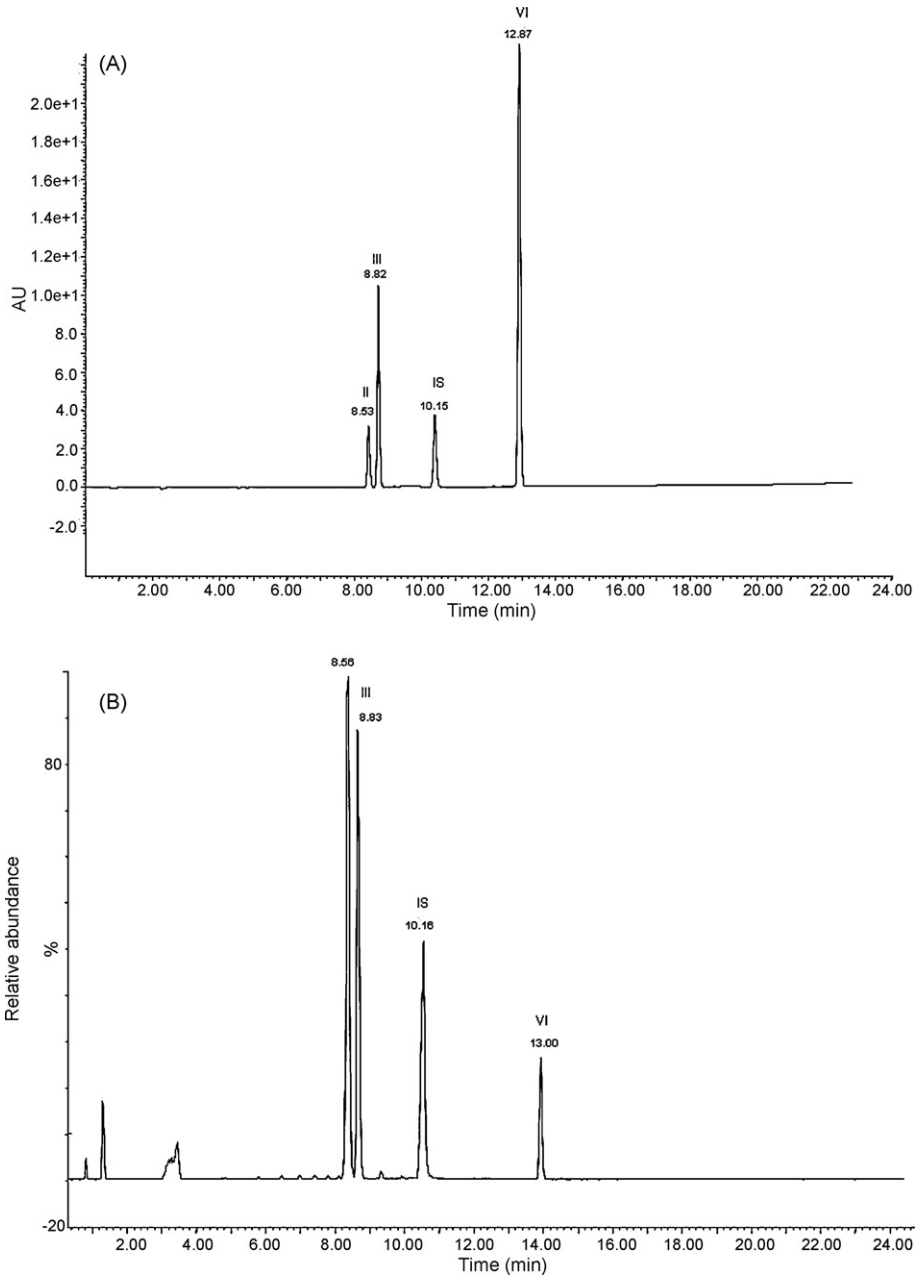

Fig. 3. Overlay of (A) HPLC–UV-DAD chromatogram at 255 nm and (B) HPLC–ESI-MS chromatogram of an extract of commercial cosmetic cream (diluted 1:100 for UV-DAD and 1:100,000 for ESI-MS) containing 21 mg/g lidocaine (II), 25 mg/g prilocaine (III), 9.8 mg/g sildenafil (VI) and IS.

