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Short communication

A method for fast determination of psoralens in oral solutions of phytomedicines using liquid chromatography

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Abstract

A method for sample preparation and analysis by high performance liquid chromatography with UV detection (HPLC-UV) has been developed for routine analysis of psoralen and bergapten, photosensitizing compounds, in oral solutions of phytomedicines employed in Brazil for some illnesses. The linearity, accuracy, the inter- and intra-day precision of the procedure were evaluated. Calibration curves for psoralen and bergapten were linear in the range of 1.0–600.0 $\mu g\,ml^{-1}$ and 1.0–400.0 $\mu g\,ml^{-1}$ respectively. The recoveries of the psoralens in the oral solutions analysed were 94.43–99.97%. The percentage coefficient of variation (CV) of the quantitative analysis of the psoralens in the products analysis was within 5%. In inter-equipment study was employed gas chromatography–flame ionization (CG–FID) detection.
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Keywords: Liquid chromatography; Quantification; Psoralens; Furanocoumarins

1. Introduction

Dorstenia species are known for their ability to synthesis linear and angular psoralens [1]. In preliminary analysis psoralen and bergapten were identified in samples analysed in this study. The formula composition of oral solutions analysed showed the presence of *Dorstenia multiformes* and others associations of plants. The oral solutions employed in this study are indicated, according to their technique information, for the treatment of menstrual irregularities, abdominal and menstrual cramps, vaginal discharge, daily pre-menstrual tension, disturbances related to menopause, inflammation of the lymphatic nodes, digestive problems. However, most of them have not information on the exact composition and dosages in the products analysed used in Brazil, imposing serious risks to public health.

Psoralens or furanocoumarins are well known as photoreactive compounds [2], psoralen and bergapten (Fig. 1) are used in pharmaceutical and cosmetic products because of their UV light absorbing properties [3]. 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 [4,5]. The psoralens are currently employed in dematology (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) [6–10].

The biological activity of these compounds are attributable to their ability intercalate into DNA, where they form mono and di-adducts in the presence of long-wave UV light [11]. Furthermore, the use of psoralens in medicine has been associated with higher incidence of skin cancer [11–13]. Several studies have been demonstrated that the psoralens are carcinogenic, mutagenic and photodermatitic [14,15]. Linear psoralens cause phototoxicity [16,17]. For this reason, is very important to know precisely the levels of psoralens in oral solutions consumed by humans.

High performance liquid chromatography (HPLC) technique has shown itself a very efficient system for separation of such complex mixtures. HPLC methods have been

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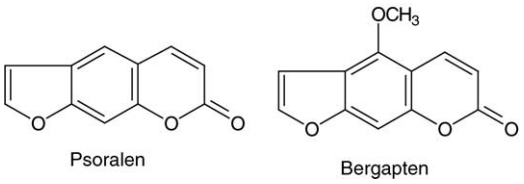

Fig. 1. Chemical structure of the psoralen and bergapten.

reported for the determination of psoralens in callus cultures, vitro culture, serum, dermis, plants, citrus essential oils, phytomedicines, but only the most recent published methods report assay validation [8,18–26]. There are no previous descriptions in the literature about sample preparation and HPLC analysis of psoralen and bergapten (Fig. 1) present in phytomedicines (oral solutions) employed in this studies. Liquid and gas chromatography (GC) method has been shown the determination of psoralens in phytomedicines for topical use [8,27].

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 content in oral solutions. The validation of the procedure was carried out according to the International Conference on Harmonisation (ICH) guidelines [28]. The sensibility, specificity, linearity, accuracy, the inter- and intra-day precisions and inter-equipment variability of the assay method were evaluated. The efficiency of the analytical procedure was assessed by the calculation of recovery values.

2. Experimental

2.1. Products

Three oral solutions (A–C) were used for method development and validation. A and B of the same manufacturer (three lots of A and two lots of B) and C of another manufacturer (three lots). The formula composition of oral solutions: A (*Dorstenia multiformis–Davilla rugosa–Plumeria lancifolia 1:2:1, v/v/v*), B (*Dorstenia multiformes–Plumeria lancifolia 1:1, v/v*) and C (*Dorstenia multiformes–Cereus jamacaru–Plumeria lancifolia–Erythrina mulungu 2:5:1:2, v/v/v/v*).

