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Sequential-injection determination of traces of disodium phenyl dibenzimidazole tetrasulphonate in urine from users of sunscreens by on-line solid-phase extraction coupled with a fluorimetric detector

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Abstract

A sensitive and selective method to determine disodium phenyl dibenzimidazole tetrasulphonate (PDT) in the urine of sunscreen users, which is suitable for studies on body accumulation/excretion is proposed. On-line solid-phase extraction allows the analyte to be retained and subsequentely eluted, using a strong anion exchange (SAX) microcolumn. Standard addition calibration was carried out with only one standard. The wavelengths of excitation and emission were 330 and 454 nm, respectively. The method allows PDT to be determined in both, spiked and unspiked human urine samples, without any pre-treatment. Results obtained for spiked urine samples (40–200 ng ml^{-1}) showed the accuracy of the method. The mean relative standard deviations (R.S.D.) of the results was 7%. Five volunteers applied a sunscreen lotion containing 5% PDT and their urinary excretion was controlled from the moment of application until the excreted amounts were no longer detectable. The sensitivity of the proposed method is in the order of 1900 ml μg^{-1} and the detection limit ($3S_{y/x}/b$) is in the order of 5 ng of PDT, which means 10 ng ml^{-1} for a 500 μl injected volume, and this is suitable for the PDT levels found in the urine.

Keywords: Disodium phenyl dibenzimidazole tetrasulphonate; Sunscreen; Urine; Sequential-injection; Solid-phase extraction; Fluorescence spectrometry

1. Introduction

Although small doses of solar radiation have a beneficial effect on humans, excessive exposure to UV radiation has side effects, some of which are harmful. Because of this, sunscreen cosmetics have become necessary daily products. As they are applied to the skin, it is important to find out to what extent they are absorbed and excreted, because unlike some dermopharmaceuticals, they are not designed to be absorbed through the skin but to prevent the solar radiation coming through the skin.

Different in vivo studies carried out either by volunteers [1–5] or animals [6] have shown how the body absorbs some organic UV filters from the skin and, consequently, the possible long-term effects must be studied, such as systemic toxicology. Therefore, from a health standpoint it is of interest to develop analytical methods capable of controlling the bioaccumulation and excretion mechanisms of these compounds.

There are still few articles devoted to the determination of traces of UV filters in the urine of human volunteers after topical application of sunscreens. Felix et al. [7] determined benzophenone-3 in urine using GC–MS; the method was sensitive and results revealed that concentrations in the order of 200 ng ml^{-1} of benzophenonone-3 were excreted after sunscreen application. Our group proposed a fluorimetric method to determine phenyl benzimidazole sulphonic acid in urine [8] which revealed that the urinary levels of this UV filter increased with time at least 12 h after sunscreen application, maximum concentration levels being in the order of 140 ng ml^{-1}. Sarveiya et al. [9] found levels of benzophenone-3 and its metabolites in the order of 1% of the applied dose.

Disodium phenyl dibenzimidazole tetrasulphonate (PDT) is a compound that has recently been authorized for use as a UV filter in sunscreen cosmetics, at concentrations <10%. It has a band that covers both, the complete UV-B (290–320 nm) range and practically all the UV-A (320–400 nm) range.

To our knowledge there are no published reports focusing on the determination of PDT in any type of sample.

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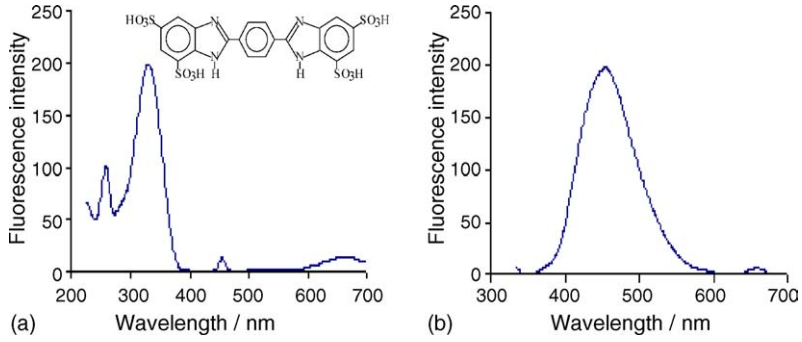

