ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 1191–1199

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

On-line post-column photochemical derivatization in liquid chromatographic–diode-array detection analysis of binary drug mixtures[1]

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Received for review 13 September 1995; revised manuscript received 30 October 1995

Abstract

HPLC methods were developed for the analysis of pharmaceutical creams containing binary drug mixtures (betamethasone valerate–chlorocresol; hydrocortisone–miconazole nitrate; desonide pivalate–chlorhexidine; dexamethasone–clotrimazole; triamcinolone acetonide–econazole nitrate). The chromatographic separations were performed on C-18 and cyano columns under reversed-phase conditions. A post-column on-line photochemical reactor (irradiation at 254 nm) was arranged between the analytical column and the diode-array detector to enhance the performance of the method. Two UV spectra (photoreactor on and off) were obtained for each analyte and these additional sources of information proved to be useful for the unambiguous identification of the various analytes. The method was applied to the quality control of commercial creams using a solid-phase extraction procedure for the sample clean-up.

Keywords: Diode-array detection; Drug analysis; High-performance liquid chromatography; Pharmaceutical creams; Photochemical derivatization; Solid-phase extraction

1. Introduction

Binary drug mixtures are currently used in the therapy of a variety of diseases [1,2]. The quality control of these pharmaceutical dosage forms may present some difficulties that are mainly due to poor detectability and/or to similar analytical properties of the analytes; for certain formulations, excipients and preservatives can also interfere with the analysis. An adequate sample clean-up procedure generally enables excipient interference to be eliminated, but the detection system often has to be enhanced to achieve the required selectivity and sensitivity. Moreover, reliable quality control calls for an information-rich detection able to provide an unambiguous identification of the analytes.

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[1] Presented at the Fifth International Symposium on Drug Analysis, September 1995, Leuven, Belgium.

SSDI 0731-7085(95)01695-3

To this end, in the present study post-column on-line photochemical derivatization was combined with a diode-array detection (DAD) to improve the intrinsic selectivity of the liquid chromatography (HPLC) in the analysis of binary drug mixtures. On-line photoderivatization is recognized as an effective way of selectively transforming the analytes into compounds with altered spectral and electrochemical properties of analytical utility [3–6]. When a photoreactor is arranged on-line between the analytical column and the DAD, the column effluent can be subjected to UV irradiation at 254 nm and two UV spectra (photoreactor switched on and off) can be achieved for each analyte under the same chromatographic conditions. These additional sources of information on the analyte structure and photoreactivity are useful to support the chromatographic retention data in respect of the positive identification of the chromatographic peaks. In previous studies, this simple approach proved to be able to enhance both the selectivity and sensitivity in the HPLC analysis of pharmaceutical [7] and cosmetic [8] formulations. Thus, in order to evaluate better the potential of this approach for quality control purposes, in the present work its application was extended to the HPLC analysis of binary drug mixtures of antimycotics (clotrimazole, econazole, miconazole) or antiseptics (chlorocresol and chlorhexidine) with steroidal anti-inflammatory drugs (betamethasone valerate, hydrocortisone, desonide pivalate, dexamethasone, triamcinolone acetonide) in commercial dosage forms (creams and a milk) of complex composition.

2. Experimental

2.1. Materials

Betamethasone valerate (Schering-Plough, Italy), miconazole nitrate (Janssen, Belgium), clotrimazole (Bayer, Italy), econazole nitrate (Cilag, Italy), budesonide (IDI Farmaceutici, Italy), fluocinonide (Recordati, Italy), flumethasone pivalate (Zyma, Italy), desonide pivalate (Laboratorio Farmacologico Milanese, Italy), dexamethasone (Merck Sharpe Dohme, Italy) and propyphenazone (Bracco, Italy) were kindly supplied by their manufacturers. Biphenyl, chlorhexidine and triamcinolone acetonide were obtained from Sigma Chimica (Italy), hydrocortisone from Merck (Italy) and all other chemicals from Carlo Erba (Italy). For chromatographic separations methanol, acetonitrile and tetrahydrofuran (THF) were of HPLC grade for Mallinckrodt (Germany) and water was de-ionized and doubly distilled. Buffer solutions (pH 3.0 and 7.0) were prepared by adding 85% (w/w) phosphoric acid to 0.05 M triethylamine (TEA) aqueous solutions to the desired pH values: phosphate buffer solution (pH 7.4) was prepared according to standard methods.

