Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 287–293
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

High-performance liquid chromatographic method for the quantification of anthranilic and 3-hydroxyanthranilic acid in rat brain dialysate☆

G. Cannazza [a,*], M. Baraldi [a], D. Braghiroli [b], A. Tait [a], C. Parenti [a]

[a] *Dipartimento di Scienze Farmaceutiche, Università degli Studi di Modena e Reggio Emilia, Via Campi 183, 41100 Modena, Italy*
[b] *Dipartimento di Scienze del Farmaco, Università degli Studi "G. D'Annunzio" Chieti, Via Dei Vestini, 66013 Chieti Scalo (CH), Italy*

Received 22 June 2001; received in revised form 5 August 2002; accepted 9 August 2002

Abstract

Anthranilic acid (ANA) and 3-hydroxyanthranilic acid (3-HANA) have attracted considerable attention as two of the L-tryptophan kynurenine pathway metabolites in the central nervous system. In this study, a highly sensitive and accurate method for the quantification of ANA and 3-HANA has been developed using reversed-phase high performance liquid chromatography (HPLC) with fluorimetric detection. The HPLC assay was carried out using a C_{18} column (5 μm, 250 × 4.6 mm i.d.). The mobile phase consisted of a mixture of 25 mM sodium/acetic acid buffer (pH 5.5) and methanol (90:10 v/v). Fluorimetric detection at $\lambda_{ex} = 316$ nm and $\lambda_{em} = 420$ nm was used. The assay was applied to the measurement of ANA and 3-HANA acid in rat brain dialysate following administration of L-tryptophan or L-kynurenine. 3-HANA and ANA levels were progressively increased during 90 min following administration of L-tryptophan, then decreased progressively to basal levels. 3-HANA levels were significantly higher than ANA levels after L-kynurenine administration. These findings suggest that the assay developed should provide an improved means for investigation of neurobiology of kynurenine pathway.

Keywords: Kynurenines; Anthranilic acid; 3-hydroxyanthranilic acid; Microdialysis; Rat brain

1. Introduction

The opening of the tryptophan indole ring by tryptophan 2,3-dioxygenase (EC1.13.1.2) in the liver or by indoleamine 2,3-dioxygenase (EC 1.13.11.14) elsewhere in the body, initiates the formation of a series of compound called kynurenines [1]. Several kynurenines, including quinolinic acid (QUIN), kynurenic acid (KYNA), 3-hydroxykynurenine (3-HK), L-kynurenine (KYN)

☆ Presented at the 9th International Meeting on Recent Developments in Pharmaceutical Analysis (RDPA'01), Lipari, Aeolian Islands, Messina, Italy.

* Corresponding author. Tel.: +39-59-2055136; fax: +39-59-371590.
E-mail address: pippi@unimo.it (G. Cannazza).

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00091-8

and 3-hydroxyanthranilic acid (3-HANA), have been shown to elicit significant behavioral effects when injected intracerebroventricularly in experimental animals [2]. Moreover, as kynurenines are endogenous brain metabolites, it has been suggested that imbalances in their production, transport or catabolism may lead to discrete brain dysfunction and may be causally related to the pathogenesis of some neuropsychiatric disorders [3–7]. The source and metabolism of brain kynurenines have not been clearly elucidated. As reported in Fig. 1, KYN is a substrate of several enzymes and, in particular, it may be metabolized into anthranilic acid (ANA) and 3-HK by the action of the enzymes kynureninase (EC 7.3.1.3) and kynurenine hydroxylase (EC 1.14.13.9). Subsequently ANA and 3-HK may be metabolized in 3-HANA by the action respectively of liver microsomal hydroxylase enzymes and kynureninase.

Chiarugi et al. suggested that the hydroxylation of kynurenine and the subsequent hydrolysis of 3-HK are the preferred pathway of kynurenine metabolism in brain [8]. In peripheral organs, however, KYN seems to be hydrolyzed rapidly to ANA, which is subsequently hydroxylated to 3-HANA [8].

