Journal of Chromatography A, 1218 (2011) 3013–3021

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Targeting metabolomics analysis of the sunscreen agent 2-ethylhexyl 4-(*N*,*N*-dimethylamino)benzoate in human urine by automated on-line solid-phase extraction–liquid chromatography–tandem mass spectrometry with liquid chromatography–time-of-flight/mass spectrometry confirmation

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ARTICLE INFO

Article history:
Received 2 February 2011
Received in revised form 15 March 2011
Accepted 17 March 2011
Available online 26 March 2011

Keywords:
2-Ethylhexyl
4-(*N*,*N*-dimethylamino)benzoate
Percutaneous absorption
Urine
Metabolism
On-line solid-phase extraction
LC–MS/MS

ABSTRACT

The *in vivo* metabolism of the xenobiotic agent 2-ethylhexyl 4-(*N*,*N*-dimethylamino)benzoate (EDP), a UV filter commonly used in sunscreen cosmetic products, was studied by targeting metabolomics analysis in human urine. The metabolomic study involved the use of urine from male and female volunteers before and after application of an EDP-containing sunscreen cosmetic. The metabolism of EDP in urine was studied by using the triple quadrupole detector in a combination of Precursor Ion Scanning and Neutral Loss Scanning modes, with and without enzymatic hydrolysis. Detected metabolites were subsequently confirmed as glucuronide conjugates of 4-(*N*,*N*-dimethylamino)benzoic acid and 4-(*N*-methylamino)benzoic acid by liquid chromatography–time-of-flight/mass spectrometry (LC–TOF/MS) in the accurate mass mode. In this way, the existence of phase II metabolism in the detoxification of EDP by effects of the lipophilic character of this sunscreen agent was confirmed. Hence, to study the *in vivo* metabolism of EDP, a fully automated method using a solid-phase extraction (SPE) workstation connected on-line to a liquid chromatograph and a triple quadrupole mass analyzer (LC–MS/MS) was developed. The ensuing hyphenated method is very simple and requires minimal human intervention. Following thorough optimization of the SPE and LC–MS/MS conditions, the analytical procedure was validated and standard addition calibration used for the quantitative correction of matrix effects. The proposed method was applied to determine the phase I metabolites of EDP in urine samples and afforded limits of detection from 0.1 to 1.1 ng and accuracy of 91–107% with relative standard deviations in the range 1.5–8.7% (sample volume: 100 μL). Based on the results of *in vivo* percutaneous absorption of a single application of the sunscreen, about 0.5% of the amount of the applied EDP is excreted in urine.

1. Introduction

Sunbathing is known to have beneficial physical and psychological effects. According to experts, the former are a result of stimulated synthesis of vitamin D [1], promotion of blood circulation [2] and effective action against some skin diseases such as psoriasis [3]; and the latter to sunbathing acting on the brain neurotransmitters responsible for mood to promote a sense of wellness [4]. However, excessive exposure to ultraviolet (UV) radiation without adequate protection can cause rashes and burns at short-time. Also, because the effects of UV radiation are cumulative, excessive long-term exposure to sunlight can lead to degeneration of dermal connective tissue (skin aging) and to the development of skin carcinomas, among other fatal consequences [5].

UV filters are commonly used in a wide range of sunscreen cosmetics to reduce the deleterious effects of UV radiation. One of the most effective authorized UV filters by virtue of its effective protection from UV radiation-induced edema [6] and photocarcinogenesis [7] is 2-ethylhexyl 4-(*N*,*N*-dimethylamino)benzoate, also known as ethylhexyl dimethyl PABA or EDP, which can absorb sunlight in the UV-B range (290–320 nm). Although sunscreens are designed for external application to the outermost layers of the skin [8], EDP has been ascribed some systemic effects such as antiandrogenic and estrogenic activity [9–12], and percutaneous absorption [13–16].

The adverse effects of UV filters on health have led national regulatory agencies to issue guidelines aimed at facilitating an optimum compromise between adequate protection and minimal side effects for users. As per European legislation and the US Food and Drug

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doi:10.1016/j.chroma.2011.03.045

Administration (FDA), EDP can be used up to a maximum concentration of 8% in cosmetic products; by contrast, Japanese legislation allows up to 10% EDP to be used [17].

Biotransformation processes, which involve changes in chemical and physical properties of xenobiotics entering the body, are catalyzed by a limited number of enzymes with broad substrate specificities [18]. The catalyzed reactions encompass hydrolysis (*e.g.* carboxylesterase, which occurs in microsomes, cytosol, lysosomes and blood), reduction (*e.g.* carbonyl reduction, which takes place in cytosol, microsomes and blood), oxidation (*e.g.* cytochrome P450, which is present in microsomes) and conjugation (*e.g.* UDP-glucuronosyltransferase, also present in microsomes). Whereas the first three reactions, which are often called "phase I reactions", result in slightly increased hydrophilicity, conjugation reactions, which are often referred to as "phase II reactions", give much more hydrophilic products, thereby greatly promoting excretion of the target compounds – mainly in urine. The conjugation reactions include glucuronidation, sulfonation, acetylation, methylation and conjugation with glutathione or amino acids.

The *in vitro* biotransformation of EDP in the presence of various cofactors was recently studied and two phase I EDP metabolites were identified by GC–MS and LC–MS as a result [19]. Although phase II metabolism was also investigated via glucuronidation and acetylation in incubated human and rat liver microsomal and cytosolic solutions containing various cofactors, no corresponding *in vitro* phase II metabolites for EDP were detected. Since urinary excretion is the primary release pathway for some UV filters and their metabolites [20], the development of analytical methods to determine both types of compounds in human urine samples has become a necessity. However, the only authorized sunscreens that have so far been determined together with all their metabolites in human urine are 2-hydroxy-4-methoxybenzophenone [21] and 3-(4-methylbenzylidene) camphor [22].