Solid-phase extraction (SPE) was performed on C-18 and diol sorbent cartridges (500 mg) from Analytichem International (Harbor City, CA, USA) using a Baker 10 SPE vacuum manifold connected to a water aspirator. Before use, the diol SPE columns were conditioned by rinsing with 6 ml of methylene chloroide and the C-18 SPE columns by rinsing with 3 ml of methanol and then with 3 ml of phosphate buffer solution (pH 7.4)–methanol (60:40, v/v).

2.2. Apparatus

The HPLC system comprised a Varian 5020 chromatograph and a photometric diode-array detector (HP 1040A) connected to an HP 78994A work station. A Beam Boost Model C6808 photoreactor (ICT, Frankfurt) was arranged on-line between the analytical column and the detector. The eluate was irradiated on-line in capillary PTFE tubing (10 m × 0.3 mm i.d.) in crocheted geometry by an 8 W low-pressure mercury lamp with the main spectral emission at 254 nm. The chromatographic conditions used are reported in Table 2.

2.3. Calibration graphs

Mixed standard solutions (concentration range in Table 1) were as follows: betamethasone valerate and chlorocresol, containing hexylresorcinol (0.180 mg ml^{-1}) as the internal standard, were prepared in methanol; hydrocortisone and mi-

conazole, containing clotrimazole (0.60 mg ml^{-1}) as the internal standard, were prepared in methanol–TEA phosphate (0.05 M) (pH 7.0) (88:12, v/v); desonide pivalate and chlorhexidine dihydrochloride, containing biphenyl (0.010 mg ml^{-1}) as the internal standard, were prepared in methanol–TEA phosphate (0.05 M) (pH 3.0) (75:25, v/v); dexamethasone and clotrimazole, containing propyphenazone (0.027 mg ml^{-1}) as the internal standard, were prepared in methanol–TEA phosphate (0.05 M) (pH 7.0) (85:15, v/v); triamcinolone acetonide and econazole nitrate, containing clotrimazole (0.075 mg ml^{-1}) as the internal standard, were prepared in methanol–isopropyl alcohol–TEA phosphate (0.05 M) (pH 7.0) (58:28:14, v/v/v).

The mixed standard solutions were injected in triplicate and the peak-area ratios of analytes to the internal standard were plotted against the corresponding analyte concentration to obtain the calibration graphs.

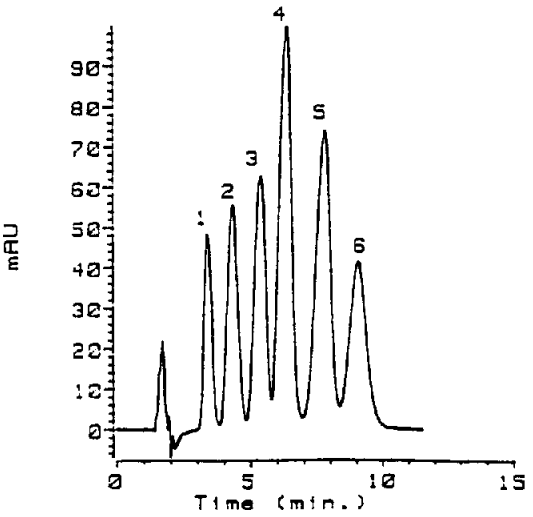

Fig. 1. Representative HPLC separation of triamcinolone acetonide (1), budesonide (2), fluocinonide (3), betamethasone valerate (4), flumetasone pivalate (5) and desonide pivalate (6). Column: 5-μm Spherisorb-CN (150 × 4.6 mm i.d.). Mobile phase: 0.05 M TEA phosphate (pH 3.0)–acetonitrile (75:25, v/v) at 1 ml min^{-1}. UV detection at 240 nm.

2.4. Sample preparation

The commercial formulations I–IV (creams) were subjected to a preliminary SPE to obtain purified and concentrated analytical solutions.

2.4.1. Formulation I

A sample, equivalent to about 0.366 mg of betamethasone valerate and 0.300 mg of chlorocresol, in a 10.0-ml volumetric flask was treated with 5 ml of hexane–methylene chloride (7:3, v/v) under ultrasonication for 5 min at ambient temperature. After dilution to volume with the same solvent system, a 5.0-ml aliquot was applied to the conditioned diol SPE column. After washing with 1 ml of the same solvent, the retained drugs were eluted with two 1.0-ml portions of methanol and 0.5 ml of the internal standard solution (0.180 mg ml^{-1} hexylresorcinol) was added to the eluate.

