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SYNTHESIS AND ENANTIOMERIC RESOLUTION OF TRITIATED (*D,L*)-3-HYDROXYKYNURENINE

John R. Lever*, Karen A. Canella, Clifford L. Eastman and Tomas R. Guilarte

Department of Environmental Health Sciences, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland 21205-2179

Summary

The synthesis, purification and enantiomeric resolution of tritiated (*D,L*)-3-hydroxykynurenine are described. Bromination of racemic 3-hydroxykynurenine, catalytic dehalotritiation and purification by ion pair, reverse phase HPLC afforded tritiated material of high radiochemical purity (99.8%) and moderate specific radioactivity (3.64 Ci/mmol). Enantiomerically pure (*D*)- and (*L*)-stereoisomers were isolated using chiral HPLC conditions. The stability of the label toward isotopic exchange was assessed to be sufficient for biochemical studies.

Keywords: 3-hydroxykynurenine, tritium, amino acid, seizure, vitamin B_6

Introduction

3-Hydroxykynurenine (3-HK), a putative endogenous convulsant (1-3) which also is cytotoxic to a neuronal cell line (4), may be linked to neonatal seizure disorders and central nervous system (CNS) insult associated with vitamin B_6 deficiency. In normal brain, levels of metabolites from the tryptophan-kynurenine pathway such as 3-HK, kynurenine and quinolinic acid are not high enough to cause seizures (5,6). However, these agents do cause convulsions after intracerebroventricular injection in mature rodents (7-9), and elevated cerebral concentrations of 3-HK are temporally related to the onset of the neurological sequelae which result from perinatal vitamin B_6 deprivation of rat pups (2). Moreover, elevated CNS levels of 3-HK have been measured in postmortem brain tissues from patients with Huntington's disease (10), and in rodent brain tissues as a consequence of exposure to bacterial endotoxin (11). 3-HK appears to exert a modulatory effect upon the supramolecular

**Author for correspondence:* John R. Lever, Room 2001, The Johns Hopkins University School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore MD 21205-2179.

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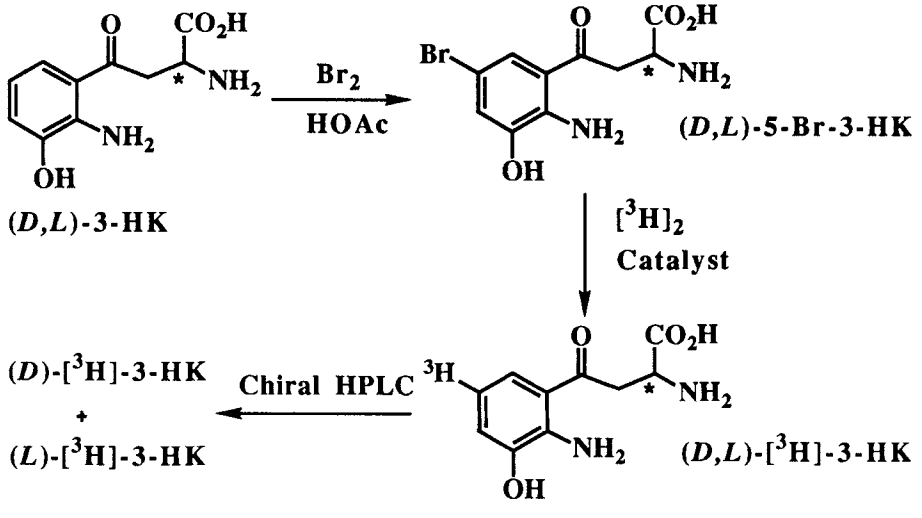
GABA/benzodiazepine/barbiturate complex (3), and altered GABAergic neurotransmission may be a component of pertinent mechanisms of action.

