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Unique pentafluorobenzylation and collision-induced dissociation for specific and accurate GC–MS/MS quantification of the catecholamine metabolite 3,4-dihydroxyphenylglycol (DHPG) in human urine☆

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ABSTRACT

In the human body, the catecholamine norepinephrine is mainly metabolized to 3,4-dihydroxyphenylglycol (DHPG) which therefore serves as an important biomarker for norepinephrine's metabolism. Most data on DHPG concentrations in human plasma and urine has been generated by using HPLC-ECD or GC–MS technologies. Here, we describe a stable-isotope dilution GC–MS/MS method for the quantitative determination of DHPG in human urine using trideutero-DHPG (d_3-DHPG) as internal standard and a two-step derivatization process with pentafluorobenzyl bromide (PFB-Br) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Two pentafluorobenzyl (PFB) trimethylsilyl (TMS) derivatives were obtained and identified, i.e., two isomeric DHPG-PFB-$(TMS)_3$ derivatives and the later eluting DHPG-tetrafluorobenzyl-$(TMS)_2$ derivative, i.e., DHPG-TFB-$(TMS)_2$. To our knowledge the DHPG-TFB-$(TMS)_2$ derivative and the underlying reaction have not been reported previously. In this reaction both vicinal aromatic hydroxyl groups of DHPG react with PFB-Br to form a heterocyclic seven-membered [1,4]dioxepin compound. The DHPG-TFB-$(TMS)_2$ derivative was used for quantitative GC–MS/MS analysis in the electron-capturing negative-ion chemical ionization mode by selected-reaction monitoring of *m/z* 351 from *m/z* 401 for DHPG and of *m/z* 352 from *m/z* 404 for d_3-DHPG. Validation experiments on human urine samples spiked with DHPG in a narrow (0–33 nM) and a wide range (0–901 nM) revealed high recovery (86–104%) and low imprecision (RSD; 0.01–2.8%). LOD and relative LLOQ (rLLOQ) values of the method for DHPG were determined to be 76 amol and 9.4%, respectively. In urine of 28 patients suffering from chronic inflammatory rheumatic diseases, DHPG was measured at a mean concentration of 238 nM (38.3 μg/g creatinine). The DHPG concentration in the respective control group of 40 healthy subjects was measured to be 328 nM (39.2 μg/g creatinine). Given the unique derivatization reaction and collision-induced dissociation, and the straightforwardness the present method is highly specific, accurate, precise, and should be useful in clinical settings.

1. Introduction

The majority of the norepinephrine released from adrenergic nerve endings is taken up again through the neuronal norepinephrine transporter or is metabolized to 3,4-dihydroxyphenylglycol (DHPG or DOPEG). Norepinephrine's amino group is oxidized by monoaminooxidase (MAO) producing the aldehyde intermediate 3,4-dihydroxyphenylglycoladehyde (DOPEGAL) which is subsequently reduced by aldehyde reductase to form DHPG; in addition, the aromatic 2-hydroxyl group of norepinephrine is methylated by catecholamine *O*-methyl transferases (COMT) to form normetanephrine [1] (Fig. 1). DHPG concentration in urine, being a biomarker of norepinephrine metabolism, is useful to assess disorders associated with altered adrenergic activity. In addition, DHPG measurements provide insights in the activity of the neuronal norepinephrine transporter, and DHPG may be an indirect measure of MAO activity [2–5]. Most published methods for DHPG measurements apply HPLC coupled to electrochemical (HPLC-ECD) or fluorescence (HPLC-FL) detection [6–9], and GC–MS [10–12] without or after derivatization of the aromatic and aliphatic hydroxyl groups of DHPG.

The versatile derivatization agent pentafluorobenzyl (PFB) bromide (PFB-Br) has been used for the derivatization and analysis of different substance classes including thiols, amines and car-

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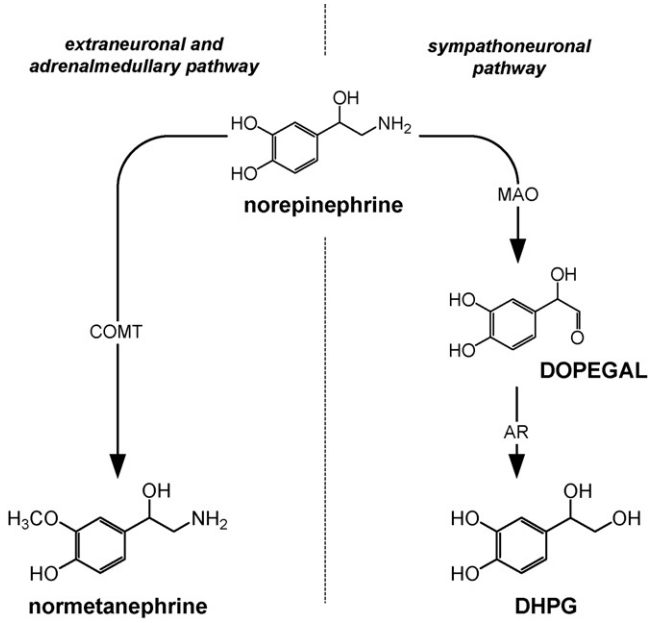

Fig. 1. Schematic of a part of the norepinephrine metabolism leading to DHPG and normetanephrine: MAO, monoamine oxidase; AR, aldehyde reductase; COMT, catechol *O*-methyl transferase.

boxylic acids [13]. The aim of the present work was to develop new extraction and derivatization methods using PFB-Br and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as the derivatization reagents and the commercially available trideutero-DHPG (d_3-DHPG) as internal standard for rapid and highly specific quantitative determination of DHPG in human urine by GC–MS/MS. We discovered a new derivatization reaction with PFB-Br in acetonitrile. This derivatization produces unusual PFB derivatives (Fig. 2) which have unique tandem mass spectrometry features. The method was validated in human urine and used for the quantification of DHPG in urine of healthy subjects and of patients suffering from chronic inflammatory rheumatism.

2. Experimental

2.1. Chemicals and materials

Unlabeled DHPG (d_0-DHPG), i.e., DL-4-(1,2-dihydroxyethyl) benzene-1,2-diol, and 2,3,4,5,6-pentafluorobenzyl bromide were purchased from Sigma–Aldrich (Munich, Germany). 4-(1,2-Dihydroxyethyl)-[1,2,2-$^2H_3$]-benzene-1,2-diol (d_3-DHPG), chemical purity declared as 95%, isotopic purity declared as 98% at ^2H, was from Medical Isotopes (Pelham, NH, USA). *N*,*O*-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce/Thermo Fisher Scientific (Rockford, IL, USA). All organic solvents were purchased from Mallinckrodt Baker (Griesheim, Germany). Distilled water (Aqua Spüllösung) was delivered by DeltaSelect (Pfullingen, Germany). All glass vials used for derivatization and chromatography came from Macherey-Nagel (Düren, Germany). Polypropylene tubes and test tubes for blood sampling were manufactured by Sarstedt (Nümbrecht, Germany).

2.2. Human urine samples

From the sample portfolio of a recently performed study at our institute [14], a collective of urine specimens (spontaneous morning urine) was used to measure concentrations of DHPG. The study involved 40 healthy subjects as controls (16 women, 24 men, aged 26–82 years, mean age 47 years) and 28 patients (18 women, 10 men, aged 23–82 years, mean age 55 years) suffering from different chronic inflammatory rheumatic diseases (10 patients with classic or definite rheumatoid arthritis fulfilling the American College of Rheumatology criteria, 10 patients with undifferentiated rheumatic arthritis, 5 patients with spondyloarthropathy and 3 patients with vasculitis). The studies were performed with local Ethics Committee approval and in accordance with the guidelines of the Declaration of Helsinki and of Good Clinical Practice (GCP).

2.3. Derivatization and extraction procedures

The extraction and derivatization procedures were performed on 100 μL sample aliquots, i.e., urine or aqueous DHPG standard solutions, which were vortex-mixed with 500 μL of ethyl acetate for 2 min. After centrifugation (800 × *g*, 5 min, 4 °C) about 400 μL aliquots of the upper organic phase were transferred to glass vials and the solvent was evaporated to dryness under a gentle stream of nitrogen gas. Subsequently, 100 μL acetonitrile, 10 μL methanol, 10 μL *N*,*N*-diisopropylethylamine as the catalyst and 10 μL of a 30 vol.% solution of PFB-Br in acetonitrile were added and heated to 30 °C for 1 h. Then the reaction mixture was evaporated to dryness under a stream of nitrogen gas, the residue was taken up in 50 μL of BSTFA and the sample was heated at 60 °C for 1 h. After cooling to room temperature, 1 μL aliquots of the solution were injected into the GC–MS/MS system. Stock acetonitrile and methanol were dried over molsieve before usage.

