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Simple and rapid determination of psoralens in topic solutions using liquid chromatography

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Abstract

A method for sample preparation and high-performance liquid chromatography detection and quantification has been developed for the routine analysis of psoralen and bergapten, photosensitizing compounds, in topic solutions employed in Brazil for treatment of vitiligo and psoriasis. The linearity, accuracy, the inter- and intra-day precision of the procedure were evaluated. The calibration curve for psoralen and bergapten were linear in the range 1.0–20.0 μg ml^{-1}. The best recoveries of the psoralens in the topic solutions analysed were 96.3–98.2%. The percentage coefficient of variation of the quantitative analysis of the psoralens in the product analyses was within 5%.

Keywords: Liquid chromatography; Psoralens; Furanocoumarins; Quantification

1. Introduction

Psoralens or furanocoumarins are well known photoreactive compounds [1], psoralen and bergapten (Fig. 1) are used in some pharmaceutical and cosmetic products because of their UV light absorbing properties [2]. Psoralens are also increasingly used in dermatology for the photochemotherapy of diseases such as vitiligo, psoriasis, mycosis fungoides, atopic eczema and alopecia areata among others [3,4]. The biological activity of these compounds are attributable to their ability to intercalate into DNA, where they form mono and di-adducts in the presence of long-wave UV light [5]. Furthermore, the use of psoralens in medicine has been associated with higher incidence of skin cancer [6,7]. Linear psoralens cause phototoxicity [8,9]. Several studies have demonstrated that the psoralens are carcinogenic, mutagenic and photodermatitic [10,11].

The psoralens are currently employed in dermatology (orally or topically), associated with Ultraviolet A (UVA) irradiation. The combination of these previous compounds with UVA irradiation is known as PUVA therapy (Psoralens plus UVA irradiation) [12–14].

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Popular phytomedicines are used in Brazil against skin diseases. However, most of them have not informations on the exact composition and dosages in the topic solutions used in Brazil, imposing serious risks to public health. The most of the topic solutions employed in the studies have not indications of their composition. Only one topic solution had indications of the presence of bergapten, nevertheless without exact dosages. Therefore, it is important to know the levels of psoralens in topic solutions consumed by humans.

The HPLC technique has shown itself a very efficient system for separation of such complex mixtures. Several HPLC methods have been reported for the determination of psoralens [15–22] in callus cultures, vitro culture, plants, citrus essential oils. However, most of the published methods not report assay validation, or reported assay validations were incomplete or in the methods was interference in relation to the formulation composition. There are not previous descriptions in the literature on sample preparation and HPLC analysis of psoralen and bergapten present in phytomedicines (topic solutions) employed in Brazil for the treatment of vitiligo and psoriasis. The gas chromatography method as been showed for determination of psoralens in phytomedicines [23].

The HPLC is an equipment wide disseminated in Brazil. In this study, was developed an analytical procedure suitable for sample preparation and HPLC–UV analysis of psoralen and bergapten (Fig. 1), content in topic solutions. The validation of the procedure was carried out according to the International Conference on Harmonisation (ICH) guidelines [24]. The linearity, accuracy, the inter- and intra-day precision of the procedure were evaluated. The efficiency of the analytical procedure was assessed by the calculation of recovery values.

2. Experimental

2.1. Products

Four topic solutions (A, B, C and D) of different manufacturer (six lot each) were used for the method development and validation. The formula composition of topic solutions: A (*Brosimum gaudichaudi* plus phenol), B and C (Natural product) and D (Juice of plant plus phenol). Only the topic solution A contains indications of the presence of bergapten in *B. gaudichaudi*, in its technique information. The others topic solutions have not information about the psoralens content in its composition.

2.2. Chemicals

Spectroscopy-grade methanol and acetonitrile were purchased from Merck (Germany). Water treated using a Milli-Q system (Milllipore) was used during sample preparation procedures and HPLC analysis. Analytical-grade ethyl acetate also was used to sample preparation. Psoralen and bergapten standards were obtained from a collection in our laboratory. Stock mixtures of these standards were made up from the individual solutions in acetonitrile and used as external standards.

