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Determination of traces of pyrethrins and piperonyl butoxide in biological material by high-performance liquid chromatography

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ABSTRACT

A liquid chromatographic method for the determination of pyrethrins and of the synergist piperonyl butoxide in human plasma after C_{18} solid-phase extraction is described. UV detection was found to be sensitive enough to determine concentrations far below the limit of toxicity. With respect to future investigations concerning studies in biological materials, a column-switching system for sample preparation was developed and compared with solid-phase extraction. Both methods show comparable limits of detection, but the column-switching technique has the advantage of fully automating the system.

INTRODUCTION

Pyrethrin extract is a biological product with low mammalian toxicity compared with synthetic pyrethroids. Pyrethrins are frequently used insecticides, especially in repellents. One of the disadvantages of pyrethrin extract is its rapid breakdown, which makes a combination with synergists necessary. A very important synergist that acts by delaying metabolic breakdown or by enhancing the penetrability of cell membranes is piperonyl butoxide (Pbo).

Recently, the possible absorption of pyrethrins by topical application and consequently its questionably harmless effect on humans, especially children, has been discussed. We tried to find a method to detect pyrethrins, particularly the main ingredients pyrethrin 1 and 2, and the synergist Pbo in plasma. Many methods for the determination of pyrethrins have been developed, including GC [1–5] and other techniques [6–11], but none of them is sensitive enough for quantification in biological material. The aim of this work was the development and optimization of an HPLC system that would permit the detection of trace concentrations of pyrethrins and Pbo in plasma.

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EXPERIMENTAL

Chemicals and reagents

Methanol from Loba (Vienna, Austria) was distilled twice and water was purified with a Milli-Q reagent water system (Millipore). Pyrethrin extract (25% in isoparaffin) was obtained from Fettrelet (Delemont, Switzerland) and piperonyl butoxide (Pbo) (technical 90%, purification per distillation 99%), from Aldrich.

Standard solutions of pyrethrin extract (100 μl of pyrethrin extract, density 0.20774 g/ml, diluted with mobile phase to 100 ml) and Pbo (93.2 mg of 99% Pbo, diluted with mobile phase to 100 ml) were stored at 4°C. Flasks were wrapped in aluminium foil because of the sensitivity of pyrethrins to UV light [12].

For sample preparation, the following columns were tested: C_{18} (500 mg) and C_{18} (100 mg), Bond Elut, ICT; C_{18} (400 mg), Bakerbond, J.T. Baker; and C_{18} Adsorbex (400 mg), Merck. For ion exchange, samples were applied to Bond Elut SCX (500 mg), SAX (500 mg) and CN (100 mg) columns, ICT. For developing the HPLC method, human citrate–plasma (blood mixed 10:1 with sodium citrate) was centrifuged at 5500 *g* for 10 min. In order to carry out pharmacokinetic studies, citrate was added to 5 ml of blood from healthy persons, which was then centrifuged for 10 min at 5500 *g* and the supernatant plasma was finally stored at −20°C.

Two different pharmaceutical preparations, the repellent Tyrasan and the louse shampoo Aescalon forte, were applied to the skin of volunteers. One spray flask containing 110 ml of Tyrasan (Pharma Diffusion, Bienne Switzerland) contains 1% pyrethrins, 1% Pbo, some essential oils, 20% 2-propanol and water; 1 g of the louse shampoo (Aesca, Traiskirchen, Austria) contains 3.3 mg of pyrethrins 1 and 2, 33 mg of Pbo and shampoo base.

