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13C/15N-Enriched L-Dopa as a Triple-Resonance NMR Probe to Monitor Neurotransmitter Dopamine in the Brain and Liver Extracts of Mice

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In an attempt to monitor μm-level trace constituents, we applied here 1H-(13C,15N) triple-resonance nuclear magnetic resonance (NMR) to 13C,15N-enriched L-Dopa as the inevitable precursor of the neurotransmitter dopamine in the brain. The perfect selectivity (to render endogenous components silent) and μm-level sensitivity (700 MHz spectrometer equipped with a cryogenic probe) of triple-resonance allowed the unambiguous and quantitative metabolic and pharmacokinetic analyses of administered L-Dopa/dopamine in the brain and liver of mice. The level of dopamine generated in the brain (within the range 7–76 μm, which covers the typical stimulated level of ~30 μm) could be clearly monitored ex vivo, but was slightly short of the detection limit of a 7T MR machine for small animals. This work suggests that μm-level trace constituents are potential targets of ex vivo monitoring as long as they contain N atoms and their appropriate 13C/15N-enrichment is synthetically accessible.

Multiple-resonance NMR is a powerful technique,³–⁵ by which particular protons in the sequence ¹H–¹³C–¹⁵N (¹H–¹³C–¹⁵N triple resonance) or ¹H–¹³C (¹H–¹³C double resonance) can be detected highly selectively as a result of magnetic coherence transfer ¹H→¹³C→¹⁵N→¹³C→¹H or ¹H→¹³C→¹H. Although the application of multiple-resonance NMR (double resonance in many cases,²,³) mostly dealing with main metabolic sources such as glucose, amino acids, and fatty acids, and triple resonance in some⁶ to metabolic analysis is by no means rare, little is known, to the best of our knowledge, about its applicability to hormone-like trace (μm-level) constituents. In the present work, we applied triple-resonance NMR to 13C,15N-enriched L-Dopa (L-3,4-dihydroxyphenylalanine) as the inevitable precursor of neurotransmitter dopamine (2-(3,4-dihydroxyphenyl)-ethylamine) in the brain.

Dopamine plays important roles in motivation, reward, and motor control,¹⁷ and problems with its metabolism can trigger several neurological/psychological disorders such as Parkinson’s disease, schizophrenia, and depression.¹⁸–²¹ Scheme 1 summarizes the L-Dopa-to-dopamine metabolism and its inhibition. The brain is not directly accessible by dopamine, which cannot pass through the blood–brain barrier (BBB). Instead, dopamine is generated in situ in the brain upon decarboxylation of its precursor, L-Dopa which is BBB-permeable, by the enzyme AAAD (aromatic L-amino acid decarboxylase). Dopamine thus generated in the brain undergoes rather rapid deactivation upon oxidative deamination by the enzyme MAO (monoamine oxidase, types A and B).

In clinical practice, L-Dopa as the precursor of dopamine is often administered together with inhibitors of enzymes AAAD²² and MAO-A and MAO-B²³ as codrugs to maintain appropriate high concentrations of dopamine in the brain. Another way to achieve high dopamine levels (~30 μm)³⁰,¹¹,¹⁵ or ~2 μm²⁹,¹² is to electrically stimulate the brain. In this work, we took 13C,15N-enriched L-Dopa as a triple-resonance probe to monitor dopamine in mice with a stimulated level of ~30 μm in the brain taken as a criterion to evaluate the per-
formance of the present method. We use the selectivity and sensitivity of triple-resonance high resolution NMR with a cryogenic probe to perform quantitative metabolic/pharmacokinetic analysis of L-Dopa/dopamine in the extracts of brain and liver of mice, showing that the stimulated dopamine level (∼30 μM) in the brain can be detected ex vivo. This work also illustrates where we are on the path to direct in vivo MR spectroscopic (MRS) monitoring of this neurotransmitter system.

15C/15N-enriched L-Dopa (15C/15N-L-Dopa, Figure 1a) with a 1H-15C-15N sequence involving the asymmetric center was obtained starting from 15C/15N-glycine in an optical yield of 94% ee, as detailed in the Supporting Information. 15C/15N-enriched dopamine (15C/15N-dopamine, Figure 1b) was also prepared from K15C15N. One-dimensional (1D) 1H-(15C/15N) triple-resonance spectra (15C-decoupled) of 15C/15N-L-Dopa and 15C/15N-dopamine showed a single peak at 3.85 ppm or at 3.14 ppm for the methylene proton (1H-15C-15N) of L-Dopa or the methylene protons (1H-15C-15N) of dopamine, respectively. The enzymatic decarboxylation of 15C/15N-L-Dopa (δ = 3.85 ppm) to dopamine (δ = 3.14 ppm) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing the decarboxylation enzyme AAAD was completed in 1h, as revealed by the triple-resonance analysis (Figure 1c) in accord with the results of high-performance liquid chromatography (HPLC) monitoring (Figure 1d).