2.4.2. Formulation II

A sample, equivalent to about 5.00 mg of hydrocortisone and 10.0 mg of miconazole nitrate, was treated with 30 ml of methylene chloride in a 50.0-ml volumetric flask, under ultrasonication for 5 min at ambient temperature. After dilution to volume with the same solvent, a 2.0-ml aliquot of the extract was applied to the diol SPE column; two washings with 3 ml of hexane–methylene chloride (4:1, v/v) were performed and the drugs were eluted with three 1-ml portions of methanol–0.05 M TEA phosphate (pH 7.0) (4:1, v/v). The eluate was collected in a 5.0-ml volumetric flask, 1.0 ml of internal solution (0.060 mg ml^{-1} clotrimazole) was added and the solution was diluted to volume with methanol.

2.4.3. Formulation III

A sample, equivalent to about 0.600 mg of desonide pivalate and 1.00 mg of chlorhexidine gluconate, was treated with 20 ml of methanol in a 50.0-ml volumentric flask under ultrasonication for 20 min at 60°C and the mixture was then brought to volume with phosphate buffer solution (pH 7.4). An aliquot of the resulting suspension was subjected to centrifugation and 5.0 ml of the extract was applied to a C-18 SPE column. After washings with three 1-ml portions of methanol–

phosphate buffer solution (pH 7.4) (40:60, v/v), the retained drugs were eluted with four 1-ml portions of methanol–0.05 M TEA phosphate (pH 3.0) (75:25, v/v). The eluates were collected in a 5.0-ml volumetric flask, 0.5 ml of the internal standard solution (0.010 mg ml^{-1} biphenyl) was added and the volume was adjusted with the elution solution.