Further investigation of the neuronal activity of 3-HK would be facilitated by the availability of isotopically radiolabelled forms. (*L*)-[^{14}C]-3-HK of low specific activity (0.64 Ci/mol) has been prepared from (*D,L*)-[^{14}C]-tryptophan through a series of enzymatic conversions utilizing *Pseudomonas* tryptophan pyrrolase and kynurenine formamidase to provide (*L*)-[^{14}C]-kynurenine as a substrate for hydroxylation at the 3-position by rat liver mitochondrial kynurenine hydroxylase (12). Since detailed biochemical studies generally call for labelled material which is readily synthesized with higher specific radioactivity, we now describe chemical methods for the synthesis and enantiomeric resolution of (*D,L*)-[^{3}H]-3-HK.

Results and Discussion

3-HK is a diamino acid containing an aromatic ring which is activated toward electrophilic aromatic substitution. As a consequence, the synthetic route envisioned for the preparation of (*D*)- and (*L*)-[^{3}H]-3-HK embodied halogenation followed by tritiodehalogenation and resolution of the enantiomers (Scheme 1). Similar methodology has proved particularly useful for the preparation of other ring-labelled aromatic compounds, including amino acids (13). We elected to perform the resolution of the enantiomers after the catalytic halogen-tritium exchange step because racemization of an optically pure precursor might occur during the tritiolysis reaction.



Scheme 1. Route to (*D*)- and (*L*)-[^{3}H]-3-HK.

In the first step of the sequence, treatment of 3-HK with bromine in glacial acetic acid followed by reverse phase column chromatography provided (*D,L*)-5-Br-3-HK in 64% yield (14). The position of halogenation was deduced by inspection of the [^{1}H]-NMR spectrum which displayed two one-hydrogen doublets in the aromatic region

with a coupling constant (J = 2.1 Hz) characteristic for *meta*-orientation (15). Other distinguishing NMR spectral features were in accord with those of related compounds such as kynurenine (16).

(*D,L*)-5-Br-3-HK (25 mg) was utilized as the substrate for catalyzed halogen displacement with tritium gas (10 Ci) which was carried out under proprietary conditions (method TR3) by personnel from Amersham International, plc. Catalyst and labile tritium were removed, and the crude product was supplied in aqueous ethanol. A portion (11.79 mCi) was purified by semi-preparative ion pair, reverse phase HPLC using aqueous formic acid (0.12 M) as eluent (17). The material which corresponded to an authentic sample of (*D,L*)-3-HK (R_t = 5.58 min; k' = 1.3) was collected for a 78% recovery of radioactivity (9.25 mCi). Chemical and radiochemical purity (98.2% and 98.7%, respectively) were determined by analytical ion pair, reverse phase HPLC using the same mobile phase where (*D,L*)-[^{3}H]-3-HK eluted at 6.00 min (k' = 3.9). The analytical HPLC conditions were used for final purification of aliquots (*ca.* 800 μCi) of the stock solution to give (*D,L*)-[^{3}H]-3-HK of 99.8% radiochemical purity (Figure 1) with no chemical impurities observed by ultraviolet absorbance detection.