For EDP, the presence of its metabolites in urine would confirm the results of its *in vitro* study and provide further insight into its percutaneous absorption in humans. Also, some biotransformation processes activate rather than inactivate or detoxify the xenobiotic. The phase I metabolites of EDP are structurally very similar to *p*-aminobenzoic acid, a UV filter widely used in the past but currently banned by European legislation owing to the high incidence of adverse dermatological effects, DNA damage included [23]. Therefore, the widespread use of EDP warrants a thorough systematic toxicological study of its metabolites with a view to preventing potential long-term effects.

EDP has scarcely been determined in human biological fluids. For example, it was determined in human urine by using GC–MS after preconcentration by microporous membrane liquid–liquid extraction [24,25], all with operational simplicity and low consumption of organic solvent. Also, it was determined with minimum limits of detection of 50 ng/mL by LC–UV in human plasma [26]. Finally, an analytical method allowing the determination of this sunscreen agent and its phase I metabolites in human urine samples by use of off-line solid-phase extraction (SPE) in combination with LC–MS was also developed but applied to no human biofluids [19].

Similarly to off-line SPE, during on-line SPE–LC the sample is loaded onto an SPE cartridge where the analytes are retained while unretained matrix components are washed away. Then, the analytes are automatically transferred from the SPE cartridge to the analytical column for chromatographic separation. In general, on-line connection facilitates development of the SPE procedure [27] and minimizes the need for manual operations such as evaporation and reconstitution, which are usually required in off-line SPE and sources of error in the analysis of biofluids.

The primary aim of this work was to develop and validate an on-line SPE–LC–MS/MS analytical method allowing the determination of EDP and its phase I metabolites in human urine. The *in vivo* formation of phase II metabolites was also examined by ultrasound-accelerated enzymatic hydrolysis. One other major aim was to identify and characterize phase II metabolites of EDP by using triple quadrupole (QqQ) and time-of-flight (TOF) mass spectrometry.

2. Materials and methods

2.1. Chemicals and stock solutions

2-Ethylhexyl 4-(*N*,*N*-dimethylamino)benzoate (EDP, 98%) from Aldrich (Milwaukee, WI, USA), and 4-(*N*,*N*-dimethylamino)benzoic acid (DMP, 98%) and 4-(*N*-methylamino)benzoic acid (MMP, 97%) from Sigma–Aldrich (Schnelldorf, Germany), were used as standards. Trihexylamine (THA) from Sigma–Aldrich was used as internal standard.

Methanol, acetonitrile, formic acid and ammonia [32% (m/v) extra pure solution] were purchased from Scharlab (Barcelona, Spain). Deionized water was obtained by using a Milli-Q water purification system from Millipore (Billerica, MA, USA). Ammonium formate and acetate were supplied by Sigma (St. Louis, MO, USA), and citric acid was purchased from Panreac (Barcelona, Spain). All chemicals were LC-grade unless otherwise stated.

β-Glucuronidase from *Helix pomatia* (Type H-1) (≥300,000 U/g activity) with sulfatase activity ≥10,000 U/g was obtained from Sigma–Aldrich. The enzyme solution was prepared by dissolving the amount of commercial product required to ensure thorough hydrolysis of glucuronide conjugates (0.01 g) in 5 mL of 1 M ammonium acetate (pH 5) and kept at 4 °C.

Multicomponent (EDP, DMP, MMP) and internal standard (THA) stock solutions were prepared separately at a 200 μg/mL concentration in methanol.

2.2. Samples and standard addition calibration

The urine samples used to develop and validate the proposed method were obtained from volunteers using no EDP-containing cosmetics.

A laboratory-made sunscreen cosmetic product containing 8% EDP was applied topically to the body of two male and two female volunteers after informed consent. Typical cosmetic-grade ingredients such as emollients, surfactants, smoothing agents, hydrating agents, preservatives and perfumes from Guinama (Valencia, Spain) or RNB (Valencia, Spain) were used to prepare the cream according to an adapted protocol [28].

Urine samples were collected immediately before and after application of the sunscreen. For post-application samples, the volunteers were instructed to collect the whole volumes of urine excreted over periods of 24 h in commercially available sterile containers. During the study, which lasted 4 days, urine samples were stored at 4 °C until analysis –which was always done within 72 h of collection (see Section 3.5). Additional urine samples were collected 8 days after application of the cream in order to check for the presence of EDP and/or its metabolites. For this purpose, two urine pools were prepared each day from male and female volunteers, but the total volume collected daily was this time used to estimate the percutaneous absorption kinetics of EDP.

An internal standard solution of THA at a 2 μg/mL concentration in deionized water and a multicomponent solution of EDP, DMP and MMP at 2 μg/mL in urine were prepared from the respective stock solutions.

The solutions for standard addition calibration were prepared by spiking five aliquots of 650 μL of urine with 0, 20, 40, 60 and 80 μL of the multicomponent solution (2 μg/mL), to which 100, 80, 60, 40 or 20 μL of urine, respectively, was added in order to have an iden-

tical volume of urine in all calibration solutions. A volume of 50 μL of internal standard (2 μg/mL) was also added to each calibration solution.

2.3. Ultrasound-assisted enzymatic hydrolysis

In order to determine the contents of analytes in their free forms or in both free and conjugated forms, 100 μL of 1 M ammonium acetate (pH 5) or β-glucuronidase solution (prepared in 1 M ammonium acetate, pH 5) was added to each urine calibration solution. The samples were subjected to ultrasound-assisted enzymatic hydrolysis in order to deconjugate any phase II EDP metabolites potentially formed. The dramatically shortened time required for deconjugation relative to traditional methods based on time-consuming enzymatic incubation justifies the use of ultrasound here [29].

Ultrasound was applied by means of a Branson 450 digital sonifier (20 kHz, 450 W) with tunable amplitude and duty cycle which was equipped with a cylindrical titanium alloy probe 12.7 mm in diameter.