Fig. 1. (a) Excitation spectrum (λ_{exc} = 330 nm) and (b) emision spectrum (λ_{em} = 454 nm) of a 0.1 μg ml^{-1} PDT solution in 0.1 M HCl.

No data have been found in the literature on the percutaneous absorption of PDT nor expected levels to be found in human urine after sunscreen products use. The aim of this work is to obtain analytical data on the urinary excretion of PDT through analysis of urine from human volunteers after using a sunscreen lotion containing PDT.

Different analytical methods have been successfully proposed for determination of some UV filters in sunscreen cosmetics (not including PDT); most of them were based on liquid chromatography (LC) [10–20]. A LC method for determination of 18 UV filters (including PDT) in sunscreen samples have been recently developed by our group [21], but the sensitivity is not enough to determine traces of PDT in urine from volunteers after using sunscreen cosmetics.

The chemical structure of PDT provides fluorescence properties (see Fig. 1) that can be used for analytical purposes with high sensitivity. An on-line solid-phase extraction using a strong anion exchange (SAX) microcolumn allows the retention and subsequent elution of PDT, thus enabling its separation from the urine matrix. A sequential-injection (SI) system has been used that provides a high level of automation.

2. Experimental

2.1. Apparatus

A FP-6200 Jasco fluorescence spectrophotometer equipped with a Xe lamp was used.

The SI system (Fig. 2) was constructed with the following components: a Crison 2030 eight-channel automatic valve (Alella, Barcelona, Spain) connected to a personal computer via an RS 232C interface and controlled by home-made software, a Crison 2031 autoburette equipped with a 5 ml syringe and a Hellma fluorescence flow-through cell (Hellma, Müllheim/Baden, Germany) with 1.5 mm optic length and 25 μl internal volume. PTFE tubing with 0.5 mm internal diameter was used to connect the system. A 25 cm mixing coil length was used, this being the lowest possible allowed by the manifold geometry.

A SAX microcolumn was home-made by introducing 150 mg of packing SAX LiChrolut® (trimethylaminopropyl chloride) (Merck, Darmstadt, Germany or Varian®, Barcelona, Spain) into a 20 mm long × 3 mm i.d. PTFE tube.

A Crison micropH 2001 pHmeter was used for the pH measurements.

2.2. Reagents and samples

Disodium phenyldibenzimidazole tetrasulphonic acid >99% (Haarmann & Reimer, Holzminden, Germany) was used. The following analytical grade reagents were also used: sodium hydroxide (Probus, Badalona, Spain), hydrochloric acid 37% (Scharlab, Barcelona, Spain), ammonium hydroxide 25% (Probus), sodium dihydrogen phosphate monohydrate (Merck). Ethanol absolute HPLC grade (Scharlab) was also used.

The urine samples were taken from five volunteers after to apply themselves 10 ml of a home-made lotion prepared according to a protocol provided by the R & D section of the cosmetic laboratory of the enterprise RNB-Cosméticos (Paterna, Valencia, Spain). This lotion contained a 5% PDT. Their urinary excretion was controlled from the moment of application until the excreted amounts of PDT were no longer detectable. Eleven PDT-free human urine samples were also taken from volunteers and spiked with 40–200 ng ml^{-1} of PDT before the analysis.

2.3. Proposed method

Urine samples were filtered through an empty microcolumn cartridge with two frits inside (paper cannot be used because it retains PDT).

