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Derivatization of carboxylic acids with 4-APEBA for detection by positive-ion LC-ESI–MS(/MS) applied for the analysis of prostanoids and NSAID in urine☆

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ABSTRACT

In order to develop a generic positive ionization ESI LC–MS method for a variety of interesting substance classes, a new derivatization strategy for carboxylic acids was developed. The carboxylic acid group is labeled with the bromine containing 4-APEBA reagent based on carbodiimide chemistry. The derivatization reaction can be carried out under aqueous conditions, thereby greatly simplifying sample preparation. In this paper, the derivatization of carboxylic acids is exemplified for the determination of prostanoids and non-steroidal anti-inflammatory drugs (NSAID). Optimization of the derivatization conditions was studied. In order to prove the applicability of the presented approach, we applied the described protocol to urine samples from complex regional pain syndrome (CRPS) patients and were able to detect several prostanoids not visible in the urine of healthy volunteers. Further, the determination of the non-steroidal anti-inflammatory drug ibuprofen in a urine sample was possible.

1. Introduction

Chemical derivatization is routinely employed to improve the chromatographic and/or mass spectrometric characteristics of an analyte [1–5]. In gas chromatography (GC), derivatization is often required to achieve the necessary volatility. Specific derivatization of analytes can simplify the separation from interferences in the sample during liquid chromatography (LC), for example by reversed-phase separation or ion-exchange methods after the introduction of a non-polar group or a charged function, respectively. A permanent charge in a molecule, such as the positive charge of a quaternary ammonium moiety, increases the ionization efficiency and thus the sensitivity in electrospray ionization (ESI) mass spectrometry (MS) [6,7]. Furthermore, by modification with a defined structural element, derivatization often enables the prediction of specific fragmentation reactions in tandem MS (MS/MS), which enhances the specificity of the method.

Carboxylic acids form a large group of biologically significant molecules [8], including fatty acids from lipid metabolism, bile acids and prostaglandins. Oxidation products from unsaturated fatty acids originating from oxidative stress are also of considerable importance [9,10]. Of this group of compounds, isoprostanes have received particular attention since their discovery by Morrow et al. [11], and have been widely acknowledged as representative biomarkers of oxidative stress [10,12]. Prostaglandins are produced enzymatically, and most important, stereospecifically by the body. Isoprostanes are prostaglandin-like compounds that originate from the attack of reactive oxygen species to arachidonic acid in a non-stereoselective process. Up to 32 isomers are possible for each prostaglandin.

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used drugs against inflammations and for pain relief. Prominent among these are the propionic acid derivatives ibuprofen (IBF), ketoprofen (KPF) and naproxen (NPX). They are thus part of an important group of non-endogenous compounds which also share the carboxylic acid function.

The analysis of free carboxylic acids by ESI-MS is subject to a number of challenges. While positive ionization is the method of choice for the large majority of compounds in biomolecular analysis, free carboxylic acids need to be detected in negative ionization mode, and ionization efficiencies are generally rather low [4,7]. It is therefore difficult, if not impossible, to achieve simultaneous detection of carboxylic acids together with a large number of different analytes, such as within a whole set of biomarkers.

Various derivatization reagents, including diethyl-aminoethyl chloride or bromide [13], 2-nitrophenylhydrazine [1], pyridinium compounds [2], benzofurazan reagents [3,5] and tris(trimethoxyphenyl)phosphonium compounds [4] have been used to solve this problem and make carboxylic acids compatible with positive ionization ESI-MS.

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The analysis of prostaglandin-like compounds in biological samples has previously been achieved by either GC–MS [14,15], LC-ESI(−)–MS/MS [14–18], or immunoaffinity methods [19]. NSAIDs have been previously analyzed in plasma or urine by GC–MS/MS [20], LC-DAD [21] or LC–MS(/MS) [22,23].

A common disadvantage of these existing methods is an often elaborate sample pretreatment with various consecutive purification, derivatization and enrichment steps [14]. Further, the need for incubation at high temperatures over a longer period of time can lead to problems with unstable analytes in biological samples.

The coupling of a carboxylic acid function with a primary amine mediated by a carbodiimide like 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is well known and often employed in organic synthesis, peptide synthesis, polymerization, protein immobilization and also derivatization [1,5,24]. In most of these applications, organic solvents like dichloromethane are used, which are not always compatible with biological samples. However, carbodiimide mediated amide formation has also been considered under aqueous conditions, although an amount of EDC is inherently deactivated under such conditions [25].

Recently, the design and development of a novel derivatization reagent called 4-amino-phenoxycholine (4-APC) has been described by Eggink et al. [26] for the selective derivatization of aldehydes. Next, a further development towards a bromine-labeled 4-APC-analog called (2-(4-aminophenoxy)ethyl)(4-bromophenethyl)-dimethylammonium bromide hydrobromide (4-APEBA) was reported [27].

In the present publication, the development and optimization of a protocol for EDC mediated amide coupling of a carboxylic acid with the amine group of 4-APEBA is demonstrated with a set of prostanoids and NSAIDs. The derivatization is performed under mild conditions and can be carried out in aqueous solution in a single incubation step. The influence of the concentrations of derivatizing agent and EDC, different temperature and different pH values, as well as the effects of the co-reagents *N*-hydroxysuccinimide (NHS) and 4-pyrrolidinopyridine (PPY) on the reaction speed and total yield were investigated.

The potential of the developed method is demonstrated by the analysis of prostanoids in human urine samples from healthy volunteers as well as from patients suffering from complex regional pain syndrome (CRPS), a chronic disease marked by serious inflammation [28]. Additionally, the analysis of NSAIDs in human urine is demonstrated, proving the broad applicability of the described protocol. Sample pretreatment comprises C_{18} solid-phase extraction of the prostaglandins from urine, subsequent derivatization of analytes by 4-APEBA under aqueous conditions and finally LC–MS/MS determination.

