Monitoring of the deuterated and nondeuterated forms of levodopa and five metabolites in plasma and urine by LC–MS/MS

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To compare pharmacokinetics, metabolism and excretion of levodopa and a triply deuterated form, which is being developed as an improved treatment for Parkinson’s disease, methods were needed for quantification of the deuterated and nondeuterated forms of levodopa and five metabolites in human plasma and urine. Results: The natural heavy isotopes in the nondeuterated compounds caused an absolute contribution of up to 100% in the response of the deuterated compounds. Similarly, heavy isotopes in the deuterated analytes contributed to the response of the internal standards, but this did not affect the reliability of the results. Conclusion: Deuterated and nondeuterated analytes can be quantified together by LC–MS/MS, but overestimation of the concentrations of the deuterated molecules may be unavoidable and a careful interpretation of the concentration data is essential.

First draft submitted: 7 September 2018; Accepted for publication: 17 December 2018; Published online: 21 February 2019

Keywords: deuterated drugs • interference • LC–MS/MS • levodopa • natural heavy isotopes

It has been long known that the covalent bond between carbon and deuterium (C-D) in organic molecules usually is more stable than the corresponding carbon–hydrogen (C-H) bond. One of the consequences of this phenomenon is that the selective replacement of one or more hydrogen atoms in a drug molecule by deuterium may slow down its metabolism, if breaking of that particular C-H bond is a rate-limiting step in the metabolic process [1,2]. This so-called kinetic isotope effect (KIE) offers potential benefits such as an increased half-life of the drug, and thus improved exposure, and/or decreased formation of toxic metabolites, while the pharmacological effect of the drug typically remains unaltered.

Although the success of applying deuterium in drug development has proven rather unpredictable because of the occurrence of competing effects that can mask the kinetic isotope effect in biological systems, there has been increasing interest to explore the potential of deuterated drugs, notably over the last decade [3,4]. In particular, the targeted deuteration of registered drugs, whose efficacy and safety profiles are well-known, may provide an efficient and relatively fast approach to develop new medicines with significantly improved properties. Several deuterated drug candidates are now being investigated in clinical trials and recently the first deuterated drug, deutetrabenazine, was approved by the US FDA [5].

To evaluate the in vivo fate of deuterated drugs and to compare their pharmacokinetics and excretion to that of the corresponding nondeuterated molecules, the availability of proper bioanalytical strategies is essential. The ideal analytical method allows the quantification of the deuterated drug and its relevant metabolites as well as their nondeuterated counterparts in several biological fluids. Since the most notable difference between the deuterium- and hydrogen-containing molecules is their molecular mass, the use of mass spectrometry to distinguish the two
forms is indispensable. In this paper, we report an approach based on LC–MS/MS for the monitoring of a triply deuterated drug candidate and five of its metabolites plus their nondeuterated forms in human plasma and urine.

The drug of interest in this investigation is levodopa, which has been the standard treatment of Parkinson’s disease for several decades. Levodopa is able to cross the blood–brain barrier (BBB) and is converted to dopamine locally to supplement the reduced endogenous levels of dopamine in the basal ganglia of patients [6]. Despite the proven efficacy of levodopa, there is need for improved therapeutic options, since about half of the patients treated with levodopa develop motoric complications within 5 years of treatment [7]. Like the nondeuterated form, deuterated levodopa crosses the BBB and is converted to deuterated dopamine, which is anticipated to exhibit identical receptor binding characteristics as nondeuterated dopamine and thus to have the same pharmacological effect. Results in animals have shown that, compared with levodopa, the administration of triply deuterated levodopa enhanced dopamine levels in the striatum and improved locomotor activity [8], which might be due to reduced degradation of deuterated dopamine in the brain. Consequently, treatment with triply deuterated levodopa has the potential to result in longer exposure of the brain to dopamine and may thus improve its potency and allow for dose reduction relative to nondeuterated levodopa [9]. Levodopa is extensively metabolized to several other metabolites (Figure 1) both in the brain and peripherally, and monitoring the concentrations of these metabolites in plasma and urine is important to investigate the disposition of deuterated levodopa relative to the normal form [10].

There are some aspects of the quantification of deuterated compounds that need special attention when developing an analytical method. Because of the natural occurrence of heavy stable isotopes, in particular $^{13}$C, in organic
molecules, a small but significant percentage of any nondeuterated (small molecule) drug will have a molecular mass that is one or more units higher than the nominal, monoisotopic mass. Such a heavy form of the nondeuterated drug may generate an interfering response in the MS/MS mass transition of the corresponding deuterated drug, in particular if the number of deuterium atoms is small. This is especially important if the nondeuterated and deuterated forms of a drug are present together in a sample, such as in the case of drugs that also occur endogenously like levodopa. Related to this, the choice for a proper internal standard (IS) for response normalization becomes more complicated. Ideally, the IS is a stable-isotope labeled (SIL) form of the drug with sufficient heavy isotope atoms to allow its distinction from both the nondeuterated and the deuterated drug, but it may be a challenge to obtain such a compound. The current paper describes how these issues were addressed in a study that was executed to compare the pharmacokinetics, metabolism and excretion of unlabeled and deuterated levodopa.