The samples containing the enzyme were placed in a container and immersed in a water bath. The temperature was kept at 37 °C throughout the enzymatic hydrolysis process by means of a thermostated water-bath from Selecta (Barcelona, Spain). The probe tip was also immersed in the water-bath, at the same distance from every vial and a fixed distance from the bottom of the container. Hydrolysis of EDP conjugated metabolites was essentially performed under the optimum conditions previously reported by Álvarez-Sánchez et al. [29]. Thus, ultrasonic irradiation was applied for 50 min at 35% duty cycle (the fraction of ultrasound application per second) and 50% output amplitude (converted applied power, 400 W). The mixture used for conventional hydrolysis of EDP conjugated metabolites was prepared as described above for ultrasound-assisted enzymatic hydrolysis and incubated overnight at 37 °C.

2.4. Automated analytical platform

2.4.1. Sample treatment

Samples were analyzed by using an automated Prospekt 2 SPE workstation (see Supplementary data, Fig. S1) from Spark Holland (Emmen, The Netherlands), which includes an automatic cartridge exchanger (ACE) and a high-pressure syringe dispenser (HPD) for SPE solvent delivery. The automated system was coupled to a Midas autosampler furnished with a 100 μL sample loop. Peek tubing of 0.25 mm i.d. from VICI (Houston, TX, USA) was used for all connections between valves. The extraction step was fully automated via the software Sparklink v. 2.10.

Various HySphere cartridges from Spark Holland were initially tested for development of the SPE step, namely: CN-SE (silica-based cyanopropyl phase, particle size 7 μm), C2-SE (silica-based ethyl phase, particle size 7 μm), C8 EC-SE (end-capped silica-based octyl phase, particle size 10 μm), C18 HD (end-capped silica based phase with a high density of octadecyl chains, particle size 7 μm), Resin GP (polymeric polydivinyl-benzene phase, particle size 5–15 μm), Resin SH (strong-hydrophobic modified polystyrene-divinylbenzene phase, particle size 20–50 μm), MM anion (mixed mode anion exchanger with a polydivinyl-benzene backbone, particle size 10 μm) and MM cation (mixed mode cation exchanger with a polydivinyl-benzene backbone, particle size 10 μm). All cartridges were 10 mm × 2 mm i.d. Sample acidification was also considered in accordance with pK_a for each compound [about 2.5 for EDP, and about 2.5 (pK_{a1}) and 5 (pK_{a2}) for MMP and DMP] and the nature of the extraction mechanisms. pK_a values were obtained from the SciFinder Scholar Database (http://www.cas.org/products/sfacad/).

In the optimized procedure, a volume of 100 μL of a 1 M citric acid/citrate buffer solution adjusted to pH 3 with ammonia was added to each sample vial on the Midas autosampler and mixed thoroughly for 2 min before automated extraction. The final volume of each urine solution was 1 mL. Then, it was applied the experimental protocol described in Fig. S1.

2.4.2. LC–MS/MS determination

The SPE workstation was on-line connected to an Agilent 1200 Series LC system from Agilent Technologies (Palo Alto, USA). Complete automation of the Prospekt 2 and LC systems was accomplished by programming appropriate analysis sequences. The LC system was interfaced to an Agilent 6460 Triple Quad LC/MS detector from Agilent Technologies (Palo Alto, USA) equipped with a Jet Stream Technology electrospray ion source, also from Agilent. Nitrogen was supplied by a high purity generator from CLAN Tecnológica (Sevilla, Spain) and used as the source gas. Nitrogen (99.999%) from Carburos Metálicos (Córdoba, Spain) was used as collision gas. Data acquisitions, and qualitative and quantitative analyses, were controlled via the Agilent MassHunter Workstation software

Analytes were separated on a Mediterranea SEA C18 analytical column (3 μm, 4.6 mm × 150 mm) from Teknokroma (Barcelona, Spain), using a flow rate of 0.8 mL/min at 20 °C. In the final method, eluent A consisted of deionized water and eluent B of a 1:1 (v/v) mixture of methanol and acetonitrile, both phases containing 0.2% formic acid. The gradient was as follows: 0–1 min, 40% eluent B; 1–3 min linear gradient to 100% eluent B, held for 12 min. The analytical column was equilibrated with a post-run time of 5 min.

Analytes were determined by ESI-MS/MS selected reaction monitoring (SRM) in the positive ion mode. The triple quadrupole MS and spray chamber conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 4450 V. The dwell time was set at 70 ms.

Calibration curves were obtained as plots of the peak area/internal standard peak ratio for each analyte against its added concentration. THA was used as internal standard.

2.5. LC–TOF/MS analysis

The analyses conducted with a view to confirming phase I metabolites of EDP and elucidating phase II metabolites were performed on the above-described Agilent 1200 Series LC system. The LC system was interfaced to an Agilent 6540 UHD Accurate-Mass TOF LC/MS detector, also from Agilent Technologies and equipped with an Agilent Jet Stream Technology electrospray ion source operating in the positive ion mode. The separation conditions were identical with those for the LC–MS/MS determination except for the use of the respective LC–MS/MS grade solvents. The injected sample volume was 100 μL.

The operating conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 4450 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 Hz) in both the centroid and profile modes was governed via the Agilent MassHunter Workstation software. The mass range and detection window were set at m/z 100–1100 and 100 ppm, respectively. Reference mass correction on each sample was performed with a continuous infusion of Agilent TOF biopolymer analysis mixture containing purine (m/z 121.0508) and hexamethoxyphosphazyne (m/z 322.0481). A resolution of 20,000 was used to examine the isotopic profiles for the EDP metabolites. Analytes were identified by accurate mass detection.

Table 1
SRM transitions in ESI-MS/MS in the positive ion mode for the determination of EDP and its metabolites in urine.

