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Automated method for targeting analysis of prostanoids in human serum by on-line solid-phase extraction and liquid chromatography–mass spectrometry in selected reaction monitoring

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ABSTRACT

Prostanoids are potent biologically active lipid molecules demanding for analysis methods combining precision, sensitivity and high-throughput for pharmacological and clinical applications. The present research describes the development and validation of an on-line automated method based on solid-phase extraction liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS) for the quantification of prostanoids in human serum. This approach overcomes the main limitation of previous methods involving manual protocols, such as analyte losses, metabolites degradation and time-consuming protocols, are minimized. Human serum (100 μL) was directly injected into an automatic solid-phase extraction workstation for cleanup and preconcentration of the target metabolites. The eluate was on-line transferred to a reversed-phase analytical column for chromatographic separation prior to mass spectrometry detection in selected reaction monitoring mode. The detection limits for the target analytes ranged from 2.3 to 63.3 pg on column. The precision (expressed as relative standard deviation) was within 3.30 and 6.15% for repeatability and from 4.16 to 11.11% for within-laboratory reproducibility. Accuracy was evaluated with spiked and non-spiked serum samples to estimate concentration differences that could be affected by matrix effects or inefficient SPE performance. Accuracy, estimated as recovery factor, was from 87.7 to 100% for the target compounds. The proposed method is reliable and has an excellent potential for high-throughput use in both clinical and research laboratories by minimizing analyst intervention.

1. Introduction

Cyclooxygenase (COX) enzymes, also termed prostaglandin H synthases, catalyze the rate-limiting steps in the synthesis of prostaglandins (PGs) and thromboxane (TX) [1]. At present, there are three known COX isoenzymes, COX-1, COX-2, and COX-3, although COX-3 is considered a splice variant of COX-1 [2]. COX-1 is found in most mammalian cells, while COX-2 is practically undetectable in most normal tissues [3]. Although both enzymes possess a similar mechanism of action, selective inhibition can make a difference in terms of side-effects. A particular fact is that COX enzymes are the target of most non-steroideal anti-inflammatory drugs (NSAID) such as aspirin, ibuprofen and naproxen [4].

Depending on COX activity, different prostanoid metabolites derived from C-20 polyunsaturated fatty acids (PUFAs) can be synthesized, mainly dihomo-γ-linoleic (20:3n-6), arachidonic (20:4n-6) and eicosapentaenoic (20:5n-3) acids [5] (Supplementary data, Fig. 1). As a response to specific physiological and pathophysiological stimuli, PUFAs are released from cell membranes into the cytoplasm [6,7]. Subsequently, they are converted into PGs and TX by COX to generate three series of compounds depending on the original fatty acid. Thus, COX-1 will catalyze the synthesis of 1-series PGs from γ-linoleic acid (20:3n-6), COX-2 will be involved in the formation of TX and 2-series PGs from arachidonic acid (20:4n-6), and COX-3 will participate in the synthesis of 3-series PGs from eicosapentaenoic acid (20:5n-3) [8–10]. Therefore, the antagonic role of prostanoids in the pathogenesis of diseases could be understood by assessing the exact balance of PGs.

Thromboxane, produced in platelets, is a vasoconstrictor, potent hypertensive agent that facilitates the clumping of platelets. Prostaglandins are potent biologically active lipid molecules produced from omega-3 and omega-6 essential PUFAs in cellular tissues. Prostaglandins of the 2- and 3-series are of clinical inter-

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est because they are derived from two competitive pathways (n-3 and n-6 PUFAs), which could justify their opposite actions [11]. Thus, 2-series PGs produced from arachidonic acid possess pro-inflammatory, pro-aggregating, vasoconstriction action and immunosuppressive properties [12–14]. On the other hand, PGs of the 3-series have anti-inflammatory, anti-aggregating, vasodilatory and antiarythmic actions and immuno-modulating properties [15,16]. Lesser known 1-series PGs, synthesized from γ-linoleic acid, are characterized by their beneficial properties based on an improved circulation, lowered blood pressure, anti-inflammatory effect, regulation of calcium metabolism and arachidonic acid metabolism, which is the precursor of undesired 2-series PGs [17].

It is difficult to describe the involvement of prostanoids in a specific pathology due to the complexity pathway ascribed to COXs. Prostanoids take part in many physiological and pathophysiological processes in practically every organ, tissue and cell, including the vascular, renal, gastrointestinal and reproductive systems. For this purpose, sensitive and selective assays to measure the levels of prostanoids in complex matrices such as biological fluids are demanded. Immunological assays (radioimmunoassay or enzyme immunoassay) are the most widely used methods for qualitative/quantitative analysis of prostanoids. The main drawbacks of these assays are their lack of specificity for complex biological fluids such as plasma and urine [18], the trend to overestimate the levels of metabolites due to cross-reactivity, variability in the quantification of sequential samples, limitation to the detection of a single product at one time [19,20] and, in most cases, a commercial antibody is not available, particularly for new or unstable metabolites [21].