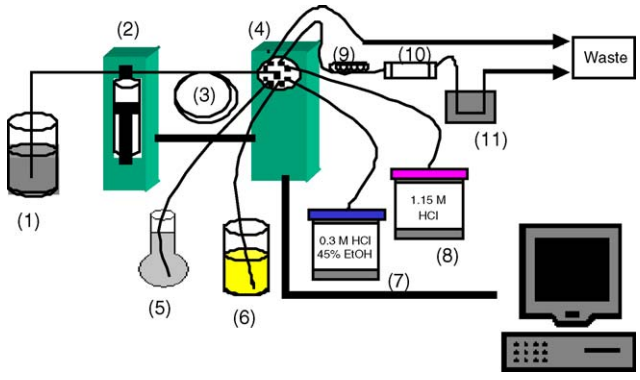

Fig. 2. SI system for fluorimetric determination of PDT in urine. (1) Carrier, water; (2) autoburette with 5 ml syringe; (3) holding coil (2.5 m); (4) eight-channel automatic valve; (5) standard solution of PDT; (6) urine samples; (7) washing solution; (8) elution solution; (9) mixing coil (25 cm); (10) SAX microcolumn and (11) fluorescence flow cell.

The pH was measured and adjusted between 4 and 7 with 2 M NH_4OH (if necessary).

Fluorescence intensity was measured at $\lambda_{em} = 454$ nm ($\lambda_{exc} = 330$ nm) using the SI system shown in Fig. 2. Both, the emission and excitation slits were 10 nm, the response time was 0.02 s, the sensitivity was medium and the acquisition data was 0.5 s.

A 0.2 μg ml^{-1} of PDT in buffer solution pH 6 (NaH_2PO_4 0.5 M/NH_3) was used as *standard solution*. A 1.15 M HCl solution was used as *elution solution*. A 0.3 M HCl:ethanol (55:45) was used as *washing solution*.

First the column was conditioned with 0.250 ml ethanol. The following aspiration/propulsion cycle for the standard addition calibration wascarried out: a 3.5 ml volume of carrier (H_2O nanopure), 0.150–0.500 ml of urine and V ml of the PDT *standard solution* ($V = 0$, 0.100, 0.200, 0.300, 0.400, 0.500) were aspirated. A 4.5 ml volume was propelled from the holding coil through the microcolumn (*retention step*). A 3.0 ml volume of carrier and 1.0 ml of the *washing solution* were aspirated. They were propelled through the microcolumn (*washing step*). A 3.0 ml volume of carrier and 1.0 ml of the *elution solution* were aspirated. They were propelled through the microcolumn to the detector (*elution step*). This cycle was repeated three times for each volume of the standard solution.

3. Results and discussion

3.1. Study of experimental variables: selection of the retention/elution conditions

Direct fluorimetric determination of PDT in urine is not possible due to the complexity of the matrix in which interfering compounds are present. Therefore, a solid-phase extraction is proposed, based on the use of a SAX microcolumn. PDT is in anionic form due to the deprotonation of its four sulphonic groups. Thus, it is selectively retained inside the microcolumn cartridge, whereas most of the interfering compounds pass through the cartridge without being retained. A washing step with a HCl:ethanol solution completely eluted the interfering compounds before analyte elution with a more concentrated HCl solution.

3.1.1. Preliminary PDT elution conditions

A preliminary experiment was carried out with the following cycle of aspiration/propulsion: a 3.5 ml volume of carrier (H_2O nanopure) and 0.5 ml of a 0.2 μg ml^{-1} PDT standard were aspirated. Then, they were propelled from the holding coil through the microcolumn. A 3.0 ml volume of carrier and 1.0 ml of the studied HCl solution were aspirated. Then, they were propelled through the microcolumn (elution step). A 3.0 ml volume of carrier and 1.0 ml 3 M HCl were aspirated. They were propelled through the microcolumn to the detector (after-elution washing step).