2. Materials and methods

2.1. Reagents

Hexanoic acid, heptanoic acid and benzoic acid were purchased from Merck (Darmstadt, Germany). Octanoic acid, nonanoic acid, hippuric acid, hydrochloric acid, prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$, ibuprofen, ketoprofen, naproxen, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysulfosuccinimide (NHS), 4-pyrrolidinopyridine (PPY), TRIS, NaH_2PO_4, Na_2HPO_4 and dichloromethane (DCM) were purchased from Sigma–Aldrich (Schnelldorf, Germany). 3,4-Dimethoxycinnamic acid (DMCA) was purchased from Acros Organics (Geel, Belgium). 8-Isoprostane $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) and deuterated prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$-d_4), which was used as internal standard, were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). The isotopic purity of the internal standard was checked and no non-deuterated impurities were detected.

Methanol (MeOH) and formic acid (FA) were purchased from Biosolve (Valkenswaard, The Netherlands). All chemicals were of analytical grade purity.

Synthesis of 4-(2-((4-bromophenethyl)-dimethylammonio)-ethoxy)-benzenaminium dibromide (4-APEBA) was performed as recently described by Eggink et al. [27].

Urine samples were obtained from complex regional pain syndrome (CRPS) patients via the VU Medical Center (Amsterdam, The Netherlands), as well as from 5 healthy volunteers. Samples were aliquoted after sampling and stored at −20 °C. Before analysis, they were thawed at room temperature, centrifuged at 16.100 × *g* in a bench top centrifuge to remove any precipitations; the supernatant was used without further clean-up.

IST Isolute C_{18} solid-phase extraction cartridges (sorbent mass 100 mg, reservoir volume 1 mL) were purchased from Biotage (Uppsala, Sweden).

2.2. Equipment

2.2.1. HPLC system settings

All samples were analyzed using an Agilent Model 1100 HPLC system (Agilent Technologies, Waldbronn, Germany), consisting of an online degasser, a binary pump and an autosampler, controlled by Chemstation Rev B.01.09 software, with a Phenomenex Luna C18(2) column (100 × 2.1 mm, particle diameter 3 μm) including a Phenomenex C_{18} SecurityGuard precolumn (4 × 2 mm, particle diameter 5 μm; Phenomenex, Utrecht, The Netherlands). The injection volume was 10 μL for optimization experiments and 25 μL for the analysis of standards and urine samples. The interior of the autosampler was thermostated to 20 °C. The chromatographic column was thermostated to 30 °C using a Shimadzu LC10 column oven (Shimadzu, 's Hertogenbosch, The Netherlands). Gradient separation was performed at a flow rate of 150 μL/min with 1% MeOH + 0.1% FA in H_2O as mobile phase A and 1% H_2O + 2% tetrahydrofuran + 0.1% FA in MeOH as mobile phase B. For optimization experiments and the analysis of derivatized prostanoids, the gradient started at 50% B and was increased to 95% B in 20 min followed by a 5 min hold and subsequent re-equilibration at 50% B for 8 min. For the analysis of derivatized NSAIDs, the gradient started at 40% B and was increased to 60% B in 20 min, then quickly increased to 95% B and held for 5 min before re-equilibration at 40% B for 8 min. Total analysis time was 35 min in both cases. The effluent from the column was directed to the mass spectrometer, where the first 5 min and the final 10 min were directed to the waste via a three-port switching valve, to avoid unnecessary contamination of the ion source.

2.2.2. Mass spectrometric conditions

MS detection for optimization experiments and for analysis of NSAIDs was done on a Micromass Q-Tof Ultima mass spectrometer (Micromass, Wythenshawe, Manchester, UK) equipped with a Z-spray ESI source and controlled by MassLynx 4.0 software. The instrument was operated in positive ion mode. Specific settings of the ESI source were: capillary voltage 1.5 kV, cone voltage 35 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas flow 50 L/h, and desolvation gas flow 350 L/h. Collision energy was set to 20 V for optimization experiments and to 30 V during NSAIDs analysis. Acquisition of optimization experiments was done in full spectrum mode with an *m*/*z* range from 100 to 1000 at a scan time of 1 s. For the analysis of NSAIDs, a segmented MS/MS method was used, were the *m*/*z* values of the analytes were selected in a window based on the retention time of the analyte. The specific segments were: 13–15.5 min *m*/*z* 553 (DMCA, IS), 15.5–17.5 min *m*/*z* 599 (KPF), 16–18.5 min *m*/*z* 575 (NPX), 18.5–21.5 min *m*/*z* 551

(IBF). The m/z range of the MS/MS products that was measured in each segment was 100–700 and the scan time was 0.75 s.

Analysis of prostanoids in urine samples was performed on an Agilent Technologies Model 6410 triple quadrupole mass spectrometer, operated in positive ion ESI mode and controlled by MassHunter version B.01.03 software. Ion source parameters were source temperature 325 °C, gas flow 8 psi, nebulizer pressure 45 psi, and capillary voltage 4 kV. Acquisition was performed by selected reaction monitoring (SRM) with the following transitions: m/z 697.3–183.0 for PGE_2 (^{79}Br isotope); m/z 701.3–185.0 (^{81}Br isotope) for PGE_1, $PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$; m/z 705.3–185.0 for $PGF_{2\alpha}$-d_4. The ^{81}Br isotope was chosen for these analytes for better distinction, since m/z 699.3 is shared as the ^{81}Br isotope signal of derivatized PGE_2 and ^{79}Br isotope signal of derivatized PGE_1, $PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$. All transitions were measured at a fragmentor setting of 150 V, collision energy of 55 V, deltaEMV of 700 V and at a dwell time of 333 ms for each transition; the resolution was set to "wide" in Q1 as well as in Q3. Settings were adjusted to maximize signal intensity for m/z 183.0 and 185.0.

2.3. Experimental part

2.3.1. Optimization experiments

Preliminary optimization experiments were performed with a test set of short chain aliphatic carboxylic acids with a chain length from C_6 to C_9, as well as with benzoic acid and hippuric acid, which were present at a final concentration of 0.5 μM in the sample. This mixture was used for experiments examining the effects of the pH and the use of NHS and PPY as co-reagents.

Influence of the pH was tested in non-buffered samples (pH 5), in TRIS and phosphate buffer (each pH 7.0, 100 mM), and with addition of HCl to the sample (pH 3.9). Samples were tested for pH drift by measuring the pH at the start of the reaction and after 2 h of incubation.