Gas chromatography–mass spectrometry (GC–MS) provides suitable sensitivity and selectivity for prostanoids analysis, but this hyphenated technique requires time-consuming sample preparation protocols including derivatization steps [22]. To overcome the limitations of GC–MS and immunoassays, LC–MS/MS [23,24] evolves as a powerful tool for qualitative/quantitative analysis of prostanoids in biological samples because of the high sensitivity and excellent selectivity. Nevertheless, the main limitation of previously reported methods is the use of manual protocols for preparation of biological samples. Generally, these protocols start with a liquid–liquid extraction step, preconcentration by off-line SPE or simple evaporation and reconstitution prior to chromatographic analysis. These off-line configurations usually involve analyte losses, degradation of intermediate metabolites and time-consuming protocols. According to the pharmacological and clinical interest of prostanoids analysis, an automated method based on SPE coupled on-line with LC–MS/MS is here proposed to improve the precision and sensitivity in combination with high-throughput for application in systematic epidemiological studies, routine analyses and, in general, when determination of these metabolites in a high number of samples is required.

2. Experimental

2.1. Chemicals

Deionized water (18 mΩ cm) from a Millipore (Billerica, MA 01821 USA) Milli-Q water purification system was used to prepare all aqueous solutions. 9-Oxo-11α,15S-dihydroxy-prost-13E-en-1-oic acid (PGE_1), 11-oxo-9α,15S-dihydroxy-prosta-5Z, 13E-dien-1-oic acid (PGD_2), 9-oxo-11α,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid (PGE_2), 9α,11α,15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid ($PGF_{2\alpha}$), 9α,11α-dihydroxy-15-oxo-prosta-5Z,13E-dien-1-oic acid (15-keto $PGF_{2\alpha}$), 9-oxo-11α,15S-dihydroxy-prosta-5Z,13E,17Z-trien-1-oic acid (PGE_3) and 9α,11,15S-trihydroxythromba-5Z,13E-dien-1-oic acid (TXB_2) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Chromatographic grade methanol, ethanol, acetonitrile and formic acid from Scharlab (Barcelona, Spain) were used for the development of the analytical method.

2.2. Serum extraction from human individuals

Venous blood was collected into a plastic Vacutainer® tube from Becton Dickinson (Franklin Lakes, NJ, USA) without additives (red top). The tube was not opened to ambient air and kept refrigerated until processing. Blood samples were processed within 1 h after collection and centrifuged at 4000 × *g* for 10 min to separate serum, which was placed in plastic tubes and stored at −80 °C until analysis.

2.3. Standard solutions and spiked serum samples

Stock standard solutions (500 μg/mL) of all compounds were prepared in ethanol and stored in amber vials at −80 °C under nitrogen atmosphere. Working solutions were prepared by dilution of the appropriate volume of stock ethanolic solution in serum pool aliquots to obtain spiked solutions. These spiked solutions were used for optimization of the sample preparation and chromatographic steps by coupling SPE and LC separation in an automated manner.

2.4. SPE–LC–MS/MS configuration

A Midas autosampler (AS in Supplementary data, Fig. 2) furnished with a 100-μL sample-loop and Prospekt 2 SPE workstation (Spark Holland, Emmen, The Netherlands) consisted of an automatic cartridge exchanger (ACE) and a high-pressure syringe dispenser (HPD) was used for full automation of the extraction step controlled by Sparklink version 2.10 software. The Prospekt 2 system was on-line connected to an Agilent (Palo Alto, CA, USA) 1200 Series LC system, which consists of a binary pump, a thermostated column compartment and a vacuum degasser. Both the Prospekt 2 and LC systems were configured for complete automation of analysis sequences. The chromatographic eluate was directly introduced in an Agilent 6410 triple quadrupole detector (QqQ) furnished with an electrospray ion (ESI) source in negative mode. Agilent MassHunter Workstation was the software for data acquisition, qualitative and quantitative analysis. Hysphere C8 (EC) (end-capped silica-based octadecyl phase) cartridges (8 μm, 10 mm × 2.0 mm, Spark Holland) for SPE and Mediterranea Sea C18 analytical column (3 μm, 150 mm × 4.6 mm, Teknokroma, Barcelona, Spain) for chromatographic separation were used. The column compartment was thermostated at 25 °C. Other Hysphere cartridges (10 mm × 2.0 mm) were assayed in the optimization of the SPE step to evaluate their retention capability. The tested phases were CN (silica-based cyanopropyl phase), C8 EC (end-capped silica-based octyl phase), C18 (EC) (end-capped silica-based octadecyl phase), C18 HD (spherical shape, end-capped, silica-based phase with a high loading of octadecyl chains) Resin GP (polymeric polydivinylbenzene phase) and Resin SH (strong-hydrophobic modified polystyrene-divinylbenzene).