In the continuing efforts to elucidate the role of kynurenine pathway metabolites in cerebral function and dysfunction, it became necessary to

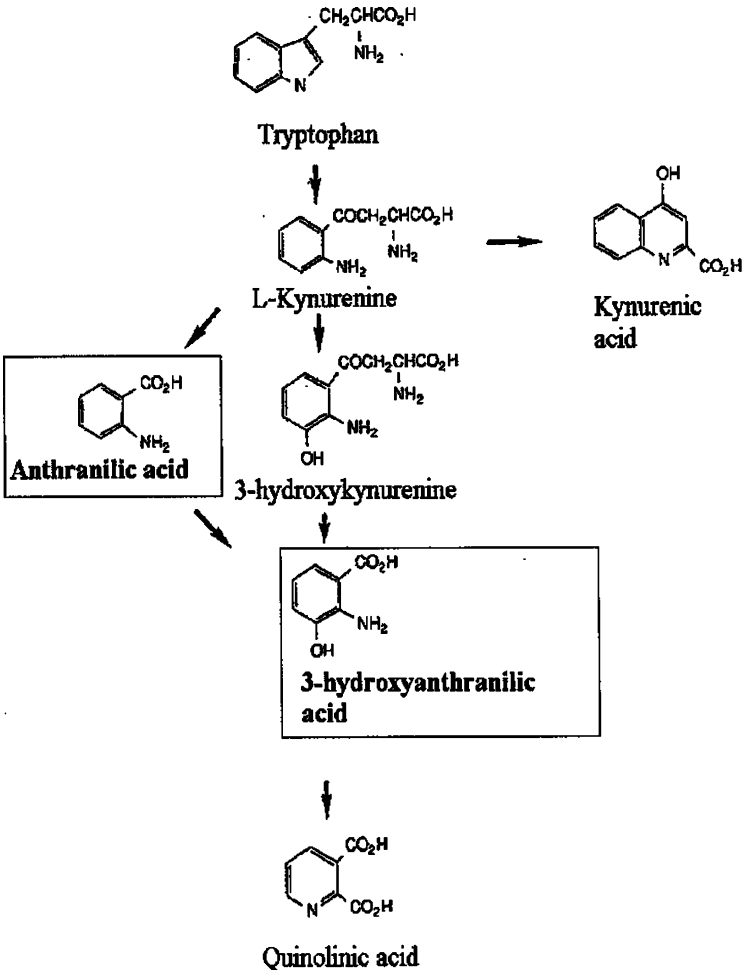

Fig. 1. Kynurenines pathway.

dispose a valuable assay to quantitate ANA and 3-HANA in the brain.

The usual method for the determination of ANA and 3-HANA in rat brain tissue are high performance liquid chromatography (HPLC) with fluorescent detection. Guidetti et al. [9] proposed an HPLC method for the determination of ANA in rat serum, urine and in several organs with a sensitivity limit of 50 fmol. Fomstedt-Wallin et al. [10] performed the measurements of 3-HANA in rat serum and brain by HPLC with fluorimetric detection to study the effects on 3-HANA after treatment with the 3-hydroxyanthranilic acid 3,4-dioxygenase (EC 1.13.11.6) inhibitor. Carpenedo et al. [8,11] quantified simultaneously ANA and 3-HANA in rat mice to study the regulation of the synthesis of QUIN and KYNA after administration of kynurenine.

No reference has been found in the literature to the determination of ANA and 3-HANA in rat brain dialysate.

In this paper, it is described an HPLC method which is applicable to the determination of small amounts of ANA and 3-HANA in both rat brain tissue and dialysate. The simultaneously measurements of concentrations of ANA and 3-HANA in rat hippocampal microdialysate was performed following acute administration of precursors tryptophan or KYN.

2. Experimental

2.1. Chemicals

ANA, 3-HANA and perchloric acid were obtained from Sigma-Aldrich (St. Louis MO). Methanol was HPLC grade (Sigma-Aldrich). Triple deionized water (Millipore, Bedford, MA) was used for all preparations.