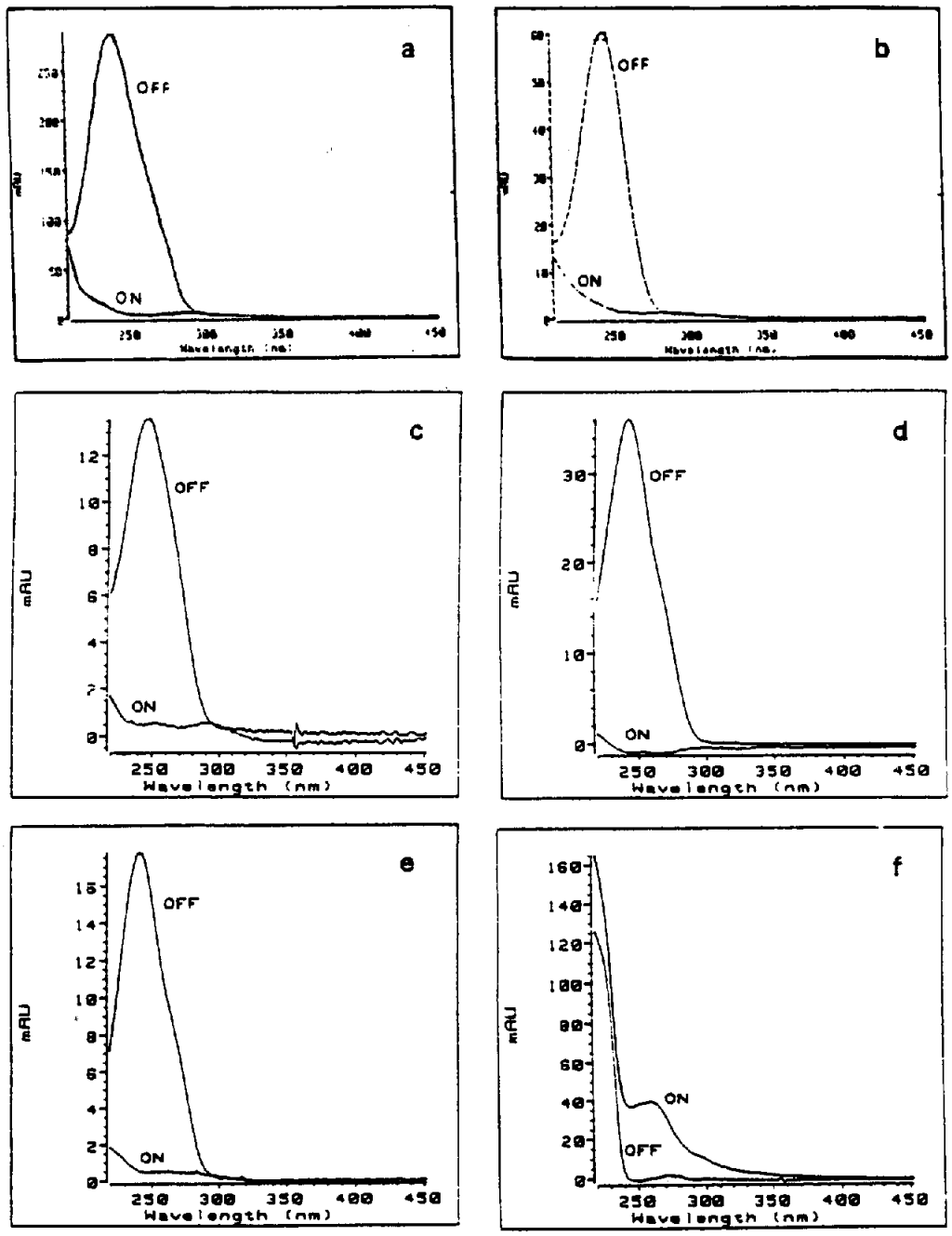

Fig. 2(a)–(f).

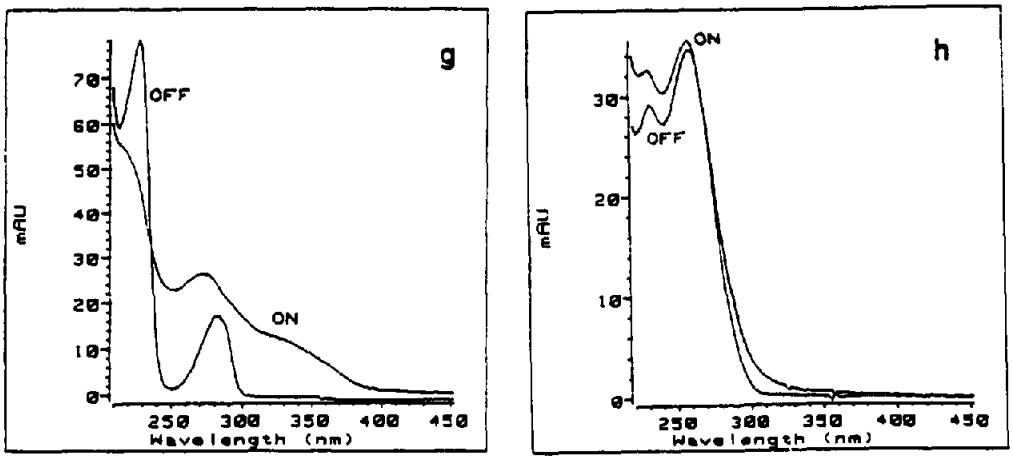

Fig. 2. UV spectra of betamethasone valerate (a), hydrocortisone (b), desonide pivalate (c), dexamethasone (d), triamcinolone acetonide (e), miconazole (f), chlorocresol (g) and chlorhexidine (h), obtained with photoreactor switched on and off. Chromatographic conditions: C-18 column with mobile phase A (spectra a, b, g); CN column with mobile phase C (spectra c, d, h) and mobile phase D (spectra e, f).

2.4.4. Formulation IV

A sample, equivalent to about 1.00 mg dexamethasone and 3.00 mg of clotrimazole, was treated with 40 ml of hexane in a 50.0-ml volumetric flask under ultrasonication for 5 min at ambient temperature; 2 ml of isopropyl alcohol was then added and the volume adjusted with methylene chloride. A 6.0-ml aliquot was applied to a diol SPE column, three washings with 1-ml portions of hexane–methylene chloride (4:1, v/v) were carried out and the retained drugs were then eluted with four 1-ml portions of methanol–0.05 M TEA phosphate (pH 7.0) (85:15, v/v); the eluates were collected into a 5.0-ml volumetric flask, 0.5 ml of the internal standard solution (0.026 mg ml^{-1} propyphenazone) was added and the volume was adjusted with the elution solvent.

