Journal of Chromatography, 428 (1988) 340–344
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4201

Note

Quantification of 3-hydroxykynurenine in brain by high-performance liquid chromatography and electrochemical detection

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(First received January 5th, 1988; revised manuscript received March 1st, 1988)

3-Hydroxykynurenine (3-HKYN) is a well recognized excretory product of systemic L-tryptophan (L-TRP) catabolism [1–3]. The synthesis and excretion of 3-HKYN is increased in a variety of states including L-TRP administration, cancer, and vitamin B_6 deficiency [1,4–6]. 3-HKYN is synthesized via the kynurenine pathway and, as such, is an intermediate in the synthesis of nicotinamide-containing nucleotides. Recent studies have indicated that L-TRP is metabolized through the kynurenine pathway in the central nervous system and that 3-HKYN is present in brain [7,8]. However, virtually no studies have investigated the regulation of L-TRP metabolism through the kynurenine pathway in brain. Direct injection of 3-HKYN into the brains of experimental animals has been reported to induce seizures [9,10]. Clearly, investigations of brain 3-HKYN levels in neuropathologic states and studies of how 3-HKYN levels are regulated within the central nervous system may be important. The development of a simple, accurate, and sensitive assay for 3-HKYN is a useful step in this investigation. To date, 3-HKYN had been quantified by high-performance liquid chromatography (HPLC) in systemic tissues [2,3,6,11] and in brain from vitamin B_6-deficient rats [8].

We have developed a simple assay for 3-HKYN in brain which requires minimal sample preparation and is sufficiently sensitive to quantify the low levels of 3-HKYN in normal brain. The method is based on HPLC separation of 3-HKYN

0378-4347/88/$03.50

in perchloric acid extracts of brain and electrochemical detection. We demonstrate that brain 3-HKYN is sensitive to the availability of systemic L-TRP.

EXPERIMENTAL

Reagents

All solvents were A.C.S. certified grade. Reference compounds were obtained from Sigma (St. Louis, MO, U.S.A.). Remaining compounds were obtained from Fisher (Fair Lawn, NJ, U.S.A.).

Chromatographic system

The reversed-phase HPLC system consisted of a 75 mm×4.6 mm, 3-μm Ultrasphere ODS column, either an LC-4B or an LC-4A (BAS, LaFayette, IN, U.S.A.) amperometric detector and glassy carbon electrochemical detector cell. The reference electrode was Ag/AgCl. The pump was a Waters (Milford, MA, U.S.A.) Model 510 and samples (20 μl) were injected with a Waters WISP 710B autoinjector. Oxidation current was recorded on a Waters 740 data module chart recorder set at 1.6 or 0.8 nA full scale. The mobile phase consisted of 950 ml deionized water, 20 ml acetonitrile, 9 ml triethylamine, 5.9 ml phosphoric acid, 100 mg sodium EDTA, and 1.8 g heptane sulphonic acid. The mobile phase was filtered through 45-μm membrane filters and degassed under vacuum immediately prior to use. The flow-rate was 1.0 ml/min. Oxidation voltage was varied between 0.40 and 0.80 V. For routine assay of 3-HKYN oxidation the voltage was either 0.55 or 0.60 V.

Preparation of brain samples

Samples of brain (50–150 mg) were sonicated in 1 ml of 1 *M* perchloric acid (PCA) using a Kontes cell disruptor. Some samples were sonicated in 1 ml PCA containing 250 pmol of dihydroxybenzylamide (DHBA) as internal standard (I.S.). Samples were centrifuged at 10 500 *g* at 4°C for 20 min and aliquots (100 μl) of the supernatants were stored at −70°C until assayed. A 10-μl volume of ascorbic acid oxidase (Boehringer-Mannheim, Indianapolis, IN, U.S.A.; 1 mg per 10 ml) was added to each sample at least 30 min prior to injection. Standards were dissolved in 0.1 *M* PCA and diluted with 1 *M* PCA containing DHBA.