2.3. Apparatus and chromatography conditions

The analyses were performed on a Shimadzu liquid chromatography, a ternary solvent delivery system—Model LC-6AD—combined with a fixed wavelength UV–Vis detector—Model SPD-6AV (Shimadzu)—a Rheodyne loading valve filted with a 100-μl sample loop. A microcomputer equipped with a Microquimica-MQI18PCA software was used for recording chromatograms and measuring peak areas. HPLC separation of the psoralens was performed using a Shimadzu octadecyl Shim-pack CLC-ODS (4.6 mm ID × 25 cm long and 5 μm particle diameter) reversed-phase column with a small pre-column (4.6 mm ID × 2.5 cm long) containing the same packing, used to protect the analytical column. Before use, the solvents were filtered through a 0.45-μm HV filter (Millipore) then degassed for 30 min in an ultrasonic bath. Chromatography was performed at temperature (22 °C). Elution was carried with acetronitrile/water 55:45 (v/v) at a flow-rate of 1.0 ml min^{-1}. Samples of 10 μl were injected with a

25-μl Hamilton syringe and detection of the peaks was recording at 223 nm (optimized wavelength).

2.4. Sample preparation of topic solutions

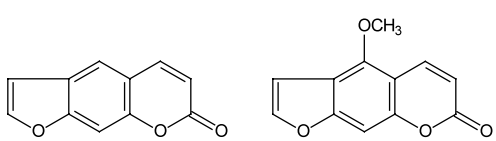
Each sample (2 ml) was extracted with 4 ml of water and 6 ml of ethyl acetate in sonication for 5 min. After repose by 10 min the two phases were separate. Both the phase was filtered in a filter paper and the solvents were evaporated to dryness in a nitrogen stream. Each residue obtained was dissolved in 3 ml of acetonitrile, filtered through a 0.45-μm Millex filter and the solution diluted in acetonitrile in a 10-ml volumetric flask and analysed by HPLC.

2.5. Determination of the limits detection and quantification.

The HPLC limits detection were determined by injecting ($n = 5$) solutions of psoralen and bergapten of know concentration (10 μl each), and lowering the concentration of the sample until the detection of the peak with three times the height of baseline noise; the corresponding concentration was considered as being the minimal amount detectable by HPLC. The limits quantification was determined by multiplication ten times the height of baseline noise.

2.6. Extraction recovery

The extraction efficiency (recovery) was determined from samples of topic solutions spiked with psoralen and bergapten corresponding to low, medium and high concentration. The spiked samples were submitted to the same procedure, described above.



Psoralen Bergapten

Fig. 1. Chemical structure of the psoralen and bergapten.

2.7. Calibration curves

Estimation of the content of psoralen and bergapten in the topic solutions was performed by external calibration. The compounds were dissolved separately in spectroscopy-grade acetonitrile in order to obtain the stock solutions, which were appropriately diluted to concentrations ranging from 1–20 μg ml^{-1} for both compounds. Aliquots (10 μl) for ten dilutions of each standard were analysed by HPLC. Each determination was carried five times. For each standard was obtained the corresponding chromatogram and constructed a graphical plotting the means of areas against the concentration for each furanocoumarin. Linear least squares regression of the peak areas ratio as a function of the concentrations was performed to determine correlation coefficients. The equation parameters (slope and intercept) of each standard curve were used to obtain concentration values for quality control samples and unknown samples of topic solutions. Specimens with an analyte concentration exceeding the calibration curve were reassayed upon appropriate dilution of the samples.

2.8. Accuracy and precision

The accuracy of the assay method was evaluated by performing replicate analyses against a calibration curve and calculating the mean percent differences between theoretical values and measured values. The accuracy values in intra-day variation studies at low, medium and high concentrations of psoralen and bergapten in topic solutions.

The precision of a method is expressed as the percentage coefficient of variation (CV) of the replicate measurements. In this work, the precision of the methods was tested for both intra-day and inter-day reproducibility in topic solutions. The intra-day variability of the assay method was determined by the repeated analysis of the quality control samples ($n = 5$) at low, medium and high concentrations on the sample day. The inter-day variability of the assay methods were determined by the repeated analysis of the quality control samples ($n = 5$) at low, medium and high concen-

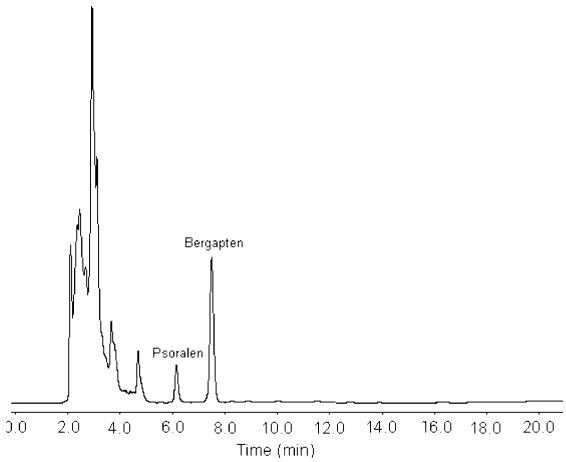


Fig. 2. Chromatogram of a typical topic solution analysis by HPLC. For chromatographic conditions see Section 2.

trations on three different days. The quality control samples were prepared on the same day at each concentration, and then divided in aliquots that were stored at −20 °C until analysis.