Compound	Retention time (min)	Precursor ion (*m*/*z*)	Q1 voltage (V)	Collision energy (V)	Quantification SRM transition	Confirmation SRM transition
MMP	5.16	151.8	90	15	151.8 → 136.8	151.8 → 119.9 (60)[b]
DMP	5.70	165.8	90	25	165.8 → 149.8	165.8 → 133.9 (20)[b]
EDP	9.11	277.8	100	15	277.8 → 165.8	277.8 → 150.8 (80)[b]
THA[a]	5.31	270.1	120	20	270.1 → 185.8	270.1 → 102.0 (20)[b]

[a] Internal standard.
[b] Intensity ratio between quantification and confirmation SRM transitions, expressed as relative percentage and considering the quantification transition as 100%.

3. Results and discussion

3.1. LC–MS/MS

The ESI-MS/MS collision-induced dissociation (CID) of all analytes under study was investigated by direct injection of individual standard solutions containing an approximate concentration of 5 μg/mL in MeOH, using the positive and negative ESI modes and various ionization agents. Based on the ESI-MS/MS ionization and fragmentation patterns for EDP, DMP and MMP, the positive ESI mode was selected for their determination.

The effect of adding a modifier to the LC mobile phase was examined in terms of ESI-MS/MS sensitivity and peak shape. Variable concentrations of formic acid and two volatile salts (ammonium acetate and formate) were tested for this purpose. The best results were obtained by adding 0.2% formic acid to both mobile phases.

Under these conditions, tandem mass spectrometry parameters were optimized for efficient isolation of the precursor ions, and their sensitive, selective fragmentation. The optimum operating conditions as regards quantification and confirmation SRM transitions are compiled in Table 1.

3.2. Ultrasound-assisted enzymatic hydrolysis

Based on the fact that glucuronidation and sulfation are two of the major phase II biotransformation pathways, samples were subjected to enzymatic hydrolysis with glucuronidase/sulfatase dual activity. As stated before, conjugated EDP metabolites were hydrolysed under the optimum conditions previously established by Álvarez-Sánchez et al. [29]. However, the ultrasonic irradiation time was extended to 50 min in order to improve the deconjugation efficiency. In fact, the efficiency of ultrasound-assisted hydrolysis was comparable to that obtained by conventional overnight incubation of EDP conjugated metabolites at 37 °C. The shortened time required for enzymatic hydrolysis and the operational simplicity of the procedure justify the use of ultrasound (see Supplementary data, Fig. S2).

3.3. Automated solid-phase extraction

The different variables related to automated sample preparation were optimized by using a univariate approach to assess clean-up and retention–elution capabilities.

For primary screening, the suitability of various sorbent materials (see Section 2.4) for SPE of the analytes in a single extraction was assessed. Acidification of samples was also tested with a view to improving retention.

Analyte-free urine samples were spiked at a 1 μg/mL concentration and acidified to pH 3 with 1 M citric acid/citrate buffer before on-line SPE. Potential precipitation by effect of the urine samples standing at pH 3 on the autosampler during the whole analysis sequence was avoided by *in situ* acidification. To this end, variable volumes of 1 M citric acid/citrate or formate/formic acid buffer (pH 3) and formic acid were added by the autosampler to the urine samples immediately before automated SPE was started. The best, most reproducible results were obtained by adding a volume of 100 μL of 1 M citric acid/citrate buffer (pH 3). In this way, SPE was efficiently conducted with *in situ* acidification.

The MMP retention capacity (expressed as relative percentage of peak areas) of the sorbents decreased in the following sequence: C18 HD (100) > C8 EC-SE (80) > Resin SH (70) > Resin GP (65) > MM anion (60) > MM cation (40) > C2-SE (15) > CN-SE (5) for MMP. The sequence for DMP was identical except that C2 cartridges performed on a par with C18 HD cartridges. As regards EDP, the sorbents exhibited only slight differences between materials; in any case, the best results were also obtained with HySphere C18 HD cartridges. Therefore, C18 HD cartridges were adopted for further testing as they provided the best overall recoveries in these preliminary tests.

The loading solvent was optimized by testing methanol–water mixtures in variable proportions and pure, deionized water was selected since the presence of even small amounts of methanol caused the most polar analyte (MMP) to be eluted. The influence of the loading volume was examined over the range 0.5–8 mL by setting the minimum value as twice the combined volume of the sample loop, transport tubing and internal cartridge. A volume of 3 mL of deionized water was adopted since higher volumes caused partial elution of MMP. Similarly, variable loading flow-rates ranging from 0.5 to 2 mL/min were examined and the best recoveries found to be those obtained at 1 mL/min.

Deionized water and aqueous methanol [2.5% and 5% (v/v)] were evaluated as washing solutions to remove polar interfering compounds present in the urine samples from the SPE cartridges. Because even the smallest amount of deionized water had adverse effects on retention of MMP in the cartridges, the washing step was suppressed in subsequent tests. It should be noted that high volumes of loading solvent resulted in partial elution of the more polar analytes from the cartridges. As a consequence, the effect on the washing solvent was dependent on the weak interaction between MMP and the SPE sorbent.

Retained analytes were eluted by pumping the LC mobile phase through the SPE sorbent instead of pumping the solvents via the Prospekt pump. In this way, the time the LC mobile phase was pumped through the SPE system to the analytical column (*i.e.* the elution time) became the key variable. Since the selected time should allow quantitative elution of the analytes while avoiding elution of interferents potentially causing ionization suppression in the mass analyzer electrospray, and excessively broad chromatographic peaks were to be avoided, the effect of the elution time was also examined. A time of 4 min was selected, after which the LC mobile phase was pumped directly to the analytical column.