For quantitative analyses, to 100 μL aliquots of human urine the internal standard d_3-DHPG was added to a fixed final concentration of 500 nM. No pH correction of the urine samples was performed and extraction and derivatization were carried out as described above.

For generation of MS and MS/MS spectra, 1 μL aliquots of stock solutions (each 100 ng/μL in 40 mM HCL) of synthetic unlabeled DHPG (d_0-DHPG) and d_3-DHPG HCl were used. After evaporation to dryness, derivatization with PFB-Br and BSTFA was performed as described above.

2.4. GC–MS and GC–MS/MS conditions

Analyses were performed in electron-capture negative-ion chemical ionization (ECNICI) mode on a triple-stage quadrupole (TSQ) mass spectrometer ThermoElectron TSQ 7000 (Finnigan MAT, San Jose, CA, USA) directly interfaced with a Trace 2000 series gas chromatograph equipped with an autosampler AS 2000 (CE Instruments Austin, TX, USA). Chromatographic separation was carried out on a DB-5ht fused silica column (15 m × 0.25 mm i.d., 0.1 μm film thickness) from Agilent (Santa Clara, CA, USA). The following oven temperature program was used: 1 min at 70 °C, then increased to 360 °C at a rate of 20 °C/min, and hold for 1 min at 360 °C. Interface and ion-source were kept at 320 and 180 °C, respectively. Electron energy and electron current was set to 70 eV and 300 μA, respectively. Methane (530 Pa) and argon (0.2 Pa pressure in the collision chamber) were used as reagent and collision gases, respectively. Collision energy and electron multiplier voltage were set to 10 eV and 2800 V, respectively, in quantitative analyses. Aliquots (1 μL) were injected in the splitless mode by using a BEST PTV injector, starting at an injector temperature of 120 °C which was increased to 320 °C at 5 °C/s. Quantitative measurements were conducted on the DHPG-TFB-$(TMS)_2$ derivatives by selected-reaction monitoring (SRM) of the product ions $[M-TMS-CH_2O]^-$, generated by collision-induced dissociation (CID) of the precursor ions at $[M-TMS]^-$ (TMS, trimethylsilyl). The mass transitions for DHPG were *m*/*z* 401 to *m*/*z* 351 for d_0-DHPG and *m*/*z* 404 to *m*/*z* 352 for d_3-DHPG (see Section 3). The dwell time was 100 ms each.

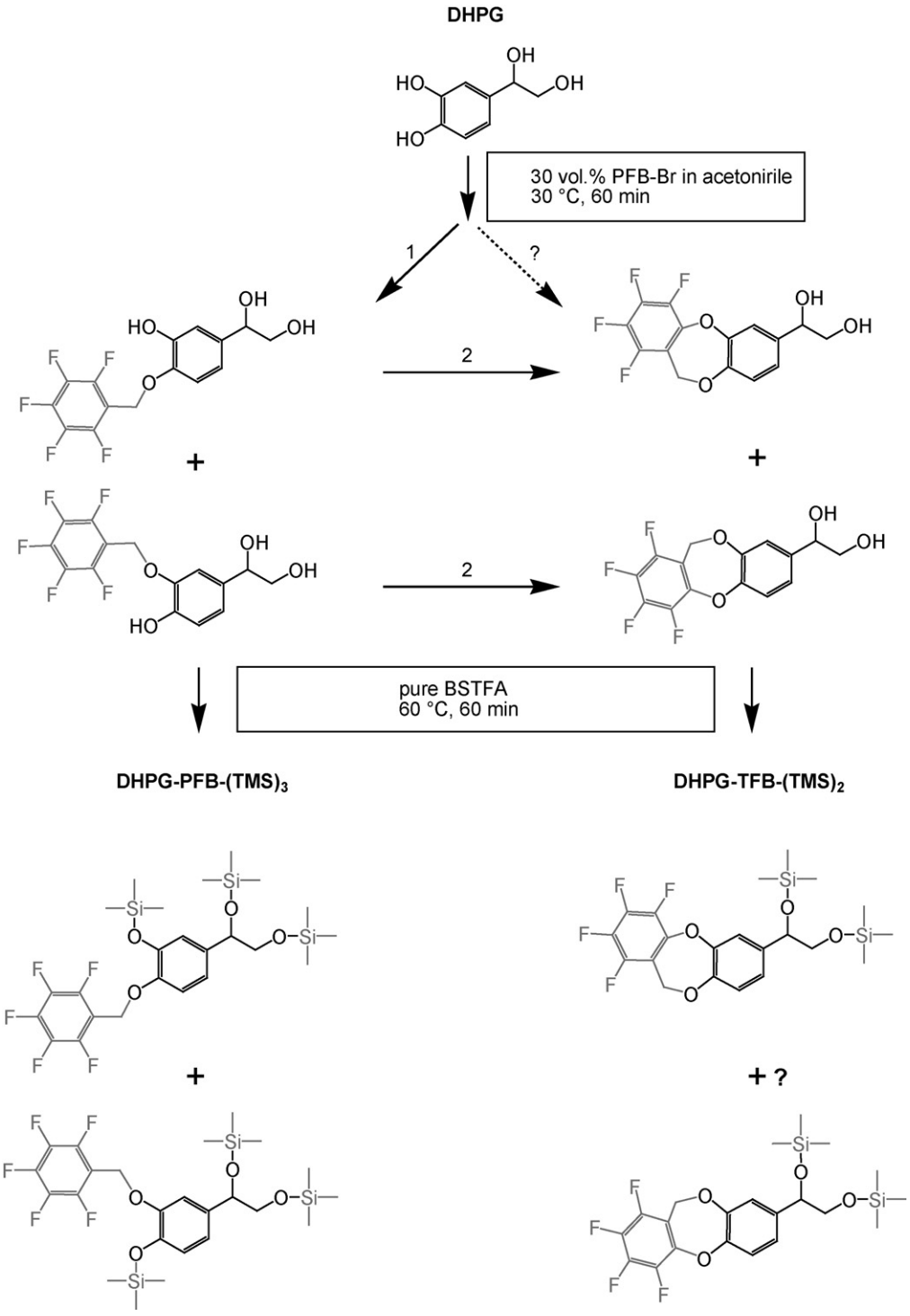

Fig. 2. Derivatization procedures for DHPG used in the present study and proposed structures for the derivatives formed. The left panel shows the formation of the DHPG-PFB-$(TMS)_3$ derivative that appears as a double peak in the chromatograms, indicating the existence of two isomers. The right panel shows the formation of DHPG-TFB-$(TMS)_2$ derivatives that appear as a single distinct peak in the chromatograms. Presumably both shown isomers are generated but not chromatographically separated on the GC column.

2.5. Linearity and standardization of the internal standard d_3-DHPG

In order to test linearity and to standardize the internal standard d_3-DHPG, varying amounts of d_3-DHPG (0, 50, 100, 200 and 500 pmol from a 10-μM ethanolic solution) were added to a fixed amount of d_0-DHPG (500 pmol from a 50-μM ethanolic solution). The dried residue of these mixtures was derivatized with PFB-Br and BSTFA as described in Section 2.3.

2.6. Validation of the method

For the purpose of method validation, a healthy volunteer donated about 50 mL of urine by spontaneous micturition. After