Fig. 3 shows the effect of the HCl concentration used in the elution step on the analytical signal obtained in both, elution and after-elution washing steps. A 1 M HCl concentration led to a quantitative elution of PDT.

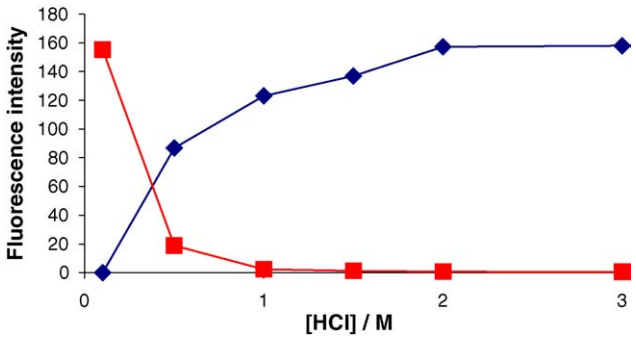

Fig. 3. (♦) Effect of the concentration of HCl in the elution step and (■) fluorescence intensity obtained after elution washing step with 3 M HCl.

3.1.2. Interference from urine matrix study

An aqueous PDT calibrate and a standard addition PDT calibrate were prepared. Aqueous calibration (a) was obtained by measuring five solutions containing between 0.1 and 0.5 μg ml^{-1} of PDT in a 0.5 M NaH_2PO_4 solution that was buffered at different pH (between 4 and 7) by using 2 M NH_3 and 1 M HCl, thus imitating the urine conditions. The standard addition calibration (b) was carried out by using 9.5 ml of urine sample (PDT-free) to which 0.5 ml of water containing PDT was added in order to get concentrations of between 0 and 0.5 μg ml^{-1}.

An experiment was carried out with the following cycle of aspiration/propulsion: a 3.5 ml volume of carrier (H_2O nanopure) and 0.5 ml of standard or spiked urine were aspirated. Then, they were propelled from the holding coil through the microcolumn (retention step). A 3.0 ml volume of carrier and 1.0 ml of 1 M HCl were aspirated. Then, they were propelled through the microcolumn to the detector (elution step). This cycle was repeated for the different solutions of each calibrate in order to obtain both calibration lines.

Fig. 4 shows the SI peaks obtained for three measurements of each solution using aqueous (a) and standard addition (b) calibration, with standard solutions at pH 7. Fig. 4b shows that two peaks for each solution of the standard addition calibration were obtained: the first peak is due to fluorescent components of the matrix which were not retained in the microcolumn and the second peak corresponds to the components eluted with 1 M HCl. These second peaks differ from the peaks obtained from the aqueous solutions shown in Fig. 4a. The following calibration equations make these differences clear:

$$y = -(0 \pm 1) + (628 \pm 4)x$$

($r^2 = 0.9998$, $N = 6$; aqueous calibrate)

$$y = (41 \pm 2) + (475 \pm 7)x$$

($r^2 = 0.9992$, $N = 6$; standard addition calibrate)

where y is the fluorescence intensity and x is the PDT concentration in μg ml^{-1}. Comparable results were obtained working with standard solutions at remaining pH levels studied.

The different slopes indicate the presence of matrix interferences which can be solved by using the standard addition calibration. As the urine sample used in the standard addition