2.4.5. Formulation V (a milk)

A sample, equivalent to about 0.300 mg of triamcinolone acetonide and 3.00 mg of econazole nitrate, in a 25.0-ml volumetric flask was treated with 7 ml of isopropyl alcohol and 5 ml of methanol under ultrasonication for 10 min at ambient temperature. Then, 2.5 ml of the internal standard (0.0757 mg ml^{-1} clotrimazole) solution in 0.05 TEA phosphate (pH 7.0) and 3.5 ml of the same TEA phosphate solution were added. The mixture was diluted to volume with methanol and the resulting suspension was filtered through a 0.45-μm Phenex nylon 66 filter (25 mm i.d.).

2.4.6. Assay procedure

The sample solutions obtained as above described were subjected to HPLC analysis (conditions in Table 2) and the drug content in each sample was calculated by comparison with an appropriate standard solution.

3. Results and discussion

3.1. Chromatography

All the formulations examined, with the exception of formulation I, contain a steroidal antiinflammatory drug together with a basic antimycotic or antiseptic agent (Table 2). To develop a versatile liquid chromatographic system, suitable for both neutral and basic compounds, a cyano column under reversed-phase conditions was used. In Fig. 1 a representative separation of a standard steroid mixture is shown. These chromatographic conditions, with minor adjustment of the mobile phase composition (Table 2), proved to be suitable for the analysis of the commercial formulations. In contrast, an RP-18 column was chosen for the analysis of be-

tamethasone valerate and chlorocresol (formulation I).

3.2. Detection

In order to provide an information-rich detection, a diode-array detector (DAD) was used in combination with post-column on-line photochemical derivatization. Thus, the recognized efficacy of DAD in confirming the peak identity [9] was enhanced by recording two UV spectra (photoreactor on and off) for each analyte. In Fig. 2 the UV spectra (on and off) of the various analytes are compared. As shown in Fig. 2, on-line UV irradiation at 254 nm induced significant alterations in the analyte chromophore, and therefore modifications in spectral properties. As a result, the absorption maximum at about 240 nm (conjugated dienone) is suppressed in the UV spectrum of the steroid; the residual weak absorption at higher wavelengths (290–300 nm) can be ascribed to an isolated carbonyl group ($n \rightarrow \pi^*$ transition). This suggests that rearrangements in-