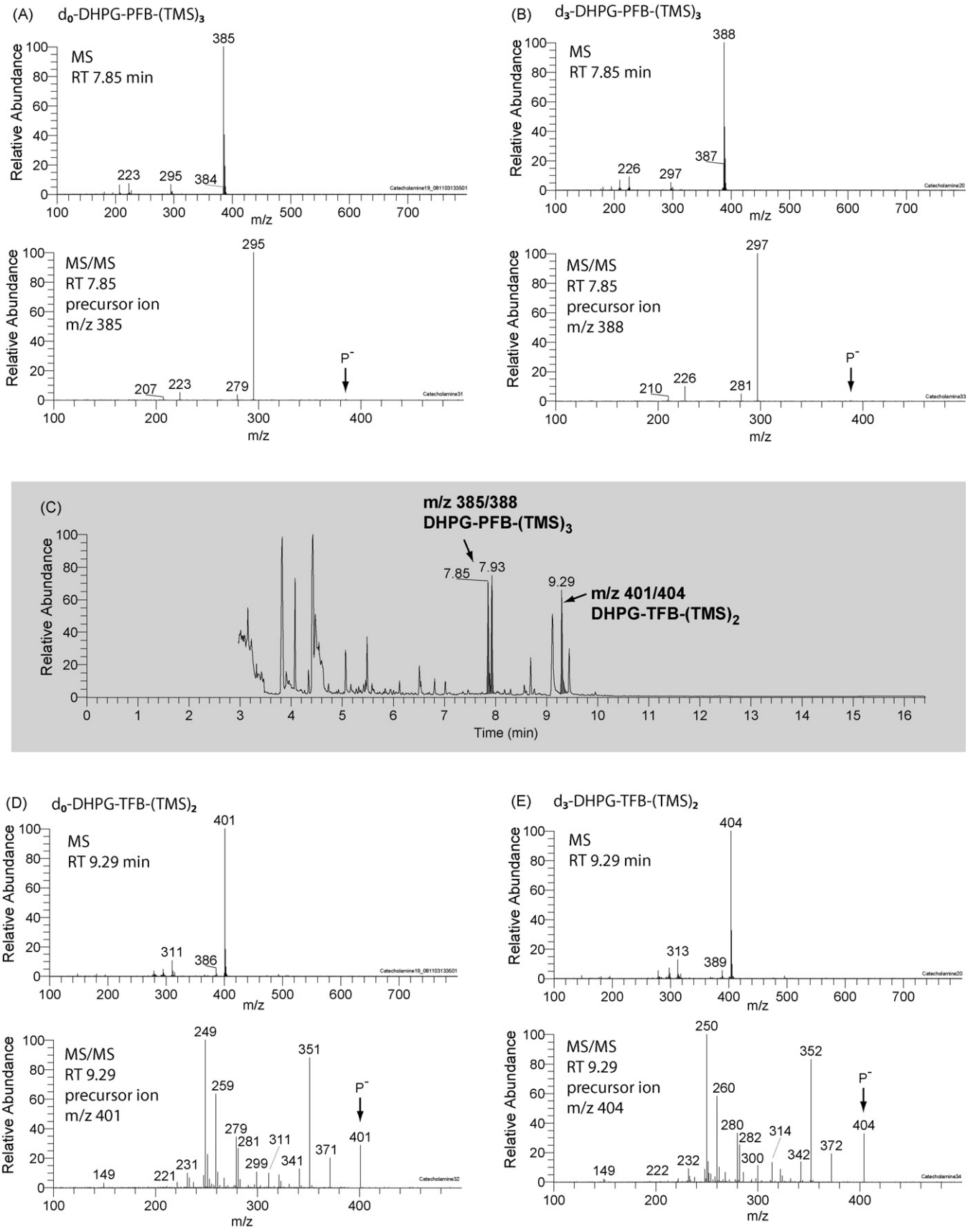

Fig. 3. Total ion current GC–MS chromatogram (middle panel, C) and mass spectra (lower and upper panel) derived from the peaks labeled as "*m*/*z* 385/388" and "*m*/*z* 401/404". A mixture consisting each of unlabeled DHPG (d_0-DHPG) and deuterium-labeled DHPG (d_3-DHPG) were derivatized with PFB-Br followed by BSTFA. Derivatized d_0-DHPG and d_3-DHPG (each 2 ng) were injected and analyzed by GC–MS in the ECNICI mode. Full scans were performed in the *m*/*z* range 100–800 at a rate of 0.4 s per scan. Peaks at 7.85 and 7.93 min were identified as the two isomeric DHPG-PFB-$(TMS)_3$ derivatives, the peak at 9.29 min was identified as the DHPG-TFB-$(TMS)_2$ derivative. P^-, precursor ion.

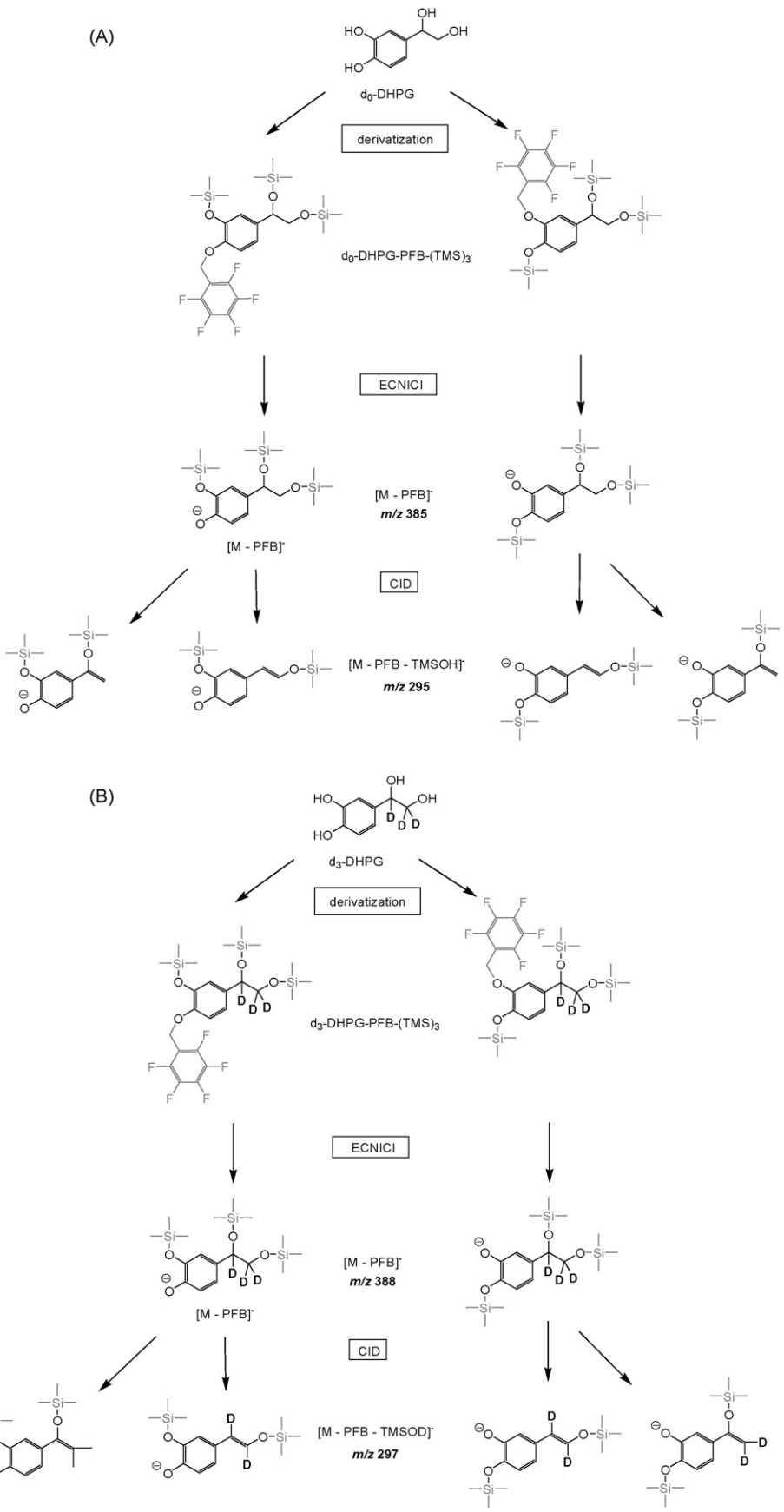

Fig. 4. Simplified schematic of the GC–MS/MS method including derivatization, ECNICI and CID of the d_0-DHPG-PFB-$(TMS)_3$ (A) and d_3-DHPG-PFB-$(TMS)_3$ (B) derivative. Extracts from urine samples are derivatized by PFB-Br in the presence of a base as a catalyst followed by BSTFA to form the DHPG-PFB-$(TMS)_3$ derivative. Depending on whether the 3- or 4-hydroxyl group of the aromatic ring is pentafluorobenzylated first, two isomers are produced during the derivatization process. Under ECNICI conditions both products ionize to form $[M-PFB]^-$. CID of these ions results in the generation of the product ions $[M-PFB-TMSOH]^-$.

Table 1
Precursor ions and their respective product ions present in GC–MS and GC–MS/MS spectra of unlabeled and deuterium-labeled DHPG-PFB-Br/BSTFA derivatives.