Instrumentation and chromatography

HPLC was performed using a Perkin-Elmer Binary 250 LC pump. For column switching a Milton Roy LC Constametric 3000 pump in combination with an LDC/Milton Roy Minimetric Tm II as an auxiliary pump was utilized. For the Aescalon study a Nucleosil RP-18 (10 μm) column (250 × 4.6 mm I.D.) was used and for the Tyrasan study a Nucleosil RP-18 (5 μm) column (250 × 4.6 mm I.D.) (Forschungszentrum Seibersdorf, Seibersdorf, Austria). Column switching was carried out using a LiChrosorb RP-18 (7 μm) column (250 × 4 mm I.D.) (Merck) and a precolumn (5 × 4 mm I.D.) packed in the laboratory with Perisorb RP-18, 30–40 μm (Merck). Samples were injected with a Rheodyne Model 7125 valve equipped with a 6-μl loop or alternatively with a Rheodyne Model 7125 valve in combination with a Rheodyne Model 7010 six-port valve and a 100-μl loop. Peaks were measured at a wavelength of 254 mm using a Perkin Elmer LC-15 UV detector (range 0.008) or a Perkin-Elmer LC-235 diode-array detector (210–260 nm) and printed by a LKB Model 2210 recorder or by an Epson PCAX2 personal computer in combination with an Epson printer. The mobile phase was methanol–water in different proportions (as described later) and the washing phase for column switching was methanol–water (10:90, v/v). All eluents were degassed with helium before use and the flow-rate was set at 0.5 ml/min.

Clean-up

Solid-phase extraction. A 1-ml volume of centrifuged human plasma (10 min, 5500 *g*) diluted with 3 ml of water, mixed on a vortex mixer for 10 s, was transferred to solid-phase extraction (SPE) columns preconditioned with 10 ml of methanol and 10 ml of water. The columns were washed with water (3 × 10 ml) and then with a wash solution of methanol–water (5:95, v/v) (1 × 12 ml). Finally, the pyrethrins were eluted with methanol (2 × 0.5 ml). After removing the solvent with a stream of nitrogen, the residue was dissolved in 100 μl of the mobile phase [methanol–water (75:25, v/v)] and 6 μl of this solution were injected into the HPLC system.

Column switching. A 1-ml volume of human plasma was diluted with 1 ml of water and spiked with pyrethrin extract and Pbo. A 100-μl volume of this solution were transferred to the precolumn by the auxiliary pump (pump 1) and cleaned from plasma substances by washing with

water; after 5 min, the analytes were transferred from the precolumn by switching over to the analytical column. The mobile phase was methanol–water (82:18, v/v).

RESULTS AND DISCUSSION

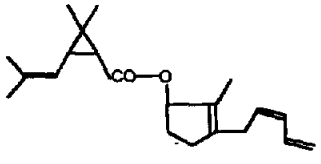
Pyrethrin extract contains pyrethrin 1 (35%), pyrethrin 2 (33%), jasmolin 1 (5%), jasmolin 2 (4%), cinerin 1 (10%) and cinerin 2 (14%). The detection is focused on the main ingredients pyrethrin 1 and 2 and on the synergist Pbo. As previously assumed and proved by several investigations, the structures of the compounds (Fig. 1) indicate that there is no intrinsic fluorescence or electrochemical activity and, further, no common functional groups for derivatization. Therefore, UV detection was applied. Pyrethrins show an absorption maximum at 210 nm. Additionally, the same concentration was measured at 220, 230, 240 and 254 nm. However, the best signal-to-noise ratio could be observed at 254 nm, which also permits the determination of Pbo.

The aim was a common determination of the main ingredients of the repellent Tyrasan and the shampoo Aescalon forte in plasma to detect possible absorption after topical application. Many different columns and mobile phases were tested in order to develop a simple isocratic HPLC technique. Utilizing a reversed-phase system, many by-products originating from plasma are to be expected during the first few minutes of the chromatographic run. Therefore, the analytes should be retained for at least 5 min.