2.2. Chemicals

Spectroscopy-grade acetonitrile, methanol and chloroform were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore). 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 methanol and used as external standards.

2.3. Apparatus and chromatography conditions

The analysis 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 i.d. × 25 cm long and 5 μm particle diameter) reversed-phase column with a small pre-column (4.6 mm i.d. × 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 20 min in an ultrasonic bath. Elution was performed with acetronitrile–water (55:45, v/v) at a flow-rate of 1.0 ml min^{-1}. Aliquots of 10 μl were inject with a 25 μl Hamilton syringe and detection of the peaks was recording at 223 nm. All chromatography analysis were performed at 22 °C.

2.4. Sample preparation

Several forms for extraction of psoralen and bergapten in the oral solutions were tested like, changing the kind and volume of solvent and the times in sonication and in centrifugation. The optimized procedure found was: each sample (5 ml) was extracted with 7 ml of chloroform in sonication for 10 min. Then centrifuged for 10 min in order to make the extraction and the phases separation. The top phase was re-extracted with 5 ml of chloroform remaining the same time in sonication and centrifugation. The two bottom phases have been joined and the solvent evaporated to dryness in a stream of nitrogen. Each residue was dissolved in 5 ml of methanol, filtrated through a 0.45 μm Millex filter and the solution diluted in methanol on a volumetric flask (10 ml) in order to be analysed by HPLC.

2.5. Determination of the detection and quantification limits

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

2.6. Extraction recovery

The extraction efficiency (recovery) was determined from samples of each oral solutions spiked with psoralen and bergapten corresponding to low, medium and high concen-

tration. The spiked samples were submitted to the same procedure described in sample preparation.

2.7. Calibration curves

Estimation of the content of psoralen and bergapten in the oral solutions was performed by external calibration. The compounds were dissolved separately in spectroscopy-grade methanol in order to obtain stock solutions, which were appropriately diluted to concentrations ranging from 1 $\mu g\,ml^{-1}$ to 600 $\mu g\,ml^{-1}$ for psoralen and ranging from 1 $\mu g\,ml^{-1}$ to 400 $\mu g\,ml^{-1}$ for bergapten. Aliquots (10 μl each) of 10 dilutions for 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 psoralen. 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 oral solutions. Specimens with an analyte concentration exceeding the calibration curve were reassayed upon appropriate dilution of the samples.

2.8. Linearity

The linearity of the assayed method was evaluated crossing the range of the analytical procedure. It was evaluated by analyzing each oral solutions spiked with known amount of the analytes at low, medium and high concentrations. Aliquots (10 μl) were analysed by HPLC as described above. Each determination was carried out five times. For each spiked sample was obtained the corresponding chromatogram and constructed a graphical plotting the means of areas against their concentrations. Linear least squares regression was performed to determine correlation coefficients.

2.9. Accuracy and precision

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

The precision of a method is expressed as the percentage of the coefficient of variation (CV) of the replicate measurements. The precision of the method was tested for both intra-day and inter-day repeatability in oral solutions by HPLC. The intra-day variability of the assay method was determined by the repeated analysis ($n = 4$) for each sample of oral solution with addiction of known amount of analyte at low, medium and high concentrations. The inter-day variability was verified by the same procedure above on 3 different days ($n = 5$). In inter-equipment variability was employed gas chromatography–flame ionization (CG–FID) detection [7].

2.10. Stability study

The stability of working standards solutions was tested at 22 °C (working temperature), 4 °C and −15 °C (storage temperature).

The stability of psoralen and bergapten in oral solutions was inspected during all the storage steps (i.e. at room temperature, at 4 °C and at −15 °C). Spiked samples were analysed, against the calibration curves, immediately after preparation (reference values) and after storage.

Stability was defined as being less than 3% loss of the initial drug concentration.