Derivative	Precursor ion *m/z*	Ion assignment	Product ion *m/z* (intensity, %)	Ion assignment
GC peaks 7.85/7.93 min				
d_0-DHPG-PFB-$(TMS)_3$	385	$[M-PFB]^-$	295 (100)	$[M-PFB-TMSOH]^-$
			279 (<10)	N.A.
			223 (<10)	N.A.
d_3-DHPG-PFB-$(TMS)_3$	388	$[M-PFB]^-$	297 (100)	$[M-PFB-TMSOD]^-$
			281 (<10)	N.A.
			226 (<10)	N.A.
GC peak 9.29 min				
d_0-DHPG-TFB-$(TMS)_2$	401	$[M-TMS]^-$	249 (100)	$[M-TMS-2\times CH_2O-92]^-$
			351 (90)	$[M-TMS-CH_2O-HF]^-$
			259 (65)	$[M-TMS-CH_2O-TMS-2\times HF]^-$
			279 (35)	$[M-TMS-CH_2O-TMS-HF]^-$
			281 (30)	N.A.
			401 (30)	$[M-TMS]^-$
			371 (20)	$[M-TMS-CH_2O]^-$
			341 (10)	$[M-TMS-2\times CH_2O]^-$
			311 (10)	N.A.
			299 (10)	N.A.
d_3-DHPG-TFB-$(TMS)_2$	404	$[M-TMS]^-$	250 (100)	$[M-TMS-CD_2O-CH_2O-92]^-$
			352 (90)	$[M-TMS-CD_2O-HF]^-$
			260 (65)	$[M-TMS-CD_2O-TMS-2\times HF]^-$
			280 (35)	$[M-TMS-CD_2O-TMS-HF]^-$
			282 (30)	N.A.
			404 (30)	$[M-TMS]^-$
			372 (20)	$[M-TMS-CD_2O]^-$
			342 (10)	$[M-TMS-CD_2O-CH_2O]^-$
			314 (10)	N.A.
			300 (10)	N.A.

N.A., not assigned.

centrifugation (4600 × *g*, 5 min, 4 °C), urine was divided into aliquots (1 mL) which were frozen at −20 °C. After thawing of seven of these 1 mL aliquots, the urine samples were spiked with 0, 10, 20, 40, 60, 80 and 100 μL of a 10-μM solution of d_0-DHPG in 40 mM HCl to achieve final added concentrations of 0, 98, 194, 381, 561, 734 and 901 nM, respectively. Subsequently, the samples were spiked with 10 μL aliquots of a 50-μM solution of d_3-DHPG in 40 mM HCl to achieve final concentrations of 495, 490, 485, 476, 467, 459 and 451 nM, respectively (wide range). Similarly was proceeded with a urine sample donated by another healthy volunteer with final added concentrations of 0, 6.7, 13.3, 20.0, 26.7 and 33.3 nM of d_0-DHPG and a fixed added concentration of 500 nM of d_3-DHPG (narrow range). From these samples, each 100 μL aliquots were withdrawn in triplicate, and analytes were extracted and derivatized as described in Section 2.3. GC–MS/MS analyses were carried out within a single run on the day of sample preparation for each DHPG concentration range.

In the validation experiments, the concentration of measured d_0-DHPG (C_m) was calculated utilizing the following formula (F1):

$$C_m = \left[\left(\frac{PA_m}{PA_{m,IS}}\right) - \left(\frac{PA_b}{PA_{b,IS}}\right)\right] \times C_{IS} \quad \text{(F1)}$$

whereas PA_m and $PA_{m,IS}$ are the measured peak areas of d_0-DHPG and the internal standard (IS) d_3-DHPG in the spiked urine samples, respectively, PA_b and $PA_{b,IS}$ are the respective measured peak areas in the unspiked samples, and C_{IS} is the respective concentration of the internal standard in the urine samples.

Accuracy (recovery, %) was calculated by using the formula (F2):

$$\text{Recovery} = \left[\frac{C_m - C_0}{C_+}\right] \times 100 \quad \text{(F2)}$$

whereas C_m is the measured DHPG concentration in a spiked sample, C_0 is the measured basal concentration of DHPG in the unspiked sample, and C_+ is the concentration of d_0-DHPG added to urine.

The relative lower limit of quantification (rLLOQ) was calculated as described elsewhere [15] using the formula (F3):

$$\text{rLLOQ} = \left[\frac{C_{LLOQ}}{C_{0,Ln}}\right] \times 100 \quad \text{(F3)}$$

whereas C_{LLOQ} is the lowest added DHPG concentration and $C_{0,Ln}$ is the basal DHPG concentration in the urine analyzed.

3. Results

3.1. GC–MS and GC–MS/MS of DHPG derivatives

GC–MS analysis of synthetic d_0-DHPG and d_3-DHPG derivatives from their ethanolic stock solutions revealed three main products with retention times (t_R) of 7.85, 7.93 and 9.29 min and with quite similar peak areas (Fig. 3C).

The GC peaks with t_R 7.85 and 7.93 min possess virtually identical mass spectra and were identified to be isomeric DHPG-PFB-$(TMS)_3$ derivatives (Fig. 3A and B; see Fig. 2). Given the acidity of the aromatic hydroxyl groups, their vicinity and different position with regard to the aliphatic glycol side chain, it is reasonable to assume that each ring hydroxyl group is etherified by PFB-Br during the first derivatization reaction to form the two isomeric 3- and 4-PFB ethers. In the subsequent derivatization step with BSTFA, the three non-reacted hydroxyl groups are etherified to form the TMS ethers (Fig. 4). Under ECNICI conditions, the DHPG-PFB-$(TMS)_3$ derivatives form stable phenolate anions at *m/z* 385, i.e., $[M-PFB]^-$. This ion fragments to a minor extent due to neutral loss of a trimethylsilanol (TMSOH, 90 Da; TMSOD, 91 Da) group from the glycol side chain to form the ion at *m/z* 295 (i.e., $[M-PFB-TMSOH]^-$) for d_0-DHPG and *m/z* 297 (i.e., $[M-PFB-TMSOD]^-$) for d_3-DHPG. The DHPG-PFB-$(TMS)_3$ derivatives were further examined by GC–MS/MS analysis. CID of the precursor ion at *m/z* 385 (i.e., $[M-PFB]^-$) of the d_0-DHPG-PFB-

$(TMS)_3$ derivative revealed a product ion mass spectrum with an intense ion at m/z 295 due to neutral loss of a TMSOH group (90 Da), i.e., $[M-PFB-TMSOH]^-$ from the glycol side chain (Figs. 3A and 4A, Table 1). CID of the precursor ion at m/z 388 of the d_3-DHPG-PFB-$(TMS)_3$ derivative revealed a similar mass spectrum with the most intense ion being m/z 297 due to the neutral loss of a TMSOD group (91 Da; Figs. 3B and 4B, Table 1). The $[M-PFB-TMSOH]^-$ and $[M-PFB-TMSOD]^-$ anions at m/z 295 and 297 seem to be very stable as they loose an oxygen atom to a very minor extent.

The mass spectra of the d_0-DHPG and d_3-DHPG derivatives eluting at 9.29 min contain the most intense ion at m/z 401 and m/z 404, respectively (Fig. 3D and E). This finding and the prod-