2.5. Analytical protocol for determination of target analytes

A serum sample was transferred to an amber injection vial placed in the autosampler tray, thus being ready for analysis. 100 μL of the liquid in the vial was injected into the system following an automated sequence of operations based on SPE–LC–MS/MS analysis. Basically, the sample preparation process started by activating the stationary phase by solvation with methanol (1 mL), conditioning and equilibration with water (2 mL) and then, sample loading into the cartridge with water (1 mL). Under these conditions, the

Table 1
Optimization of the MS/MS step for qualitative and quantitative determination of eicosanoids.

Analyte	Precursor ion (*m*/*z*)	Voltage MS1 (eV)	Product ions (*m*/*z*)	Collision energy (eV)	Quantitation transition
TXB_2	369.3	80	169.3, 195.3	10	369.3 → 169.3
PGE_3	349.3	90	269.3, 313.3	10	349.3 → 269.3
$PGF_{2\alpha}$	353.3	90	193.3, 309.3	25	353.3 → 193.3
PGE_2	351.3	70	315.3, 271.3	10	351.3 → 271.3
15-Keto $PGF_{2\alpha}$	351.3	80	315.3, 219.3	10	351.3 → 219.3
PGE_1	353.3	70	317.3, 235.3	10	353.3 → 235.3
PGD_2	351.3	90	315.3, 233.3	5	351.3 → 233.3

target compounds were retained in the sorbent that was washed with 10% methanol aqueous solution (1 mL) to remove protein interferences. The chromatographic step started by switching the left clamp valve and putting the content of the cartridge into contact with the isocratic mobile phase 66:34 (v/v) water–acetonitrile containing 0.02% formic acid pumped by the chromatographic pump at 1.0 mL/min. The last step was carried out by purging the tubes of the SPE workstation with methanol and deionized water.

2.6. Mass spectrometry operating conditions

Mass spectrometry detection was performed by an Agilent 6410 triple-quadrupole tandem-mass spectrometer in negative ESI mode. The electrospray capillary voltage and the source temperature were set at 2.7 kV and 315 °C, respectively. The pressure nebulizer was 45 psi with nitrogen as desolvation gas flowing at 10 mL/min. The selected voltage for efficient filtration of precursor ions in the first quadrupole, collision energy to fragment the precursor ions by collision induced dissociation (CID) to generate the product ions selected as quantification and qualifier ions in the selected reaction monitoring (SRM) method for each analyte are shown in Table 1. A dwell time of 200 ms was applied to monitor all SRM transitions by virtue of the chromatographic separation of the target compounds. Both filter quadrupoles were adjusted at 0.7 mass units as full width at half maximum (FWHM) resolution. The data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis. The entire analytical process is completed within 25 min.

2.7. SRM-based quantitation of target analytes

Standard solutions were run by LC–MS/MS to build the corresponding calibration curve for each compound using the peak area as a function of the standard concentration of each compound. The concentrations of target analytes at low and high level were 0.075 and 1000 ng/mL, respectively. Ten calibration levels were prepared with the following dilution sequence 1:2:2:2:2:2:2:2:2:2:2 with triplicate injection of three of them to set confidence levels. This calibration model was selected because of the correction of matrix effects occurring during sample preparation as it will be exposed.

3. Results and discussion

3.1. Optimization of LC–MS/MS analysis

Mass spectrometry optimization was initiated with a design aimed at finding the best ionization conditions for the target prostanoids. Ionization operating conditions were studied by direct injection of individual standard solutions using positive and negative ESI modes with different ionization agents and three levels of capillary voltage in the range 2600–3400 V. Negative ESI mode clearly showed a more efficient ionization by generation of $[M-H]^-$ precursor ions providing the best sensitivity for all prostanoids. The electrospray variables temperature, pressure and nebulizer voltage were set with a multivariate response surface design in the ranges 185–320 °C, 10–32.5 psi and 2600–4000 V, respectively. Different ionization agents such as acetic acid and formic acid, and two volatile salts as ammonium acetate and formate were tested at different concentrations in the concentration range 0.01–1% (w/v) and pH between 6.2 and 6.7. Fig. 1 compares the different ionization agents at their optimum concentrations by monitoring the precursor ion of PGE_3 *m*/*z* 349.2. As can be seen, formic acid led to the highest ionization efficiency and, therefore, to the best sensitivity by setting at the concentration 0.02% (v/v). This behaviour was shared by the rest of the analytes.

This study was followed by chromatographic tests under isocratic mode using acetonitrile–water mixtures at different proportions. Resolute separation with minimum chromatographic time was achieved by using a 34:66 (v/v) acetonitrile–water solution. With an isocratic protocol, time is not required for equilibration of the chromatographic column, thus increasing sample throughput.

Tandem mass spectrometry parameters were optimized for efficient isolation of the precursor ions and their sensitive and selective fragmentations. The collision energy was varied from 5 to 60 eV after isolation of the target precursor ion for each analyte. The optimum collision energy for most analytes was 10 eV, except for PGD_2 and $PGF_{2\alpha}$ which were better fragmented at 5 and 25 eV, respectively. A unique transition was selected for each PG with quantification purposes due to secondary transitions were extremely low-sensitive. Table 1 shows the target precursor and product ions selected for each prostanoid and optimum values found for filter voltage of the first quadrupole and collision energy. This study was completed by optimizing the influence of the dwell time in the range 50–250 ms, setting 200 ms for all transitions.