2.11. Specificity

To evaluate the specificity of the method, two different others psoralens (pimpinellin and isobergapten), usually present in the genus *Dorstenia* were assayed by the same procedures using HPLC, and the retention times of compounds were compared with those of psoralen and bergapten in the samples.

3. Results and discussion

Oral solutions A and B indicate the presence of coumarins in their technique information, but none of products studies have information about psoralens in their compositions. 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 eight minutes (psoralen 6.15 min and bergapten 7.45 min) (Fig. 2). The relative short elution 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 oral solutions tested employed sample preparation optimized (Fig. 2). The identification of psoralen and bergapten in the oral solutions were performed by comparison of their retention time with the of authentic standards and standards addiction in the samples.

No changes of psoralen and bergapten were detected in working solutions after 24 h at 22 °C, 2 months at 4 °C and 6 months of storage at −15 °C. Psoralen and bergapten were stables in the oral solutions after 24 h at 22 °C, on month of storage at 4 °C and 6 months storage at −15 °C. Thus this validated method for the assay of psoralens may be regarded as a stability of the solutions.

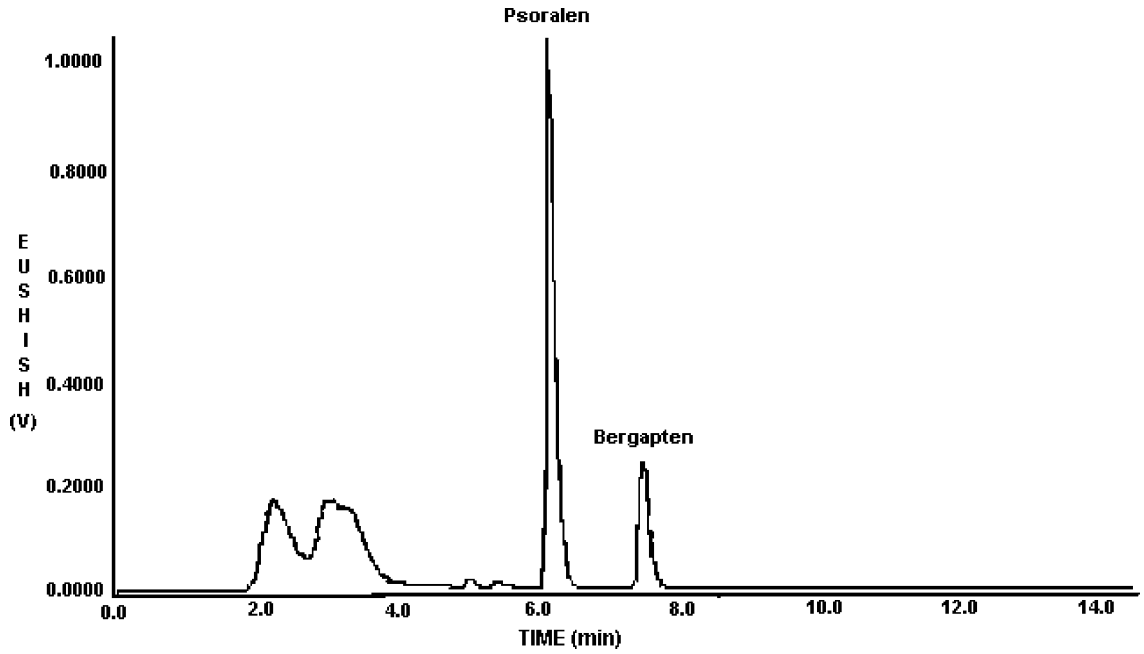

Fig. 2. Chromatogram of a typical oral solution analysis by HPLC. For chromatographic conditions see Section 2.

The calibration curves were determined by linear regression. The calibration curve for psoralen was linear in the range of 1.0–600.0 μg ml^{-1} and 1.0–400.0 μg ml^{-1} for bergapten (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.