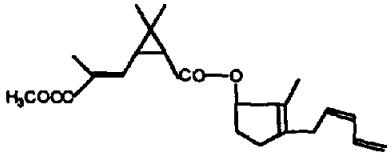
In order to obtain sufficient sensitivity and selectivity necessary for the pharmacokinetic study, great attention was paid to the sample preparation. Consequently, we used the off-line technique of solid-phase extraction. As the analyte compounds have different polar and nonpolar groups, the best sorbent for SPE had to be found. After testing different columns from different producers, Bond Elut C_{18} was found to be the most appropriate sorbent. In addition to hydrophobic interactions, the formation of hydrogen bonds between the ester groups of the pyrethrins and the residual silanol groups of the sorbent can be assumed.

As human plasma contains many substances with acidic, basic and amphoteric groups, another way to clean plasma samples is ion-exchange SPE. Plasma ingredients are retained on the columns by interactions with the sorbent. The insecticides and the synergist are neutral and therefore are not retained by the sorbent material.

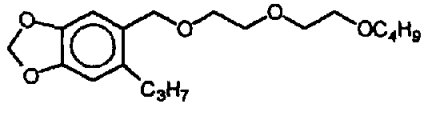
Strong ion-exchange columns, *e.g.*, SCX columns at pH 4, 6 and 8 and SAX columns at pH 6, 9 and 12, were used to decrease the amounts of plasma by-products as much as possible. Although ion-exchange columns showed good cleaning results, the Bond Elut C_{18} columns



PYRETHRIN I



PYRETHRIN II



PIPERONYL BUTOXIDE

Fig. 1. Structures of the main ingredients of Tyrasan spray and Aescalon forte shampoo.

TABLE I

SCHEME FOR APPLICATION OF PHARMACEUTICAL PREPARATIONS TO HUMAN SUBJECTS

	No. of volunteers		Mass (kg)		Age (years)	Application	Sampling
	Male	Female	Male	Female			
Tyrasan repellent spray	3	3	61–85	49–60	22–31	Spraying an area of 25 × 25 cm on the back	Blank, 30, 60, 90, 150, 210, 330 min
Aescalon forte louse shampoo	4	3	68–81	47–60	23–33	On the head	Blank, 30, 60, 90, 120, 180, 240 min

proved to be even more efficient. Applying 60 ng of each substance, the recovery was 81.5% Pbo, 72% pyrethrin 1 and 70% pyrethrin 2. The reproducibility of this method was tested by spiking human plasma blanks with 60 ng per sample and relative standard deviations ($n = 8$) of 4.8%, 5.2% and 5.4% were obtained. Linearity was observed over a range of one order of magnitude starting at the detection limit. The correlation coefficients were between 0.998 and 0.999.

Two different pharmacokinetic studies were performed. The first was carried out with the repellent Tyrasan. Six healthy persons, non-smokers, were treated with nine sprayed strokes from a distance of 10 cm on the back over an area of 25 × 25 cm. After sampling (see Table I) the plasma was centrifuged (4°C, 10 min, 5500 *g*), the supernatant was transferred into test-tubes and stored at −20°C. All samples, injected twice, did not show absorption of either pyrethrins or Pbo. Fig. 2 shows the chromatogram