3.2. Optimization of SPE as sample preparation approach

Optimization of on-line sample-preparation was aimed at obtaining maximum extraction efficiency and sample cleanup. The SPE workstation enabled to prepare automated sequences of analysis to test the influence of SPE sorbent, sample volume, loading solution, type of washing solvent and elution time by a univariate approach due to their discontinuous character. Additionally, loading and washing volume and flow rate variables were set by a

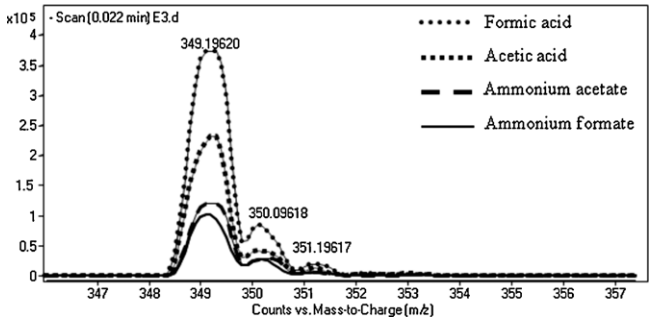

Fig. 1. Effect of ionization agents (acetic acid and formic acid) and two volatile salts (ammonium acetate and formate) on ESI.

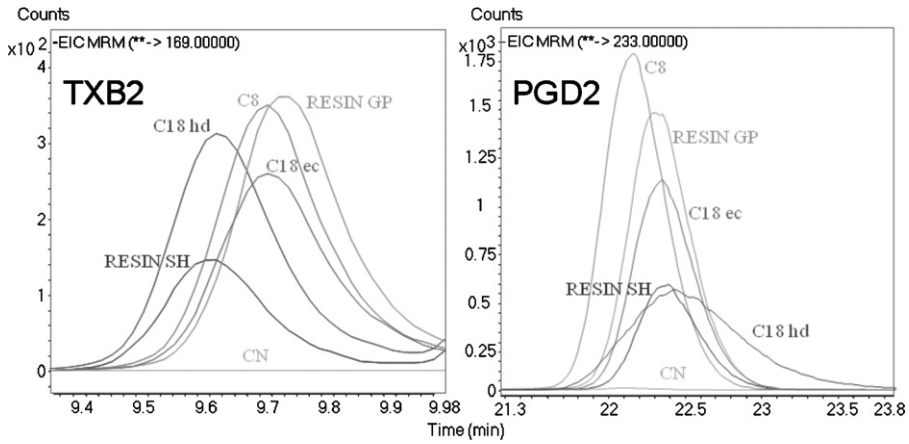

Fig. 2. Comparison of SPE sorbents with different retention capabilities: CN (silica-based cyanopropyl phase), C8 (EC) (end-capped silica-based octyl phase), C18 (EC) (end-capped silica-based octadecyl phase), C18 (HD) (high density), Resin GP (polymeric polydivinylbenzene phase) and Resin SH (strong-hydrophobic modified polystyrene-divinylbenzene).

multivariate response surface design consisting of 16 experiments and 2 central points. A serum pool, either spiked with 500 ng/mL of each analyte or as blank, and standard solutions were used for these tests that revealed the following results.

3.2.1. SPE sorbent

Six types of SPE sorbents with different retention capabilities: CN (silica-based cyanopropyl phase), C8 (EC) (end-capped silica-based octyl phase), C18 (EC) (end-capped silica-based octadecyl phase), C18 (HD) (high-density), Resin GP (polymeric polydivinylbenzene phase) and Resin SH (strong-hydrophobic modified polystyrene-divinylbenzene). With this panel, the range of retention mechanisms from medium to low-polarity, including mixed mechanisms associated to resin phases, is covered. Fig. 2 shows SRM chromatograms of two representative analytes, TXB_2 and PGD_2, eluted at the beginning and end, respectively, of the chromatographic profile, obtained from a spiked pool serum. The parameter used to select the optimum sorbent was the pair peak area–peak height to obtain optimum sensitivity with minimum peak dispersion. Each chromatographic analysis was preceded by SPE preparation for the different sorbents tested. This assay enabled to evaluate the retention capability of the binomial prostanoid–sorbent material. However, it is well-known that the efficiency of an SPE test should be a compromise between retention capability and elution with the chromatographic mobile phase. As can be seen, the general sequence for the target compounds was as follows: C8 (EC) > Resin GP > C18 (EC) > C18 (HD) > Resin SH > CN. As expected, a medium polarity sorbent such as CN phase provided a low retention capability due to non-polar character of the target prostaglandins. The rest of the sorbents are suited for retention of non-polar metabolites, but they had a different behaviour. As can be seen, the sequence follows an order from less hydrophobic materials such as C8 (EC) or Resin GP with mixed mechanisms based on polydivinyl–benzene interactions, to highly-hydrophobic materials such as Resin SH or C18 (HD), which strongly retain non-polar metabolites by π–π and non-polar interactions, respectively. Therefore, it should be expected a similar retention for these materials but the critical variable should be the elution from the sorbent. This theoretical fact would explain the better behaviour of C8 (EC) than the rest of sorbents.