The dialysis perfusate used was artificial cerebrospinal fluid (aCSF) and consisted of 147 mM of NaCl, 4 mM of KCl and 2.3 mM of $CaCl_2$. All reagents used in the preparation of buffer solutions were of analytical reagent grade or better and were used as received.

2.2. Fluorimetric measurements

Aqueous solutions of 100 mM of ANA and 3-HANA were prepared and the pH of the solutions was varied over the range 4–6, by the addition of trace amounts of hydrochloric acid or sodium hydroxide. Fluorimetric spectra of single solutions of ANA and 3-HANA at different pH were measured.

Fluorescence spectra was made on a L-7480 Merck-Hitachi fluorimeter (Bracco, Italy) and measured at $\lambda_{ex} = 316$ and $\lambda_{em} = 420$ nm.

2.3. Chromatography

The chromatographic apparatus consisted of a L-6200A Merck-Hitachi Pump (Bracco, Italy), a 7125 Reodhyne manual injector (Bracco, Italy) equipped with a 50 μl sample loop, a L-7480 Merck.-Hitachi fluorimeter (Bracco, Italy) was used as detector. Chromatograms were recorded with a Spectra Phisics Integrator (Waters, Italy). The separation was obtained using a column LiChrospher 100 ODS, 5 μm, 250 × 4.6, Merck (Bracco, Italy).

The eluent was prepared by mixing an aqueous solution of 20 mM of sodium acetate/acetic acid (pH 5.5) with methanol in a 90:10 (v/v) proportion. The eluent was filtered through a 0.45 urn cellulose acetate filter and degassed before use. The flow-rate was 1 ml/min.

2.4. Brain tissue analysis

Routinely, Sprague–Dawley male rats (Harlan, Italy) of different age were anesthetized with chloral hydrate (400 mg/kg/i.p.) and the brain was quickly removed. Hippocampus and frontal cortex were dissected at 4 °C. Brain and dissected tissues were frozen on dry ice and stored at −80 °C until the assay. All tissue samples were homogenised in perchloric acid 0.4 M (1:10 w/v) and the resulting homogenates were split into 1 ml aliquots which were processed either alone or after addition of ANA and 3-HANA. The samples were sonicated and centrifugated (20 min at 3000 × *g*). 50 μl of the resulting supernatant were subjected to HPLC analysis.

2.5. Microdialysis

Sprague–Dawley male rats (250–300 g) were anaesthetised with chloral hydrate (400 mg/kg/i.p.) the skull was exposed and a guide cannula (CMA/10, Carnegie Medicine, Stockholm, Sweden) was implanted stereotaxically 2 mm above the middle of the right hippocampus. The coordinates (A–P −5.8 mm, M–L −4.8 mm, from the bregma D–V +2.4 mm, from the dura) were calculated according to the atlas of Paxinos and Watson [12]. The guide cannula was fixed to the skull of the rat with dental cement and anchor screws. After 24 h recovery period the animal was placed in a system for freely moving animals (CMA/120). The concentric microdialysis probe (CMA/10) was slowly inserted into the hippocampus via the guida cannula. The tip of the probe extended 2 mm below the end of the guida cannula to reach the middle of the hippocampus. The probe was connected to a slow-speed syringe pump (CMA/100) and perfused with artificial CSF (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 2.3 mM) at a flow rate of 2 μl/min. Dialysate samples were collected into polyethylene microcentrifuge vials every 30 min following a 3 h stabilisation period of 3-HANA and ANA levels in the dialysate after probe insertion, aqueous suspension of tryptophan or L-kynurenine (100 mg/kg/i.p.) was acutely administered. Collected perfusates were directly injected into the HPLC system.

A baseline level, defined as three consecutive peak areas differing by $\leqslant 10\%$, was usually obtained 2–3 h after probe insertion. The concentrations of ANA and 3-HANA in dialysate was confirmed by their retention times with those from authentic standard samples.

Before implantation of the microdialysis probe, the in vitro recovery was determined by placing the probe into a stirred (37°) CSF solution 10 nM each of ANA and 3-HANA.