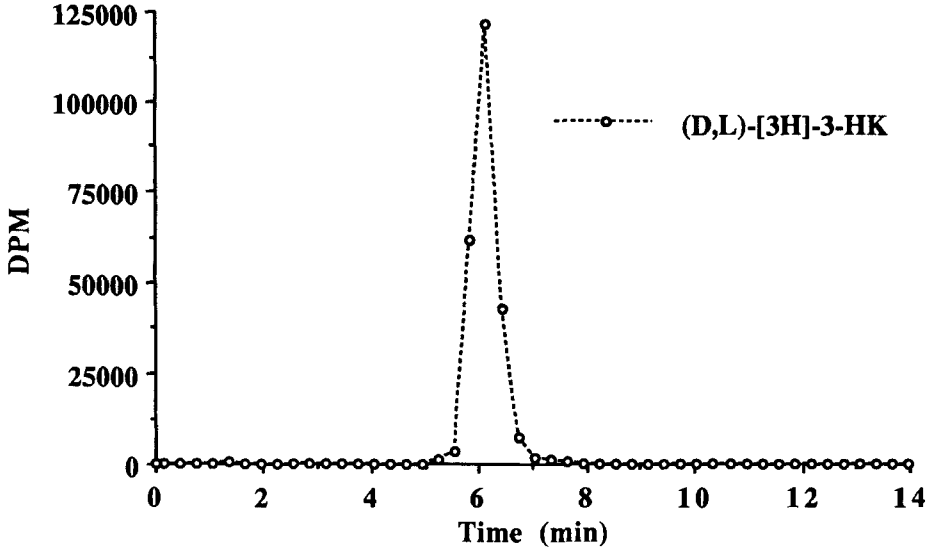


Figure 1. Ion pair reverse phase HPLC radiochromatogram of (*D,L*)-[^{3}H]-3-HK.

Although the location of the tritium has not been determined explicitly, (*D,L*)-[^{3}H]-3-HK should be labelled principally at the 5-position since the majority of compounds prepared by catalytic tritiodehalogenation have relatively small proportions of tritium in positions other than those which had been halogenated (13).

The specific radioactivity of (*D,L*)-[^{3}H]-3-HK was calculated to be 3.64 Ci/mmol, which is significantly less than the theoretical value and lower than that of some labelled aromatic compounds prepared in similar fashion (13,18-21). This may

result from a much slower rate of catalytic tritiodebromination as compared to the rate of isotopic dilution of the tritium gas by solvent or substrate (13,19). Alternatively, catalyst-mediated transfer of labile hydrogen which is independent of isotopic dilution of the tritium gas also might occur (22).

Isotopic exchange subsequent to the tritiodebromination step is a more pressing concern with direct impact upon not only the specific radioactivity of the radiotracer but also its suitability for use in biological assays. Isotopic exchange would be expected to occur to some degree since the aromatic ring of (*D,L*)-[^{3}H]-3-HK is activated toward electrophilic substitution by two electron-donating substituents. Accordingly, aliquots of (*D,L*)-[^{3}H]-3-HK were incubated under a variety of conditions, and subsequently checked by analytical HPLC in order to assess the stability of the label (Table 1).

Table 1. Radiochemical purity of (*D,L*)-[^{3}H]-3-HK after incubation under various storage and biological assay conditions.

pH[a,b]	Time	Temperature	Radiochemical Purity[c]
2.5	72 hr	8 °C	99.8%
2.5	5 weeks	8 °C	97.1%
2.5	20 weeks	8 °C	87.5%
2.5	60 hr	22 °C	76.5%
2.5	4 hr	37 °C	88.4%
no solvent[d]	4 weeks	8 °C	99.3%
7.2	2.5 hr	22 °C	98.9%
7.2	60 hr	22 °C	81.5%
7.2	2 hr	37 °C	98.1%
7.2	4 hr	37 °C	92.9%
7.2	2 hr	45 °C	97.3%
7.8	1 hr	37 °C	92.0%
7.8	2 hr	37 °C	86.1%

[a] pH 2.5, 0.12 M formic acid
[b] pH 7.2 and 7.8, *ca.* 0.1 M phosphate buffers
[c] 99.8% initial radiochemical purity
[d] 0.12 M formic acid removed *in vacuo*

In all cases, only minor chemical impurities were detected by HPLC with ultraviolet absorbance detection, and the major radiochemical impurity eluted at the solvent front. These results are consistent with isotopic exchange as the principle confounding reaction. Stock solutions of (*D,L*)-[^{3}H]-3-HK in 0.12 M formic acid, the HPLC eluent used for purification, were reasonably stable when kept at 8 °C but showed substantially greater exchange at elevated temperatures. Upon prolonged storage (20 weeks) at 8 °C, 9.6% of the radioactivity eluted at the solvent front, and an

additional 2.9% of the radioactivity was associated with two minor radiochemical impurities. A sample of this material was repurified, and the specific radioactivity had fallen from 3.64 Ci/mmol to 3.29 Ci/mmol, a decline of 9.6% which corresponds to the HPLC results. In contrast, samples from which the formic acid solvent had been removed *in vacuo* showed negligible exchange or decomposition for a period of at least 4 weeks. Thus, the radiotracer can be stored in the laboratory refrigerator at 8 °C or below for short intervals in acidic solution, or for longer periods without solvent under argon. High pH, elevated temperatures and exposure to light should be avoided because (*D,L*)-3-HK is converted to xanthurenic acid, 4,8-dihydroxyquinoline and 2-carboxy-2,3-dihydro-8-hydroxyquinolone-4 by air oxidation in alkaline solution (23).