Breakthrough as a measure of sorbent capacity was assessed by using a two-cartridge configuration in the SPE automated workstation [30]. This involved connecting two cartridges of the same type serially in order to have the second retain the portion of analyte not retained in the first during the loading step. In the event of breakthrough on the first cartridge, the analyte was trapped by the second. In the absence of breakthrough, no analytes reached the second cartridge. Breakthrough was assessed by triplicate analysis of urine samples at three different concentration levels. The retention efficiency was calculated as the ratio of analyte response

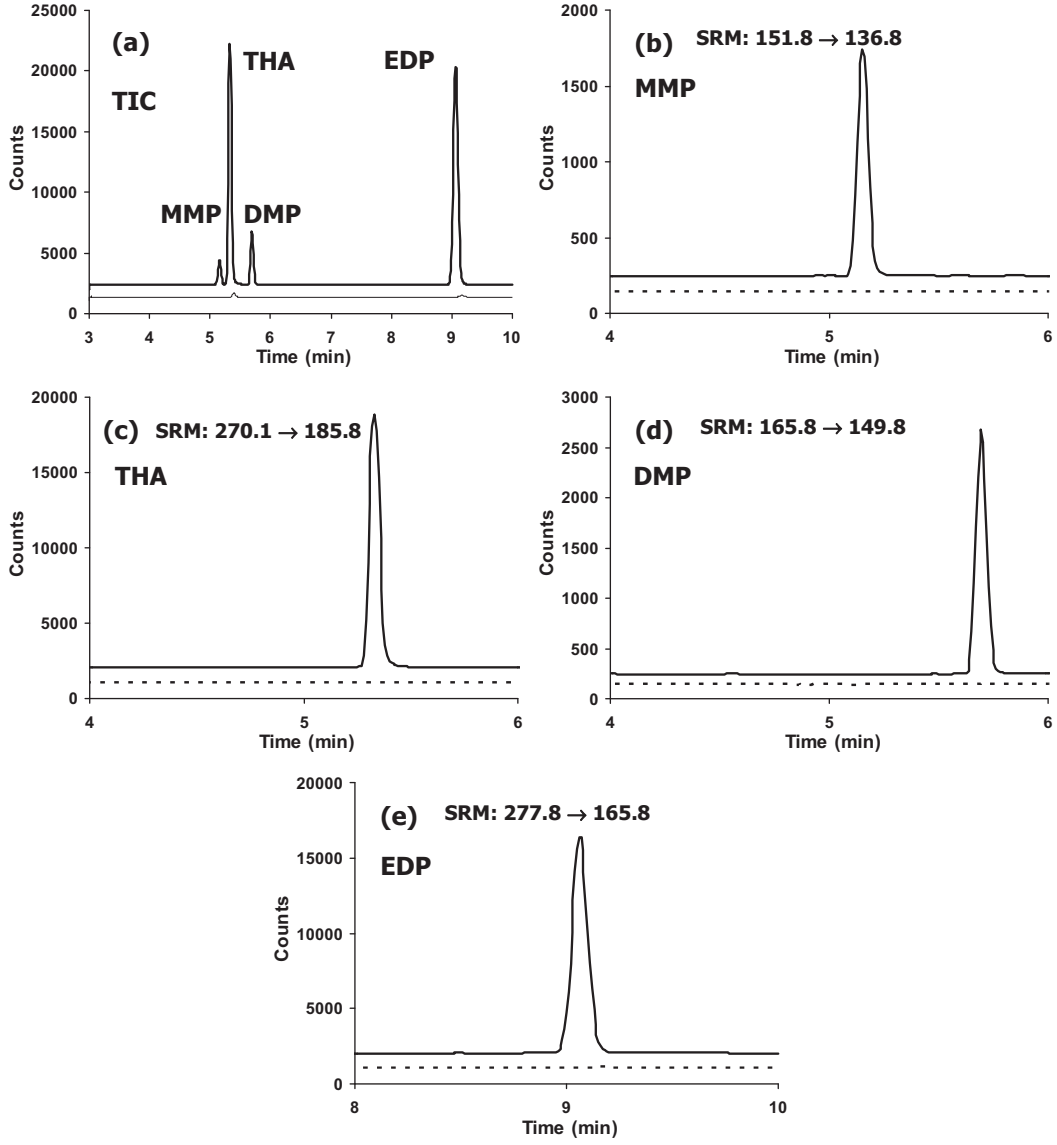

Fig. 1. Total ion chromatograms (TICs) in the positive ionization mode for EDP, DMP, MMP and THA (a); and SRM chromatograms for each target compound (b–e) as obtained by analyzing an analyte-free urine sample (dotted line) and a urine sample spiked at a 40 ng/mL concentration (solid line). The experimental conditions are described under Section 2.

in cartridge 1 to the combined response in both. Tests conducted under the selected experimental conditions showed that no breakthrough occurred for any target analyte at any concentration level (see Supplementary data, Table S1). No tubing analysis for analyte adsorption was needed since recoveries with C18 HD cartridges were near-quantitative. As a result, carry-over by effect of adsorption of hydrophobic analytes (EDP) onto the tubing [31] was negligible thanks, mainly, to the thorough post-extraction washing step.

3.4. Performance of the proposed analytical method

Matrix effects occurring during the on-line SPE–LC–MS/MS determination of EDP and its metabolites were assessed by comparing the differences between the responses of a calibration set prepared in single analyte-free urine with those of samples prepared in pool free-analyte urine from several volunteers. Statistically different slopes ($P_0 = 0.05$) between both calibrations were obtained for all analytes. Accordingly, we chose to use the standard addition method for quantitation in order to correct matrix interferences and measure the levels of the targeted compounds in urine samples. Fig. 1 shows the SRM chromatograms obtained by monitoring selected ions of the target analytes in unspiked and spiked (40 ng/mL) analyte-free urine samples subjected to the above-described on-line SPE–LC–MS/MS procedure.

Accuracy was evaluated by using the proposed method to analyze urine samples from male and female volunteers who were known to use no EDP-containing cosmetic. The samples were spiked with known amounts of EDP, DMP and MMP. The results for each sample, and their standard deviations, are shown in Table 2. The standard deviation was calculated as the variability of the extrapolated value in the standard addition curve as described in detail elsewhere [32]. Student's *t*-test confirmed the absence of significant differences between the found concentrations obtained with the proposed method and the concentrations added to the samples, which testifies to the accuracy of the proposed method. Analyte recoveries ranged from 91 to 107%, which indicates that matrix effects were efficiently corrected (Table 2).