Samples of dialysate were collected at 30 min intervals and injected into the HPLC system to determine concentrations of ANA and 3-HANA. The in vitro recoveries were calculated using the expression:

$$\%\text{Recovery}_{\text{invitro}} = (C_{\text{dialysate}}/C_{\text{sample}}) \times 100 \quad (1)$$

where $C_{\text{dialysate}}$ is the concentration of analyte in the dialysate and C_{sample} is the concentration of analyte in the solution around the probe.

3. Results and discussion

3.1. Fluorimetric properties of 3-HANA and ANA

A study of the influence of pH on the fluorescence intensity of both 3-HANA and ANA was carried out. The pH of the solutions was varied over the range 4–6 by addition of trace amounts of acetic acid or sodium hydroxide. The results obtained for 3-HANA were presented in Fig. 2.

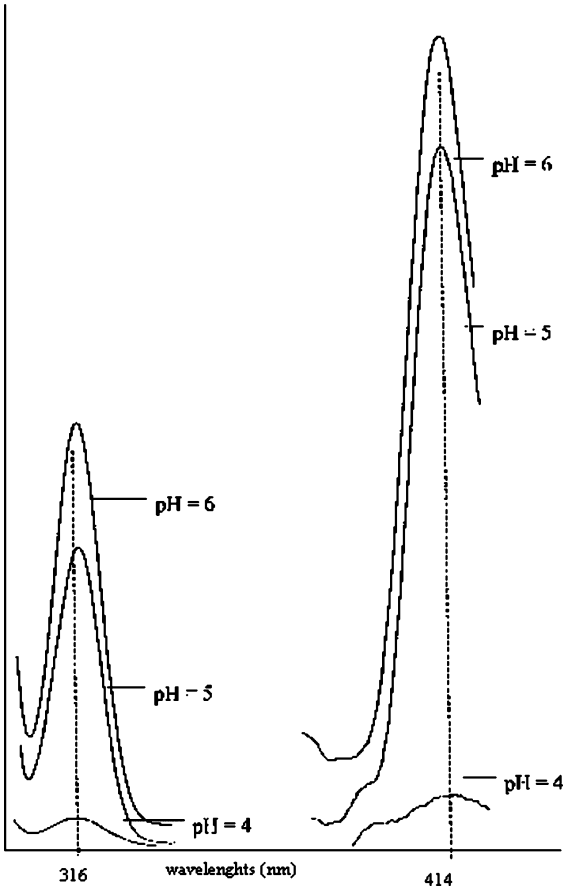

Fig. 2. Influence of pH on the excitation and emission spectra of the 3-HANA solution.

Acidification caused a decrease of fluorescence intensity for 3-HANA, being practically negligible at pH < 4. Fluorescence spectra measured at pH > 4 revealed an excitation maximum at 316 nm and a fluorescence maximum at 414 nm.

3.2. Chromatographic analysis

A chromatographic analysis of an aqueous standard solution was presented in Fig. 3a and a typical chromatographic analysis of rat brain tissue extract alone and after addition of references substances in Fig. 3c and b, respectively. The retention times of 3-HANA and ANA were respectively 6.6 and 12.0 min.

Using rat brain tissue as starting material, two peaks with identical retention times as standard 3-HANA and ANA were detected. Addition of references substances to cerebral tissue increased peaks height without creating a second peak. Further identification of tissue 3-HANA and ANA were accomplished by analysing the fluorescence spectra measured under stop flow conditions at maximum of HPLC peaks revealed for authentic substances as well as for isolated components the same excitation and emission maximum.

Recovery tests were performed in triplicate by spiking rat brain tissues samples with 50 fmol/mg of standards 3-HANA and ANA. The mean recovery of 3- HANA and ANA added to tissues sample were 103 ± 4.1% and 106 ± 2.6% (mean ± S.D.).

The intra- and inter-assay coefficients of variation measured ($n = 12$) were respectively 2.6 and 3.8% for 3-HANA (10 nM) and 2.1 and 3.2% for ANA(10 nM).