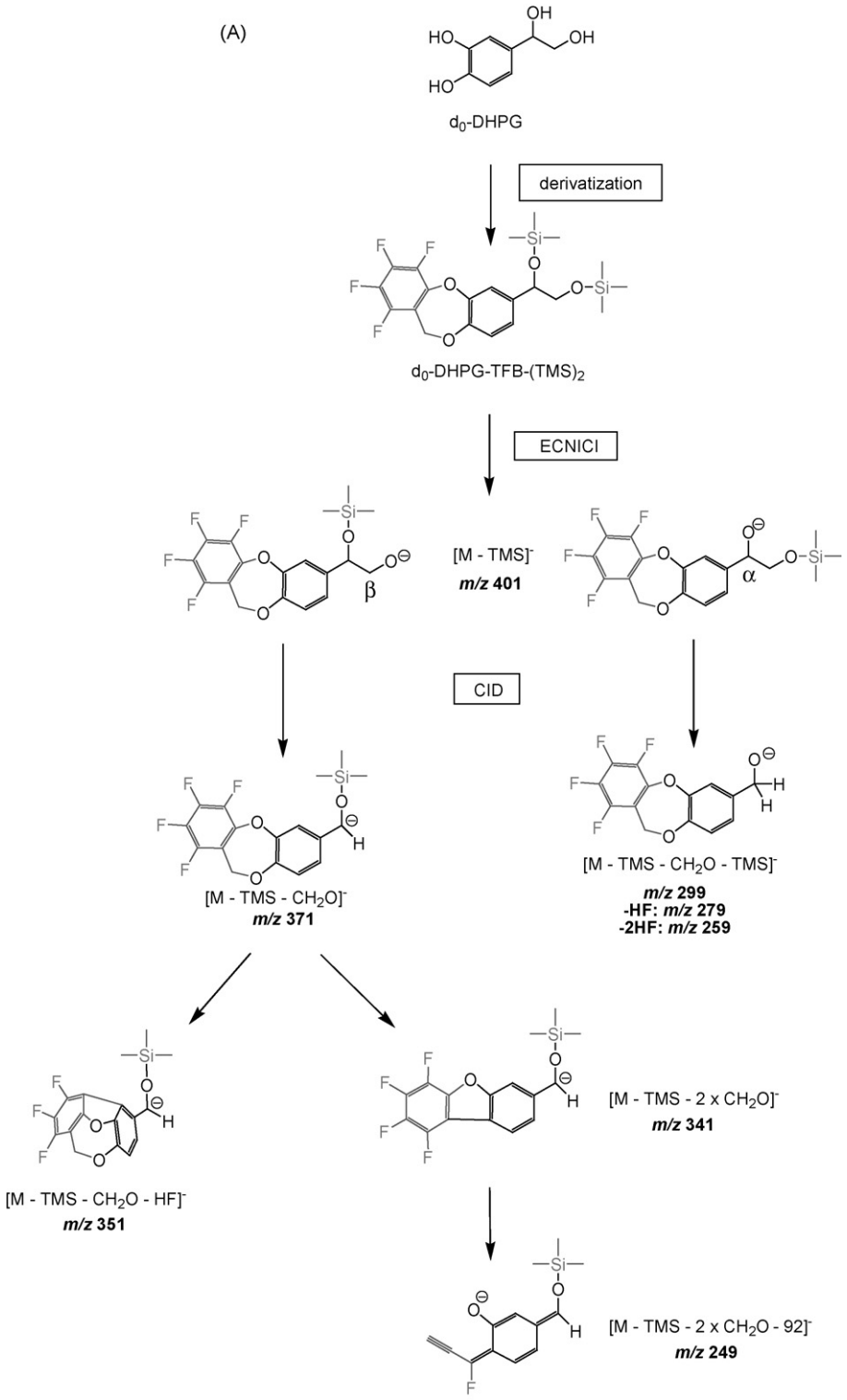

Fig. 5. Simplified schematic of the GC–MS/MS method including derivatization, ECNICI and CID of the d_0-DHPG-TFB-$(TMS)_2$ (A) and d_3-DHPG-TFB-$(TMS)_2$ (B) derivative. Extracts from urine samples are derivatized by PFB-Br in the presence of a base as a catalyst followed by BSTFA to form the DHPG-TFB-$(TMS)_2$ derivative. Under ECNICI conditions the derivative ionizes to form $[M-TMS]^-$. CID of these ions results in the generation of many product ions due to the neutral loss of formaldehyde and TMS group or due to opening of the ring structure. Subsequent loss of one or two HF molecules produces further product ions.

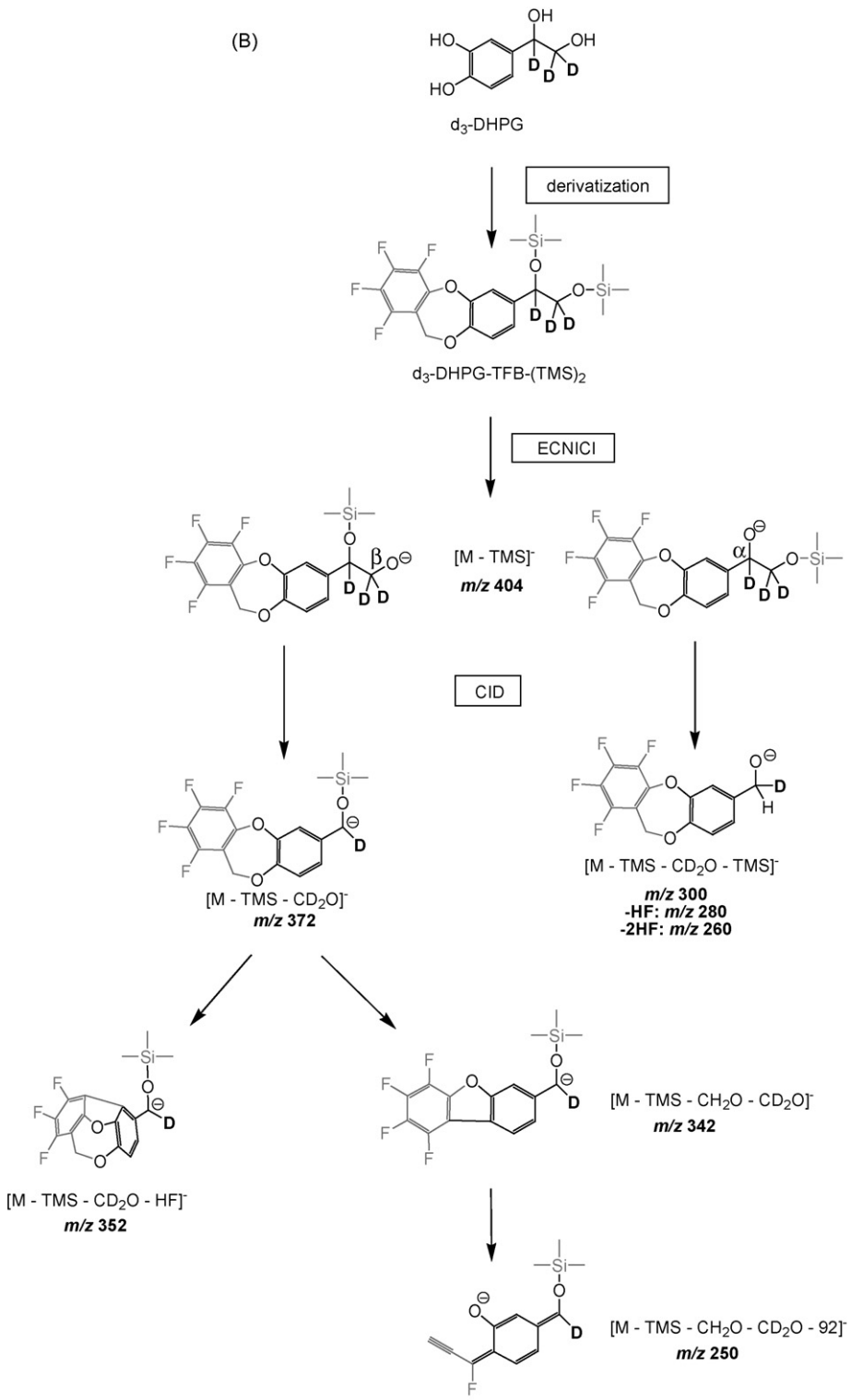

Fig. 5. (*Continued*).

uct ion spectra obtained by CID of *m*/*z* 401 and *m*/*z* 404 suggest that the peak eluting at 9.29 min represents a tetrafluorobenzyl (TFB)-$(TMS)_2$ derivative of DHPG. Despite the lower molecular mass of DHPG-TFB-$(TMS)_2$, as compared to the DHPG-PFB-$(TMS)_3$, the DHPG-TFB-$(TMS)_2$ derivative emerges about 1.5 min later from the GC column than the former. Subjecting the DHPG-TFB-$(TMS)_2$ derivative to CID, a more diverse pattern of product ions was obtained as compared to the DHPG-PFB-$(TMS)_3$ derivatives. The ion at *m*/*z* 401 of d_0-DHPG-TFB-$(TMS)_2$ unveils the three most intense product ions at *m*/*z* 249 (100%), *m*/*z* 351 (90%) and *m*/*z* 259 (65%) (Figs. 3D and 5A, Table 1). The three most intense ions generated by CID of the precursor ion *m*/*z* 404 of d_3-DHPG-TFB-$(TMS)_2$ are *m*/*z* 250 (100%), *m*/*z* 352 (90%) and *m*/*z* 260 (65%) (Figs. 3E and 5B, Table 1).

Taken together, we suggest that the DHPG-TFB-$(TMS)_2$ derivative is produced by reaction of both aromatic hydroxyl groups with

Table 2
Intra-assay accuracy (recovery) and imprecision of the GC–MS/MS method for DHPG in human urine in a wide concentration range.

DHPG added (nM)	DHPG found (nM, mean ± SD, $n = 3$)	Precision (RSD, %)	Accuracy (recovery, %)
0	156 ± 0.62	0.39	N.A.
98	246 ± 0.95	0.39	91.2
194	337 ± 0.11	0.03	93.2
381	483 ± 1.26	0.26	85.9
561	670 ± 0.12	0.01	91.7
734	830 ± 1.71	0.21	91.8
901	959 ± 8.57	0.89	89.2

N.A., not applicable.

one molecule of PFB-Br to form an apparently stable tetrafluoro-[1,4]dioxepin structure in the first derivatization step. In the subsequent silylation reaction with BSTFA the aliphatic hydroxyl groups of the tetrafluoro-[1,4]dioxepin are silylated to form the TMS ethers of the glycol group (Fig. 2; see Section 4).