2.9. Stability study

The stability of working standards solutions was tested at temperature 22 (working temperature), 4 and −20 °C (storage temperature).

The stability of psoralen and bergapten in topic solutions was inspected during all the storage steps (i.e. at room temperature, at 4 and at −20 °C). Spiked samples were analysed, against a calibration curve, immediately after preparation (reference values) and after storage.

Stability was defined as less than 10% loss of the initial drug concentration.

2.10. Specificity

To evaluate the specificity of the method, three different others usual psoralens (pimpinellin, isopimpinellin, isobergapten), usually present in plants employed in Brazil for treatment of vitiligo and psoriasis, were assayed through the same procedure, and the retention times of compounds were compared with those of psoralen and bergapten in samples.

3. Results and discussion

There are indications that some topic solutions sold in Brazil for the treatment of vitiligo and psoriasis contains bergapten. Thus, they were selected for the development of the analytical method for the present study. A number of preliminary sample preparation and HPLC experiments employing topic solutions were performed to establish optimal conditions for sample preparation and HPLC analysis of psoralen and bergapten.

HPLC analysis showed baseline separation for the compounds of interest, which could be analysed in a satisfactory time interval of less than 8 min in the case of the standard mixture (psoralen 6.05 min and bergapten 7.33 min) (Fig. 2). The relative short time of psoralen and bergapten allows the analysis of a large number of samples. In the time intervals, where the compounds eluted, were free of interference in all the topic solutions tested employed sample preparation optimized (Fig. 2).

The identification of psoralen and bergapten in the topic solutions were performed by comparison of their retention time with the authentic standards and standards addiction in the samples.

No changes of psoralen and bergapten were detected in working standard solution after 24 h at 22 °C, one month of storage at 4 °C and 6 months storage at −20 °C. Psoralen and bergapten were stable in topic solution after 24 h at 22 °C, one month of storage at 4 °C and 6 months storage at −20 °C. Thus validated method for the assay of psoralens may be regarded as a stability-indicating.

The calibration curves were determined by a linear regression. The calibration curves for psoralen and bergapten were linear in the range 1.0–20.0 μg ml^{-1} (Table 1). Average standard errors for the peak areas of replicate injections ($n = 5$) were smaller than 2% showing good repeatability of the calibration curve.

The limits detection were 0.030 μg ml^{-1} for psoralen and 0.070 μg ml^{-1} for bergapten. The limits quantification were 0.10 μg ml^{-1} for psoralen and 0.23 μg ml^{-1} for bergapten.

Table 1
Regression data of calibration curves for quantitative determination of psoralen and bergapten by HPLC

	Substances	
	Psoralen	Bergapten
LR (μg ml^{-1})	1.0–20.0	1.0–20.0
b	0.6709	0.5689
a	−0.0110	0.0097
S_a	0.0175	0.0125
S_b	0.0032	0.0023
R	0.9999	0.9999
n	10	10

LR, linear range; b, slope; a, intercept; S_b, standard deviation of the slope; S_a, standard deviation of the intercept; R, correlation coefficient; n, data number. Linear regression: formula, $y = b + ax$, where y = peak areas ratio, x = concentration (μg ml^{-1}), a = slope and b = intercept.

The recovery experiments with the topic solutions gave mean ($n = 10$) averaged 98.2 ($\pm$ 1.3)% for psoralen and 96.3 ($\pm$ 1.8)% for bergapten in the top phases. The extraction efficiency was not statistically different over the range of concentrations studied. On the bottom phase was detected less then 3% of psoralen and bergapten. The results showed that the procedure used is good for extraction of psoralen and bergapten in the topic solutions. The recovery in 11 other experiments tested, were 63–88% for psoralen and 59–71% for bergapten and was obtained a mixture complexes for separation by HPLC.

The accuracy values were less then 5% (Table 2). Regarding the assay precision, intra- and inter-day CVs were generally lower than 5%. In this work precision of the methods was tested for both intra-day and inter-day reproducibility in topic solutions. The intra- and inter-day variability of the assay method were determined at low, medium and high concentrations. The results are shown in Tables 3 and 4. These data indicate that the assay methods are reproducible within the same day and three different days.