Analytical recoveries were calculated by comparing the peak areas obtained when calibration samples were analyzed by adding the QC samples and the IS in diluted blank creams prior to and after the dilution procedure. The recoveries were assessed by QC samples using four replicates at each level.

For an evaluation of matrix effects, the peak areas of extracted blank creams spiked with QC 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 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 (SD) of the mean noise level over the retention time window of each analyte was used to determine detection limit (LOD = 3 SD) and the quantification limit (LOQ = 10 SD). To be accepted, the calculated LOQ had to show precision and accuracy within the 20% relative SD and relative error, respectively.

A total of five replicates at each of QC samples added to blank creams and over-curve samples, diluted to optimal levels, extracted as reported above were analyzed for the determination of intra-assay precision and accuracy. Inter-assay parameters were calculated in three different assays using five replicates per assay. Precision was expressed as the relative SD (RSD) of concentrations calculated for QC samples and accuracy as the relative error of the calculated concentrations. Both parameters had to be within 20% of RSD or error for both QC and over-the-curve samples.

The effects of three freeze-thaw cycles (storage at −20 °C) on the stability of compounds in blank creams were evaluated by repeated analysis ($n = 3$) of QC samples. In addition, mid-term stability test was performed for QC and real samples stored at −20 °C. Three replicates of both QC and real samples were analyzed once a month