To examine the effect of NHS and PPY, each co-reagent was added to the sample at a concentration of 0% (control), 5%, 25% and 100% relative to the 4-APEBA concentration of 2 mM. These experiments were performed with an incubation time of 2 h at 40 °C. Kinetics experiments were performed by repeatedly injecting from the same sample during an incubation period of 12 h starting immediately after mixing analytes and derivatization reagents.

Further optimization was done with a mixture of the prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$ and the isoprostane 8-iso-$PGF_{2\alpha}$ as model analytes.

The influence of the amount of 4-APEBA was tested at concentrations of 0.5 mM, 1 mM, 2 mM and 3 mM, corresponding to 750–4500 fold excess relative to the total analyte concentration in the standard samples.

The concentration of EDC in the sample that was used in the initial protocol [27] was 20 mM. Influence of the EDC concentration was measured at 40 mM, 20 mM, 10 mM, 4 mM, 2 mM and 1 mM.

The influence of temperature was investigated at 10 °C, 20 °C and 30 °C by adjusting the temperature inside the autosampler.

The derivatized analytes were found to be stable at 20 °C for at least 24 h.

2.3.2. Solid-phase extraction of urine samples

Urine samples were subjected to solid-phase extraction (SPE) prior to derivatization in order to remove salts and other interferences.

For prostanoid standards, pooled urine from five healthy volunteers was spiked with a mixture of PGE_1, PGE_2 and $PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ in a concentration range from 0 to 50 nM. $PGF_{2\alpha}$-d4 was added as IS to all samples at a concentration of 25 nM. NSAID standards were prepared by similarly spiking the urine with KPF, NPX and IBF in a concentration range from 6 to 120 nM and adding DMCA as IS at a concentration of 120 nM.

All urine samples were acidified to pH 3 by addition of FA prior to SPE.

A C_{18} SPE cartridge was activated with 5 mL of a mixture of DCM and MeOH (1:1) to ensure the absence of lipophilic impurities, and then equilibrated with 5 mL of 30% MeOH in water. Problems due to potentially immiscible solvents were not observed. After equilibration, 1 mL of urine sample was loaded on the cartridge and washed with 3 mL of 30% MeOH. Elution was accomplished with 2 mL of DCM/MeOH (1:1). The eluate was collected and evaporated under a gentle stream of nitrogen at room temperature. The residue was reconstituted in 0.3 mL of MeOH and used for derivatization.

To determine the recovery of the SPE procedure and assess matrix effects, blank urine was subjected to the same procedure. After the evaporation step, the residue was reconstituted with a solution of the analytes in MeOH. The concentration in this solution was corrected for the volume reduction from 1 mL loading volume to 0.3 mL for reconstitution. The resulting residue was then derivatized and analyzed.

2.3.3. Derivatization procedure

Based on the results of the optimization, the following protocol was established for derivatization: 150 μL of analyte solution in MeOH were mixed with 300 μL of an aqueous solution of 4-APEBA (2 mg/mL), followed by addition of 150 μL of an aqueous EDC solution (24 mg/mL). This results in final concentrations of 2 mM 4-APEBA and 40 mM EDC in the sample vial. The reaction mixture was then incubated at 20 °C for 1 h before analysis.

3. Results and discussion

Preliminary optimization of reaction conditions, namely the influence of pH and the use of co-reagents, was performed using a series of small carboxylic acids. Based on these results, further optimization regarding the necessary excess of the derivatizing reagents 4-APEBA and EDC as well as the influence of temperature was done with target analytes, the prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$ and the isoprostane 8-iso-$PGF_{2\alpha}$.

3.1. Optimization of the derivatization reaction

3.1.1. Influence of pH

The derivatization reaction employed is a two step reaction. First, the carboxylic acid is activated using EDC to generate an unstable O-acyl-isourea, which is subsequently converted into the 4-APEBA derivative (Fig. 1). In organic solvents, where the concept of pH is not directly applicable, the protonation of EDC that is crucial to the onset of the reaction (Fig. 1) is usually achieved by the carboxylic acid itself [5]. In mostly aqueous solution, however, the influence of pH requires closer examination, particularly because the reaction of the amine group in 4-APEBA proceeds preferably at higher pH [26,27]. The activation of the acid and the nucleophilic attack of the amine have indeed different pH optima [25].

Derivatization at a pH of 7.0 shows no product formation at all with the use of TRIS buffer and only a minor amount of product in phosphate buffer. The most important reason for this is probably an insufficient amount of protonated EDC at neutral pH, since EDC has been shown to be more reactive at low pH [25].

Buffer systems involving acetate or formate, which are commonly applied in LC–MS, cannot be used because they are also derivatized, while bicarbonate apparently inhibits the derivatization (data not shown). Therefore, the selection of suitable buffers for the lower pH region is rather limited. Experiments at lower pH were thus performed in non-buffered solvent standard solutions, by adding HCl to a final concentration of 0.5 mM to the sample.