Fig. 3 shows the contents of the psoralen and bergapten in each solution topic for different lots analysed by GC-FID [23] and HPLC. The variance analyses revealed no statistically differences among data obtained by CG and HPLC for a 5% level significance (Fig. 3 and Table 5). The GC-FID method showed higher detection limits and

Table 2
Accuracy of HPLC method for determination of psoralen and bergapten in topic solutions samples ($n = 5$)

	Psoralen		Bergapten	
C added (μg ml^{-1})	C found (μg ml^{-1}) (mean $\pm$ SD)	Accuracy (%)	C found (μg ml^{-1}) (mean $\pm$ SD)	Accuracy (%)
1	1.0 $\pm$ 0.04	3.92	1.0 $\pm$ 0.03	3.24
9	8.9 $\pm$ 0.37	3.76	8.9 $\pm$ 0.28	2.88
20	19.0 $\pm$ 0.69	3.24	19.0 $\pm$ 0.81	4.01

C, concentration; SD, standard deviations.

Table 3
Intra-day variability of HPLC method for determination of psoralen and bergapten in topic solutions samples ($n = 5$)

	Psoralen		Bergapten	
C added (μg ml^{-1})	C found (μg ml^{-1}) (mean $\pm$ SD)	CV (%)	C found (μg ml^{-1}) (mean $\pm$ SD)	CV (%)
1	1.0 $\pm$ 0.04	4.00	1.0 $\pm$ 0.03	3.00
9	8.9 $\pm$ 0.37	4.16	8.9 $\pm$ 0.28	3.15
20	19.0 $\pm$ 0.69	3.63	19.0 $\pm$ 0.81	4.26

C., concentration; CV, coefficient of variation; SD, standard deviations.

Table 4
Inter-day variability of HPLC method for determination of psoralen and bergapten in topic solutions samples ($n = 5$)

	Psoralen		Bergapten	
C added (μg ml^{-1})	C found (μg ml^{-1}) (mean ± SD)	CV (%)	C found (μg ml^{-1}) (mean ± SD)	CV (%)
1	1.0 ± 0.04	4.00	1.0 ± 0.04	4.00
9	8.9 ± 0.43	4.83	8.9 ± 0.31	3.48
20	19 ± 0.83	4.37	19 ± 0.91	4.79

C, concentration; CV, coefficient of variation; SD, standard deviations.