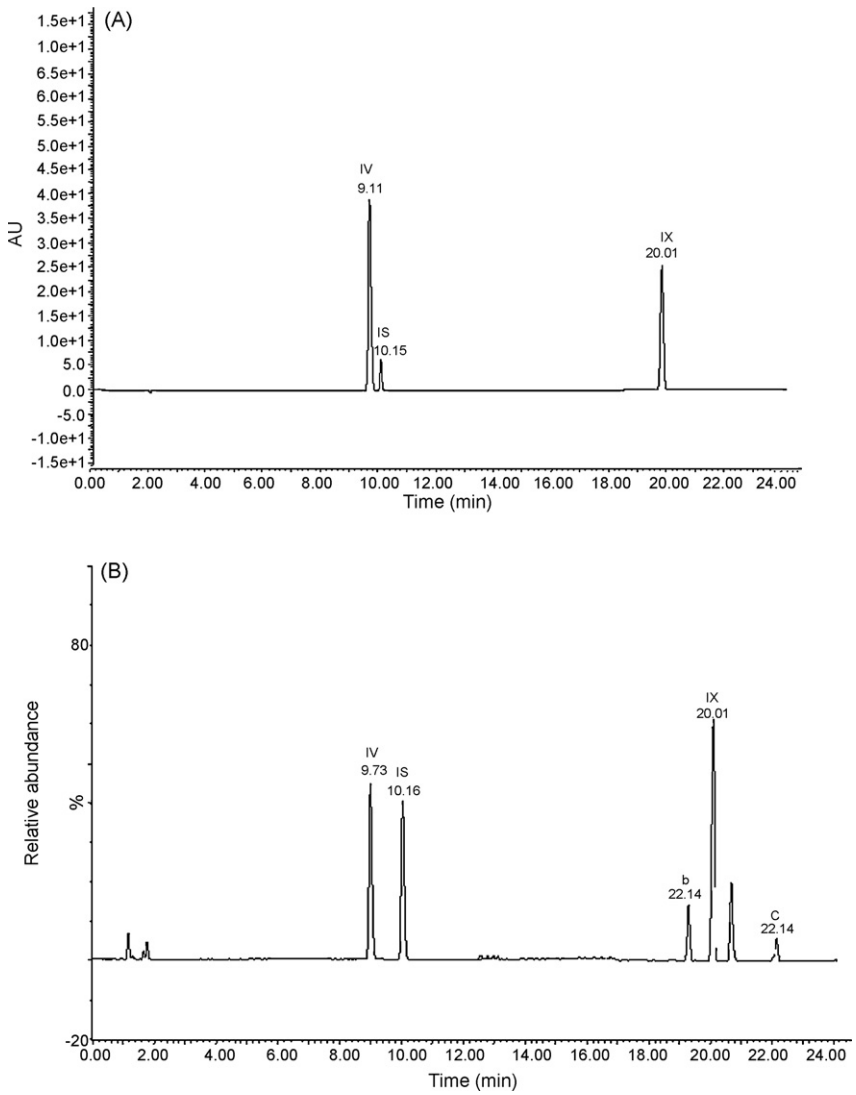

Fig. 4. Overlay of (A) HPLC–UV-DAD chromatogram at 255 nm and (B) HPLC–ESI-MS chromatogram of an extract of commercial cosmetic cream (diluted 1:100 for UV-DAD and 1:100,000 for ESI-MS) containing 9.5 mg/g vardenafil (IV), 9.8 mg/g testosterone (IX), and IS.

during a four-month period. The stability was expressed as a percentage of the initial concentration (first analyzed batch) of the analytes both in QC and real samples.

3. Results and discussion

3.1. Chromatography and validation results

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 electrospray ionisation ESI-MS is also suitable.

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. Under these analytical conditions, the retention times of the analytes under investigation are those shown in Figs. 2–4 while the relative retention time (RTT) are the following: procaine 0.51, lidocaine 0.84, prilocaine 0.87, vardenafil 0.96, tetracaine 1.21, sildenafil 1.28, benzocaine 1.35, tadalafil 1.83 and testosterone 1.97.

Fig. 2 presents an overlay at 255 nm and ESI-MS total ion current chromatograms indicating the coincidence of signals of the analytes under investigation; Figs. 3 and 4 show the UV DAD at 255 nm and ESI-MS chromatograms of extracts of two different analyzed creams containing lidocaine, prilocaine and sildenafil and vardenafil and testosterone, 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 creams 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 and ESI-MS chromatograms, in any case at retention times different from those of analytes under investigation, were identified as 4-hydroxybenzoate esters (ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate and butyl 4-hydroxybenzoate) used as preservatives in cosmetic products. With respect to the matrix effect, the comparison between peak areas of analytes spiked in diluted blank creams ver-

Table 1
Method calibration data and recovery of analytes under investigation.

Analytes	Determination coefficient (r^2)[a]	LOD (μg/g)	LOQ (μg/g)	Mean recovery (%)[b]		
HPLC–UV-DAD				10 μg/g	80 μg/g	200 μg/g
Procaine	0.9988 ± 0.004	1.2	3.9	95.7 ± 2.5	97.7 ± 2.6	93.8 ± 2.4
Lidocaine	0.9985 ± 0.004	2.3	7.8	96.3 ± 2.2	95.7 ± 2.4	93.7 ± 1.5
Prilocaine	0.9989 ± 0.003	1.9	6.2	95.1 ± 2.8	95.5 ± 2.1	95.0 ± 2.7
Vardenafil	0.9955 ± 0.003	1.1	3.6	95.9 ± 2.5	97.8 ± 2.2	94.5 ± 2.9
Tetracaine	0.9983 ± 0.004	1.7	5.6	94.8 ± 2.6	94.7 ± 2.1	93.9 ± 2.6
Sildenafil	0.9984 ± 0.003	0.8	2.5	94.7 ± 2.6	98.7 ± 2.1	94.7 ± 2.5
Benzocaine	0.9988 ± 0.002	1.8	5.9	97.9 ± 2.4	94.6 ± 2.7	95.5 ± 2.7
Tadalafil	0.9989 ± 0.003	1.1	3.8	95.6 ± 2.8	95.9 ± 1.9	94.7 ± 2.2
Testosterone	0.9957 ± 0.002	1.7	5.5	96.7 ± 2.4	96.9 ± 2.7	92.2 ± 2.1
Analytes	Determination coefficient (r^2)[a]	LOD (ngg)	LOQ(ngg)	Mean recovery (%)[b]		
HPLCESIMS				10 ng/g	80 ng/g	200 ng/g
Procaine	0.9998 ± 0.004	1.4	4.6	97.2 ± 3.5	97.4 ± 2.5	95.1 ± 2.5
Lidocaine	0.9985 ± 0.005	1.6	5.3	94.0 ± 2.8	94.7 ± 2.8	95.6 ± 2.6
Prilocaine	0.9985 ± 0.003	1.0	3.3	91.5 ± 2.4	95.8 ± 2.3	94.0 ± 2.9
Vardenafil	0.9955 ± 0.003	2.7	8.9	92.8 ± 3.2	96.9 ± 2.6	93.1 ± 2.7
Tetracaine	0.9983 ± 0.004	1.9	6.3	92.7 ± 2.9	94.8 ± 2.3	95.5 ± 2.4
Sildenafil	0.9984 ± 0.003	2.0	6.6	95.7 ± 3.1	97.6 ± 2.0	94.8 ± 2.8
Benzocaine	0.9986 ± 0.002	1.7	5.6	93.6 ± 2.7	95.7 ± 2.1	94.9 ± 2.4
Tadalafil	0.9979 ± 0.003	2.4	7.9	96.7 ± 2.1	96.7 ± 2.2	92.6 ± 2.8
Testosterone	0.9979 ± 0.002	2.2	7.3	96.0 ± 2.5	95.7 ± 2.3	95.9 ± 2.5