Establishment of the assay

The stability of 3-HKYN in water and PCA was tested. Oxidation current as a function of applied voltage was determined in 3-HKYN standards dissolved in PCA. The same brain sample extract was quantified six times to determine injection reproducibility. Cerebral cortex which had first been frozen on dry ice and pulverized by mortar and pestle was aliquoted into six samples, and the 3-HKYN concentrations measured in the same assay to determine the intra-assay variability. The inter-assay variability was determined by measuring 3-HKYN levels in a further six aliquots of cerebral cortex in six separate assays. Six samples of brain were spiked with 4.5 pmol of 3-HKYN to determine the recovery of 3-HKYN.

Procedures

Samples of cerebral cortex from C57BL/6NCR mice and Sprague–Dawley rats were used in the development of the assay. Thirty-two male Sprague–Dawley rats (200–250 g) were used to determine the effects of systemic L-TRP loading on brain 3-HKYN concentrations and investigate the contribution of 3-HKYN in blood on the measured response to L-TRP. Sixteen rats were given an intraperitoneal injection of 6 ml of 150 mmol/l saline as control and sixteen rats were given an intraperitoneal injection of 1 mmol/kg body weight L-TRP dissolved in saline. After 2 h, rats were rapidly anesthetized by an intraperitoneal injection of sodium pentobarbital (75 mg/kg). The brains of half of the rats given saline and half of the rats given L-TRP were flushed with ice cold 150 mmol/l saline via intracardiac perfusion (30 ml per 2 min) to remove blood and then decapitated. The total elapsed time from induction of anesthesia to death was 3.5 min. The remaining rats were decapitated 3.5 min after induction of anesthesia. All brains were rapidly removed and samples of frontal cerebral cortex collected. Samples were immediately frozen in tared 1.5-ml polypropylene tubes.

RESULTS AND DISCUSSION

The identity of 3-HKYN in brain was verified by ranging the concentration of heptane sulphonic acid from 0.8 to 1.8 g (five concentrations) and demonstrating that the retention time of the peak identified as 3-HKYN in brain tracked with authentic 3-HKYN. A typical reversed-phase HPLC separation of 1 pmol 3-HKYN standard is presented in Fig. 1A. The time of elution of 3-HKYN in standards and brain was 8.8 min. 3-HKYN was quantified by measuring peak heights. Peak oxidation current for 3-HKYN was achieved at approximately 0.65–0.70 mV [11]. Optimum oxidation voltage for analysis of brain was 0.55–0.60 mV, which ensured minimal interference from baseline noise or adjacent peaks and only 17% loss of the 3-HKYN signal. Fig. 1B shows a chromatogram from the cerebral cortex of an untreated mouse. The 3-HKYN peak in this brain sample corresponds to 213 fmol (59.9 fmol/mg brain). Systematic L-TRP administration resulted in a marked increase in 3-HKYN in cerebral cortex (Fig. 1C). At an oxidation voltage of 0.55 mV, the minimum detection limit for 3-HKYN at a signal-to-noise ratio of 5:1 was 175 fmol. Over a 15-h period, there was only 4% loss of 3-HKYN when dissolved in 6% PCA, whereas in water, 18% loss occurred.

The standard curve of 3-HKYN with both external and internal standards was linear over the 0–2000 fmol range (peak height 0–50 mm). Typical regression analysis by the method of least squares were: external standard (E.S.): $r=0.989$, slope = 39.5 fmol/mm, y-intercept = -23.5 fmol, x-intercept = 0.59 mm; I.S.: $r=0.999$, y-intercept = -2.3 fmol. There was no significant difference in variance of slopes between I.S. versus E.S. standard curves. No oxidation currents were found at the time of DHBA elution in samples sonicated in PCA alone (Fig. 1D; L-TRP-treated mouse). Therefore, DHBA can be used as I.S. Fig. 1E shows a chromatogram from a sample of cortex sonicated in PCA containing DHBA. Results of regression analysis of standard curves with DHBA as I.S. were very similar to the values obtained using E.S.