Table 2
Determination of EDP, DMP and MMP in spiked analyte-free urine samples.

Analyte	Parameter	Urine samples				
		1	2	3	4	5
EDP	μ (ng/mL)[a]	12	40	52	110	120
	$C \pm s$ (ng/mL)[b]	11 ± 1	40 ± 2	56 ± 4	108 ± 8	115 ± 7
	$t_{calculated}$[c]	2.33	0.46	2.17	0.44	1.54
	Accuracy (%)	91 ± 9	101 ± 6	107 ± 8	99 ± 7	96 ± 6
	On column LOD (ng)[d]	0.3	0.5	0.7	1.1	0.7
	On column LOQ (ng)[e]	0.9	1.5	2.2	3.5	2.2
DMP	μ (ng/mL)[a]	13	36	50	107	116
	$C \pm s$ (ng/mL)[b]	13 ± 1	33 ± 2	47 ± 4	111 ± 6	111 ± 8
	$t_{calculated}$[c]	0.05	2.71	1.99	1.49	1.24
	Accuracy (%)	100 ± 10	92 ± 6	93 ± 8	104 ± 6	96 ± 7
	On column LOD (ng)[d]	0.3	0.5	0.7	0.7	0.9
	On column LOQ (ng)[e]	1.1	1.5	2.3	2.5	3.1
MMP	μ (ng/mL)[a]	11	38	52	103	113
	$C \pm s$ (ng/mL)[b]	10 ± 1	35 ± 3	51 ± 1	102 ± 4	107 ± 5
	$t_{calculated}$[c]	3.04	2.18	1.93	0.80	2.73
	Accuracy (%)	92 ± 6	92 ± 8	99 ± 1	98 ± 4	95 ± 4
	On column LOD (ng)[d]	0.2	0.6	0.1	0.5	0.5
	On column LOQ (ng)[e]	0.6	2.1	0.3	1.6	1.8

[a] Spiked concentration.
[b] Concentration found by standard addition calibration.
[c] Tabulated t (0.05, $n-2=3$) = 3.18.
[d] Estimated as $3.3\,s_A/b$, where b is the slope of the calibration curve and s_A the standard deviation of the intercept y of the calibration curve. Injected volume, 100 μL.
[e] Estimated as $10\,s_A/b$ (see note d).

Calibration graphs ($n=5$) exhibited excellent linearity for EDP, DMP and MMP, with regression coefficients higher than 0.995 over the concentration range from 20 to 100 ng/mL in all cases. The limits of detection (LOD) and quantitation (LOQ) were determined according to the International Conference on Harmonization (ICH) guidelines [33]. Table 2 summarizes the procedure used for validation. Worth special note are the low values of the on-column LODs, which ranged from 0.1 to 1.1 ng. This allows the sample volume (100 μL) to be increased if more sensitivity is required, provided potential breakthrough under those conditions should be investigated.

Within-day variability (*i.e.* repeatability) was evaluated by extracting the analytes from five different urine solutions spiked at a 50 ng/mL concentration in a single analytical run. Between-day variability (*i.e.* reproducibility) was determined by extracting the analytes from a 50 ng/mL spiked urine solution over five consecutive days. Within-day and between-day variability ranged from 1.5 to 8.7% and 3.8 to 11.2% as relative standard deviation (RSD), depending on the analyte.

3.5. Stability considerations

The stability of EDP and its metabolites in human urine was studied at 4 °C. The target analytes were found to remain stable for at least 72 h under the studied conditions (see Supplementary data, Fig. S3). Beyond that time, however, the analytes exhibited slight instability in the urine samples – particularly EDP. Therefore, the samples were stored at 4 °C and always analyzed within 72 h after collection, which provided ample time for the intended study. Also, the analytes remained stable during the time the samples were on the autosampler. Only the citric acid/citrate buffer required special care: fresh preparation at 4 h intervals, approximately, and placement in the autosampler plate holder in order to maintain the optimum conditions for the analytical method.

3.6. In vivo metabolic pathway for EDP

To the best of our knowledge, ours is the first reported instance of the presence of EDP metabolites in human urine samples. The first test conducted here was the analysis with the proposed SPE–LC–MS/MS method without enzymatic hydrolysis of a urine sample from a volunteer after application of an EDP-containing sunscreen cosmetic; neither DMP nor MMP was detected, however. This result supports the existence of phase II biotransformation pathways for the detoxification of EDP resulting from the low polarity of DMP and MMP. However, a peak corresponding to the same SRM transition as that for DMP was observed at 4.9 min (compared to 5.7 min in DMP) which exhibited a similar fragmentation pattern and could be ascribed to a conjugated form. We used various MS modes to identify potential metabolites and selected the Precursor Ion Scanning (PI) mode for use in the QqQ analyzer in order to monitor any compound generating product ions with m/z 165.8 or 151.8 (*viz.* the molecular mass of DMP and MMP, respectively) upon fragmentation. This approach revealed that the glucuronide conjugate of DMP (DMP-Glu, m/z 341.8) yielded the ion product at m/z 165.8 observed at 4.9 min (Fig. 2a). The presence of glucuronide forms led us to use the Neutral Loss Scanning (NL) mode in the QqQ analyzer to detect any compound whose fragment loss after MS/MS activation would be 176.0 (m/z), which corresponds to the characteristic neutral loss from a glucuronide conjugate [34]. The results, shown in Fig. 2b, confirm the involvement of phase II metabolism in the detoxification of EDP.

Next, the same urine sample was analyzed with the proposed methodology, using SRM. The procedure included enzymatic hydrolysis with β-glucuronidase possessing sulfatase activity. Surprisingly, both DMP and MMP were detected (Fig. 3). Whereas detecting DMP in the enzyme treated sample with both PI and NL was to be expected, the presence of MMP was quite unexpected.