3.2.2. Loading solution

Methanol–water and acetonitrile–water mixtures in different ratios were used as loading solutions. Organic solvent concentration in the loading solution must be below 20% (v/v) to minimize protein precipitation in the SPE-workstation tubes. Retention tests showed that pure water provided the best extraction efficiency. The initial loading volume should be at least twice the sum of the loop, transport tubing and cartridge volume, and, it was studied in the range 1–4 mL. There was no influence of this parameter on prostanoids retention, so 1 mL was selected to load the serum sample. The loading flow rate was varied between 0.5 and 4 mL/min to evaluate how the retention capability was affected. The highest flow rate without affecting retention, 1.2 mL/min, was selected to load the sample in the SPE workstation.

3.2.3. Washing solution

Different aqueous solutions with methanol or acetonitrile were tested as washing solution to remove salts, clean proteins and other interferents with polarity higher than that of the target analytes present in human serum. The volume of washing solution and its organic concentration were studied in the range 1–4 mL and from 0 to 30% methanol and acetonitrile, respectively. Fig. 3 shows that a single washing step using an aqueous solution containing 10% methanol was optimum to clean the extract. This behaviour was assessed by evaluation of ionization suppression in the ESI after analysis of blank serum (non-spiked serum), spiked serum and a multistandard solution at the same concentration. Ionization efficiencies above 96% were obtained for all prostaglandins by SRM experiments. A volume of 1 mL and flow rate of 1.8 mL/min of the washing solvent provided the best results.

3.2.4. Elution time

The last step of the SPE optimization was aimed at assaying the chromatographic mobile phase as elution solution to transfer the target analytes from the cartridge, which starts by switching the clamp valve. Therefore, the elution time was the only parameter studied in this step, of particular interest to protect the chromatographic column from undesirable interferents. The time required for elution of prostanoids from the cartridge sorbent was 2 min, after which the left clamp valve was switched to the loading position to continue with the chromatographic process. In this way, the elution of non-polar compounds retained in the cartridge is avoided. The entire analytical process is completed in 25 min, which enables to synchronize the SPE protocol with the chromatographic step.

3.2.5. Re-usability of the SPE cartridges

One of the main advantages of SPE cartridges is their capability to be re-used without efficiency losses. Tests carried out with

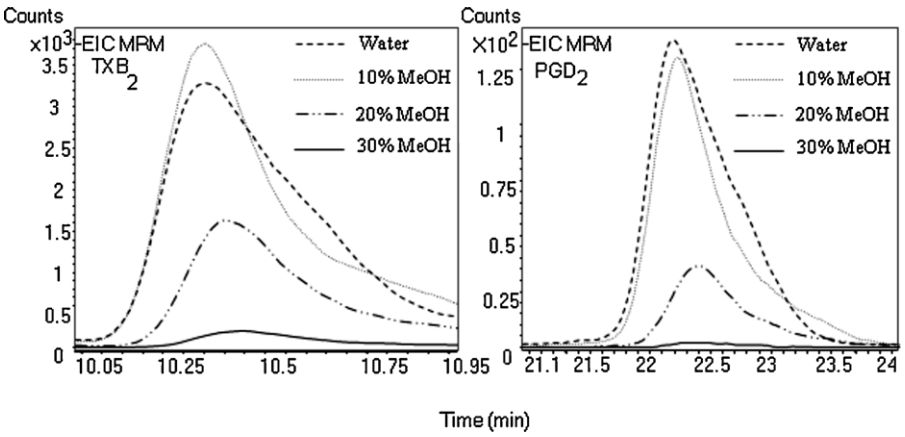

Fig. 3. Influence of the composition of aqueous solutions on the SPE washing step.

Table 2
Repeatability (Sr), within-laboratory reproducibility (Swr) and recovery for each analyte calculated with a two-cartridge configuration (1), and single-cartridge configuration (2).

Analyte	Repeatability	Reproducibility	Recovery[a]	Recovery[b]
TBX_2	4.04	8.25	92.27	90
PGE_3	5.18	11.11	87.74	89.72
$PGF_{2\alpha}$	4.36	9.77	95.51	100
PGE_2	3.35	4.16	97.84	100
15-Keto $PGF_{2\alpha}$	5.08	5.73	99.81	99.73
PGE_1	3.30	4.99	90.13	100
PGD_2	6.15	11.01	96.52	93.48

[a] Expressed as percent of material retained in cartridge 1/[material retained in cartridge 1 + material retained in cartridge 2].
[b] Expressed as percent of [final concentration – initial concentration]/added concentration.

spiked serum samples ensured the re-usability of SPE cartridges for four analyses; thus allowing a drastic reduction of the analysis costs taking into account that direct serum analysis is carried out without protein precipitation.