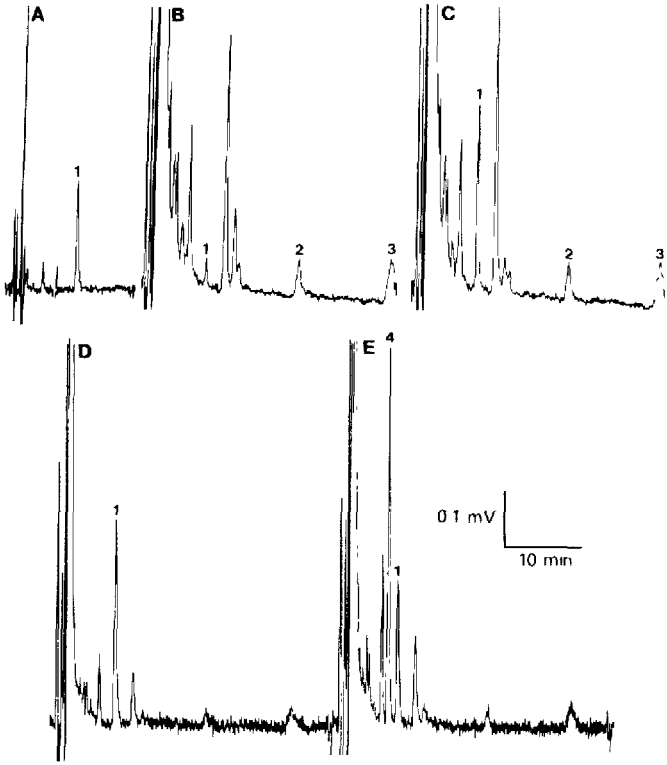

Fig. 1. Reversed-phase chromatograms of a 1-pmol 3-HKYN (peak 1) standard (A), a sample of cerebral cortex from an untreated mouse (B) and a mouse treated with 1 mmol/kg L-TRP (C). Samples were sonicated in 1 ml of PCA alone. Peaks 2 and 3: serotonin and 5-hydroxyindoleacetic acid, respectively. (D) Chromatogram of a sample of cerebral cortex from a mouse treated with L-TRP showing the position of DHBA elution. (E) A sample of the same cerebral cortex as D, but sonicated in 1 ml PCA containing 250 pmol DHBA (peak 4) as internal standard.

Repeated injections of the same brain sample gave a coefficient of variation (standard deviation/mean $\times 100\%$) of 1.10% (E.S.) and 0.78% (I.S.). The intra-assay coefficient of variation was 4.0% (E.S.) and 4.6% (I.S.). The inter-assay coefficient of variation was 13.9% (E.S.) and 11.8% (I.S.). The recovery of 3-HKYN from five samples of brain was 103% (E.S.) and 102% (I.S.).

Systemic injection of 1 mmol/kg body weight of L-TRP increased cortical 3-HKYN from 62.4 ± 21.4 to 198.8 ± 36.6 fmol/mg ($p < 0.005$; unpaired t-test) in rat brains not flushed with blood. Removal of blood from the brain had no significant effect on brain 3-HKYN concentrations in untreated rats (58.8 ± 11.6 fmol/mg) and did not affect increases in 3-HKYN in response to systemic L-TRP (217.1 ± 86.0 fmol/mg). These observations are consistent with the notion that catabolism of L-TRP through the kynurenine pathway occurs in brain. However, from the present data we cannot exclude the possibility that some of the 3-HKYN in brain was accumulated from the blood prior to sample collection.

We conclude that 3-HKYN can be accurately quantified in untreated rat and

mouse brain by HPLC and electrochemical detection and that brain 3-HKYN concentrations are sensitive to increases in systemic L-TRP availability.

ACKNOWLEDGEMENTS

We acknowledge the careful technical assistance of P. Kim and thank J. Harvey-White for helpful discussion.

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