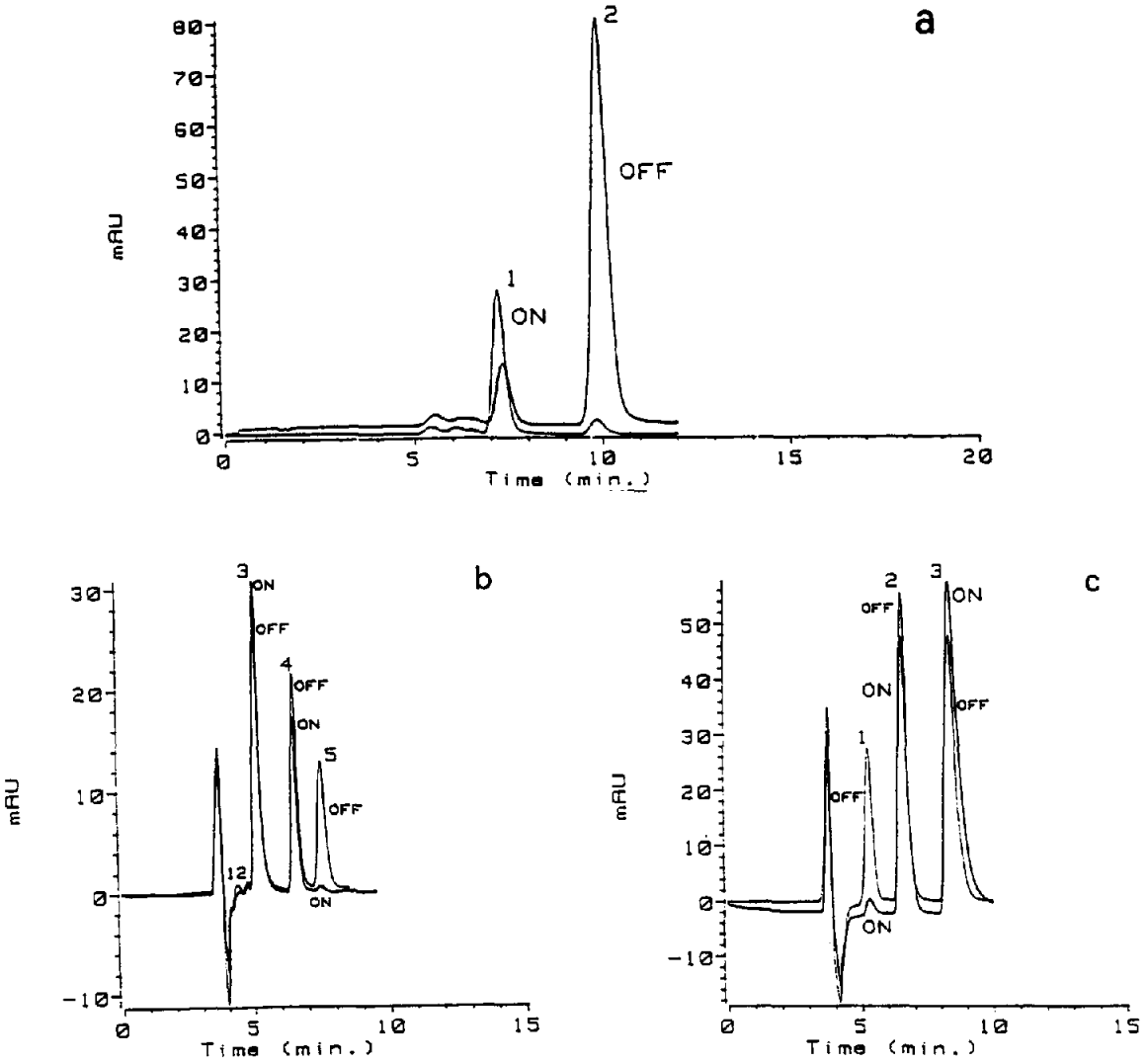

Fig. 3(a)–(c).

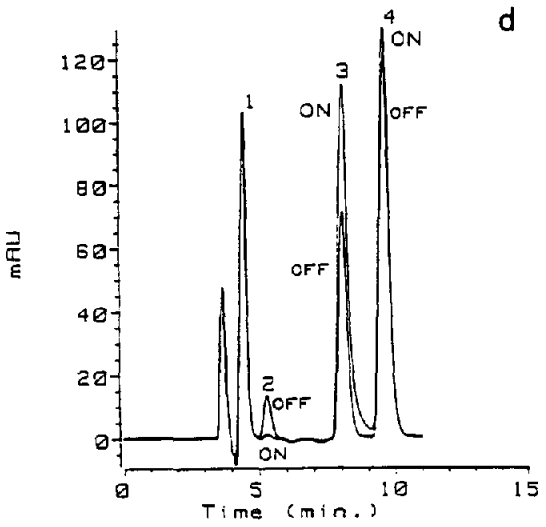

Fig. 3. Commercial samples of (a) chlorocresol (1) and betamethasone valerate (2); (b) methylparaben (1), propylparaben (2), chlorhexidine (3), biphenyl (4) and desonide pivalate (5); (c) dexamethasone (1), propyphenazone (2; internal standard) and clotrimazole (3); (d) benzoic acid (1), triamcinolone acetonide (2), clotrimazole (3; internal standard) and econazole (4). Off = photoreactor switched off; On = photoreactor switched on. Chromatographic conditions as in Table 2.

volving the conjugated double bond in the ring A, were photoinduced; these results are consistent with previous reports on the photostability of steroidal drugs [10–12]. In the present study, specific investigations on the steroid photodegradation products were not conducted.