[a] Mean ± SD of three replicates.
[b] Mean ± SD of four replicates.

sus those for pure diluted standards showed less than 10% analytical signal suppression due to coeluting endogenous substances.

Absolute analytical recoveries (mean ± standard deviation, SD) obtained after dilution procedure of both water-in-oil and oil-in-water creams 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 and adequate for the purposes of the present study (Table 1). Particularly, the calculated LOQ tested for precision 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 [18,19] (Table 2). Over-curve samples, tested for accuracy and precision after diluting 10 and 50 times, gave values always better than 10% relative standard deviation (RSD) 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

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 (RSD)			Accuracy (% error)			Precision (RSD)			Accuracy (% error)		
	10 μg/g	80 μg/g	200 μg/g	10 μg/g	80 μg/g	200 μg/g	10 μg/g	80 μg/g	200 μg/g	10 μg/g	80 μg/g	200 μg/g
HPLC–UV-DAD												
Procaine	10.0	11.8	8.7	1.2	1.1	1.8	3.8	4.3	2.4	10.9	8.3	9.6
Lidocaine	8.1	8.7	5.2	4.2	7.5	4.9	4.7	1.7	8.5	8.6	9.2	10.8
Prilocaine	2.8	1.7	4.6	9.0	1.5	1.0	3.2	4.3	6.4	2.8	7.6	4.4
Vardenafil	5.2	3.2	3.9	3.8	4.0	4.4	7.5	1.6	9.1	4.7	11.9	5.3
Tetracaine	5.8	12.7	9.3	5.1	4.7	4.1	3.2	5.6	3.1	5.9	10.4	10.2
Sildenafil	9.1	9.8	8.4	5.9	4.4	2.7	5.9	6.3	7.2	11.0	8.5	2.4
Benzocaine	8.8	7.8	9.3	7.9	9.4	1.3	1.9	7.6	11.1	5.9	11.5	9.7
Tadalafil	9.9	8.3	5.3	9.2	4.3	3.3	4.9	8.0	7.3	2.5	6.3	4.6
Testosterone	7.8	9.4	7.6	3.9	9.4	1.2	1.3	9.8	6.2	5.3	4.1	4.5
	Precision (RSD)			Accuracy (% error)			Precision (RSD)			Accuracy (% error)		
	10 ng/g	80 ng/g	200 ng/g	10 ng/g	80 ng/g	200 ng/g	10 ng/g	80 ng/g	200 ng/g	10 ng/g	80 ng/g	200 ng/g
HPLC–ESI-MS												
Procaine	6.4	7.6	3.4	9.0	13.1	7.7	4.3	5.2	3.7	6.5	9.8	11.2
Lidocaine	9.7	2.8	10.1	7.1	2.1	1.7	7.3	7.5	3.3	10.5	7.2	4.9
Prilocaine	12.6	6.3	7.9	6.5	9.9	9.9	5.8	6.6	1.9	3.4	7.6	1.9
Vardenafil	12.4	6.7	2.2	1.0	5.8	9.9	2.1	8.2	7.4	1.8	5.8	3.6
Tetracaine	14.8	9.9	2.0	15.2	3.7	1.3	8.4	2.7	9.6	1.3	7.5	9.8
Sildenafil	9.6	9.8	7.4	2.9	5.6	7.3	1.6	6.3	7.3	2.0	2.1	1.2
Benzocaine	6.4	1.4	2.5	5.7	6.9	10.8	7.6	5.5	6.3	2.6	8.0	12.5
Tadalafil	9.7	6.7	8.3	8.9	1.9	5.6	8.6	1.7	2.1	9.7	4.8	6.2
Testosterone	2.1	5.2	3.5	1.5	5.0	3.7	1.2	8.2	4.6	9.7	8.5	3.7