Because of the unique structure of the DHPG-TFB-$(TMS)_2$ derivative and its superior chromatographic and mass spectrometric properties as compared to the DHPG-PFB-$(TMS)_3$ derivatives, we decided to use the TFB-$(TMS)_2$ derivative of DHPG for quantitative analyses (see also Section 4).

3.2. Standardization of the internal standard and linearity

Discrepancy between declared and measured concentration of commercially available stable-isotope labelled substances is common [16]. Therefore, the concentration of d_3-DHPG used in our study was determined by analysis of mixtures of d_3-DHPG and d_0-DHPG. Linear regression analysis of the peak area ratio (y) of d_3-DHPG to d_0-DHPG from GC–MS/MS analyses of these mixtures against the amount (x) of d_3-DHPG in the mixtures resulted in a straight line ($r^2 = 0.9994$) with a slope value of 0.00248/pmol and a y-axis intercept of 0.00055. The slope value 0.00248 is by a factor of 1.238 higher than the expected theoretical value of 0.002. Therefore, the declared concentration of the d_3-DHPG in the commercially available solution was corrected by this factor.

3.3. Method validation

Tables 2 and 3 summarize the results from the validation experiments in an extended and in a narrow DHPG concentration range, respectively. The values comply with the generally accepted ranges for accuracy and precision. Linear regression analysis between the measured d_0-DHPG concentration (y) and the d_0-DHPG concentration added to human urine samples (x) revealed high linearity ($r^2 = 0.998$), a y-axis intercept of 157 nM and a slope value of 0.901, indicating an overall recovery of 90.1% inthe wide concentration range (Table 2). The corresponding data from the linear regression analysis for the narrow concentration range of added DHPG were 0.879, 73 nM and 0.925 (Table 3).

The rLLOQ, which is calculated by dividing the lowest added DHPG concentration by the basal concentration [15] in the urine, is determined to be 64% in the wide (Table 2) and 9.4% in the narrow (Table 3) DHPG concentration range. Table 3 suggests that DHPG concentrations differing each by 6.7 nM in the range 71–101 nM can be discriminated by the present GC–MS/MS method. Furthermore, in the narrow concentration range we found close correlations between the two DHPG-PFB-$(TMS)_3$ derivatives ($r^2 = 0.995$), as well as between DHPG-TFB-$(TMS)_2$ and the DHPG-PFB-$(TMS)_3$ derivatives ($r^2 = 0.933$ each).

Fig. 6 shows a partial chromatogram acquired from GC–MS/MS analysis of an unspiked urine sample except for the internal standard d_3-DHPG (495 nM). The measured DHPG concentration in this unspiked sample was 156 nM (i.e., 26.2 μg/L), representing the individual's basal DHPG concentration in the urine. Using this chromatogram, the LOD of the method was roughly estimated by taking into account a signal-to-noise ratio (S/N) of 3:1. The peak of the d_0-DHPG-TFB-$(TMS)_2$ derivative in Fig. 6 has a S/N value of 701:1, hence the suggested value of the method's LOD under consideration of an injection volume of 1 μL would be of the order of 200 amol injected DHPG-TFB-$(TMS)_2$. The LOD value was determined by injecting mixtures of synthetic d_0-DHPG (10, 100, 200, 1000 and 2000 fmol) and 2000 or 10000 fmol of d_3-DHPG. A linear relationship was found between the S/N for the DHPG-TFB-$(TMS)_2$ peak and the amount injected (Supplemental Fig. 1), suggesting that in average 1 fmol of DHPG increases the S/N value by about 30:1. On the basis of an S/N of 3:1 the LOD of the method is estimated as 100 amol. In fact, injection of 10 fmol of the DHPG-TFB-$(TMS)_2$ derivative produced a peak with a mean S/N value of 395:1, suggesting an LOD value of 76 amol DHPG.

3.4. Biomedical application of the method

In the present study we applied the GC–MS/MS method to quantify DHPG in urine samples from healthy and ill subjects, which had been collected in a previous study [14]. In all of the 61 analyzed urine samples, endogenous DHPG was detected and quantified. For the benefit of better comparison, DHPG concentration in spot urine is normalized to the urine's creatinine concentration which was measured in our laboratory by GC–MS as previously described [17]. Fig. 7 shows the results from theses DHPG measurements. The mean DHPG excretion rate was measured to be 328 ± 239 nM (range, 43–964 nM), i.e., 39.2 ± 21.9 μg/g creatinine in the healthy subjects and 238 ± 204 nM (range, 9–509 nM), i.e., 38.3 ± 24.2 μg/g creatinine in the patients suffering from chronic inflammatory rheumatism. There was no statistically significant difference in the DHPG excretion rates measured in these groups (Mann–Whitney test).

Table 3
Intra-assay accuracy (recovery) and imprecision of the GC–MS/MS method for DHPG in human urine in a narrow concentration range.

DHPG added (nM)	DHPG found (nM, mean ± SD, $n = 3$)	Precision (RSD, %)	Accuracy (recovery, %)	P value between neighbours
0	71.3 ± 0.35	0.49	N.A.	N.A.
6.7	77.5 ± 2.18	2.81	93.1	0.00315
13.3	84.8 ± 2.25	2.65	101.5	0.01547
20.0	92.0 ± 1.32	1.43	103.5	0.00898
26.7	95.7 ± 1.26	1.32	91.4	0.00238
33.3	100.7 ± 2.36	2.34	88.3	0.03302

N.A., not applicable.

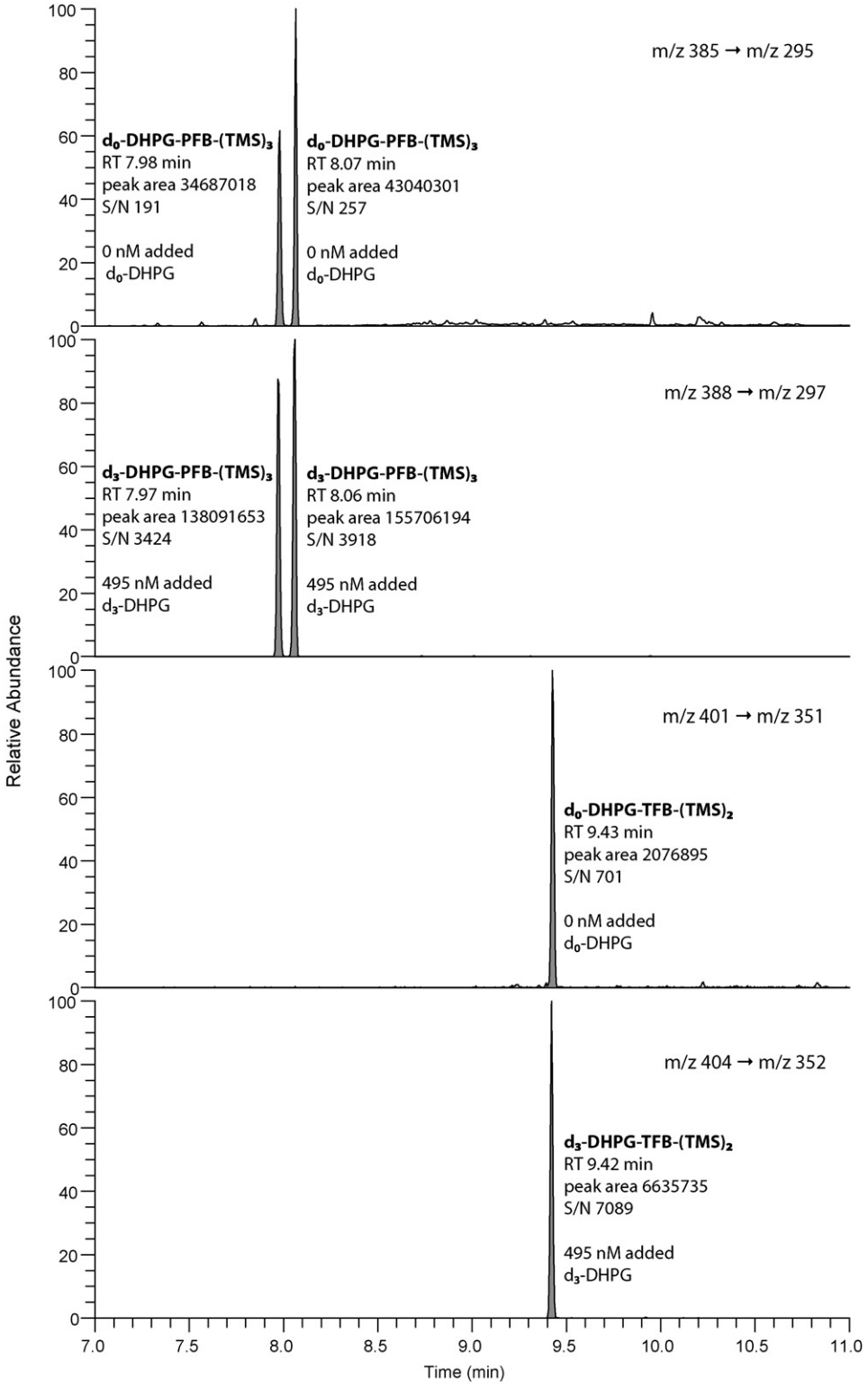

Fig. 6. Partial GC–MS/MS chromatograms derived from an unspiked urine sample (100 μL). The internal standard d_3-DHPG had been added externally to the sample at a final concentration of 495 nM. Mass transitions and other relevant information are provided on the chromatograms: RT, retention time; *S/N*, signal-to-noise ratio; peak area is given in arbitrary units.