Marked spectral UV modifications were also observed on irradiation of miconazole and chlorocresol, whereas chlorhexidine was found to be essentially photostable (Fig. 2). Clotrimazole and econazole undergo photochemical modifications like miconazole [7]. These photochemically alterated spectral properties are favourable to measurements at higher wavelengths (antimycotic drugs) or to enhanced sensitivity (chlorocresol) in HPLC analyses. The chromatograms obtained with the on-line photo reactor on and off exhibit different profiles with selectively altered peak heights. These results are illustrated in Fig. 3(a) (betamethasone valerate–chlorocresol), Fig. 3(b) (desonide pivalate–chlorhexidine), Fig. 3(c) (dexamethasone–clotrimazole) and Fig. 3(d) (triamcinolone acetonide–econazole). In each instance, as expected, at the selected detection wavelength the steroid peak was suppressed with the lamp turned on; this allows easy confirmation of the peak identity and the monitoring of potentially co-eluting components. Moreover, the suppression of a peak can offer the opportunity of selectively determining a second partly overlaping peak. These possibilities, although only in part exploited in a given application, constitute an important basis for optimizing the experimental conditions for practical and reliable quality control.

In the photoderivatization step, various products were obtained for each analyte, as verified when the irradiated column effluent was collected and subjected to HPLC analysis. Preliminary investigations carried out on chlorocresol showed that two major photoderivatives were obtained, of which one proved to be *m*-cresol (elimination of the chloro substituent), as indicated by its retention time and UV spectrum.

3.3. Analysis of pharmaceutical formulations

Routine analyses of the drugs in their commercial dosage forms were performed by direct UV detection without photochemical derivatization. To this end, a linear relationship between the peak-area ratio (analyte to internal standard) and the drug concentration was obtained for each drug (Table 1). As can be seen, the within-run precision (repeatability) of the HPLC assay, expressed as RSD of the peak-area ratios from replicate ($n = 8$) analyses of the same standard solution, was better for the antimycotic drugs (RSD = 0.40–0.60%).

The subsequent analysis of the commercial formulations involved a preliminary, accurate sample preparation in order to remove interfering matrix components and to avoid overloading of the analytical column. According to previous experience [7,13], the cream samples were subjected to SPE on a diol sorbent. The sample dissolved in *n*-hexane–methylene chloride of appropriate composition was applied to a conditioned diol SPE column and, after selective washings, the retained drugs were quantitatively eluted with methanol or methanol–TEA phosphate buffer. However, this

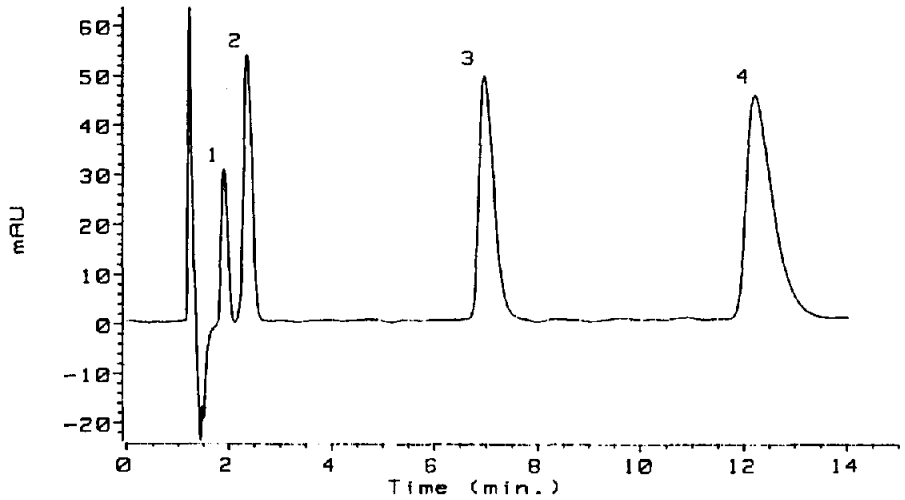

Fig. 4. HPLC of a commercial sample of benzoic acid (1) hydrocortisone (2), clotrimazole (3; internal standard) and miconazole (4). Chromatographic conditions as in Table 2.

approach was found to be inadequate for the analysis of formulation III, giving a low recovery of desonide pivalate; thus, for this application a C-18 sorbent was preferred. When formulation V (a milk) was analysed, the SPE step was omitted and the properly diluted sample was filtered through a nylon filter to give a clear solution. Under the described chromatographic conditions (Table 2; Fig. 3(a)–3(d) and Fig. 4), the sample solutions were then subjected to HPLC analyses and the drug content in each commercial dosage form was found to be in agreement with the claimed content with good between-run precision (reproducibility) (RSD = 1.05–1.80%). The accuracy of the whole method (extraction and HPLC assay) was verified by analysing samples fortified with 30% and 50% of the claimed drug content; the recoveries were 98.80–101.3%. The efficacy of the SPE step is illustrated in Fig. 3(b), where peaks 1 and 2 are due to residual methyl- and