**Experimental section**

**Reagents & materials**

Levodopa and its metabolites 3-O-methyldopa (3-OMD), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), dopamine and 3-methyltyramine (3-MT) were obtained from Sigma (MO, USA). Triply deuterated levodopa was provided by Teva (PA, USA), triply deuterated 3-OMD, dopamine and 3-MT by Chiracin (Luekenwalde, Germany) and doubly deuterated forms of DOPAC and HVA by Pharmaron (Beijing, China). The ISs levodopa-d6, DOPAC-d5, HVA-d5 and dopamine-13C6 were purchased from CDN Isotopes (Pointe Claire, MON, Canada), 3-OMD-d6 from Cephalon (Malvern, PA, USA) and 3-MT-13C-d5 from Alsachim (Illkirch, France). Structures of all compounds are presented in Supplementary Figure 1. Sodium metabisulfite (SMB), butylated hydroxytoluene (BHT), dansylchloride, propionic anhydride and pyridine were obtained from Sigma. All other chemicals came from Merck (Darmstadt, Germany). LC grade water was prepared using a Milli-Q purification system (Merck). Blank human plasma (on dipotassium EDTA) was obtained from Seralab (West Sussex, UK) and blank human urine was donated locally by healthy volunteers after giving appropriate written consent.

**Standards & samples**

All solutions and samples were prepared and handled on wet ice and under yellow light. Separate stock solutions (1.00 mg/ml for all 12 analytes and all six ISs) were prepared in stock solvent: ice-cold 1.5% aqueous acetic acid containing 5 mg/ml SMB. They were divided into multiple, single-use aliquots and stored at -70°C. Appropriate dilutions in the same solvent were freshly prepared directly before use. The stock and diluted solutions were used to prepare calibration standards by adding small (<5% v/v) volumes to ice-cold analyte-free calibration matrix, that had been stabilized with 5 mg/ml SMB and 1 mg/ml BHT (plasma methods) or 0.1 M hydrochloric acid (urine methods). Levodopa, 3-OMD, DOPAC and HVA and their deuterated forms were quantified together; the calibration matrices were doubly charcoal-stripped human K2EDTA plasma (obtained from Seralab) and synthetic urine (14.1 g/l of sodium chloride, 2.8 g/l of potassium chloride and 17.3 g/l of urea in water). For dopamine, 3-MT and their deuterated forms, that were coquantified, the calibration matrices were human plasma and urine that had been incubated overnight at 37°C under stirring. Quality control (QC) samples were prepared in the same way. For the lower limit of quantitation (LLOQ) level, stabilized calibration matrix was used, for the other levels human plasma and urine that had been incubated overnight at 37°C under stirring and subsequently stabilized. The following calibration ranges were covered (values rounded to three significant digits): levodopa, HVA and their deuterated forms in plasma: 5.00–5000 ng/ml; 3-OMD, DOPAC and their deuterated forms in plasma: 10.0–5000 ng/ml; dopamine, 3-MT and their deuterated forms in plasma: 50.0–20,000 pg/ml; levodopa, 3-OMD, DOPAC, HVA and their deuterated forms in urine: 0.100–50.0 μg/ml, dopamine and deuterated dopamine in urine: 0.100–50.0 μg/ml. An overview of the individual calibration and QC levels is presented in Supplementary Tables 1 and 2. All calibration and QC samples were divided into aliquots, which were frozen in acetone/dry ice and stored protected from light at -70°C. IS working solutions were prepared in stock solvent at 500 ng/ml (plasma method) and 5000 ng/ml (urine method) for each of the ISs for levodopa, 3-OMD, DOPAC and HVA. The IS working solution for dopamine and 3-MT was made in stock solvent at 2.00 and 2.50 ng/ml (plasma method) and at 2000 ng/ml (urine method, dopamine only), respectively. All working solutions were divided into single-use aliquots and stored at -70°C.
Collection of study samples
The clinical study with EudraCT number 2015-004525-13 was approved by the Independent Ethics Committee BEBO (Beoordeling Ethiek Biomedisch Onderzoek) in Assen, The Netherlands; and written informed consent was given by all subjects (healthy volunteers). An oral dose of 150 mg levodopa or triply deuterated levodopa was administered, each in combination with 37.5 mg carbidopa. Blood samples (6 ml) were collected in prechilled K$_2$EDTA vacuum tubes, to which 60 $\mu$l of an aqueous 250 mg/ml SMB solution had been added. After mixing, the tubes were placed on wet ice, transported to a room with yellow light and centrifuged for 10 min at 1500 $\times$ g at 4°C. Subsequently, 2.4 ml of the resulting plasma was transferred to a tube containing 48 $\mu$l of an ice-cold 50 mg/ml ethanolic BHT solution. The stabilized plasma samples were split into six aliquots, which were frozen in acetone/dry ice and stored at -70°C protected from light. Immediately after voiding, an aliquot of 5 $\mu$l of each urine sample was mixed with 75 $\mu$l of ice-cold 6 M hydrochloric acid and stored at -70°C and protected from light.