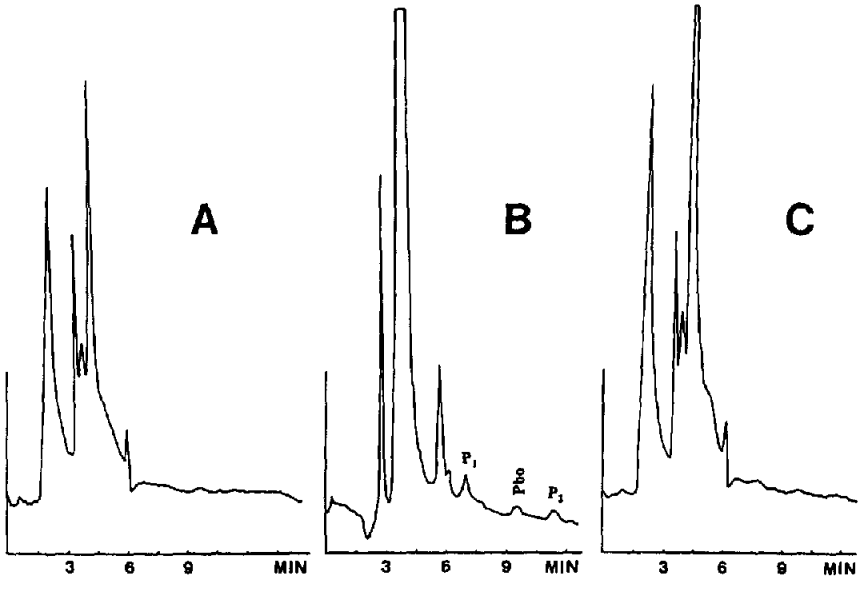


Fig. 2. Typical chromatogram of human plasma specimen after solid-phase extraction. (A) Plasma blank; (B) plasma spiked with 10 ng of pyrethrin 1, 10 ng of pyrethrin 2 and 10 ng of Pbo; (C) Human plasma 2 h after Tyrasan application. Detector, LC-15 UV detector; mobile phase, methanol–water (75:25, v/v); column, Seibersdorf RP-18, 10 μm; injection volume, 6 μl; flow-rate, 0.5 ml min^{-1}.

for a sample from a volunteer compared with spiked plasma.

The second study with the louse shampoo Aescalon forte gave similar results. Seven healthy volunteers, male and female, applied a large amount (*ca.* 30 g) of shampoo to their hair. The incubation time was 15 min, then the hair was washed. No reddening of the skin on the head became visible. Further, the chromatographic investigation of the plasma samples from all the volunteers showed that no absorption had occurred. As the detection limit of 10 ng in 6 μl corresponds to 167 ng pyrethrins/ml plasma (*ca.* 0.17 μg/kg), absorption of larger amounts would have been detected. As particularly the $LD_{50\ dermal}$ for pyrethrins is more than 2.0 mg/kg in the rat, there is a difference of the factor 10 000 between the detection limit and the $LD_{50\ dermal}$. No $LD_{50\ dermal}$ values for piperonyl butoxide could be found in literature, but the $LD_{50\ oral}$ is 6150–7500 mg/kg in the rat, indicating the low toxicity of this synergist. As a result, the application of insecticides containing pyrethrin extract seems to be allowable for children. Irritations and allergies did not appear, which can be traced back to the use of a purified extract [12].

As additional pharmacokinetic studies are planned, a column-switching method was developed with the aim of automating the system. As Fig. 3 shows, a switching valve including the injection valve with loop and precolumn and two pumps allows a parallel performance of the wash and analytical process. A schedule for a complete chromatographic cycle is given in Table II. In order to optimize the wash process, different wash solutions were tested. First pure water was used, and subsequently the methanol content was increased continuously from 5% up to 40%. Although no significant difference in the chromatographic behaviour could be observed, water–methanol (90:10, v/v) was used in further investigations. Higher contents of methanol may include the risk of protein precipitation, thereby producing enhanced pressure.

A critical point for attaining adequate recoveries is the duration of the wash process. Different wash intervals from 1 to 20 min were tested; a period of 5 min was found to be optimum, providing recoveries of almost 100%. This period is also sufficient if the injection volume is increased from 6 to 20, 75 and 175 μl.

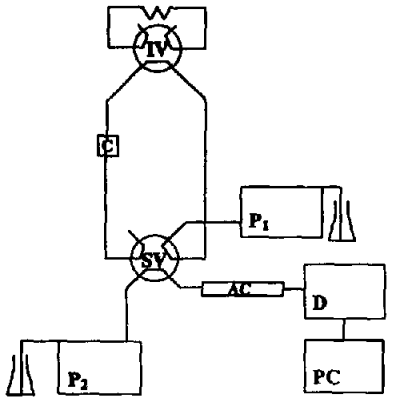


Fig. 3. Column-switching system. IV = injection valve; SV = switching valve; P_1 = Milton Roy LC Constametric 3000 pump; P_2 = LDC/Milton Roy pump; D = LC-15 UV detector; AC = analytical column, LiChrosorb RP-18, 7 μm; C = precolumn packed with Perisorb RP-18, 25–30 μm; PC = Epson PCA × 2.