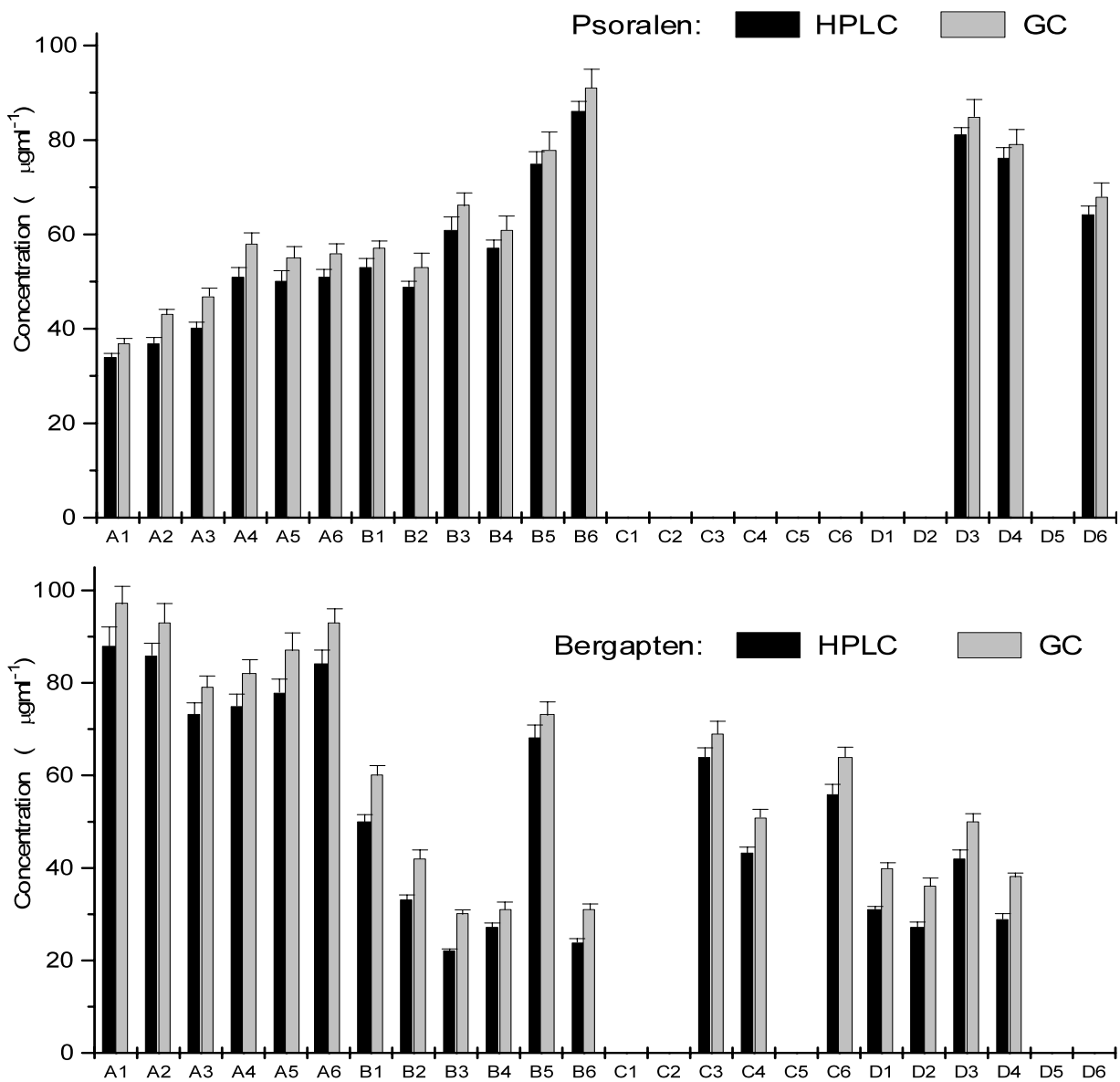


Fig. 3. Quantification of psoralen and bergapten by HPLC and GC. The results are expressed in mean of five samples to a same lot and sticks represent the standard deviations.

quantification, while procedure HPLC using ultraviolet detection method presents lower quantification and detection limits. Both methods showed good recovery, accuracy, precision and linearity. Then, the two methods can be used for analyses of psoralens in topic solutions.

Literature reports that the concentration of psoralens in topic preparations used against vitiligo must be between 0.001–1% [25] and 1% with possible dilutions to 1:1000 and 1:10 000 [26]. In this study, were found concentrations between 0.0027–0.0143%. Presence of psoralens was not detected in some samples (Fig. 3 and Table 5).

Only the topic solution A showed no psoralens discrepancies among the analysed lots. The other solutions presented significant differences in psoralens contents, of lot for lot (Fig. 3 and Table 5). The lots of the topic solutions B presented discrepancies principally in the bergapten contents. The samples of topic solution C presented only bergapten in three lots. In the other lots of C no psoralens were detected (Fig. 3 and Table 5). The lots of the topic solutions D presented composition totally different of a lot for another (Fig. 3 and Table 5). Nevertheless, each lot of topic solution ($n = 5$) showed no statistically significant difference in the contents of psoralens analysed by analysis of variance for a degree of confidence of 95%. These data showed the necessity of quality control methods to phytoterapics medicaments employed in Brazil for treatment vitiligo and psoriasis.

Table 5
Contents (μg ml^{-1}; mean ± SD) of furanocoumarins in topic solutions employing the HPLC method

Topic solutions	Psoralen	Bergapten
A1	34 ± 0.8	88 ± 4.1
A2	37 ± 1.2	86 ± 2.6
A3	40 ± 1.4	73 ± 2.7
A4	51 ± 2.0	75 ± 2.6
A5	50 ± 2.3	78 ± 2.8
A6	51 ± 1.6	84 ± 3.1
B1	53 ± 1.9	50 ± 1.5
B2	49 ± 1.1	33 ± 1.2
B3	61 ± 2.7	22 ± 0.5
B4	57 ± 1.8	27 ± 1.1
B5	75 ± 2.5	68 ± 2.9
B6	86 ± 2.2	24 ± 0.7
C1	–	–
C2	–	–
C3	–	64 ± 2.0
C4	–	43 ± 1.5
C5	–	–
C6	–	56 ± 2.1
D1	–	31 ± 0.7
D2	–	27 ± 1.3
D3	81 ± 1.6	42 ± 1.9
D4	76 ± 2.4	29 ± 1.1
D5	–	–
D6	64 ± 2.0	–

SD of five determinations; (–) not detected.

4. Conclusion

A sensitive, rapid and repeatable HPLC method with UV detection was developed for simultaneous determination of psoralen and bergapten in routine analysis of the topic solutions providing a method for their quality control. This method does not require a tedious procedure to sample preparation and eliminated the interfering materials. Validation experiments showed a very good precision and accuracy of the method with coefficients of variation and relative errors of less than 5%.

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