The previous results led us to conduct LC–TOF/MS analyses in order to confirm the presence of the glucuronide conjugate of MMP (MMP-Glu). For this purpose, two urine samples were collected from the same volunteer immediately before application of the EDP-containing cream (blank) and 24 h later (sample). Each urine specimen was split into two fractions that were treated with either 1 M ammonium acetate (pH 5) or β-glucuronidase/sulfatase solution (see Section 2) and subjected to the ultrasound-assisted protocol. The urine samples were directly injected into the LC–TOF/MS system without SPE. Molecular features were extracted from the two blank solutions (enzyme treated and untreated). Two or more ions were used as compound ion count threshold. The iso-

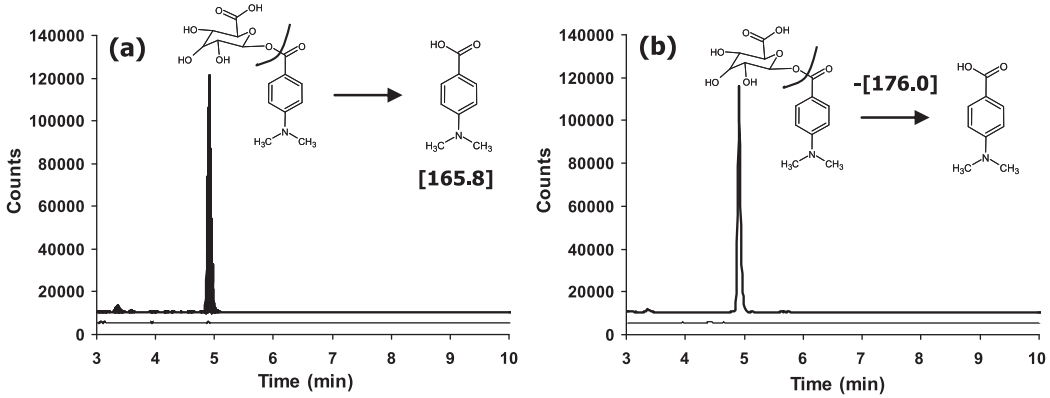

Fig. 2. Triple Quad Precursor Ion (a) and Neutral Loss (b) chromatograms as obtained by analyzing an analyte-free urine sample (dotted line) and a urine sample from a volunteer after application of the sunscreen cream (solid line). The experimental conditions are described under Section 2.

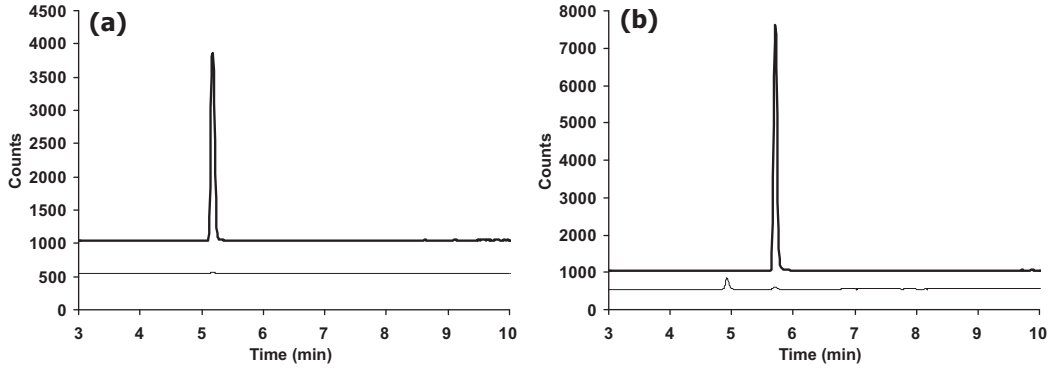

Fig. 3. SRM chromatograms for MMP (a) and DMP (b) as obtained by analyzing a urine sample from a volunteer after application of the sunscreen cream and subjected to enzymatic hydrolysis in the presence (solid line) and absence of ultrasound (dotted line). The experimental conditions are described under Section 2.

tope model corresponded to common organic molecules with peak spacing tolerance of m/z 0.0025 ± 7.0 ppm. Following subtraction of both lists of compounds from the corresponding sample solutions (enzyme treated and untreated), identification of the compounds proceeded by generation of candidate formulae with a mass accuracy limit of 5 ppm. The contribution to mass accuracy, isotope abundance and isotope spacing scores was 100.00, 60.00 and 50.00, respectively. As can be seen in Fig. 4, DMP and MMP were detected in the sample treated with β-glucuronidase, whereas DMP-Glu and MMP-Glu were identified in the enzymatically untreated sample. Retention times, formulae, experimental and theoretical masses, and errors, in ppm, were obtained by accurate mass measurements of EDP metabolites in human urine (see Supplementary data, Table S2). The retention times for the glucuronide conjugates were shorter than those for the unconjugated metabolites, consistent with the increased hydrophilicity of phase II metabolites. The hydrophilic metabolite MMP-Glu was most likely not retained by the SPE cartridge during the automated sample treatment and went undetected by the Triple Quad detector as a result. However, the proposed method aims at determining deconjugated DMP and MMP forms, so the deficient extraction of MMP-Glu with C18 HD cartridges poses no problem since ultrasound-assisted enzymatic hydrolysis ensures quantitative glucuronide deconjugation.