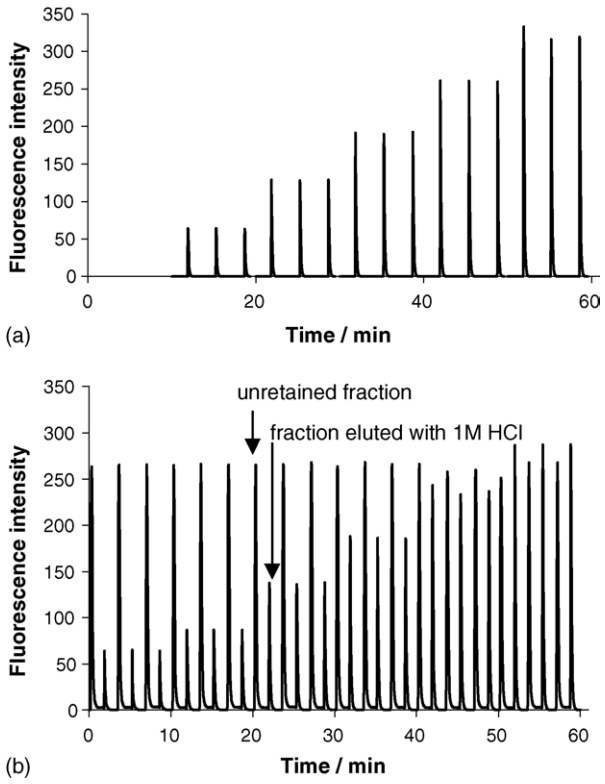

Fig. 4. SI peaks obtained using HCl 1 M as eluent. (a) Aqueous-buffered standard calibration of PDT and (b) standard addition calibration of PDT for a urine sample (PDT-free).

calibration was PDT-free, an intercept statistically different to zero indicates that some fluorescent components of the matrix were eluted together with PDT causing a constant error which must be resolved in order to separate the analyte and the interferents properly.

3.1.3. Washing and elution conditions

Assays were carried out before the elution of the analyte with 1 M HCl to try to elute the interfering compounds that were causing constant errors. Different washing conditions were assayed in a previous step to the final elution of the analyte. A 1 ml volume of different concentrations of HCl solution (0, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 M) was used in the corresponding experiments as before-elution washing solution. The signal obtained for a PDT-free urine sample in the elution step, significantly decreased in line with the concentration of HCl in the washing solution, while concentrations over 0.4 M are necessary to obtain a signal statistically similar than zero. These high concentrations of HCl in the washing step not only eluted interfering compounds but also a significant part of the analyte, with the subsequent sensitivity loss.

As ethanol cannot elute the analyte, but can elute other compounds, other conditions were studied based on using mixtures of HCl:ethanol in the washing elution and subsequent elution with 1 M HCl. Different mixtures, all them containing 0.3 M in HCl but different concentrations of ethanol (25, 50 and 75%) were assayed as washing elution solutions. For ethanol concentrations over 50%, a signal similar to zero was obtained in the elution of a PDT-free urine, with insignificant loss of the analyte. Because of the interdependence between the HCl concentrations in the elution, and in the washing step and the percentage of ethanol, a SIMPLEX multivariate study was carried out. Results showed that the optimum experimental parameters were: *washing solution*, 0.3 M HCl and 45% ethanol solution; *elution solution*, 1.15 M HCl.

To test whether the interfering compounds had been eliminated totally, an on-line standard addition calibration based on using only one PDT standard was prepared from a PDT-free urine sample and measured with the following cycle of aspiration/propulsion: a 3.5 ml volume of carrier (H_2O nanopure), 0.5 ml of urine were aspirated and a V ml volume of a 0.2 $\mu g\,ml^{-1}$ PDT solution, buffered with NaH_2PO_4 0.5 M/NH_3 at pH 6 ($V = 0$, 0.1, 0.2, 0.3, 0.4 and 0.5) were aspirated. They were propelled from the holding coil through the microcolumn (retention step). A 3.0 ml volume of carrier and 1.0 ml of *washing solution* were aspirated. They were propelled (washing step). A 3.0 ml volume of carrier and 1.0 ml of *elution solution* were aspirated. They were propelled (elution step). This cycle was repeated three times.

A calibration line $y = (2 \pm 2) + (2110 \pm 30)x$ was obtained (y, fluorescence intensity; x, μg PDT). The intercept was statistically similar to zero being $t_{experimental} = 1.02$ and $t_{theoretical} = 2.78$ (5% significance level, $N - 2 = 4$ d.f.). This proves the total elimination of the constant interference.