Decarboxylation and its inhibition in complex biological mixtures such as liver lysate could also be readily monitored by triple-resonance. 13C15N-L-Dopa (0.5 mm) in crude mouse liver lysate was incubated for 45 min. After workup, the mixture was subjected to NMR analysis. The conventional 1H NMR spectrum (Figure 1e, top) was completely useless; all 1H-containing molecules in the lysate represent their signals. The 1H-(13C15N) triple-resonance spectrum (Figure 1e, bottom) exhibited two signals at 3.85 ppm for L-Dopa and 3.14 ppm for dopamine in a ratio of 1:7 (12% and 86% of L-Dopa used, respectively), indicating that most of the L-Dopa had undergone decarboxylation by endogenous AAAD contained in the liver lysate to give dopamine. In the presence of carbidopa (BBB-impermeable), a potent AAAD inhibitor that is clinically used as a codrug together with L-Dopa (referring to Scheme 1), the build-up of dopamine was effectively suppressed (∼85%) even at [carbidopa] = 5 μM (0.01 equiv of L-Dopa (0.5 mm)) (Figure 1f, bottom) and completely suppressed at [carbidopa] = 50 μM (0.1 equiv) (Figure 1f, top). Triple resonance can thus completely suppress noise signals arising from endogenous components in complex biological mixtures to allow the unambiguous and quantitative metabolic analyses of 13C15N-enriched substrates therein.

In vitro and ex vivo spectra were obtained with a 700 MHz (16.4 T) NMR spectrometer equipped with a high-sensitivity
cryogenic probe after 256 scans (~7 min), where the detection limit, i.e., the lowest concentration to give S/N = 3, of $^{13}$C/$^{15}$N-dopamine lies at around 4 $\mu$M. Triple-resonance spectra of $^{13}$C/$^{15}$N-dopamine as phantom samples (500 $\mu$L) were also obtained with an MR machine for small animals operating at 7 T (300 MHz) without a cryogenic probe (Supporting Information); the detection limit after 3600 scans (1 h) turned out to be ~1 mM.

We proceeded to the L-Dopa/dopamine metabolic analysis in mice, focusing on the effects of inhibitors of decarboxylation (AAAD) and oxidation (MAO) enzymes. Mice (~16 g) were administered $^{13}$C/$^{15}$N-L-Dopa (0.63 mmol kg$^{-1}$) with or without carbidopa (AAAD inhibitor) and MAO inhibitors [clorgyline and selegiline (BBB-permeable MAO-A and MAO-B inhibitors, respectively; see Scheme 1)]. After 1 h, brain and liver tissues were collected and, after workup, their triple-resonance spectra (256 scans) were obtained for doubly diluted (compared with the tissue weights) solutions, as shown in Figure 2, where the signal intensities for the brain and liver samples are weight-normalized. In the absence of any inhibitors, asmall amount of $^{13}$C/$^{15}$N-dopamine was hardly detected in the liver or the brain. However, the gap between the $^{13}$C/$^{15}$N-dopamine levels in the brain increased, but only slightly (middle), probably because of its oxidative deamination by MAO to give dopal, which of course exhibited no $^{1}H$/$^{13}$C/$^{15}$N signals. Indeed, when the MAO inhibitors clorgyline (63 $\mu$mol kg$^{-1}$) and selegiline (63 $\mu$mol kg$^{-1}$) were both present, there was a 5-fold increase in the dopamine level in the brain, and a substantial amount of L-Dopa that escaped decarboxylation remained in the liver (top).$^{14,15}$ The local concentrations of dopamine in the brain (~400 mg) were estimated by calibration using an authentic specimen to be 7 $\mu$M (in the absence of any inhibitors), 15 $\mu$M (with AAAD inhibitor), and 76 $\mu$M (with AAAD and MAO inhibitors). The inhibitor-dependent dopamine levels of 7–76 $\mu$M are consistent with those of 5–120 $\mu$M$^{16}$ reported for rat based on HPLC analysis.

In this work, we investigated the usefulness of triple resonance for monitoring dopamine at a stimulated ~30 $\mu$M level. As shown above, a wide concentration range which includes this critical 30 $\mu$M could be easily accessed by a combination of L-Dopa and inhibitors. Selectivity and sensitivity are key issues in applying NMR to complex biological mixtures. In this context, the present work may be summarized as follows:

1) Triple resonance showed “perfect” selectivity. The probability of the natural occurrence of the sequence $^{1}H$/$^{13}$C/$^{15}$N is as low as 0.011 $\times$ 0.0037 = 0.0004 (0.004%), where 0.011 and 0.0037 are the natural abundance of $^{13}$C and $^{15}$N, respectively, and the mole-based selectivity factor for the $^{13}$C/$^{15}$N-enriched target over endogenous components is 1/0.0004 = 25000 (2.5 $\times$ 10$^{4}$). Thus, endogenous components may effectively compete with the enriched target at, for example, 10 $\mu$M, only when they are present in unnaturally high concentrations of 10$^{4}$ $\mu$M = 0.1 mM. An implication of this observation is that selectivity is by no means a formidable issue to deal with for any $\mu$M-level trace constituents as long as they contain N-atom(s) and their appropriate double $^{13}$C/$^{15}$N-enrichment is synthetically accessible.