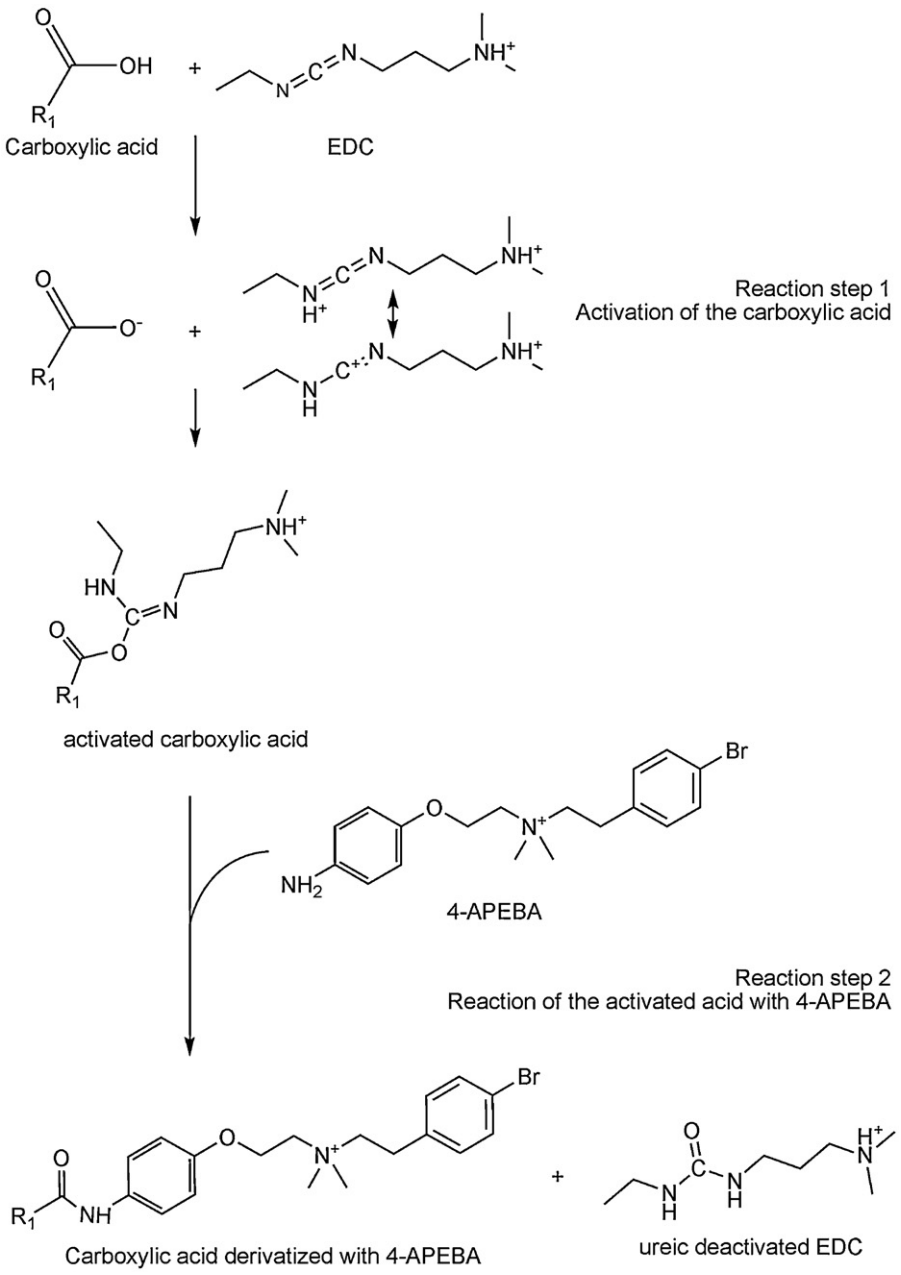

Fig. 1. Two-step reaction mechanism of the EDC mediated derivatization of a carboxylic acid with 4-APEBA.

A check for possible pH drifts during incubation showed no significant change in pH over time. However, with a pH of about 5, the measured pH values in the samples were higher than the calculated value of pH 3.3 for a solution of HCl of that concentration, which must be due to the other reagents in the sample, namely the basic 4-APEBA.

Kinetics measurements of the acidic samples showed no difference in reaction speed and total yield compared to the control without additional acid. It was found that the derivatization proceeded best at pH 5 without any additional buffers.

3.1.2. Influence of co-reagents

3.1.2.1. NHS. The presence of NHS in the reaction mixture leads to an enormous decrease in reaction speed. Judged by the number of additional peaks in the chromatograms, a large extent of unwanted side reactions seems to occur. An EDC-mediated reaction between NHS and 4-APEBA could be contributing to a strong decrease in available 4-APEBA concentration and thus in total yield of derivatives. Based on these results, NHS was discarded as co-reagent for this application.

3.1.2.2. PPY. Low concentrations of the catalyst PPY, corresponding to 5% or 25% of the 4-APEBA concentration, lead to a marginal increase of about 5–10% in yield and reaction speed. An amount of 100% of the concentration of 4-APEBA drops the total yield to about 20% of the non-catalyzed reaction, which can be explained by a significant increase in pH (see Section 3.1.1) at this concentration of the basic PPY. It was judged that the relatively small benefit of PPY does not justify an additional component in the otherwise simple reaction mixture.

Both examined co-reagents are expected to act on the second reaction step (Fig. 1), the coupling of the EDC-activated carboxylic acid to the amine group. The fact that neither of the two established co-reagents performed according to expectations gives reason to

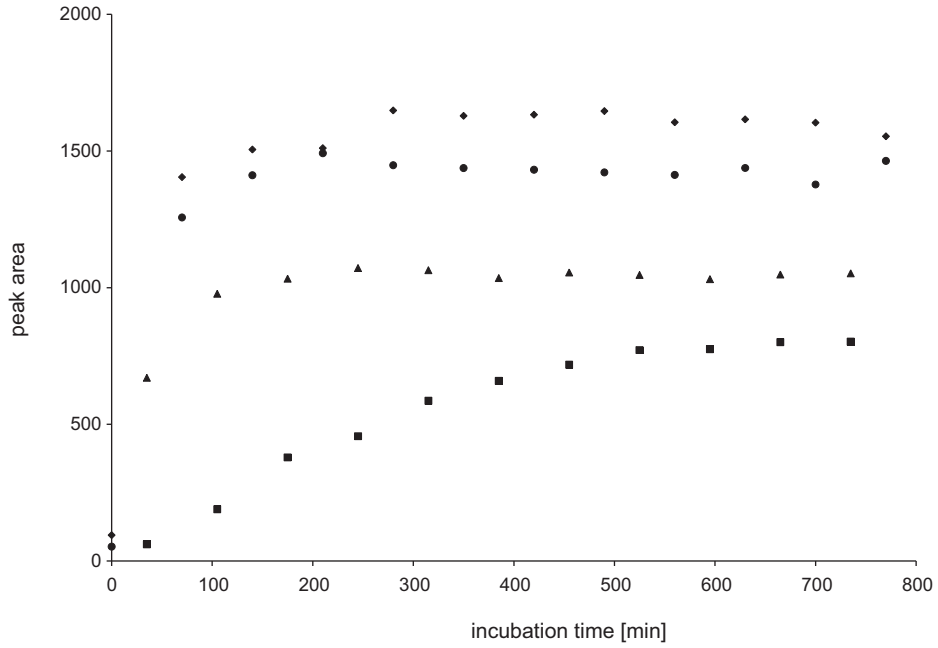

Fig. 2. Reaction kinetics of the derivatization of $PGF_{2\alpha}$ as a function of the concentration of 4-APEBA. Concentrations are: 0.5 mM (■), 1 mM (▲), 2 mM (●), 3 mM (♦).

the assumption that under the present aqueous conditions the reaction mechanism is governed by different factors than in the established organic solvents.