Sample preparation for levodopa, 3-OMD, DOPAC, HVA & deuterated forms
All sample handling was performed under yellow light. All plasma and urine samples were thawed at ambient temperature and directly after thawing transferred to wet ice. For plasma, aliquots of 100 $\mu$l were pipetted into the 1.2 ml wells of a 96-deep-well plate (VWR, Amsterdam, The Netherlands), which was kept on melting ice, and mixed with 50 $\mu$l of the IS working solution. Proteins were removed by the addition of 100 $\mu$l of ice-cold methanol, vortex-mixing, addition of 200 $\mu$l of ice-cold acetonitrile, vortex mixing and centrifugation for 5 min at 3500 $\times$ g and 4°C. Two aliquots of 150 $\mu$l of the supernatant were subsequently transferred to an Ostro (Waters, MA, USA) phospholipid removal plate. The eluate was mixed with 100 $\mu$l of 0.5 M sodium carbonate solution (pH 10) and 200 $\mu$l of a freshly prepared solution of 1 mg/ml dansyl chloride in acetone. The plate was covered with a mat and incubated at 60°C for 20 min, under vortex-mixing (1000 r.p.m.) in an Eppendorf (Hamburg, Germany) thermomixer. The acetone was evaporated from the mixture under a gentle stream of nitrogen at 40°C for 30 min and 50 $\mu$l of 20% aqueous formic acid were added. Subsequently, the derivatized extracts were placed in an autosampler kept at 4°C.

Aliquots of 50 $\mu$l urine were pipetted into a 96-deep-well plate (well volume 1.2 ml), kept on melting ice, and 50 $\mu$l of the IS working solution and 800 $\mu$l of water were subsequently added. After mixing, 300 $\mu$l was transferred to a new plate and 100 $\mu$l of 0.5 M sodium carbonate solution (pH 10) and 200 $\mu$l of a freshly prepared solution of 1 mg/ml dansyl chloride in acetone were added. The rest of the sample preparation workflow was identical to the one for plasma.

Sample preparation for dopamine, 3-MT & deuterated forms
All sample handling was performed under yellow light. All plasma and urine samples were thawed at ambient temperature and directly after thawing transferred to wet ice. For plasma, aliquots of 100 $\mu$l were pipetted into the 1.2 ml wells of a 96-deep-well plate, which was kept on wet ice, and 50 $\mu$l of the IS working solution was added. Proteins were precipitated by the addition of 250 $\mu$l of ice-cold acetonitrile, vortex mixing and centrifugation for 5 min at 3500 $\times$ g and 4°C. Subsequently, 200 $\mu$l of the supernatant was transferred to a new 96-deep-well plate in a fume hood and 200 $\mu$l of a freshly prepared mixture of acetonitrile, propionic anhydride and pyridine (45:5:1, v/v/v) was added. The plate was covered with a mat and incubated at 60°C for 45 min, under vortex mixing (1000 r.p.m.) in a thermomixer. The sample was evaporated to dryness under a gentle stream of nitrogen at 60°C for 45 min and 200 $\mu$l of 10% aqueous acetonitrile were added. Subsequently, the derivatized extracts were placed in an autosampler kept at 4°C.

In a 1.2 ml well of a 96-deep-well plate, kept on melting ice, 100 $\mu$l of urine and 100 $\mu$l of the IS working solution were mixed. In a fume hood, 200 $\mu$l of a freshly prepared mixture of acetonitrile, propionic anhydride and pyridine (45:5:1, v/v/v) was added. The plate was covered with a mat and incubated at 60°C for 30 min, under vortex mixing (1000 r.p.m.) in a thermomixer. Subsequently, an aliquot of 50 $\mu$l of the sample was transferred to a new plate and diluted with 500 $\mu$l of ice-cold acetonitrile. This dilution step was repeated in the same way. The derivatized extracts were then transferred to an autosampler kept at 4°C.

Chromatography & detection for levodopa, 3-OMD, DOPAC, HVA & deuterated forms
An Agilent (CA, USA) 1290 Infinity II pump, autosampler and column heater were used for chromatography. For plasma, aliquots of 20 $\mu$l of the derivatized plasma extracts were injected onto a Waters HSS T3 column (50 $\times$ 2.1 mm, 1.8 $\mu$m particles) maintained at 40°C. The derivatives of the analytes and ISs were separated by gradient
elution at 0.7 ml/min with a mobile phase consisting of a mixture of 0.1% aqueous formic acid (A) and acetonitrile (B). The A/B ratio changed linearly from 70:30 (v/v) to 40:60 (v/v) between 0 and 8 min after injection. The composition was then kept at 40:60 (v/v) for 1 min and at 10:90 (v/v) for 1 min and finally at 70:30 (v/v) from 10 to 11 min after injection. A Model 6500 MS/MS (AB Sciex, Concord, ON, Canada) was used for detection, using positive electrospray ionization in the multiple reaction monitoring (MRM) mode at an interface temperature of 750°C and an ionspray voltage of 5500 V. The mass transitions for each of the analytes and ISs are presented in Table 1.