Under conditions which are of biological relevance, incubation of (*D,L*)-[^{3}H]-3-HK in phosphate buffers, isotopic exchange of varying severity also occurred (Table 1). With increases in temperature, incubation time or pH, the extent of exchange became more significant. These data indicate that the stability of the label should be ascertained under a given set of biological assay conditions, and that a correction for lability of the label might be required for implementation of certain protocols.

Enantiomeric resolution of purified samples of (*D,L*)-[^{3}H]-3-HK (100 μCi) was achieved by chiral HPLC using a bovine serum albumin (Resolvosil®-BSA-7) column with phosphate buffer (0.1 M, pH 7.5) as mobile phase at a flow rate of 2 mL/min. (*D*)-[^{3}H]-3-HK (R_t = 1.26 min, k' = 0.3) eluted very near to the solvent front under these conditions, and was well-separated from (*L*)-[^{3}H]-3-HK (R_t = 9.00 min, k' = 8.2). An 86% total recovery of radioactivity was obtained by collecting heart fractions of the optical isomers. The relative stereochemistry was assigned by comparison of the chiral HPLC retention profiles with those of (*D,L*)-3-HK and an authentic sample of the natural product (*L*)-3-HK. Interestingly, (*L*)-3-HK is the major yellow pigment

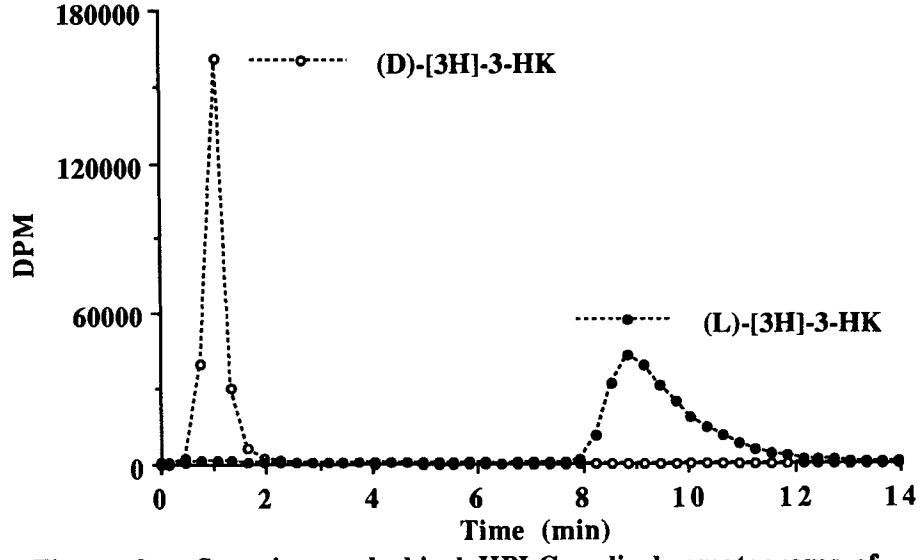


Figure 2. Superimposed chiral HPLC radiochromatograms of (*D*) and (*L*)-[^{3}H]-3-HK.

isolated from the wings and bodies of genus *Heliconius* South American butterflies (23). Resolved samples of both the (*D*)- and (*L*)-stereoisomers labelled with tritium were judged to be enantiomerically pure by chiral HPLC (Figure 2). Subsequently, resolved samples were stored at pH 2.8 by diluting the phosphate buffer with 0.3 volumes of aqueous formic acid (1.2 M). As expected, both (*D*)- and (*L*)-[^{3}H]-3-HK coeluted with either (*D,L*)-3-HK or (*L*)-3-HK under ion pair, reverse phase HPLC conditions (*cf.* Figure 1). These latter analyses also verified that little (< 0.6%) isotopic exchange had taken place during the resolution procedures.