3.2. Other studies on the experimental conditions

3.2.1. Sequences of aspiration

Different sequences of sample and standard aspiration into the holding tube were assayed to ensure greater precision. The sequence sample–standard was selected because it provided a relative standard deviation (R.S.D.) in the order of 0.4%, which was lower than other sequences, such as standard –sample–standard, standard–sample or sample–standard–sample.

3.2.2. pH of the urine samples

Experiments at different pH values, within the usual range of urine samples (pH 4–7) were also carried out, using NH_3 2 M and HCl 1 M to adjust the pH of the urine. Similar results were obtained for a urine spiked with 0.060 $\mu g\,ml^{-1}$ PDT, indicating the procedure is robust against the urine pH.

3.2.3. pH of the PDT standard solution

Experiments at different pH values were done showing that accuracy is not affected by this parameter. However, if the solution is not buffered, there is around a 10-fold increase in the R.S.D. of the determination.

3.2.4. Stability of the urine samples: intra and interday data

Due to the special features of biological samples, differences among analytical results obtained from interday studies can be due not only to the irreproducibility of the analytical method but also to sample decomposition. In order to determine both the stability of urine samples and the reproducibility of the pro-

Table 1
Study of the stability of human urine samples

Store	PDT found ± S.D. (μg ml^{-1})				
	Day 0	Day 1	Day 2	Day 3	Day 4
Room temperature (T = 28 °C)	0.103 ± 0.009	0.108 ± 0.008	0.123 ± 0.005	0.126 ± 0.009	0.123 ± 0.010
T = 11 °C	0.103 ± 0.009	0.106 ± 0.008	0.101 ± 0.004	0.134 ± 0.012	0.122 ± 0.007

S.D., standard deviation obtained as the standard deviation of the extrapolated value in the standard addition line (n = 6 points).

posed method, the following experiment was carried out. Two portions were taken from a PDT-free urine sample spiked with 0.100 μg ml^{-1} PDT. One of them was stored at room temperature (28 °C maximum) and the other one in a refrigerator (11 °C). The PDT content of both portions was analyzed over the following 4 days. Data are shown in Table 1. The values indicated that samples must be analyzed during the first day after collection or stored in the refrigerator and used within 3 days, because longer conservation times lead to errors.

3.2.5. On-line standard addition calibration into the SI system

As shown in Section 2.3 the standard addition was carried out by varying the volume of only one standard, and generating the diluted solutions into the SI system, according to the cycle described for the proposed method. Experiments using the same urine were also done with the standard addition carried out by introducing several standards of different concentrations, and results were comparable. Because of this and its greater automation, the on-line standard addition using only one standard is preferable.

3.3. Analytical figures of merit

The accuracy of the method was studied by using it to analyse 11 spiked human urine samples.

Urine samples were free of PDT and were spiked with known amounts of the analyte. The PDT concentration in the spiked samples was between 40 and 200 ng ml^{-1}. Results obtained are shown in Table 2. The standard deviations were obtained as the standard deviation of the extrapolated value in the standard addition line [22]. The correlation line for the 11 mean values of PDT obtained using the proposed method versus the added contents was studied. The line obtained was $y = (3 \pm 3) + (1.00 \pm 0.03)x$ (r^2 = 0.9917, N = 11), where x and y are expressed in ng ml^{-1}. The $t_{\text{theoretical}}$ value (5% significance level, $N - 2 = 9$ d.f.) was 2.26 and the experimental values for the intercept and the slope were 0.97 and 0.16, respectively, which shows the comparability of the results.

The R.S.D. of the concentration values obtained for the analyzed samples was in the order of 3–14%, with a mean value of 7%, indicating a good level of precision, taking into account that traces of PDT are determined.

The sensitivity of the instrumental measurements differs for each urine sample. The mean slope of the standard addition calibration curves of the analyzed urine samples was of the order of 1900 μg^{-1}.