3.3. Validation of the method

The accuracy of the method and potential matrix effects were studied by analysis of non-spiked and spiked human serum samples by setting each variable at their optimum values. First, a two-cartridge configuration was employed to estimate the recovery factor (defined as the proportion of analytes retained in the first cartridge) where non-spiked human serum samples were used. Two cartridges were put in serial; hence the amount of analyte not retained in the first cartridge could be retained in the second. Then, the compounds retained in both cartridges were sequentially eluted to the chromatographic column for independent analysis. Recovery was calculated as amount in cartridge 1/[amount in cartridge 1 + amount in cartridge 2], where the first and second cartridges were Hysphere C8 EC. Recovery factors for all prostanoids were above 87.7%, which ensures quantitative retention in the cartridge and, therefore, validates internally the analytical method for determination of prostanoids.

A pool of spiked serum with high multistandard concentration (1 μg/mL, the highest level assayed in calibration) was analyzed with the single configuration. This high concentration was used to ensure that the SPE cartridges were not saturated. In this case, the recovery was calculated as [final concentration – original concentration]/spiked concentration, evaluated by analysis of five replicates in the same day. In this case, recoveries were above 89.7% for all prostanoids, which proves the absence of ionization suppression effects with excellent precision. The results thus obtained are listed in Table 2.

Since the results of these two internal validation tests showed a low or null influence of matrix composition, calibration curves were run by direct injection of standard solutions in the LC–MS system, using the peak area as a function of the standard concentration of each compound. The limit of detection (LOD) and that of quantification (LOQ) for each analyte were calculated as the concentration that provided a signal three and ten times, respectively, higher than the noise background signal. The LODs ranged between 2.3 and 63.3 pg, while LOQs were from 7.5 to 211 pg on-column. Table 3 shows the characteristics of the method.

Within-laboratory reproducibility and repeatability were evaluated in a single experimental set-up with duplicates by experiments carried out with a serum pool. Two measurements per

Table 3
Analytical features of the method.

Analyte	Calibration equation	Intercept error	Slope error	Linear range (ng/mL)	Coefficient of regression (R^2)	LODs on column (pg)	LOQs on column (pg)
TXB_2	$y = -24640.3 + 1942.9C$	1054.6	27.3	LOQ – 1000	0.9984	2.3	7.5
PGE_3	$y = -7659.7 + 1035.3C$	5058.2	24.6	LOQ – 750	0.9960	4	14
$PGF_{2\alpha}$	$y = -494.5 + 410.2C$	110.7	5.1	LOQ – 750	0.9984	14	46
PGE_2	$y = -23754.7 + 1802.9C$	1551.7	27.9	LOQ – 1000	0.9969	41	138
15-Keto $PGF_{2\alpha}$	$y = -13370 + 1179.3C$	9222.5	22.6	LOQ – 1000	0.9974	63	211
PGE_1	$y = -21179.7 + 2709C$	1197.6	38.5	LOQ – 750	0.9967	27	92
PGD_2	$y = -12290 + 1321.2C$	7448.3	18.2	LOQ – 1000	0.9987	56	188

day were carried out on 7 days. The results obtained are listed in Table 2. The repeatability, expressed as relative standard deviation (RSD), ranged between 3.30 and 6.15%, and the within laboratory reproducibility, also expressed as RSD, from 4.16 to 11.11% for all target analytes.

3.4. Application of the method

The method was applied to the analysis of the prostanoids profile in human plasma from twenty-six obese individuals with a body mass index between 30 and 40 kg/m^2. All subjects were non-diabetics, non-smokers and showed no evidence of kidney, pancreas, lung, liver or thyroid diseases. The target cohort was composed by 17 post-menopausal women with age from 48 to 70 years and 9 men with age from 39 to 70 years. None of the subjects was taking drugs or supplementary foods with influential effect on serum levels of the target metabolites. Four blood samples were extracted to each individual with intervals between samples of fourteen days. Figs. 4 and 5 show two typical LC–MS/MS chromatograms and selected transitions corresponding to one of the serum samples of the target cohort and a spiked serum sample. Mean, minimum and maximum values and standard deviation of the concentration of each target analyte in the serum samples of the individuals of this cohort are shown in Table 4. Concentration values of target analytes found in other reported studies are also included in Table 4 [25–27]. It is worth emphasizing that the cohorts selected for previously reported studies were composed by healthy individuals. This could justify the differences in serum levels obtained in the cohort selected in this study composed by obese individuals with ages between 48 and 70 years for males and between 39 and 70 for females. Additionally, PGE_1 was not detected in the selected cohort as compared to the study reported by Cawello et al. [27] for human individuals after PGE_1 administration by intravenous infusion.

Table 4
Profile of the target analytes in the cohort under study (expressed as mean ± standard deviation, ng/mL).