For urine, the settings were the same as for plasma with the following exceptions. A Waters CSH C18 column (100 × 2.1 mm ID, 1.7 μm particles) was used and an injection volume of 10 μl. The gradient profile (0.5 ml/min) was a linear increase from 20% B to 35% B between 0 and 3 min after injection, followed by 35% B for 4 min, a step to 40% B for the next 6 min, a step to 90% B for another minute and re-equilibration at 20% B for 2 min.

### Chromatography & detection for dopamine, 3-MT & deuterated forms

The same LC and MS systems were used as for the plasma method for levodopa. For plasma, the injection volume was 15 μl, the column was an HSS T3 (1.8 μm particles, 100 × 2.1 mm) at a temperature of 45°C and the mobile phases were 0.1% aqueous formic acid (A) and methanol (B). A linear gradient of 40–45% B over 8.5 min after injection was used, followed by 1 min at 90% B and 1.5 min at 40% B, at a flow rate of 0.5 ml/min. The MS interface temperature was 650°C and the ionspray voltage 3000 V. The mass transitions are shown in Table 1.

For urine, the injection volume was 2 μl, the column was an HSS T3 (1.8 μm particles, 50 × 2.1 mm) at a temperature of 45°C and the mobile phases were 0.1% aqueous formic acid (A) and acetonitrile (B). Elution was isocratic at 30% B for 2.4 min after injection, followed by a step gradient to 90% B for 1 min and re-equilibration at 30% B until 5 min after injection, all at 0.7 ml/min. MS settings were the same as for the plasma method.

### Results & discussion

#### General

After oral administration, levodopa is converted into several metabolites, of which 3-OMD, DOPAC, HVA, dopamine and 3-MT (Figure 1) were judged most important to study the in vivo fate of the drug with and without deuteration [10]. We decided to set up two separate plasma methods, one for dopamine and 3-MT and one for the others, mainly because dopamine and 3-MT are present in plasma at about 100–200-fold lower levels and because these two metabolites are bases, while the others are acids (DOPAC and HVA) or amino acids (levodopa...
Figure 2. Stability of levodopa (A) and 3,4-dihydroxyphenylacetic acid (B) in human EDTA plasma. Under several storage conditions and in the presence of 5 mg/ml SMB and/or 1 mg/ml BHT. Average of duplicate measurements. BHT: Butylated hydroxytoluene; SMB: Sodium metabisulfite.

and 3-OMD). For urine, a similar distinction was made. Since all (nondeuterated) analytes occur endogenously in human plasma and urine, and it was deemed necessary to obtain information about the endogenous background levels, low (5–10) ng/ml sensitivity was required for levodopa, 3-OMD, DOPAC and HVA in plasma, while for dopamine and 3-MT levels down to 50 pg/ml needed to be quantified [11,12].

Sample collection

The catechol-containing analytes levodopa, DOPAC and dopamine are very sensitive to oxidative degradation and therefore several stabilization procedures were evaluated. As we reported earlier for the structurally similar compound carbidopa [13], stabilization was required directly after blood withdrawal to avoid unacceptable analyte degradation during plasma preparation. The combination of two anti-oxidants, SMB and BHT, plus storage of the plasma samples on ice water and under yellow light, gave optimal stability of all analytes; more than 90% remaining up to 4 h of storage (see Figure 2 for the two most unstable analytes). SMB was added to the vacuum tube to guarantee adequate stability during plasma collection (up to 2 h on ice water). To enhance longer-term stability, BHT was subsequently added to the plasma sample. Since BHT is insoluble in water, an ethanolic solution had
to be used, and this could not directly be added to the blood because it induces hemolysis. Acidification of urine directly after voiding (which lowers the pH to about 1.5 regardless of the initial urine pH) and immediate storage at -70°C provided sufficient stability.

Sample handling
Because of the endogenous nature of the nondeuterated analytes, an analyte-free proxy matrix was needed to prepare calibration standards with known analyte levels [14]. Background levels of the catechol-containing analytes (levodopa, DOPAC and dopamine) could be oxidatively removed from both plasma and urine by incubation of the matrix under stirring. Since endogenous 3-MT plasma levels were sufficiently low and 3-MT quantification in urine was not required, authentic plasma and urine after incubation could be used as calibration matrices for the methods for dopamine and 3-MT. For 3-OMD and HVA, background levels could not be oxidatively removed and another calibration matrix was needed. Doubly charcoal-stripped plasma and synthetic urine were found to be analyte-free, but the derivatization efficiency for levodopa and metabolites was different in charcoal-stripped plasma than in authentic plasma, leading to unacceptable accuracies for spiked plasma when quantified against calibrators in charcoal-stripped plasma. Matching results were found after phospholipids had been removed from both matrices and, therefore, a phospholipid removal step was included in the workflow for the plasma method for levodopa, 3-OMD, DOPAC and HVA.