4. Discussion

4.1. General

DHPG, a major catecholamine metabolite (Fig. 1), is used as an endogenous biochemical parameter to assess neuronal norepinephrine uptake and metabolism through MAO [18–22]. Commonly, DHGP is measured by HPLC-ECD [6–9]. This methodology is widely applied and provides solid analytical results, even though the required internal standards, such as 3,4-dihydroxybenzylamine for example, are different from DHPG in terms of chemical structure and chromatographic behaviour. The

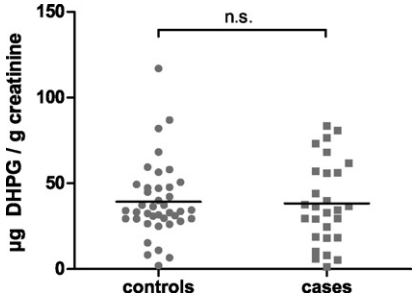

Fig. 7. Results from DHPG measurements in urine of healthy controls (controls) and patients suffering from chronic inflammatory rheumatism (cases). Measured DHPG concentrations were normalized to the creatinine concentration of the patient's urine. Horizontal lines represent the mean values 39.2 μg/g creatinine in the healthy subjects ($n = 40$) and 38.3 μg/g creatinine in the patients ($n = 28$): n.s., not significant.

aim of the present study was to develop a GC–MS/MS method for the specific quantitative determination of DHPG in human urine in the setting of clinical studies.

We report a novel derivatization method using PFB-Br and BSTFA as the derivatization reagents and its application to quantitate DHPG in urine of healthy and ill subjects. To the best of our knowledge, both the PFB-Br derivatization reaction and the mass spectrometry of the derivatives identified have not been reported before in the literature. We found that the formation of the [1,4]dioxepin structure, i.e., in case of DHPG the DHPG-TFB-$(TMS)_2$ derivative, is not limited to DHPG but also applies to aromates that contain two vicinal phenolic hydroxyl groups, such as epinephrine (data not shown). However, the presence of an amino group in the side chain, such as in norepinephrine, seems to have a remarkable influence on the derivatization reaction products, ionization and CID (data not shown). The method for the measurement of DHPG in human urine presented here features the benefits of the stable-isotope dilution technique and the tandem mass spectrometry approach, i.e., high accuracy and specificity.

4.2. PFB-Br derivatization

In gas chromatography-based methods, conversion of analytes into thermally stable, volatile and strongly electron-capturing derivatives is an indispensable requirement and a prerequisite for sensitive analysis [13]. DHPG, 3,4-dihydroxyphenylglycol, possesses two vicinal aromatic groups and two vicinal aliphatic hydroxyl groups which are accessible to derivatization (Figs. 1 and 2). All these OH groups can be silylated within a single derivatization step, for instance by using BSTFA as the derivatization reagent. However, the resulting derivative, i.e., DHPG-$(TMS)_4$, would not allow sensitive detection by GC-ECD or GC–MS in the electron ionization (EI), negative-ion chemical ionization (NICI) or electron-capture negative-ion chemical ionization (ECNICI) mode. Introduction of strongly electron-capturing atoms such as fluorine (F) in the derivatives would be necessary. PFB-Br and other (per)fluorated derivatization reagents such as pentafluoropropionic anhydride are useful reagents to derivatize various compounds including carboxylic acids, phenols, and amines for detection of as little as a few femtomoles of these analytes. This methodology has been successfully applied by us and others in the analysis of fatty acids and their oxygenated metabolites [13], 3-nitrotyrosine [23], and histamine [24] amongst others.

Given their considerable acidity, both phenolic OH groups of DHPG should be susceptible to derivatization with PFB-Br, i.e., to their conversion to PFB ether derivatives. By contrast, the two aliphatic OH groups of the glycol side chain of DHPG lack acidity and should not be susceptible to derivatization with PFB-Br. Indeed, we observed that PFB-Br is not suitable for aliphatic OH group etherification of hydroxylated fatty acids [13] and DHPG (data not shown). Therefore, highly sensitive determination of DHPG by GC–MS or GC–MS/MS in the ECNICI mode requires a two-step derivatization process, in which the first derivatization reaction is specific for the two aromatic hydroxyl groups of DHPG. The second derivatization reaction with BSTFA is then used to simply etherify the two aliphatic hydroxyl groups as well as – under certain circumstances – non-reacted phenolic OH groups for the purpose of higher thermal stability and improved gas chromatography. We found that pentafluopropionic anhydride allows for pentafluoropropionylation of DHPG. However, pentafluoropropionyl ester derivatives lack stability.

Analysis of PFB-Br/BSTFA derivatization products of unlabeled and deuterium labelled DHPG under ECNICI conditions revealed three derivatization products. The GC peaks eluting at 7.85 and 7.93 min have virtually identical MS and MS/MS spectra and were identified as isomeric DHPG-PFB-$(TMS)_3$. Apparently, during the first derivatization step with PFB-Br in acetonitrile with a base as a catalyst, the 3-hydroxy and 4-hydroxy groups of the aromatic ring are alkylated to form 3- and 4-PFB-DHPG ethers to a similar extent, given the comparable GC peak areas of the final derivatives (Fig. 3C). The remaining three hydroxyl groups, i.e., one phenolic OH group on position 3 or 4 and two aliphatic OH groups readily react during the second derivatization step with BSTFA to form tri-TMS ethers, DHPG-PFB-$(TMS)_3$. Thus, the two GC peaks at 7.85 and 7.93 min are 1,2,3-tri-TMS-4-PFB-DHPG and 1,2,4-tri-TMS-3-PFB-DHPG, respectively (Figs. 2 and 3). Our results do not allow for a definite assignment of the two isomeric DHPG-PFB-$(TMS)_3$ derivatives to the GC peaks. It is worth mentioning that these derivatives were also obtained from derivatization of DHPG directly in aqueous buffers and urine (data not shown).

The compound eluting at 9.29 min was identified as a DHPG-TFB-$(TMS)_2$ derivative. This derivative was observed when PFB-Br derivatization was performed in anhydrous acetonitrile but not in aqueous buffer or urine, unlike the isomeric DHPG-PFB-$(TMS)_3$ derivatives. This observation together with the MS and MS/MS spectra of the DHPG-TFB-$(TMS)_2$ derivative lead to the conclusion that one PFB-Br molecule reacts with both aromatic hydroxyl groups during the first derivatization in anhydrous acetonitrile to form the tetrafluoro-[1,4]dioxepin derivative of DHPG. In theory and in analogy to the two isomeric DHPG-PFB-$(TMS)_3$ derivatives, the DHPG-TFB-$(TMS)_2$ derivative could also compose of two isoforms (Fig. 2). The observation of a single GC peak at 9.29 min of a similar peak width to the GC peaks at 7.85 and 7.93 (Fig. 3C) argues for the formation of one isomer. However, formation of two isomeric coeluting DHPG-TFB-$(TMS)_2$ derivatives cannot be excluded. In our opinion, the bulky tetrafluorobenzyl residue of the DHPG-TFB-$(TMS)_2$ derivative could minimize and even diminish the influence of the 3- and 4-hydroxy groups of DHPG on GC separation.