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

	Substances	
	Psoralen	Bergapten
LR (μg ml^{-1})	1–600	1–400
b	0.35642	0.35431
a	0.05357	0.02343
Sa	0.05176	0.04773
Sb	0.00161	0.00148
r	0.9998	0.9997
n	10	10

LR: linear range, *b*: slope, *a*: intercept, *Sb*: standard deviation of the slope, *Sa*: standard deviation of the intercept, *r*: correlation coefficient, *n*: number of samples. Linear regression, formula: $y = a + bx$, where y = peak areas ratio, x = concentration (μg ml^{-1}), a = intercept and b = slope.

The linearity of the method was determined by linear regression. The analysis of samples spiked with known amounts of analyte demonstrated that response was proportional to the concentrations of the samples with the determination coefficiente r^2 = 0.9998 for the linear range of the calibration curves for samples of A–C.

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

The recovery experiments with the oral solutions gave mean (n = 5) in the bottom phases (Table 2).

The accuracy values were less than 6% (Tables 3 and 4). Regarding the assay precision, intra- and inter-day CVs were less than 5%. In this work the precision of the method was tested for both intra-day and inter-day repeatability in oral solutions. The intra- and inter-day variability of the assayed method were determined at low, medium and high concentrations. The results are shown in Tables 3 and 4. These data indicate that the assayed method is reproducible within the same day and three different days.

Fig. 3 shows the contents of psoralen and bergapten in the oral solutions for differents lots analysed by HPLC and GC.

Table 2
Recovery of psoralen and bergapten in oral solutions samples A–C (n = 5)

Conc. added (μg ml^{-1})	Psoralen (%) (mean ± S.D.)			Bergapten (%) (mean ± S.D.)		
	A	B	C	A	B	C
4	95.99 ± 1.17	97.28 ± 0.91	96.13 ± 1.16	98.76 ± 0.74	98.34 ± 1.17	96.43 ± 1.11
40	95.35 ± 0.35	96.55 ± 1.14	97.19 ± 0.99	99.89 ± 0.87	99.97 ± 0.89	97.29 ± 0.67
100	95.76 ± 0.81	99.05 ± 0.74	98.98 ± 1.01	94.43 ± 0.53	98.43 ± 0.79	98.11 ± 1.03
200	96.83 ± 0.97	95.95 ± 0.87	98.37 ± 0.78	98.26 ± 0.93	96.86 ± 1.05	97.81 ± 1.15

Conc, concentration; S.D., standard deviations.

Table 3
Intra-day accuracy and precision of method for determination of psoralen and bergapten in oral solutions A–C samples (n = 12)

C added ($\mu g\,ml^{-1}$)	Psoralen ($\mu g\,ml^{-1}$) (mean ± S.D.)			Bergapten ($\mu g\,ml^{-1}$) (mean ± S.D.)		
	C found ($\mu g\,ml^{-1}$) (mean ± S.D.)	Accuracy (%)	CV (%)	C found ($\mu g\,ml^{-1}$) (mean ± S.D.)	Accuracy (%)	CV (%)
4	3.9 ± 0.17	2.5	4.35	3.8 ± 0.18	5.0	4.73
40	41.0 ± 0.83	2.5	2.02	42.0 ± 1.13	5.0	2.69
200	202.0 ± 2.39	1.0	1.18	203.0 ± 3.05	1.5	1.50

C, concentration; CV, coefficient of variation; S.D., standard deviations.

Table 4
Inter-day accuracy and precision of method for determination of psoralen and bergapten in oral solutions A–C samples (n = 15)

C added ($\mu g\,ml^{-1}$)	Psoralen ($\mu g\,ml^{-1}$) (mean ± S.D.)			Bergapten ($\mu g\,ml^{-1}$) (mean ± S.D.)		
	C found ($\mu g\,ml^{-1}$) (mean ± S.D.)	Accuracy (%)	CV (%)	C found ($\mu g\,ml^{-1}$) (mean ± S.D.)	Accuracy (%)	CV (%)
4	3.9 ± 0.16	2.5	4.10	3.9 ± 0.15	2.5	3.85
40	42.0 ± 2.07	5.0	4.93	42.0 ± 1.79	5.0	4.26
200	204.0 ± 3.23	2.0	1.58	203.0 ± 3.91	1.5	1.93

C, concentration; CV, coefficient of variation; S.D., standard deviations.