Derivatization & LC–MS analysis
Many approaches for the determination of levodopa and structurally related compounds in biological samples have been described, in the past decade primarily LC–MS/MS was used. Since this technique has an inherently poor sensitivity and selectivity for the analytes because of their relatively low molecular mass and unselective fragmentation behavior, derivatization is required to quantify down to the clinically relevant levels [13,15–17]. For the simultaneous determination of levodopa, 3-OMD, DOPAC, HVA and their deuterated forms we selected derivatization with dansyl chloride [18], because of the simple and fast reaction (60°C for 20 min) and the resulting good sensitivity for all analytes in plasma (Figure 3) and urine. For all compounds, a protonated form of a singly dansylated derivative was most abundant and selected as precursor ion, while the protonated dansyl moiety, the predominant ion after collision induced dissociation, was selected as product ion. For levodopa and DOPAC as well as their -d3 and -d6 forms, which all contain two phenolic hydroxy groups, two analyte-related peaks showed up in the chromatograms, while for 3-OMD and HVA and their deuterated forms, which all contain one phenolic hydroxy group, just one peak was obtained. This suggests that dansylation primarily occurs at the phenolic hydroxy groups, which leads to two isobaric monodansylated derivatives for levodopa and DOPAC, that can be chromatographically separated and the first of which was used for quantification. Using a straightforward linear gradient, the eight derivatized analytes could be determined in an 11-min run. Small changes were applicable for the methods for plasma and urine, because of differences in concentration range (therefore, other injection volume) and ionization suppression (therefore, other LC conditions).

For dopamine and 3-MT, derivatization with propionic anhydride as described for dopamine in plasma [19] allowed quantification of dopamine, 3-MT and their deuterated forms down to the relevant level of 50 pg/ml in plasma (Figure 3) and 0.1 μg/ml in urine. Dopamine was derivatized to a triple propyl ester (at the amino group plus at the two phenol groups), while 3-MT, which has only one phenol group, was converted to a double propyl ester. Precursor ions for all derivatized analytes were the protonated molecules, with product ions corresponding to the loss of the propyl groups and of the amine group from the derivatives. The chromatographic run time was 10.5 min.

Method validation
The two plasma and the two urine methods for the 12 analytes were validated in accordance with the international guidelines for bioanalytical method validation that were applicable at the time [20,21]. The usual criteria for linearity, precision, accuracy, selectivity and matrix effect were met. Supplementary Table 3 shows the results for precision and accuracy. Extraction recoveries ranged from 84 to 102% for all analytes and ISs. Despite the unstable character of some of the analytes, the stabilizing precautions proved to be sufficient to allow storage at -70°C for up to 180 days, storage on melting ice for up to 23 h, up to five freeze/thaw cycles between -70°C and 0°C and storage of sample extracts at 4°C for up to 94 h. In addition, all analytes were stable for up to 2 h in whole blood at 0°C.
Figure 3. LC–MS/MS chromatograms. Levodopa at 15.0 ng/ml (A), 3-O-methyldopa at 18.7 ng/ml (B), 3,4-dihydroxyphenylacetic acid at 30.0 ng/ml (C), homovanillic acid at 16.0 ng/ml (D), dopamine at 150 pg/ml (E) and 3-methoxytyramine at 150 pg/ml (F) in plasma, after derivatization. Left panels: nondeuterated analytes, middle panels: deuterated analytes and right panels: ISs.

containing 2.5 mg/ml SMB. Stock solutions were demonstrated to be stable for up to 19 h on melting ice and for up to 12 days at -70°C.

Interference of natural heavy isotopes

About 1.1% of all carbon atoms present on earth have an atomic mass of 13 rather than the more usual 12. This means that a certain percentage of the molecules of an organic compound will have a mass that is one or more units higher than the monoisotopic mass due to the presence of one or several $^{13}$C-atoms in its structure. This percentage increases with the number of carbon atoms in the molecule. Similar effects play a role for other atoms and the heavy forms of nitrogen ($^{15}$N), oxygen ($^{18}$O) and hydrogen (deuterium) also contribute to the occurrence of molecular species with a higher mass. The natural abundance of these heavy atoms; however, is lower than for carbon and typically only has a minor effect for most organic compounds. Sulphur, on the other hand, has two natural heavy isotopes with a higher abundance ($^{33}$S: 0.8% and $^{34}$S 4.3%) that will contribute to the effect for sulphur containing molecules.
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Figure 3. LC–MS/MS chromatograms (cont.). Levodopa at 15.0 ng/ml (A), 3-O-methyldopa at 18.7 ng/ml (B), 3,4-dihydroxyphenylacetic acid at 30.0 ng/ml (C), homovanillic acid at 16.0 ng/ml (D), dopamine at 150 pg/ml (E) and 3-methoxytyramine at 150 pg/ml (F) in plasma, after derivatization. Left panels: nondeuterated analytes, middle panels: deuterated analytes and right panels: ISs.