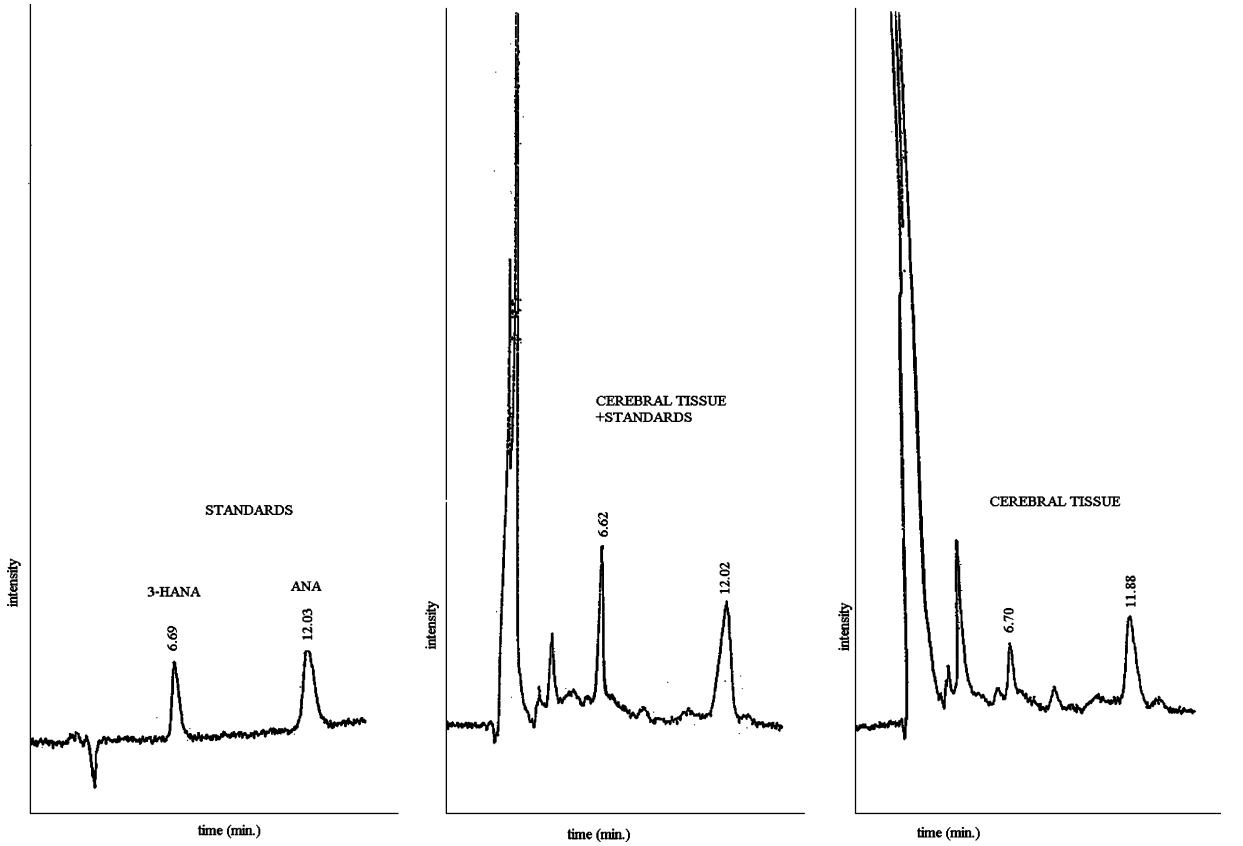

Fig. 3. HPLC profiles of (a) standard ANA and 3-HANA (50 fmol), (b) tissue extract plus standard and (c) tissue extract. See text for experimental details.

The linearity and reproducibility of the present method were tested. Linear calibration curves were obtained over the range of 5–50 nM for both 3-HANA ($r^2 = 0.996$) and ANA ($r^2 = 0.998$).

The detection limits for both 3-HANA and ANA were about 1 nM at a S/N = 3.

The cerebral tissue concentrations of ANA and 3-HANA are showed in Table 1.