We also considered acetylation and glycine conjugation reactions, which are known to occur in the structurally similar *p*-aminobenzoic acid (PABA) [35]; however, no other phase II

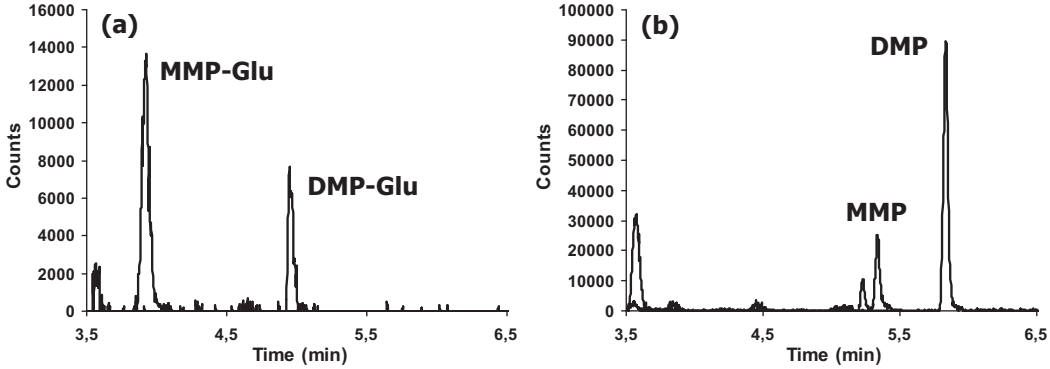

Fig. 4. Extracted ion chromatograms for accurate mass measurements as obtained from LC–TOF/MS analyses for the identification of EDP metabolites in urine from volunteers after topical application of a sunscreen product containing EDP and enzymatic hydrolysis in the absence (a) or presence (b) of ultrasound. See Section 2 for experimental conditions and Supplementary data, Table S2 for measurement details.

Table 3
Excreted amount of DMP and MMP via urine from volunteers after a single topical application of a sunscreen product containing EDP. Results are expressed in absolute excreted mass by considering the corresponding dilution factor and the total volume of sample collected.

Day	DMP excreted mass (μg)		MMP excreted mass (μg)	
	Male pool	Female pool	Male pool	Female pool
0[a]	N.D.	N.D.	N.D.	N.D.
0[b]	N.D.	N.D.	N.D.	N.D.
1[a]	17 ± 1	N.D.	N.D.	N.D.
1[b]	970 ± 15	1040 ± 15	440 ± 11	160 ± 4
2[a]	N.D.	46 ± 2	N.D.	N.D.
2[b]	1380 ± 50	1000 ± 14	680 ± 16	500 ± 20
3[a]	N.D.	N.D.	N.D.	N.D.
3[b]	690 ± 19	240 ± 5	330 ± 10	140 ± 5
4[a]	N.D.	N.D.	N.D.	N.D.
4[b]	140 ± 8	61 ± 5	129 ± 3	122 ± 3
8[a]	N.D.	N.D.	N.D.	N.D.
8[b]	51 ± 5	116 ± 7	N.D.	46 ± 4

N.D.: non detectable.
[a] Without application of enzymatic hydrolysis.
[b] With application of enzymatic hydrolysis.

metabolites of EDP were identified. These *in vivo* data confirm the *in vitro* metabolism pathway for EDP in rat microsomes [19] in spite of phase II metabolites were only detected under *in vivo* conditions, probably due to the inherent differences between *in vitro* and *in vivo* experiments.

3.7. Application to urine samples

The proposed on-line SPE–LC–MS/MS method was used to determine EDP and its metabolites in urine from male and female volunteers following body application of 13 g of a laboratory-made sun cream containing 8% EDP. This dose falls in the usual range of thickness application for sunscreens (0.5–1 mg cream/cm^2 skin), which is usually below the recommended dose for reaching the desirable SPF value (2 mg/cm^2). After measurement of its total volume in order to determine the excreted amounts of analytes, the urine from each group of volunteers was pooled separately at 24 h intervals and stored at 4 °C in a refrigerator. Then, each urine sample from the male and female pools was split into two fractions and each fraction treated with either 1 M ammonium acetate (pH 5) or β-glucuronidase solution (see Section 2) in order to determine the contents of the analytes in their free forms or in both free and conjugate forms (total analyte contents), respectively (Table 3).

EDP was found in no urine sample; also, the free forms of its phase I metabolites, MMP and DMP, contributed very little to the respective total metabolite contents as they were present mainly as glucuronide conjugates. In fact, DMP was detected at very low concentrations in free form and only in the first sample from males, which corresponded to the pooled excretions obtained during the first 24 h after cream application, and in the second sample from females, which corresponded to the pooled urine obtained over the next 24 h. The highest concentrations of DMP and MMP in both males and females were found in the urine samples collected 48 h after application of the cream. Subsequently, the metabolite contents of the urine were likely to decrease gradually. However, both compounds were still detectable in urine samples collected 8 days after application of the cream.

Based on the overall contribution of DMP and MMP to percutaneous absorption and excretion of EDP, approximately 0.5% (males) and 0.4% (females) of all applied EDP was excreted in urinary, mainly as DMP and MMP glucuronide conjugates.

4. Conclusions

A fully automated on-line SPE–LC–MS/MS method for the determination of EDP and its phase I metabolites (*i.e.* DMP and MMP) in human urine was developed. The proposed hyphenated method requires minimal human intervention and is quite simple, sensitive and selective. Ultrasound-assisted enzymatic hydrolysis is used to deconjugate phase II glucuronide metabolites of EDP.

The analytical procedure was validated statistically with accurate results, using standard addition calibration for quantitative correction of matrix effects. The ensuing method was used to determine the target compounds in urine from male and female volunteers after application of an EDP-containing sunscreen cosmetic. Only DMP and MMP were found in urine (mainly as glucuronide conjugates, which were characterized by LC–TOF/MS analysis based on accurate mass measurements and LC–MS/MS confirmation with a QqQ analyzer).

The results of an *in vivo* percutaneous absorption tests after a single application of the sunscreen revealed that around 0.5% of all EDP applied was excreted in urine.

Acknowledgments

The authors acknowledge financial support of Spain's Ministry of Science and Innovation (MICINN) (Projects CTQ2009-07430 and CTQ2009-12709). Zacarías León and Feliciano Priego are also grateful to MICINN for award of an FPI pre-doctoral grant and a "Ramón y Cajal" contract, respectively. The authors wish to thank the volunteers Beatriz Álvarez, Verónica Sánchez de Medina and Jose María Mata for their cooperation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.03.045.

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