3.1.3. Influence of 4-APEBA concentration

Following the preliminary experiments with small carboxylic acids, the concentrations of necessary derivatization reagents were optimized based on the derivatization experiments with prostaglandins.

As can be expected, an increase in the 4-APEBA concentration initially leads to higher total yields of derivatized analytes as well as higher reaction speed. However, at a 4-APEBA concentration higher than 2 mM, no further increase in reaction speed or yield was observed (Fig. 2). Hence, a concentration of 2.0 mg/mL APEBA was chosen for all subsequent experiments, corresponding to a final concentration in the sample of 2 mM. For the standard solutions used for optimization, this corresponds to an approximate 3000 fold excess of reagent.

3.1.4. Influence of EDC concentration

The final concentration of EDC in the initially adopted procedure was 20 mM. Optimization experiments were carried out in a range from 1 mM to 40 mM. At the highest concentration of 40 mM, the reaction is complete after about 1 h (Fig. 3). The observed total yield at 40 mM did not increase significantly compared to the results obtained with 20 mM EDC after 8 h. Therefore, the derivatization

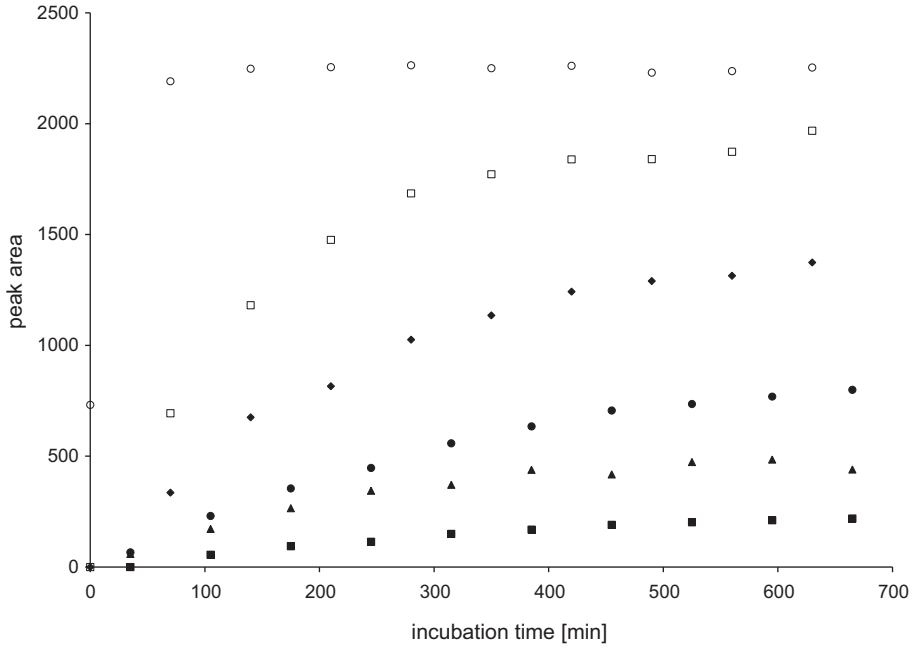

Fig. 3. Reaction kinetics of the derivatization of 8-iso-$PGF_{2\alpha}$ as a function of the concentration of EDC. Concentrations are: 1 mM (■), 2 mM (▲), 4 mM (●), 10 mM (♦), (□) 20 mM and (○) 40 mM.

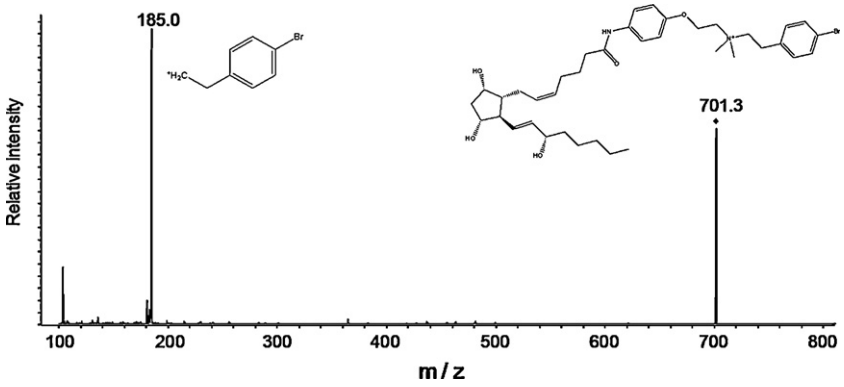

Fig. 4. Product ion spectrum of 4-APEBA-derivatized $PGF_{2\alpha}$ (^{81}Br isotope, m/z 701.3, selected as precursor ion) illustrating the obtained fragments.

is assumed to be quantitative. The necessary incubation time at 40 mM EDC was found to be reasonably short, so this concentration was adopted for the final protocol. The large excess that is necessary [5] compensates for the reported instability of EDC in aqueous solutions [25].

While a further increase in the EDC concentration could still lead to an even higher reaction speed, contamination of the MS source with excess reagent will also be enhanced further, leading to an intense signal with m/z 174.2, which corresponds to the ureic product of deactivated EDC. Since this byproduct is not retained on the column under the conditions used here, diverting the early eluting excess reagents towards the waste significantly reduced this cause of background.

3.1.5. Influence of temperature

Compared to the influence of increasing EDC concentrations on the reaction speed, the influence of temperature (10, 20 or 30 °C) was negligible. Hence, incubation of samples was possible at room temperature in acceptable time without losses neither in yield nor in speed. For this reason, reaction kinetics at higher temperatures than 30 °C were not examined.

3.1.6. Selection of reaction conditions

Based on the above results, a derivatization protocol using 2 mM 4-APEBA, 40 mM EDC and an incubation time of 1 h at 20 °C was established (also see Section 2.3.3). The large excess of reagents accounts for possibly interfering compounds which may be derivatized as well in complex matrices like urine or plasma samples. Especially the high amount of acidic compounds that is excreted in urine requires consideration.