The occurrence of these natural heavy isotopes has consequences for the mass spectrometric determination of the deuterated analytes. This is discussed for three of the six analytes below, which represent the most pronounced cases. For more details and the pharmacokinetic evaluation of all results, the interested reader is referred to [10]. Dansylated levodopa, for example, contains 21 carbon atoms and one sulphur atom and has a monoisotopic mass of 430. Due to the combined presence of $^{13}$C-, $^{33}$S- and $^{34}$S-atoms, 1.2% of dansylated levodopa molecules has a mass of 433, which is the same as the mass of dansylated triply deuterated levodopa with no further heavy atoms. Since these two forms cannot be distinguished on a triple-quadrupole MS, a sample containing levodopa but no levodopa-d$_3$ will still give a response in the mass transition for levodopa-d$_3$. This was experimentally confirmed during method validation by adding 2500 or 4000 ng/ml of levodopa (but no levodopa-d$_3$) to plasma and measuring the concentrations for levodopa-d$_3$, which were found to be 32.8 ng/ml (1.3%) and 59.0 ng/ml.
Figure 4. Levodopa (■) and levodopa-d3 (□) concentrations in plasma. After single oral dosing with 150 mg levodopa (A) or levodopa-d3 (B) to a healthy human volunteer.

Figure 5. Dopamine (♦) and dopamine-d3 (♦) concentrations in plasma. After single oral dosing with 150 mg levodopa (A) or levodopa-d3 (B) to a healthy human volunteer.

(1.5%), respectively. During study sample analysis, quantifiable levodopa-d3 concentrations were found in most of the plasma samples after dosing with levodopa, corresponding to an average of 1.3% of the corresponding levodopa concentration (range 1.2–1.5%), which is consistent both with the theoretical prediction and the validation results. Figure 4 shows examples of concentration–time curves for a representative subject. These results demonstrate that the concentrations found for levodopa-d3 after dosing with only levodopa originate from the heavy-atom containing molecules of dosed levodopa and not from any levodopa-d3 and therefore should not be reported as such.

Derivatized dopamine is a smaller molecule (17 carbon atoms) and contains no sulphur, so the interference of natural heavy isotopes is less pronounced for this analyte. In theory, only 0.2% of derivatized dopamine has a molecular mass three units above the monoisotopic mass, mainly due to the occurrence of three 13C-atoms. After spiking 16,000 pg/ml of dopamine to plasma and subsequent analysis, no quantifiable concentrations were found for dopamine-d3. With an LLOQ of 50 pg/ml, this means that the interference was less than 0.3%. Likewise, after dosing with nondeuterated levodopa, none of the plasma samples showed quantifiable concentrations for dopamine-d3 (e.g., in Figure 5), which confirms that for this analyte the analytical results are not affected by interference from heavy isotopes from the corresponding nondeuterated form.

Obviously, the effect of naturally occurring heavy isotopes is more pronounced for the analytes which contain only two deuterium atoms. Dansylated HVA (one sulphur and 21 carbon atoms) with two heavy atoms, for example, generates a response in the mass transition for dansylated HVA-d2. During validation, HVA-d2 concentrations corresponding to 6.8% of the added nondeuterated HVA were observed, which is consistent with the theoretical prediction of 6.5%. After dosing with levodopa, HVA-d3 could be quantified in most of the plasma samples at levels corresponding to 5.9–6.6% (average: 6.4%) of the corresponding HVA concentrations (exemplified in Figure 6).
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Figure 6. HVA (●) and HVA-d₂ (○) concentrations found in plasma. After single oral dosing with 150 mg levodopa (A) or levodopa-d₃ (B) to a healthy human volunteer. HVA: Homovanillic acid.

Again, although these results appear to be due to the presence of HVA-d₂ in plasma, they actually originate from nondeuterated HVA containing natural heavy atoms and should not be reported as HVA-d₂ levels.

Contribution of endogenous background levels
An additional complication in this study is that endogenous background levels occur in plasma and urine for most of the nondeuterated analytes. If these levels are high enough, the interference of their heavy isotopes may also generate responses in the mass transitions of the corresponding deuterated compounds, which cannot be distinguished from the responses of the actual deuterated analytes themselves. For low endogenous concentrations, this generally is no problem. When levodopa-d₃ was dosed, the endogenous plasma levels of levodopa were found to be below or just above 5 ng/ml and the contribution of their heavy forms to the measured levodopa-d₃ concentrations theoretically is around 0.06 ng/ml. This is far less than 1% of the total levodopa-d₃ levels and can be considered negligible.