Analyte	Values found		Values of reference
	Woman	Man	
TXB_2	0.59 ± 0.47	0.31 ± 0.19	0.05 ± 0.13 [26]
PGE_3	7.75 ± 2.65	10.01 ± 2.08	[a]
$PGF_{2\alpha}$	10.30 ± 1.84	10.79 ± 1.70	0.05 ± 0.13 [26]
PGE_2	0.17 ± 0.11	0.11 ± 0.07	0.02 ± 0.35 [27]
15-Keto $PGF_{2\alpha}$	N.D.[b]	N.D.[b]	0.07 ± 0.04 [26]
PGE_1	N.D.[b]	N.D.[b]	0.001 [27]
PGD_2	N.Q.[c]	N.Q.[c]	0.11 ± 0.12 [26]

[a] Not found.
[b] Non detected.
[c] Non quantifiable.

The concentration of target analytes *versus* different variables such as sex, weight, body mass index, height and waist perimeter was studied. The concentration data of each prostanoids were normalized by transformation to base-N logarithm. The influence of anthropometric variables on the prostanoids profile was studied by multivariate analysis of variance (MANOVA). Pearson correlation analysis showed highly significant relationship between PGE_2 and individual weight ($R^2 = -0.85$) at 99.5% of confidence level (p-value 0.05). Other relationships between the concentration of target analytes and anthropometric variables were also found. Thus, a strong correlation (level of significance $p < 0.002$) between weight and other two prostanoids such as $PGF_{2\alpha}$ and TXB_2 ($R = -0.78$ and $R = -0.77$ respectively) was observed as well as between individual sex and TXB_2 ($R = 0.79$). Other relationships were found for five pairs of variables such as body mass index and $PGF_{2\alpha}$ ($R = -0.69$), waist perimeter and $PGF_{2\alpha}$ ($R = -0.68$), waist perimeter and PGE_2 ($R = -0.71$), weight and HETE12 ($R = -0.67$), and age and $PGF_{2\alpha}$ ($R = -0.73$) at 95.0% of confidence level.

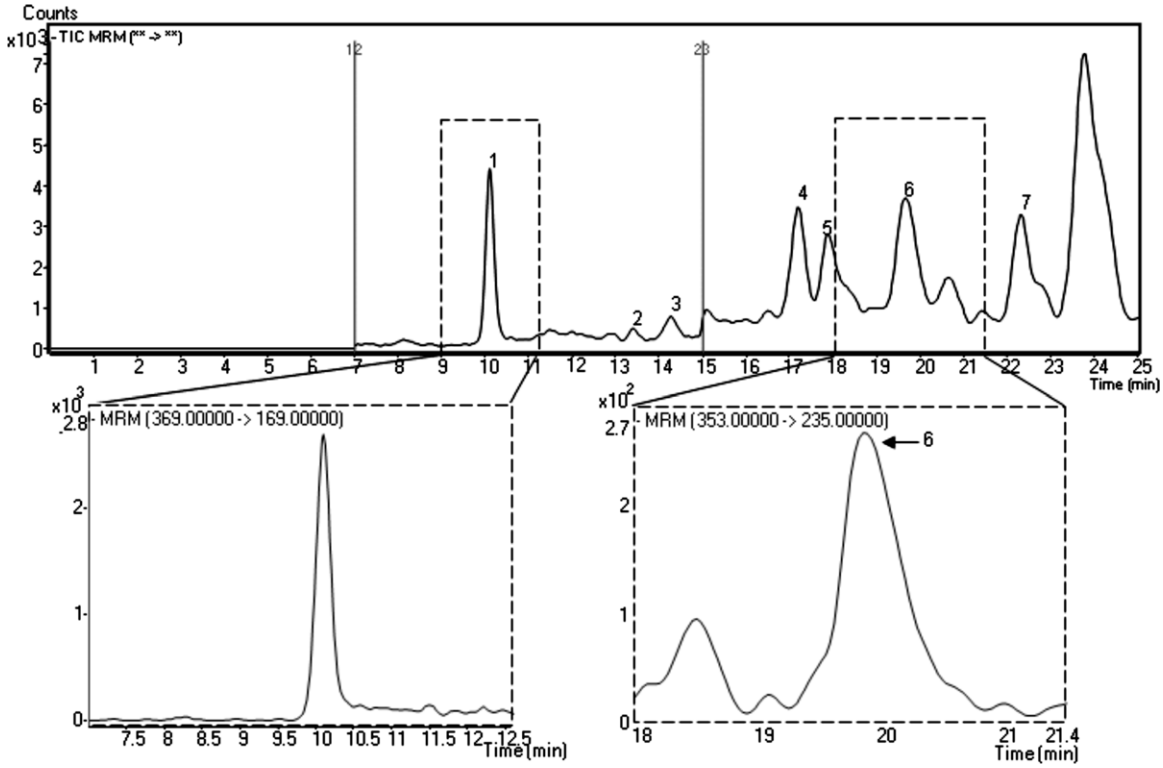

Fig. 4. Total ion chromatogram (TIC) of a serum sample. Analytes: (1) TXB_2; (2) PGE_3; (3) $PGF_{2\alpha}$; (4) PGE_2; (5) 15-keto $PGF_{2\alpha}$; (6) PGE_1; (7) PGD_2. SRM chromatograms for two metabolites are zoomed below.