The investigations with different loops were performed in order to optimize the signal-to-noise ratio and thus to improve the detection limit. Fig. 4 shows a chromatogram obtained following an injection of 20 μl. On injecting 175 μl the peaks show some band broadening; however, the detection of 130 ng/ml of pyrethrins

TABLE II

SCHEDULE FOR COLUMN SWITCHING

Time (min)	Operation
0	Injection of the sample, washing the precolumn with water by fore flushing
1	Injection valve is switched from inject to load position
6	Switching valve is switched on; the eluent transfers the compounds to be analysed to the analytical column by back flushing
6.5	Switching valve is switched to the original position. Analytes are separated on the analytical column and at the same time conditioning of the wash system with wash solution occurs
17	End of chromatographic cycle

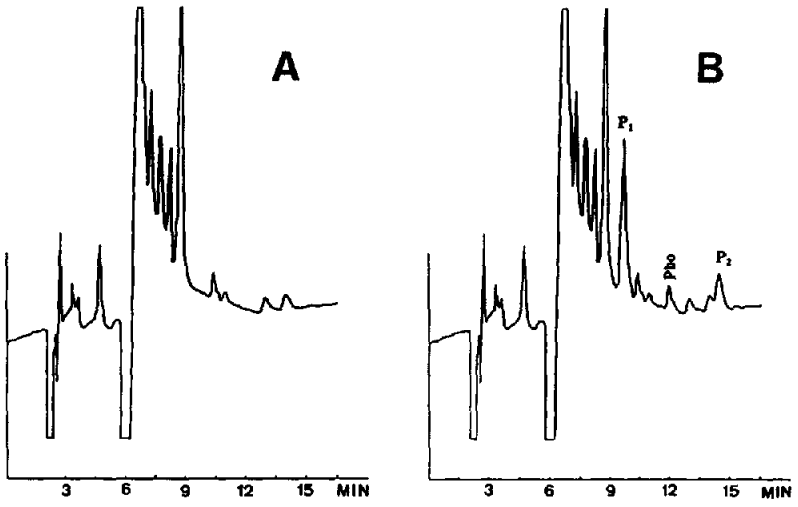


Fig. 4. Typical chromatogram obtained using column switching. (A) Plasma blank; (B) plasma spiked with 20 ng of pyrethrin 1, 20 ng of pyrethrin 2 and 20 ng of Pbo. Wash period, 8 min; wash phase, methanol–water (10:90, v/v); mobile phase, methanol–water (80:20, v/v); injection volume, 20 μl; other chromatographic conditions as in Fig. 3.

and Pbo in plasma is possible. Using this technique a slightly better detection limit is achieved compared with SPE.

CONCLUSIONS

This work has demonstrated the utility of a simple RP-HPLC system for the separation and identification of the main ingredients of natural insecticides in human plasma. Using solid-phase extraction interfering by-products of the biological material were removed, providing acceptable recoveries for pyrethrins 1 and 2 and the synergist piperonyl butoxide. Pharmacokinetic studies subsequent to the topical application of two different preparations, a repellent spray and a louse shampoo, indicate that absorption does not occur or, if it does occur, the amounts are below the detection limit. As the method developed is sensitive enough to detect amounts far below the toxicity levels of these compounds, the application of such preparations containing Pbo and pyrethrins from purified pyrethrin extract seem to be harmless, even for children. As an alternative to SPE, a column-switching system was developed for sample preparation in order to effect fully automated operation for further pharmacokinetic studies.

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