The endogenous background levels of HVA in plasma were between 5 and 20 ng/ml and the contribution of their heavy-atom forms to the observed response for HVA-d₂ (6.5%) was therefore between 0.3 and 1.3 ng/ml. This is a contribution of up to 3% of total HVA-d₂ found after a levodopa-d₃ dose and may not be negligible for some samples. The pharmacokinetic results for HVA-d₂ after dosing with levodopa-d₃ should therefore be interpreted with caution, especially at the end of the pharmacokinetic curve where HVA-d₂ levels are low and the contribution from endogenous HVA relatively high. The most extreme situation was encountered for HVA in urine, which contained the highest endogenous levels of any of the analytes. Endogenous urinary concentrations for HVA were typically between 1 and 10 μg/ml, which implies that responses corresponding to between 0.06 and 0.6 μg/ml are expected in the mass transition for HVA-d₂ because of the interference of heavy isotopes from unlabeled HVA. After dosing with unlabeled levodopa, HVA-d₂ concentrations were found in many urine samples and, like for the examples described above, these can be disregarded as they originate from the heavy isotopes of unlabeled HVA (Figure 7A). After a levodopa-d₃ dose; however, the responses found for HVA-d₂ in urine typically are a combination of actual HVA-d₂ and the heavy forms of endogenous HVA. The contribution of the latter can vary from less than 1%, for samples with a high actual HVA-d₂ concentration, to 30% for later samples with a low HVA-d₂ level and to 100% for the predose samples without any HVA-d₂ (Figure 7B). It should; therefore, be realized that the measured HVA-d₂ concentrations in urine may be considerably overestimated.

Internal standards
In contemporary bioanalysis, SIL forms of analyte molecules are the preferred ISs because of the (close to) ideal correction that they provide for fluctuations in experimental conditions. Typically, molecules containing at least three stable isotopes are used to minimize the contribution of heavy isotopes of the analyte to the IS response. For the present study, d₂- and d₃-labeled forms of levodopa and metabolites are also being monitored as analytes and, therefore, molecules with at least five to six heavy atoms were required as ISs. Even though levodopa and its metabolites are frequently being analyzed in laboratories across the globe, not all required ISs were available off-the-shelf and custom synthesis was needed for some of them. This shows that, in general, obtaining suitable ISs...
Figure 7. HVA and HVA-d2 concentrations found in individual urine samples. Collected consecutively after single oral dosing with 150 mg levodopa (A) or levodopa-d3 (B) to a healthy human volunteer. The theoretical contribution in the HVA-d2 channel originating from heavy isotopes of endogenous HVA is also indicated in panel (B).

HVA: Homovanillic acid.

to support the analysis of deuterated drugs and their metabolites might be a challenge, and the same applies for obtaining reference standards of drug metabolites with deuterium atoms at the right positions.

Analytes and SIL ISs can contribute to each other’s responses in two ways. As extensively discussed above for nondeuterated and deuterated analytes, the natural heavy atoms of an analyte can generate a response in the mass transition of its IS. The practical consequence is that the IS signal increases with increasing analyte concentration and, thus, that the peak area ratio of analyte over IS will decrease. Although this may lead to nonlinearity of the calibration line, accuracy of results is generally not impacted, because the effect is identical for spiked samples and incurred samples [22]. The best way to prevent this situation is to use an IS with more stable isotopes, but this may not always be technically possible, and the relative importance of this effect can also be reduced by increasing the IS concentration. An opposite effect is due to the potential contamination of the IS material with a nonlabeled form of the molecule, in other words, the analyte itself, due to imperfect synthesis or purification. In this case, a fixed amount of analyte is introduced in each sample along with the IS, which results in an upward shift of the entire calibration line (increase of the intercept). If no better purified IS material can be obtained, this effect can be minimized by decreasing the IS concentration.

In the current study, both effects played a role. The isotopic purity of some of the IS materials was not complete, and traces (<0.5%) of molecules with the same mass as the deuterated analyte were present in some standards. To keep the intercept of the calibration lines below 20% of the LLOQ response, the amount of IS added to each sample had to be restricted to 5% of the amount of deuterated analyte in a sample at ULOQ level. This means that there are 20-times more deuterated analyte molecules than IS molecules present in a ULOQ sample. For DOPAC and HVA, SIL ISs could be generated with a molecular mass of four units higher than the corresponding deuterated analyte, for the others this difference was three units. After derivatization, a theoretical contribution from the deuterated analytes to the IS response of 1.2% (levodopa and 3-OMD) or 0.2% (other four) can be expected for equal concentrations of analyte and IS and, thus, of 24 and 4% in the ULOQ sample, respectively. As a consequence, a small deviation from linearity was indeed observed for the calibration lines of levodopa and 3-OMD, which could be addressed by quadratic regression. For DOPAC, HVA, dopamine and 3-MT the effect was negligible. For all analytes, results for accuracy and precision, remained unaffected, which shows that even ISs containing only three extra heavy atoms and with a small percentage of isotopic impurity in their material can be successfully used for quantitative purposes.