3.2. Application of derivatization to prostaglandins

3.2.1. Analysis of prostaglandins in urine samples

Different strategies may be followed in the analysis of prostaglandins in urine samples. Initial experiments were performed by simply adding the derivatization reagents directly to the urine without prior cleanup steps. While in principle the derivatization and direct analysis was possible under these conditions, interferences were too high to obtain reliable and reproducible results. Therefore, to reduce the amount of interfering compounds from urine that may also be derivatized by the 4-APEBA label, SPE of the urine was performed first, in order to isolate the prostaglandins from the more polar urine constituents as much as possible. The SPE procedure further has the advantage that it eliminates any variations in pH of the urine samples. Subsequently, the isolated fraction was derivatized according to the procedure outlined in Section 2.3.3. MeOH was chosen as solvent for reconstitution to avoid any possibility of precipitation and adhesion to the vial walls by the rather lipophilic analytes. Still, to demonstrate the applicability of the method as originally intended in aqueous media like biological samples, water was chosen as solvent for the reagents. The derivatization was successfully performed under these conditions in a single derivatization step. Also, the mostly aqueous solution of the reaction mixture makes direct reversed phase LC analysis possible, while a high amount of organic solvent in the derivatization mixture would require the dilution of the sample with mobile phase prior to analysis. Given a less complex sample matrix than urine, an even simpler method without prior extraction would be feasible.

Full-spectrum LC–MS measurements of processed urine samples show a large number of derivatized compounds, which are easily recognized by the typical isotopic pattern originating from the bromine atom in 4-APEBA.

SRM measurements first discriminate for the correct m/z values of the analytes of 697.3 and 701.3, while the labeled part of the analyte then is confirmed by the specific fragmentations of 4-APEBA. These fragmentations have been previously described in detail [27]. Interestingly, the only major fragment that is observed with the derivatized analytes of this study is the 4-bromophenethyl cation with m/z 183 ($C_8H_8{}^{79}Br^+$) or 185 ($C_8H_8{}^{81}Br^+$) (Fig. 4). Therefore, the fragmentation of the derivatized prostanoids to the 4-bromophenethyl cation was chosen as SRM transition.

However, for improved distinction between the analytes, the ^{79}Br isotope (m/z 697.3–183.0) was chosen for PGE_2, while the ^{81}Br isotope was measured for the remaining prostaglandins and the internal standard (m/z 701.3–185.0 and 705.3–185.0, also see Section 2.2.2).

3.2.2. Extraction recovery and matrix effects

Matrix effects and SPE extraction recovery were assessed following the procedures described by Matuszewski et al. [29]. However, this procedure is somewhat complicated by the fact that the current analytical procedure involves a derivatization step in between the SPE isolation and the LC–MS analysis. No adequate derivatized prostaglandin standards are available.

To assess matrix effects and extraction recovery, three types of samples need to be measured [29]. The first is a solution of the analytes in MeOH (referred to as solvent standard). The second is a matrix standard that is spiked with analytes after the extraction procedure (referred to post-extraction spike), which was obtained by performing the SPE procedure with blank urine and then using the solvent standard solution for reconstitution of the dry residue after evaporation. The third sample is a matrix standard which is spiked before the extraction procedure (referred to as pre-extraction spike). This pre-extraction standard resembles an actual sample. To obtain comparable results, the concentration of the solvent standard has to be corrected for the pre-concentration effect that is achieved by the reduction of the sample volume dur-

Table 1
Analytical data for 8-iso-$PGF_{2\alpha}$, PGE_2, PGE_1 and $PGF_{2\alpha}$, RSD relative standard deviation, LOD limit of detection, ME matrix effect, expressed as % signal normalized to solvent standard.

	8-iso-$PGF_{2\alpha}$	PGE_2	PGE_1	$PGF_{2\alpha}$
SPE recovery (%)	80.5	83.7	81.9	84.6
RSD of recovery (%) ($n=3$)	4.3	4.1	7.7	6.9
ME (%)	92.5	97.8	116.4	107.0
LOD (nM)	10	2	5	2
R^2	0.9999	0.9975	0.9984	0.9963
RSD intra-day (%) ($n=3$)	2.3	5.3	2.8	4.1
RSD inter-day (%) ($n=3$)	13.3	18.0	6.2	8.1

ing the SPE procedure. In our procedure, this means that an analyte concentration of 25 nM in the pre-extraction spike corresponds to 83.3 nM in the solvent standard and post-extraction spike.

Matrix effects are then determined by normalizing the results from the post-extraction spike to the solvent standard (no observation of matrix effects corresponds to 100% analyte signal [29]). In this way, possible variations originating from incomplete recovery are excluded. Matrix effects were observed ranging from 92.5% for 8-iso-$PGF_{2\alpha}$ to 116.4% for PGE_1. Values for all analytes are provided in Table 1. Most likely reasons for these signal changes are ion suppression or ion enhancement due to coeluting urine constituents, respectively, in the MS source.

In a similar way, recovery is calculated by normalization of the pre-extraction spike to the post extraction spike. The presence of the urine matrix in both samples here corrects for matrix effects. Satisfying recoveries of more than 80% (+/− 4–8%, $n=3$) for all analytes (Table 1) were obtained with the SPE procedure that was employed here.

3.2.3. Calibration and validation

For calibration, pooled urine from 5 healthy volunteers was spiked with different concentrations of prostaglandins and subsequently analyzed with the complete procedure (SPE, derivatization and LC–MS analysis). To accommodate for eventual variations in SPE recovery, derivatization and MS ionization efficiency, an isotopically labeled internal standard ($PGF_{2\alpha}$-d_4) was applied and all peak areas of the analyte peaks were normalized to the peak area of the internal standard. Good linearity of normalized signal over concentration was obtained within a concentration range from 2 nM to 25 nM, covering the estimated diagnostic range of the investigated prostaglandins in urine [14]. Limits of detection were determined as a signal-to-noise ratio of 3:1 and estimated to be 2 nM for PGE_2 and $PGF_{2\alpha}$, 5 nM for PGE_1 and 10 nM for 8-iso-$PGF_{2\alpha}$ (Table 1). Because the pooled blank urine also showed a significant signal for $PGF_{2\alpha}$, measurements were corrected for this blank value. The measured signal in the blank corresponded to a concentration of 6.9 nM $PGF_{2\alpha}$.