2) This perfect, noiseless selectivity of triple resonance gives rise to a $\mu$M sensitivity (4 $\mu$M, 256 scans under less time-consuming (in minutes), one-dimensional (monitoring of $^{1}H$ signals only) conditions). This allows unambiguous and quantitative ex vivo metabolic/pharmacokinetic analyses of administered L-Dopa and its metabolite dopamine, i.e., ratiometric monitoring of their decay/build-up profiles, which clearly shows that the stimulated level of dopamine in the brain can be monitored ex vivo. 3) Unfortunately, however, the key dopamine level of 30 $\mu$M is short of the detection limit (~1 mm, 3600 scans) of the 7T MR machine (noncryogenic probe) for noninvasive, i.e., in vivo, monitoring.$^{16}$ However, the gap between them is only a factor of ~30. This appears to be significant since an increase in sensitivity of this extent (~30-fold) may be achieved by combining an existing highest-field machine and a high-sensitivity cryogenic probe equipped with a triple-resonance coil. In-brain dopamine may then become a real target of direct in vivo MRS with which we can record the dopamine spectra in the brain.

In addition to a variety of techniques, based on HPLC$^{18a}$ biological (enzyme-linked immunosorbent assay, ELISA) affinity (for the analysis of urine),$^{18b}$ microdialysis$^{18c}$ electrochemical techniques,$^{18d}$ and chemical sensing$^{18e}$ a couple of methods have recently been developed to monitor dopamine in the brain. One is positron emission tomography (PET) using $^{13}$C-raclopride, which competitively binds to the dopamine receptor to enable the (dopamine)-dependent emission of
The other is MRI using a protein-engineered heme-based contrast agent which reversibly binds to dopamine, thereby changing the relaxivity, and thus gives [dopamine]-dependent images. Both methods are highly sophisticated, but are indirect and involve complicated complexation processes. MRS is much simpler and can directly monitor the targets and their transformations with minimal noise signals which may arise from nonspecific binding, etc. Currently, the metabolic analysis of $^{13}$C-glucose in the brain has received increasing attention. The present work shows a way to detect pm-level trace constituents and has shed light on the issues to be overcome for in vivo imaging. Further work is now underway along these lines with an ultimate goal of detection of hypodopaminergy in related diseases.

**Experimental Section**

1) General methods, 2) preparation, 3) monitoring of the L-Dopa-to-dopamine conversion and subsequent dopamine oxidation, and 4) phantom MRS are included in the Supporting Information.

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**Keywords:** dopamine • L-dopa • metabolic analysis • neurotransmitters • stable isotope enrichment • triple-resonance NMR


[12] The reported values for rat are –35 μM (electrochemical),[11m] a) –30 μM (electrochemical),[11m] and –2 μM (electrochemical).[11n]

[13] The workup procedure involves treatment with 10% trichloroacetic acid (TCA). [11m] $^{14}$C-L-Dopa was independently shown to be stable when treated with 10% TCA under the same conditions and exhibited methine proton resonance at 4.19 ppm, which is shifted downfield by 0.34 ppm (probably as a result of changes in the protonation state) from that (3.85 ppm) for TCA-unreated L-Dopa.

[14] The liver spectrum (Figure 2, top, right) shows a notable amount of dopamine (3.14 ppm), indicating that 0.1 equiv of carbidopa is not enough to completely suppress in vivo decarboxylation of L-Dopa in the liver, although enzymatic in vitro decarboxylation of L-Dopa can be completely inhibited by 0.1 equiv of carbidopa (Figure 1 f).

[15] Interestingly, the L-Dopa level in the brain is higher with carbidopa and MAO inhibitors than with carbidopa alone.

[16] The sensitivity of MR machines for small animals with a wider bore diameter, a longer sample-to-coil distance, and more pronounced inhomogeneity of the magnetic field is known to be considerably lower than that of spectrometers dealing with solution samples in 5 mm tubes. Under these circumstances, recent attention has been directed to specific signal amplification techniques such as hyperpolarization and CEST (chemical exchange saturation transfer). For example, a) A. Viale, S. Aime, *Curr. Opin. Chem. Biol.* 2010, 14, 90–96 and references therein; b) A. R. Lippert, K. R. Keshari, J. Kurhanewicz, C. J. Chang, *J. Am. Chem. Soc.* 2011, 133, 3776–3779; c) S. Viswanathan, Z. Kovacs, K. N. Green, S. J. Ratnakar, A. D. Sherry, *Chem. Rev.* 2010, 110, 2960–3018 and references therein.

[17] The commercially available, highest-field MR machine for small animals operates at 21 T (Bruker, Billerica, MA, USA). The NMR sensitivity increases in proportion to $B_0$, where $B_0$ is the static magnetic field. A cryogenic probe is known to enhance sensitivity by a factor of ~5 compared with noncryogenic probes. Thus, a 21 T (900 MHz) machine with a cryogenic probe is expected to be more sensitive than a 300 MHz (7T) machine without a cryogenic probe by a factor of $(21/7)^{7/2} = 5.26$.


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