The values obtained for the contents of ANA and 3-HANA in rat hippocampus, cortex and whole brain were in good agreement with previous reports [1,3–6].

Assessment of the distribution of ANA and 3-HANA in the rat brain revealed only small regional differences.

3.3. Microdialysis

The assay described has been used to measure 3-HANA and ANA concentration in rat hippocampal microdialysate samples. In vitro recovery test was performed in a standard mixture to determine the recoveries of ANA and 3-HANA at 2 μl/min. The in vitro recoveries of a standard mixture containing 10 nM 3-HANA and ANA were 13 ± 1% and 23 ± 2%, respectively ($n = 6$).

Table 1
3-HANA and ANA rat brain concentrations

	3-HANA (fmol/mg tissue)	ANA (fmol/mg tissue)
Whole rat brain	18 ± 0.9	35 ± 4.2
Hippocampus rat tissue	12 ± 0.8	39 ± 1.3
Cortex rat tissue	11 ± 0.8	34 ± 4.5

Values were expressed as mean ± S.E.M. ($n = 5$).

In general, stable basal levels of analytes in hippocampus were obtained 2 h after implantation of microdialysis probes. Basal concentrations of ANA and 3-HANA were 1.1 ± 0.1 nM and 0.42 ± 0.08 nM, respectively ($n = 24$).

Fig. 4 shows the hippocampal fluid concentration-time profile (values were expressed as percent variation of basal levels) of ANA and 3-HANA in rat after the administration of an intraperitoneal dose of 100 mg/kg of tryptophan ($n = 4$).

ANA levels increased 18 times basal levels and 3-HANA 12 times basal levels for 120 min following tryptophan administration. The levels gradually returned to basal levels in 210 min.

The concentrations of ANA and 3-HANA in dialysate, sampled from the hippocampus ($n = 4$) of rats subjected to KYN intraperitoneal administration (100 mg/kg), are shown in Fig. 5.

3-HANA levels drastically increased (13 times basal levels) for 120 min following KYN administration. Then 3-HANA levels gradually decreased. There were no significant ANA levels changes following administration of KYN for at least 210 min. Fukui et al. [13] have demonstrated that KYN is readily taken up into brain by the large neutral amino acid carrier of the blood–

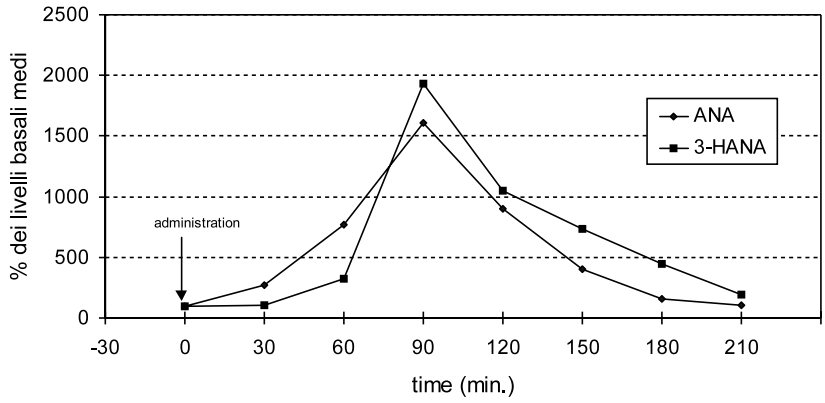

Fig. 4. Time course of 3-HANA and ANA outputs from rat hippocampus after a single administration of tryptophan (100 mg/kg/i.p.). Values were expressed as percentage variation of mean value detected before drug administration. S.E.M. were always lower then 14% ($n = 4$) and omitted from the graph.