Intra day repeatability showed a relative standard deviation (RSD) between 2–6% ($n=3$), and the RSD of inter day repeatability was within 6–18% ($n=3$) (Table 1).

3.2.4. Analysis of CRPS patient urine samples

In order to establish the potential of this procedure, some preliminary experiments were performed using urine samples from patients suffering from CRPS. Of the six patient samples analyzed, three showed presence of $PGF_{2\alpha}$, at concentrations of 9.6 nM, 3.1 nM and 14.5 nM, respectively. The last of these three also showed PGE_2 in a concentration of 2.7 nM. No response was observed for the other model compounds that were studied.

Comparison of the specific SRM traces of the individual samples (Fig. 5) shows significant differences in the obtained peak profiles, beyond the analytes that were used as standard compounds. Immediately evident in the patient samples as well as in the pooled healthy volunteer urine standards is the occurrence of additional peaks in close proximity to the actual analyte peaks, particularly eluting close to 8-iso-$PGF_{2\alpha}$ (15.4 min) and $PGF_{2\alpha}$ (18.3 min). Since these peaks are also observed in the healthy volunteer urine and at a constant area ratio in comparison to the internal standard, they cannot be related to the spiked analytes. Given the large number of potential isomers of each isoprostane and due to the specificity of the employed SRM method as well as in conjunction with the similar retention time window, these additional signals are believed to originate from different isomers of the analytes. More elaborate studies on the prostaglandins present in these CRPS patient urine samples are currently underway and results will be reported in due course.

3.3. Application of derivatization to non-steroidal anti-inflammatory drugs

3.3.1. Analysis of non-steroidal anti-inflammatory drugs in urine

In order to show the wider applicability of the developed methodology we also derivatized and analyzed NSAIDs from urine samples, namely ibuprofen (IBF), naproxen (NPX) and ketoprofen (KPF). The above stated derivatization protocol, as well as the developed SPE procedure were applied without changes. Unlike the prostanoids the NSAIDs did not only show the typical bromine pattern introduced by the label itself, but also gave characteristic MS/MS fragments. The two most intense of these were subsequently used for the quantification of the different analytes (Table 2). These fragmentations allowed the unambiguous

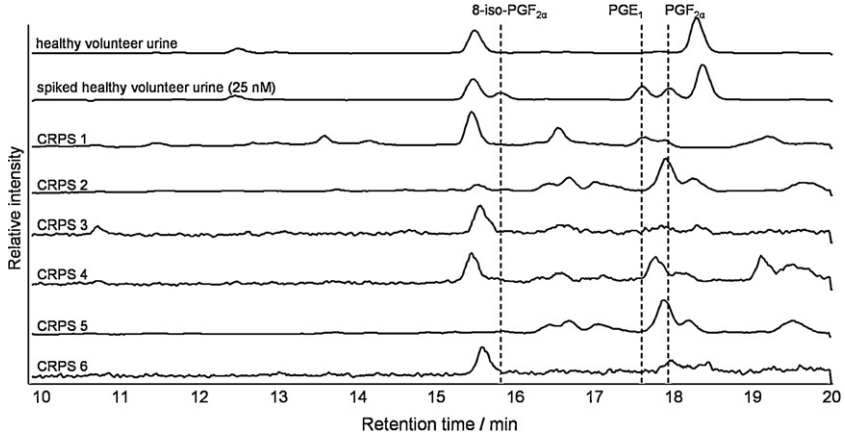

Fig. 5. Comparison of the SRM traces for the transition m/z 701.3–183.0 obtained from blank urine, spiked urine and 6 different complex regional pain syndrome (CRPS) patient urine samples. Intensities are normalized to the highest peak in each chromatogram.

Table 2
Monoisotopic masses of the derivatized analytes that were isolated for fragmentation and the specific fragments that were used quantification, DMCA dimethoxycinnamoic acid (IS), KPF ketoprofen, NPX naproxen, IBF ibuprofen.

	DMCA (IS)	KPF	NPX	IBF
Chemical formula of derivatized analyte	$C_{29}H_{34}BrN_2O_4^+$	$C_{34}H_{36}BrN_2O_3^+$	$C_{32}H_{36}BrN_2O_3^+$	$C_{31}H_{40}BrN_2O_2^+$
Monoisotopic mass (m/z)	553.2	599.2	575.2	551.2
Fragments used for quantification (m/z)	191.1	209.2	348.2	324.2
	371.2	417.2	393.2	369.3

determination of the derivatized NSAIDs. Combined extracted ion chromatograms of the respective fragments were obtained from the corresponding acquisition segment. Fig. 6 shows the resulting chromatograms as well as the MS and MS/MS spectra of derivatized ketoprofen. The proposed fragment structures as well as their high-resolution mass data for all NSAIDs and the internal standard is summarized in S1–S4 (supporting information).

3.3.2. Extraction recovery and matrix effects

Recovery and matrix effects for the analyzed NSAIDs were determined in the same way as described for prostanoids in Section 3.2.2. The concentrations in the spiked urine samples were 100 nM KPF, 110 nM NPX and 120 nM IBF. These correspond to 333 nM KPF, 366 nM NPX and 400 nM IBF in the post-extraction spiked samples and solvent standards that were used for normalization. The results are summarized in Table 3.