In summary, enantiomerically pure (*D*)- and (*L*)-[^{3}H]-3-HK are readily available in moderate specific radioactivity (3.64 Ci/mmol) from racemic (*D,L*)-3-HK *via* a sequence which involves electrophilic aromatic bromination, catalyzed tritiodebromination, and enantiomeric resolution by chiral HPLC. The tritiated compound is most likely labelled at the 5-position, and is stable for at least 4 weeks when stored at 8 °C in the absence of solvent. Although (*D,L*)-[^{3}H]-3-HK suffers varying degrees of isotopic exchange under both acidic and basic conditions, the radiotracer is sufficiently stable and of high enough specific radioactivity to permit biochemical investigations (24).

Experimental

Materials and Methods: Proton nuclear magnetic resonance (^{1}H NMR) spectra were determined in d$_4$-deuteromethanol using an IBM NR-80 (80 MHz). Chemical shifts are reported in parts per million (δ) relative to residual absorptions (δ = 3.34) of the deuterated solvent. Microanalyses were determined by Atlantic Microlab, Inc. (Norcross, GA). Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected. Column chromatography was performed under N_2 using bonded-phase octadecyl (C-18) packing (J. T. Baker, 40 μm). The high performance liquid chromatography (HPLC) equipment consisted of a Rheodyne Model 7125 automated injector, Waters Associates Model 510 EF pumps and Model 490 variable wavelength ultraviolet (UV) absorbance detector with the eluent diverted to a fraction collector (ISCO Cygnet). Areas of the UV absorbance peaks were determined with an automated integrating recorder (Chromatopac C-R3A, Shimadzu Scientific Instruments, Columbia, MD). Reverse phase analytical (4.6 x 250 mm) and semi-preparative (10 x 250 mm) HPLC columns (Econosil C-18, 10 μm) as well as the chiral HPLC column (Resolvosil®-BSA-7, 4.0 x 150 mm) were obtained from Alltech Applied Sciences (Deerfield, IL). Liquid scintillation counting (LKB Wallac 1219 RackBeta) was conducted in Budget-Solve™ (Research Products Intl., Prospect, IL) scintillation cocktail at an efficiency of 47%. (*D,L*)-3-Hydroxykynurenine was obtained from Sigma Chemical Co. (St. Louis, MO) while (*L*)-3-hydroxykynurenine was obtained from Wako Pure Chemical Industries, Ltd. (Richmond, VA).

(*D,L*)-5-Bromo-3-hydroxykynurenine ((*D,L*)-5-Br-3-HK). A solution of 3-hydroxykynurenine (101.0 mg, 0.45 mmol) and bromine (52.7 mg, 0.33 mmol) in glacial acetic acid (50 mL) was stirred under argon at ambient temperature for 12 h in

the dark. The mixture was filtered, and the reaction flask and filter were washed with benzene (50 mL). Removal of solvents under reduced pressure (20 torr, 40 °C) provided a bright yellow residue which was dissolved in water (1 mL), applied to a reverse phase column (65 g) and eluted with water. Concentration of like fractions afforded racemic 5-Br-3-HK (63.4 mg) as a bright yellow solid in 64% yield which was dried *in vacuo* (0.1 torr) over phosphorous pentoxide: mp 204 °C (dec.). ^{1}H NMR: δ 3.74 (2H, d, J = 5.2 Hz; CH_2); 4.49 (1H, m; CH); 5.82 (br s; exchangeable hydrogens); 7.04 (1H, d, *Jmeta* = 2.1 Hz); 7.48 (1H, d, *Jmeta* = 2.1 Hz). Anal. Calcd. for $C_{10}H_{11}N_2O_4Br$: C, 39.63; H, 3.66; N, 9.24; Br, 26.36. Found: C, 39.49; H, 3.73; N, 9.19; Br, 26.19. Analytical reverse phase HPLC using aqueous ammonium formate (0.5 M)/methanol (35:65 *v/v*; 1.0 mL/min) with UV detection (254 nm) showed (*D,L*)-5-Br-3-HK (R_t = 4.77 min, k' = 1.4) free of (*D,L*)-3-HK (R_t = 3.20 min; k' = 0.6).