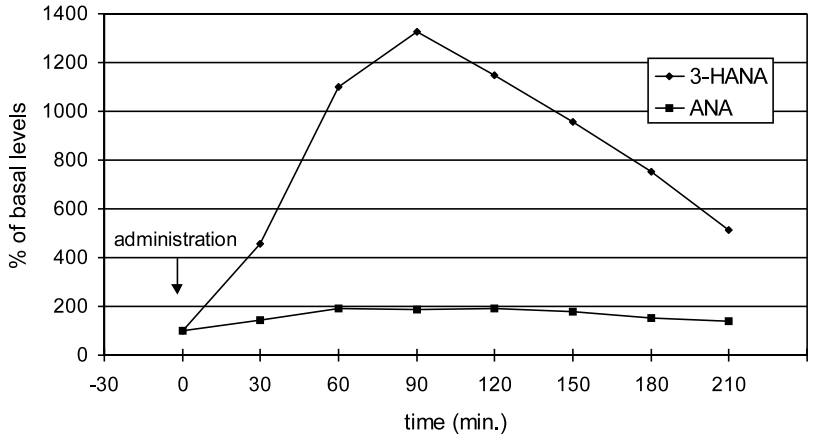

Fig. 5. Time course of 3-HANA and ANA outputs from rat hippocampus after a single administration of KYN (100 mg/kg/i.p.). Values were expressed as percentage variation of mean value detected before drug administration. S.E.M. were always lower then 14% ($n = 4$) and omitted from the graph.

brain barrier. Tryptophan inhibits the blood–brain barrier transport of KYN by $>90\%$ [13].

Moreover Chiarugi et al. suggested that the hydroxylation of kynurenine and the subsequent hydrolysis of 3-HK are the preferred pathway of kynurenine metabolism in brain [8]. In peripheral organs, however, KYN seems to be hydrolyzed rapidly to ANA, which is subsequently hydroxylated to 3-HANA [8].

Our data confirm these previous studies and suggest that the disposition of administered tryptophan or KYN preferentially occurs through hydroxylation in brain and through hydrolysis in peripheral tissues.

4. Conclusion

A sensitive and reliable HPLC-fluorimetric assay was developed for the simultaneous determination of ANA and 3-HANA in rat brain tissue.

The assay has been used to measure 3-HANA and ANA concentration in rat hippocampal microdialysate samples following acute administration of precursors tryptophan or KYN.

3-HANA and ANA levels were progressively increased during 90 min following administration of tryptophan, then decreased progressively to basal levels. 3-HANA levels were significantly higher than ANA levels following KYN administration. These findings suggest differences between brain and peripheral kynurenine metabolism.

References

[1] T.W. Stone, Pharmacol. Rev. 45 (1993) 309–379.
[2] I.P. Lapin, Epilepsia 22 (1982) 257–265.
[3] T.W. Stone, N.R. Burton, Prog. Neurobiol. 30 (1988) 333–368.
[4] R. Schwarcz, E. Okuno, R.J. White, E.D. Bird, W.O. Whetsell, Proc. Natl. Acad. Sci. USA 85 (1988) 4079–4081.
[5] M.P. Heyes, I.N. Mefford, B.J. Quearry, M. Dedhia, A. Lackner, Ann. Neurol. 27 (1990) 666–675.
[6] P. Zanoli, G. Cannazza, M. Baraldi, Brain Res. Bull. 55 (2001) 235–238.
[7] G. Cannazza, A. Chiarugi, C. Parenti, P. Zanoli, M. Baraldi, Neurochem. Res. 26 (2001) 511–514.
[8] A. Chiarugi, R. Carpenedo, F. Moroni, J. Neurochem. 67 (1996) 692–698.
[9] P. Guidetti, J.L. Walsh, R. Schwarcz, Anal. Biochem. 220 (1994) 181–184.
[10] B. Fomstedt-Wallin, J. Lundstrom, G. Fredriksson, R. Schwarcz, J. Lutham, Eur. J. Pharmacol. 386 (1999) 15–24.
[11] R. Carpenedo, A. Chiarugi, P. Russi, G. Lombardi, V. Caria, R. Pellicciari, L. Mattoli, F. Moroni, Neuroscience 61 (1994) 237–244.
[12] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, second ed., Academic Press, New York, 1986.
[13] S. Fukui, R. Schwarcz, S.I. Rapoport, Y. Takada, Q.R. Smith, J. Neurochem. 56 (1991) 2008–2017.