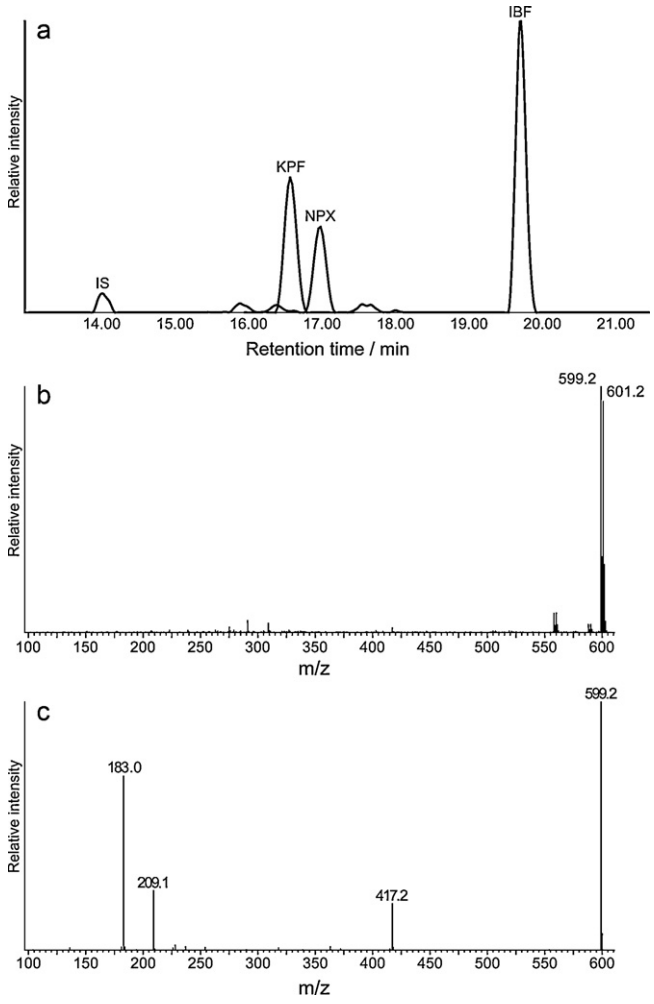

Fig. 6. (a) Combined extracted ion chromatograms of the specific fragments of the analytes, IS internal standard, KPF ketoprofen, NPX naproxen, IBF ibuprofen, (b) Mass spectrum of ketoprofen derivatized with 4-APEBA, (c) Product ion spectrum obtained from the fragmentation of derivatized ketoprofen.

Significant matrix effects were observed for KPF (36.7%) and NPX (35.9%), while IBF was affected less (68.8%), which is one explanation for the lower detection limit of IBF compared to KPF and NPX (see Section 3.3.3).

SPE recoveries were satisfying with more than 90% recovery for each analyte, namely 90.4% IBF, 94.4% KPF and 94.6% NPX (Table 3).

3.3.3. Calibration and validation

Pooled urine from 5 healthy volunteers was spiked with different concentrations of KPF, NPX and IBF and subsequently analyzed by SPE, derivatization and LC–MS as described above. Stock solutions of 2 mg/mL of the NSAIDs were prepared in MeOH, diluted to working solutions of appropriate concentration and then spiked into the urine samples. The concentration ranges were 6–100 nM for KPF, 7–110 nM for NPX and 8–120 nM for IBF. Quantification was done by normalizing the peak area under the curve to the peak area of the IS (DMCA), which was used at a concentration of 120 nM. Linearity of the normalized signal over concentration was obtained for all three analytes above the limit of detection in the investigated range. The LOD was defined as the lowest concentration that yielded a signal to noise ratio of 3:1 or higher. LODs were 13 nM for KPF, 28 nM for NPX and 8 nM for IBF (Table 3). Intra day repeatability ranged from 4.9% to 5.7%, with inter day repeatability ranging from 6.2% to 8.6% (Table 3).

3.3.4. Analysis of volunteer urine

A volunteer took 600 mg of ibuprofen and urine was collected 3 h after the intake. This urine sample was subject to the same procedure as the standards. A substantial amount of IBF was detected in the sample, and no KPF and NPX were found. The sample had to be diluted 10 times to yield a signal within the calibrated range. The concentration of IBF was then determined to be 2.1 μM in the undiluted urine.

3.4. Discussion of detection limits

The LODs in the presented method range from 2 to 10 nM for prostanoid compounds and 8–28 nM for NSAIDs.

In the most sensitive of the previously published methods for analysis of non-derivatized prostanoid compounds using negative ESI-MS/MS, a detection limit of 40 pg/mL, which corresponds to 0.1 nM, is reported [16]. This method, however, was validated for human plasma, which is an entirely different matrix. Another

Table 3
Analytical data for IBF, NPX and KPF, RSD relative standard deviation, LOD limit of detection, ME matrix effect, expressed as % signal normalized to solvent standard.

	IBF	NPX	KPF
SPE recovery (%)	90.4	94.6	94.4
RSD of recovery (%) ($n = 3$)	3.7	6.6	3.7
ME (%)	68.8	35.9	36.7
LOD (nM)	8	28	13
R^2	0.9922	0.9868	0.9937
RSD intra-day (%) ($n = 3$)	5.7	5.3	4.9
RSD inter-day (%) ($n = 3$)	6.2	8.6	6.6

recently published method, which was validated for hamster and rat urine uses the same detection mode and reaches an LOD of 100 pg/mL, corresponding to 0.3 nM [18].

For analysis of non-derivatized NSAIDs in equine urine via MRM on a QTrap instrument, an LOD of 10 ng/mL (39 nM) was reported for KPF [22]. Detection limits for related compounds like diclofenac or flufenamic acid with this method are in the range from 1 to 10 ng/mL, which is roughly 5–50 nM.

The goal of the presented research was to develop a generic approach selectively and specifically measuring carboxylic acids in positive ESI-MS(MS). While existing methods admittedly reach similar (NSAID) or even better (PGs) detection limits for the respective analytes without derivatization, it has to be stressed that these methods were specifically developed for their according analyte class, and do not comprise the generic applicability to the analysis of carboxylic acids that is provided here.

4. Conclusion

The derivatization of carboxylic acids with the novel derivatization agent 4-APEBA makes carboxylic acids accessible for ESI-MS(/MS) detection in positive ionization mode. The quaternary ammonium function of 4-APEBA provides excellent ESI efficiency, while specific fragmentation reactions in MS/MS experiments as well as the bromine isotopic signature allow the highly specific recognition of derivatized analytes. The derivatization reaction is straightforward, proceeds under mild reaction conditions and is possible in aqueous solution, while the necessary reaction time is reasonably short.

The possibilities of the described method have been demonstrated by analysis of a set of prostanoid compounds in human urine from healthy volunteers as well as from CRPS patients. Further, specific differences in peak profiles between samples were observable. The method was also applied to the analysis of NSAIDs in human urine. These examples illustrate the generic applicability of the presented method.

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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.jchromb.2010.11.028.

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