(*D,L*)-[^{3}H]-3-hydroxykynurenine ((*D,L*)-[^{3}H]-3-HK). The tritiation reaction was carried out on racemic 5-Br-3-HK (25 mg) at Amersham International, plc (Buckinghamshire, England) under proprietary (method TR3) catalyzed halogen displacement conditions using tritium gas (10 Ci). Catalyst and labile tritium were removed, and the crude product (170 mCi) was supplied in a water/ethanol (1:1, *v/v*) solution. A portion of this solution (10 mL) was taken to dryness under reduced pressure (20 torr, 45 °C). The brown residue was dissolved in aqueous formic acid (1.0 mL, 0.12 M) and clarified by passage through a 0.45 μm Nylon filter. An aliquot of this solution (0.8 mL, 11.79 mCi) was purified by semi-preparative ion pair, reverse phase HPLC using aqueous formic acid (0.12 M) as the mobile phase at a flow rate of 4.0 mL/min with UV detection (254 nm). (*D,L*)-[^{3}H]-3-HK eluted at 5.58 min (k' = 1.3), and was collected in a volumetric flask (10 mL). Dilution to the mark provided a standard solution containing 9.25 mCi (78% radioactivity recovery). The chemical and radiochemical purity were determined by analytical reverse phase HPLC using the same eluent at a flow rate of 2.25 mL/min. Under these conditions, (*D,L*)-[^{3}H]-3-HK eluted at 6.00 min (k' = 3.9). Based upon the UV absorbance chromatogram, the chemical purity was 98.2%. Fractions (675 μL) of the eluent were collected and analyzed by liquid scintillation counting to give the radiochemical purity as 98.7%. For final purification, aliquots of the (*D,L*)-[^{3}H]-3-HK (*ca.* 50 μg, 800 μCi) stock solution were submitted to the analytical conditions and the heart cut collected by hand for an 88% recovery of radioactivity. The area of the UV absorbance peak of carrier (*D,L*)-3-HK in aliquots of this formulation was measured by HPLC using an automated integrator, and compared with the area of standard samples of (*D,L*)-3-HK for determination of the concentration. Aliquots also were counted by liquid scintillation spectrometry, and the specific radioactivity was calculated to be 3.64 Ci/mmol. Standard errors for measurements (n = 4) were < 2%.

Resolution of (*D,L*)-[^{3}H]-3-HK. The 0.12 M formic acid was removed from a purified sample of (*D,L*)-[^{3}H]-3-HK (100 μCi) under reduced pressure, and the residue was taken up in phosphate buffer (0.15 mL; 0.1 M, pH 7.5). Chiral HPLC using the same phosphate buffer as mobile phase (2 mL/min) with UV detection (254

nm) allowed isolation of (*D*)-[^{3}H]-3-HK (46 μCi, 46%; R_t = 1.26 min, k' = 0.3) and (*L*)-[^{3}H]-3-HK (41 μCi, 41%; R_t = 9.00 min, k' = 8.2). The relative stereochemistry was assigned by comparison to authentic samples of (*L*)-3-HK and (*D,L*)-3-HK. Retention on the chiral column was somewhat subject to mass effects, and an equal count rate of either (*D*)- or (*L*)-[^{3}H]-3-HK was mixed with racemic 3-HK (6 μg) before analysis by chiral HPLC under the conditions employed for the resolution. Liquid scintillation counting of eluent fractions (600 μL) showed each of the resolved materials to be enantiomerically pure. The stock solutions of these samples were diluted with aqueous formic acid (0.3 volumes, 1.2 M) for storage at pH 2.8 at 8 °C. (*D*)- and (*L*)-[^{3}H]-3-HK coeluted with either (*D,L*)-3-HK or (*L*)-3-HK under ion pair, reverse phase HPLC conditions. Minor isotopic exchange (< 0.6%) occurred during resolution procedures.

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