The detection limit of each analysis estimated by $3S_{y/x}/b$ (where $S_{y/x}$ is the standard deviation of the calibration curve and b is the slope) was 5 ± 2 ng, which is 10 ± 4 ng ml^{-1} of PDT in the urine samples (for a sample injection volume of 500 μl).

3.4. Validation of the method for urine from users of sunscreens containing PDT

Five volunteers applied 10 ml of a commercial sunscreen lotion containing 5% PDT. Their urine was collected both before and after the application for a period of 1–3 days. Fig. 5

Table 2
Determination of PDT in spiked human urine samples

Sample	Added concentration (μg ml^{-1})	Found concentration ± S.D. (μg ml^{-1})
1	0.100	0.110 ± 0.019
2	0.061	0.064 ± 0.005
3	0.051	0.055 ± 0.002
4	0.202	0.198 ± 0.013
5	0.043	0.047 ± 0.002
6	0.122	0.130 ± 0.006
7	0.150	0.160 ± 0.012
8	0.079	0.081 ± 0.004
9	0.049	0.046 ± 0.004
10	0.060	0.061 ± 0.002
11	0.120	0.125 ± 0.017

S.D., standard deviation obtained as the standard deviation of the extrapolated value in the standard addition line (n = 6 points).

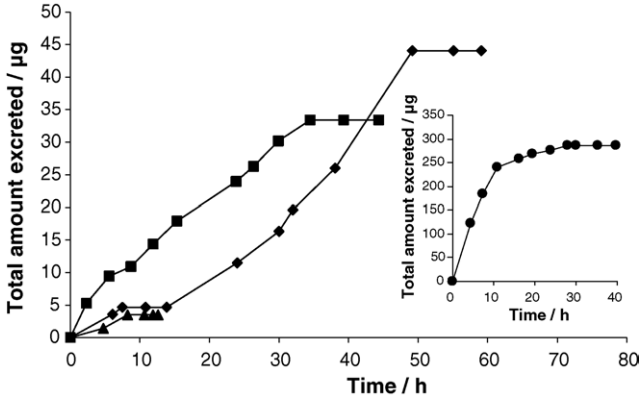

Fig. 5. Total amount excreted for four volunteers after application sunscreen product containing PDT (■) volunteer 1; (♦) volunteer 2; (▲) volunteer 3 and (●) volunteer 4.

shows the results obtained expressed as the total μg excreted. Volunteer 4 excreted around 300 μg PDT and continued excreting for 30 h after the application. Volunteers 1 and 2 excreted between 35 and 45 μg and they needed 35 and 50 h, respectively for excretion to become undetectable. However, volunteer 3 had a very low level (3.5 μg) and rapid (8 h) excretion and the urine sample of volunteer 5 (results not shown) gave undetectable values. These results shows that the absorption/excretion process of PDT in the human body depends greatly on the characteristics of each person. As the PDT is water-soluble UV filter [21], this feature could favour its rapid urinary excretion.

4. Conclusions

A fluorimetric method enabling PDT determination at ng levels is proposed here for the first time.

Direct and selective determination of PDT in urine samples can be achieved through the combination of sequential-injection with solid-phase extraction and fluorescence detection.

Automation of the method means a decrease in the amount of reagents used and residues generated. The SI system allows the required analysis sequence to be programmed using suitable software.

The applied SI–standard addition method avoids matrix interferences and it requires only one standard solution. The accuracy of the developed method has been proved by analysing 11 spiked urine samples.

The sensitivity of this method enables PDT determination in urine samples taken from volunteers after topical application of the sunscreen. Results seem to indicate that the excretion of this UV filter is both low and fast enough.

An analytical method for in vitro determination of the percutaneous absorption of PDT has been also recently developed by our research team (unpublished results). Both methods will allow an in-depth study of the biokinetics of this UV filter in the human body.

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