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 HPLC–ESI-MS (mg/g cream and in %w/w substance in cosmetic product).

Sample	Procaine	Lidocaine	Prilocaine	Vardenafil	Tetracaine	Sildenafil	Benzocaine	Tadalafil	Testosterone
Cream 1									
HPLC–DAD	n.d.	21.0 (2.1%)	25.0 (2.5%)	n.d.	n.d.	9.8 (1.0%)	n.d.	n.d.	n.d.
LC–MS	n.d.	23.4 (2.3%)	24.9 (2.5%)	n.d	n.d	10.5 (1.1%)	n.d.	n.d.	n.d.
Cream 2									
HPLC–DAD	n.d.	n.d.	n.d.	9.5 (0.9%)	n.d.	n.d.	n.d.	n.d.	9.8 (1.0%)
LC–MS	n.d.	n.d	n.d	10.1 (1.0%)	n.d	n.d	n.d.	n.d.	10.7 (1.1%)
Cream 3									
HPLC–DAD	n.d.	21.8 (2.2%)	n.d.	n.d.	n.d.	8.7 (0.9%)	n.d.	n.d.	n.d.
LC–MS	n.d.	22. 9 (2.3%)	n.d.	n.d	n.d	9.4 (0.9%)	n.d.	n.d.	n.d.
Cream 4									
HPLC–DAD	n.d.	18.7 (1.9%)	n.d.	n.d.	n.d.	9.6 (1.0%)	n.d.	n.d.	n.d.
LC–MS	n.d.	19.3 (1.9%)	n.d.	n.d.	n.d	10.1 (1.0%)	n.d.	n.d.	n.d.
Cream 5									
HPLC–DAD	20.8 (2.1%)	n.d.	n.d.	n.d.	n.d.	8.7 (0.9%)	n.d.	n.d.	n.d.
LC–MS	21.3 (2.1%)	n.d.	n.d.	n.d	n.d	8.5 (0.8%)	n.d.	n.d.	n.d.

n.d.: not detected.

stability test for both QC and real samples assuring the feasibility of stored samples analysis.

3.2. Analysis of products

The validated HPLC–UV-DAD and HPLC–ESI-MS assays have been applied to the analysis of five different cosmetic creams sold on Internet as remedies for male erectile dysfunction genital stimulation. Differently from previous reported assays, which only analyzed either local anaesthetics in after sun lotions, or testosterone in nutritional supplements or PDE-5 inhibitors in bulk products [14–17], the peculiarity of the presented method is the possibility to separate and detect substances coming from different drug classes.

Figs. 3 and 4 and Table 3 show that, in all the examined products, more than one forbidden compound was present. In details, lidocaine was the local anesthetic mainly found in the cosmetic creams (three out of fine preparation) and in one occasion together with prilocaine. Procaine was found in one out of the five products, while benzocaine was never found. Four out of the five creams contained around 1% sildenafil, in one case together with 1% testosterone. In only one case the PDE-5 inhibitor was the vardenafil, while tadalafil was never present in these illegal preparations. This is the remarkable result of the study, which demonstrates the presence of a variety forbidden substances in cosmetics sold for topical use.

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 LC-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.

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