Table 1
Data for the calibration graphs ($n = 6$) for HPLC determinations

Drug	Internal standard	Slope[a]	Intercept[a]	Correlation coefficient	Working (mg ml^{-1})	RSD[b] (%)
Betamethasone valerate	Hexylresorcinol	15.139 (0.313)	−0.0162 (0.0350)	0.9990	0.040–0.120	1.34
Chlorocresol	Hexylresorcinol	15.615 (0.318)	−0.0114 (0.0260)	0.9990	0.030–0.090	0.93
Hydrocortisone	Clotrimazole	17.088 (0.240)	−0.0247 (0.0102)	0.9997	0.020–0.060	0.45
Miconazole nitrate	Clotrimazole	23.241 (0.251)	−0.0177 (0.0230)	0.9996	0.040–0.120	0.50
Desonide pivalate	Biphenyl	63.590 (0.815)	−0.0124 (0.0208)	0.9991	0.004–0.016	1.80
Chlorhexidine	Biphenyl	96.228 (1.264)	0.0150 (0.0261)	0.9990	0.004–0.020	0.97
Dexamethasone	Propyphenazone	21.441 (0.434)	−0.0054 (0.0126)	0.9991	0.007–0.035	1.13
Clotrimazole	Propyphenazone	18.400 (0.175)	−0.0082 (0.0122)	0.9998	0.021–0.105	0.40
Triamcinolone acetonide	Clotrimazole	43.114 (0.563)	−0.0135 (0.0081)	0.9995	0.004–0.020	1.6
Econazole nitrate	Clotrimazole	12.445 (0.104)	−0.0024 (0.0140)	0.9999	0.040–0.200	0.60

[a] ± 95% confidence intervals (for the slope and intercept).
[b] From replicate ($n = 8$) analyses of the middle standard solution of the working concentration.

Table 2
Assay results for the HPLC analysis of commercial binary drug mixtures, expressed as a precentage of the claimed content (averages of five determinations)

Formulation	Components	Found (%)	RSD (%)	Column[a]	Mobile phase[b]	UV detection, λ (nm)
I	Betamethasone valerate	101.40	1.46	C-18	A	235
	Chlorocresol	104.00	1.07			
II	Hydrocortisone	104.00	1.35	CN	B	230
	Miconazole nitrate	98.50	1.80			
III	Desonide pivalate	101.30	1.60	CN	C	240
	Chlorhexidine	101.00	1.40			
IV	Dexamethasone	96.50	1.15	CN	C	230
	Clotrimazole	97.60	1.05			
V	Triamcinolone acetonide	104.00	1.50	CN	D	235
	Econazole nitrate	99.10	1.80			

[a] Column: C-18 = 5-μm Hypersil ODS (150 × 3.2 mm i.d.) with a 5-μl sample loop; CN = 5-μm Spherisorb-CN (150 × 4.6 mm i.d.) with a 20-μl sample loop.
[b] Mobile phase: A = water–methanol (25:75, v/v) at flow-rate of 0.5 ml min^{-1}; B = 0.05 M TEA phosphate (pH 3.5)–acetonitrile–THF (84:11:5, v/v/v) at 1.4 ml min^{-1}; D = 0.05 M TEA phosphate (pH 3.0)–acetonitrile (65:35, v/v) at 1 ml min^{-1}; C = 0.05 M TEA phosphate (pH 3.0)–acetonitrile (75:25, v/v) at 1 ml min^{-1}.

propylparabens; as shown, these preservatives were essentially eliminated by SPE. The application of post-column photoderivatization can enhance sensitivity in the assay of chlorocresol and antimycotic drugs and offer a simple approach for confirming the peak identity.

In summary, the results confirm the utility of the on-line post-column photochemical derivatization as a way of improving the analytical performance of HPLC methods in respect of both the qualitative and quantitative aspects. SPE based on a diol sorbent was found to be suitable for practical sample clean- up in the analysis of pharmaceutical creams.

Acknowledgements

Thanks are due to Miss Raffaella Roffi and Luciana Cenni for valuable technical assistance. This work was supported by a grant from MURST (Rome, Italy).

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