Conclusion
LC–MS/MS can be successfully used to monitor the plasma and urine concentrations of deuterated and non-deuterated pharmaceuticals and their metabolites. The main complexity is related to the natural heavy atoms (in our case mainly carbon and sulphur) that occur in the nondeuterated analytes and that causes a response in the mass transitions of the deuterated analytes. This effect is most pronounced if the number of deuterium atoms in an analyte is small and the number of carbon and sulphur atoms is large: derivatized HVA (one sulphur and 21
carbon atoms) adds 6.5% to the response of HVA-d₂, while the contribution for dopamine (17 carbon atoms and no sulphur) to dopamine-d₃ is just 0.2%. The problem would be solved if a sufficient number of deuterium atoms were present, because the interference typically is smaller than 0.2% for a fourfold deuterated molecule, but this number is defined by pharmacological considerations and typically not free to choose. Derivatization, such as applied here, increases the problem, because it adds atoms and thus also heavy atoms to the analytes of interest and should be avoided if at all possible. Still, the molecular characteristics of levodopa and its metabolites and the requirements for sensitivity and chromatographic behavior dictated derivatization with a reagent of a suitable size. This made the undesired effect of the contribution of natural heavy isotopes unavoidable, just as it will be for underivatized compounds with a relatively high molecular mass. For drugs containing chlorine (ratio of ³⁷Cl to ³⁵Cl around 1 to 3) or bromine (ratio of ⁸¹Br to ⁷⁹Br around 1 to 1), the situation is even more complicated because of the much higher contribution of the natural heavy isotopes.

The result of all this is that it can be predicted and was indeed experimentally confirmed that quantifiable concentrations may be obtained for the deuterated compounds, which are not due to the presence of these deuterated compounds but to the occurrence of their nondeuterated counterparts. Consequently, very inaccurate concentrations could be reported, if the situation is not properly evaluated. If only nondeuterated compounds are expected in a sample, such as after the dose of a nondeuterated drug, quantifiable concentrations of the corresponding deuterated forms should be disregarded. In this respect, it is advisable that the degree of interference is confirmed by analyzing samples only spiked with the nondeuterated analytes. The situation becomes more complex if the nondeuterated and deuterated forms occur together, for example, when relatively high endogenous analyte levels are present in samples taken after a dose with the deuterated drug, or in (the probably hypothetical) case that the deuterated and nondeuterated drug are dosed together. In such a situation, the concentration found for a deuterated analyte is the sum of its actual concentration plus the interference from the nondeuterated analyte. Triple-quadrupole MS has insufficient resolution to distinguish the m/z values of the deuterated and the ¹³C-containing compounds and probably the only option to improve accuracy is to subtract the theoretical contribution of the nondeuterated analyte from the experimentally observed total concentration. Heavy carbon and sulphur atoms in the deuterated analytes may have a similar contribution to the response of a SIL IS, but in many situations this has no quantitative consequences, because the IS is present at the same concentration in all samples and the contribution is the same for spiked samples and patient samples. It may be a challenge; however, to find a good source of an IS with a sufficient number of heavy atoms.

**Future perspective**

Although the development of deuterated drugs will probably remain a niche, it is important that proper bioanalytical approaches exist for monitoring the pharmacokinetics, metabolism and excretion of deuterated compounds. High-resolution mass spectrometry (HRMS), with sufficient resolving power to discriminate an organic compound containing multiple deuterium atoms from the same compound containing the same number of – for example, carbon-13 atoms – is an attractive alternative to MS/MS for the simultaneous, interference-free determination of both compounds. Now that the acceptance of HRMS for quantitative bioanalysis continues to grow and this technique is increasingly being introduced in bioanalytical laboratories around the world, it is to be expected that HRMS will eventually replace MS/MS for these applications.

**Supplementary data**

See online at: https://www.future-science.com/doi/10.4155/bio-2018-0239

**Acknowledgements**

The authors would like to thank Dr Frank Schneider for helpful discussions.

**Financial & competing interests disclosure**

SHG and RB are or were employees of Teva. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.
Summary points

Background
• LC–MS/MS methods were set up for the quantification of levodopa, a triply deuterated form of levodopa and, in total, ten nondeuterated and deuterated metabolites, in human plasma and urine.

Experimental
• Derivatization with dansyl chloride or propionic anhydride was needed to quantify down to the relevant concentrations (LLOQ between 0.05 and 5 ng/ml in plasma).
• Stabilization with two antioxidants and sample handling on ice and under yellow light was essential to avoid degradation.

Results & discussion
• The natural occurrence of heavy atoms (notably $^{13}$C and $^{33}$S and $^{34}$S) in the nondeuterated analytes caused an interfering signal in the mass transitions of the corresponding deuterated analytes.
• This resulted in a – sometimes non-negligible – overestimation of the concentrations of the deuterated analytes.

Conclusion
• Therefore, careful evaluation of the results of the deuterated analytes is essential.

Data sharing statement
The authors certify that this manuscript reports original clinical trial data. Data from the trial will not be made available.

References
Papers of special note have been highlighted as: ● of interest

   ● Thorough overview of the possibilities and limitations of deuterated drugs.


- Good overview of the different effects that analytes and stable isotope-labeled internal standards can have on each other’s signals.