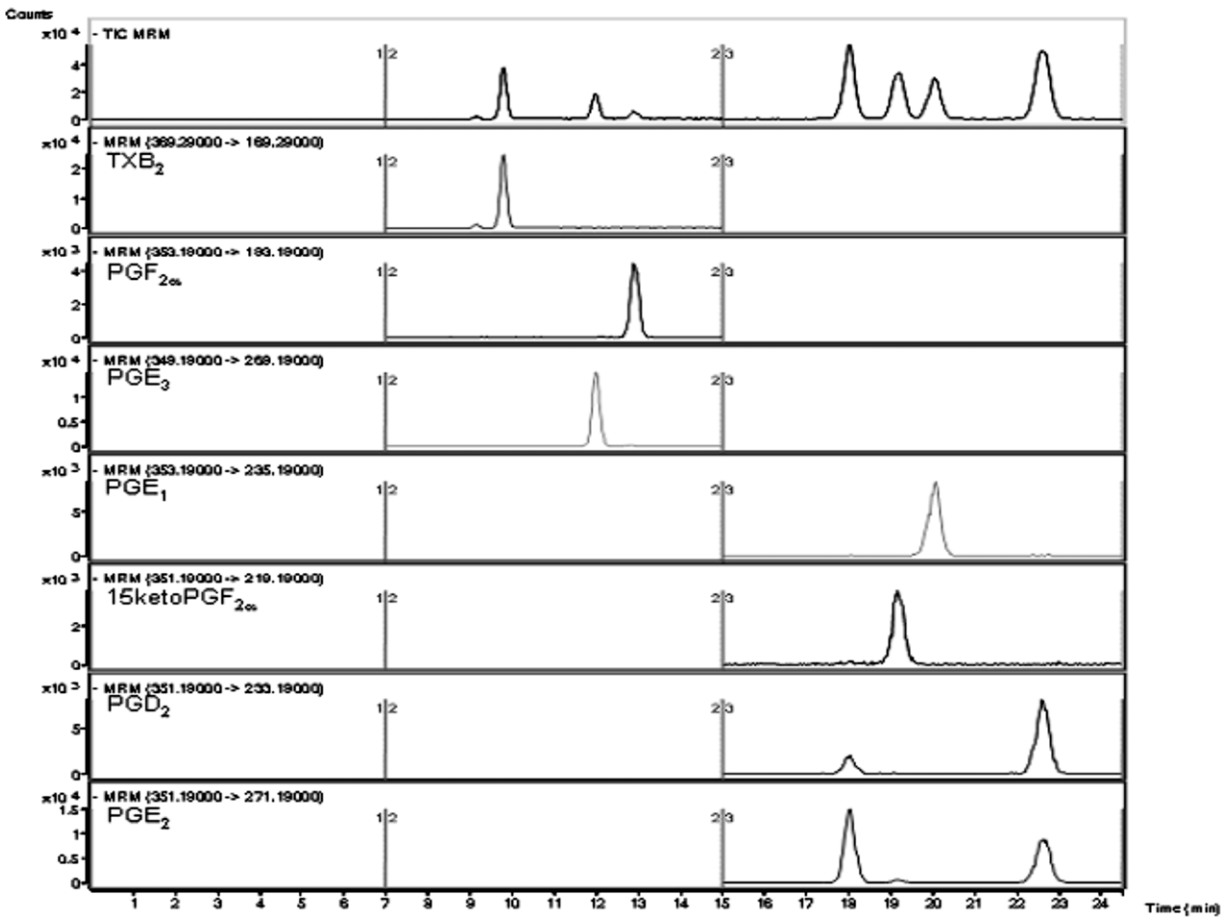

Fig. 5. TIC profile and selected quantitation transition.

4. Conclusions

The proposed method was designed to work unattended in a safe and fast manner to process serum samples for the determination of metabolites of the COX pathway. This aim was achieved by:

(1) Automation of sample preparation using on-line SPE with automatic valve switching and cartridge exchange, which proved an excellent approach for unattended preparation of human serum samples. Minimizing human intervention makes the step easy to apply and improves reproducibility and accuracy. Furthermore, a sample can be automatically prepared while running the chromatographic step of the previous one, so that continuous analysis can be done, providing a high-throughput assessment of analytes in human serum.
(2) The total and fast chromatographic separation of the eicosanoids under study between themselves and from interfering substances in serum samples.
(3) The avoidance of using an internal standard by SPE validation using tests with dual cartridge configurations to check quantitative retention of target analytes. In this way, sample spiking with internal standard – which notably increased the cost of the analysis – is not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.03.049.

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