Table 5
Contents ($\mu g\,ml^{-1}$) (mean ± S.D.) of furanocoumarins in oral solutions employing the HPLC method

Oral solutions	Psoralen ($\mu g\,ml^{-1}$)	Bergapten ($\mu g\,ml^{-1}$)
A1	274 ± 4.3	65 ± 2.9
A2	272 ± 4.1	63 ± 2.1
A3	260 ± 4.9	66 ± 2.0
B1	530 ± 3.1	134 ± 3.6
B2	522 ± 5.9	128 ± 3.2
C1	265 ± 4.9	72 ± 2.8
C2	314 ± 6.7	84 ± 3.4
C3	325 ±7.3	90 ± 2.1

S.D., standard deviations; S.D. of five determinations.

Variance analysis revealed no statistically differences among data obtained by HPLC and CG for a 5% level significance (Fig. 3 and Table 5).

The GC method has shown higher detection and quantification limits, while the HPLC procedure using ultraviolet as detection presents lower quantification and detection limits. Both methods showed good accuracy, precision, and linearity. Then, the two methods can be used to analysis of psoralens in oral solutions.

In this study, concentrations between 0.26 $mg\,ml^{-1}$ and 0.53 $mg\,ml^{-1}$ were found for psoralen and 0.06–0.13 $mg\,ml^{-1}$ for bergapten in the oral solutions (Fig. 3 and Table 5). The dose recommended by manufacturers for the phytomedicines A–C is of 15 $ml\,day^{-1}$. Thus a person of 60 kg ingest the dose of 0.065–0.132 $mg\,kg^{-1}\,day^{-1}$ of psoralen and 0.016–0.033 $mg\,kg^{-1}\,day^{-1}$ of bergapten.

The lack of knowledge of the presence of psoralens in the analysed phytomedicines offers risks to the public health, since they not adversed about the potencialization of the effects carcinogenic, mutagenic and phototoxicity of psoralens by the exposition to the sun.

The oral solution C showed psoralens discrepancies among the lots analysed, coming out 20%, while the oth-

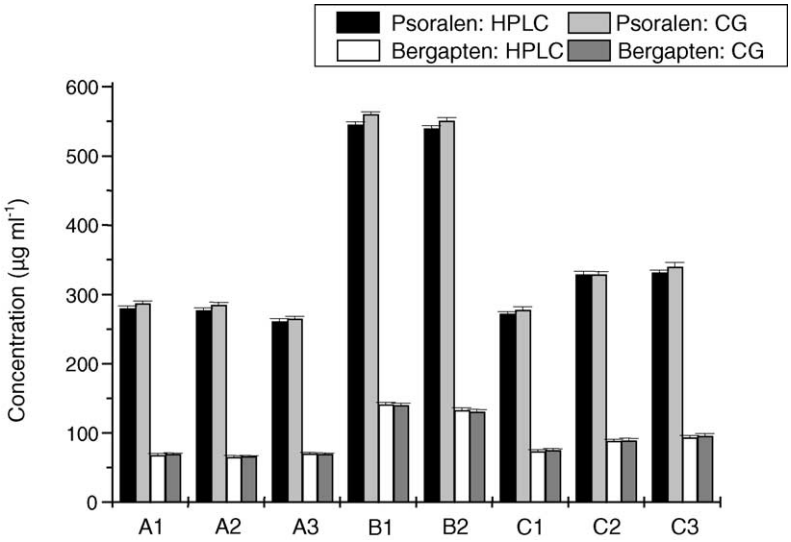

Fig. 3. Quantification of psoralen and bergapten by HPLC and CG. The results are expressed in mean of six samples to a same lot and sticks represent the standard deviations.

ers solutions present no significant differences in psoralens contents between the lots (Fig. 3 and Table 5).

4. Conclusion

A sensitive, rapid and repeatable method was developed for simultaneous determination of psoralen and bergapten in routine analysis of the oral solutions providing a method for then quality control. Validation experiments showed a very good precision and accuracy of the method with coefficients of variation and relative errors less than 6%.

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