Fig. 5 illustrates the proposed mechanism for the formation of the two isomeric DHPG-TFB-$(TMS)_2$ derivatives. In the first derivatization step PFB-Br reacts with the 3-hydroxy or 4-hydroxy groups of DHPG to form the corresponding PFB ethers. A part of these PFB ethers undergoes intra-molecular nucleophilic substitution. The phenolate anion attacks the PFB ring on position 2, whereby a fluorine anion leaves the molecule, to finally generate the [1,4]dioxepin structure. The observation that the DHPG-TFB-$(TMS)_2$ derivative is not formed in aqueous buffer of neutral pH value or in urine samples (pH about 5–7) suggests that strong alkaline conditions and non-aqueous media are required for its formation. Such conditions seem to prevail in anhydrous acetonitrile in the presence of the base *N*,*N*-diisopropylethylamine (10 vol.%). It is worth mentioning that a similar reaction has been observed under drastic reaction conditions (i.e., under reflux at 153 °C) during the synthesis of 2-nitro-11*H*-dibenzo[*b*,*e*][1,4]dioxepin, starting from the cate-

chol 1,2-dihydroxybenzene and 2-chloro-5-nitrobenzyl chloride, in anhydrous dimethylformamide as the solvent and potassium *t*-butyloxide which is a very strong base [25]. Because of the higher reactivity of the benzyl group of PFB-Br towards nucleophiles, it is likely that one phenolate group of DHPG reacts via an aliphatic nucleophilic substitution with the benzyl group of PFB-Br to form the PFB ether of DHPG, i.e., DHPG-PFB, with bromine being the leaving group. Subsequently, a part of DHPG-PFB undergoes cyclization via aromatic nucleophilic substitution of the F atom at position 2 which leaves the molecule as fluorine to finally form the [1,4]dioxepin DHPG-TFB-$(TMS)_2$.

Performance of PFB-Br derivatization in anhydrous acetonitrile requires preceding extraction of DHPG from a biological sample. Solvent extraction with water-immiscible solvents such as ethyl acetate seems to be a very practical and time-saving method. Adjustment of the pH value of the urine sample prior to extraction is not necessary.

4.3. GC–MS and GC–MS/MS of the DHPG-TFB-$(TMS)_2$ derivative

The fact that the respective product ions from d_0-DHPG and d_3-DHPG evolving from the precursor $[M-TMS]^-$ differ only by 1 Da each, indicates that this difference is exclusively due to the presence of a single deuterium atom in these product ions. Fig. 5 shows two proposed fragmentation mechanisms. In the case of a TMS loss from the TMS ether at the β-position of the glycol residue, the precursor ion $[M-TMS]^-$ (*m*/*z* 401) looses a formaldehyde molecule (HCHO, 30 Da) to form the product ion at *m*/*z* 371, from which one HF molecule (20 Da) is subsequently loosen to form the ion at *m*/*z* 351, presumably a tricyclic anion. Remarkably, in addition to this rather common CID, the product ion at *m*/*z* 371 seems to undergo two unusual fragmentations. On the one hand, rearrangement induced by loss a formaldehyde molecule from the former pentafluorobenzyl group produces the ion *m*/*z* 341 which dissociates to finally produce the highly conjugated and obviously stable major product ion at *m*/*z* 249. These considerations also apply to the d_3-DHPG-TFB-$(TMS)_2$ derivative (Fig. 5).

In the case of a TMS neutral loss from the TMS ether at the α-position of the glycol residue during ECNICI, fragmentation of the ion $[M-TMS]^-$ (*m*/*z* 401) under CID conditions is likely to include neutral losses of a second TMS (72 Da) group and subsequently a formaldehyde molecule (30 Da) resulting in a total neutral loss of 102 Da for d_0-DHPG and 104 Da for d_3-DHPG. Finally, subsequent loss of two HF molecules from *m*/*z* 299 and *m*/*z* 300 yields the ions at *m*/*z* 279 and *m*/*z* 280 as well as *m*/*z* 259 and *m*/*z* 260, respectively (Fig. 5).

It is worthy of mention, that not all of the product ions from $[M-TMS]^-$ of the DHPG-TFB-$(TMS)_2$ derivative keep their [1,4]dioxepin structure under CID conditions.

4.4. Excretion of DHPG in urine in health and chronic inflammatory disease

On a molar basis, the DHPG-PFB-$(TMS)_3$ derivatives have about 20 times higher peak areas than the DHPG-TFB-$(TMS)_2$ derivative (Fig. 6). Given the unusual structure and unusual CID of the DHPG-TFB-$(TMS)_2$ derivative the transitions *m*/*z* 401 → *m*/*z* 351 for endogenous DHPG and *m*/*z* 404 → *m*/*z* 352 for the internal standard d_3-DHPG were used to reliably quantitate DHPG in human urine. Indeed, analysis of the DHPG-TFB-$(TMS)_2$ derivative in this GC–MS/MS method was found to be accurate and precise for DHPG in human urine samples in relevant concentration ranges of up to 901 nM (Table 2). A typical chromatogram from the analysis of DHPG in a human urine sample is shown in Fig. 6. In this GC–MS/MS analysis both the DHPG-PFB-$(TMS)_3$ and the DHPG-TFB-$(TMS)_2$ derivative were monitored within the same run. In all urine samples analyzed we observed two DHPG-PFB-$(TMS)_3$ GC peaks and the DHPG-TFB-$(TMS)_2$ GC peak, indicating that the used conditions of the PFB-Br derivatization procedure constantly produced three GC peaks due to DHPG-PFB-$(TMS)_3$ (two peaks) and the DHPG-TFB-$(TMS)_2$ (one peak). It is worth mentioning the observation of an unknown interfering compounds which coeluted with the second later eluting DHPG-PFB-$(TMS)_3$ derivative (Fig. 6) and contributed artefactually to endogenous DHPG (data not shown).

The DHPG concentration in the unspiked urine sample used for method validation in the wide concentration range was measured as 156 nM (i.e., 26.2 μg/L) with a creatinine-normalized DHPG concentration in this sample of 37.1 μg/g creatinine. Based on a mean excretion rate of 10–15 mmol creatinine per day, daily urinary DHPG excretion is estimated to be in the range of 80–120 μg. This value is well in the order of previously reported data for urine DHPG [4,8,19,26–29]. In the healthy and ill volunteers of our study we observed a quite wide range for urinary DHPG concentrations. This observation agrees well with the range reported by Duncan et al. for hypertensive patients without (n = 1061) or with pheochromocytoma (200–6756 nmol DHPG per 24 h, n = 25) in 24 h collected urine samples as measured by GC–MS [29].

As an example for the utility of the present GC–MS/MS method we measured DHPG excretion rates in healthy and ill subjects. Previous studies suggested that the regulation of the sympathetic nervous system may be altered in patients with rheumatological disorders and other autoimmune diseases, such as inflammatory bowel disease [30]. The issue is potentially relevant because catecholamines released from sympathetic nerve endings may affect immune cell function which could conceivably modulate the disease course. In our study, the creatinine-normalized DHPG excretion in urine samples from 61 subjects were in the range 1.3–117 μg/g creatinine, i.e., within reported ranges. In contrast, we recently showed that nitrative stress, measured as 3-nitrotyrosine excretion, is elevated in this disease [14]. It is worth mentioning that both DHPG and 3-nitrotyrosine derive from the same amino acid, i.e., phenylalanine. In our study, patients suffering from chronic inflammatory rheumatism and healthy subjects showed similar creatinine-corrected DHPG excretion rates. The observations exclude a major difference in systemic sympathoneuronal tone in these patients. However, we cannot rule out subtle or organ specific changes in sympathetic regulation.

5. Conclusion

We developed an accurate, precise and highly specific and sensitive GC–MS/MS method for the reliable measurement of DHPG in human urine. This method involves a rapid solvent extraction of endogenous d_0-DHPG and the added internal standard d_3-DHPG from urine samples without any pH adjustment and a two-step derivatization procedure by PFB-Br and BSTFA. The PFB-Br derivatization of DHPG in anhydrous acetonitrile yields three derivatives, i.e., two isomeric DHPG-PFB-$(TMS)_3$ derivatives, and apparently a single later eluting DHPG-TFB-$(TMS)_2$ derivative. The PFB-Br derivatization involves a unique reaction mechanism that has not been demonstrated for this derivatization reagent and DHPG before. Also, the DHPG-TFB-$(TMS)_2$ derivative possesses unique structure, undergoes unusual CID and is best suitable for specific DHPG quantification in human urine.

In our experience, the present method is suitable for clinical studies on whole body catecholamine metabolism. Indeed, we successfully applied the method to assess sympathetic regulation in healthy subjects and in patients suffering from chronic inflammatory rheumatism. DHPG excretion rate measurements ranging between 10 and 100 μg/g creatinine support the validity of this GC–MS/MS method. In addition, this method could be useful for

the measurement of DHPG in plasma and other biological samples. Also, endogenous and exogenous aromatic substances that contain two vicinal acidic hydroxyl groups in their aromatic rings, i.e., a catechol structure, including norepinephrine itself, could possibly be determined by the present method, provided they are extractable from their matrixes by water-immiscible solvents.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.06.022.

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