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Time-course of kynurenic acid concentration in mouse serum following the administration of a novel kynurenic acid analog

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ABSTRACT

The changes in concentration of kynurenic acid (KYNA) in different biological samples are of great interest in the pathomechanism and medication of several disorders, and especially those affecting the nervous system. Besides the recent pharmaceutical advances targeting the kynurenine pathway, there is a constant need for further drug development through the synthesis of novel analogs. Reliable analytical methods should be set up to monitor the metabolism and effects of these analogs in both preclinical experiments and human studies. Following a sample preparation procedure based on protein precipitation, new high-performance liquid chromatographic methods with fluorescence and mass spectrometric detection were developed for the determination of KYNA and a novel KYNA analog (*N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride; KYNA amide) in mouse serum samples. The analytical parameters obtained in the validation procedure suggest that the developed method with mass spectrometric detection is simple, fast, accurate and suitable for the measurement of KYNA and its analogs. The results reveal the good *in vivo* stability of the novel KYNA amide.

1. Introduction

Kynurenic acid (KYNA) is a widely studied metabolite of tryptophan, formed through the kynurenine pathway [1]. It is known to exert endogenous protection against the toxic effects of other kynurenine pathway metabolites, such as quinolinic acid and 3-hydroxy-L-kynurenine, and those of other excitotoxins. The possibility of its therapeutic use therefore emerges, including treatment of disorders of the central nervous system [2–4]. However, the chemical and pharmacokinetic properties of KYNA hamper its use in preclinical studies. In higher doses, its solubility is a limiting factor, it penetrates the blood–brain barrier poorly [5], and it undergoes a rapid clearance from the brain and the body, this clearance being mediated by organic anion transporters [6]. To overcome these disadvantages and/or improve pharmacodynamic properties, numerous derivatives or prodrugs have been synthesized [7,8]. A promising strategy is the ester or carboxamide formation, using a water-soluble side-chain [9]. Carboxamide formation is preferred as it is probably more resistant to *in vivo* cleavage. A water-soluble side-chain, 2-*N*,*N*-dimethylaminoethylamine, was applied in the amide moiety to synthesize *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride; KYNA amide). This KYNA derivative has already been proved to be beneficial in an *in vitro* model of pentylenetetrazole-induced epileptic seizures, in *in vivo* migraine models and in an *in vivo* model of Huntington's disease [10–13]. However, it is essential to acquire deeper insight into its mechanism of action.

A number of publications have dealt with the separation and determination of KYNA and its metabolites in biological media, including pharmacokinetic and metabolic studies. The most common method applied for determination of the concentration of KYNA and its metabolites is high-performance liquid chromatography (HPLC). The HPLC methods adopted have mostly employed ultraviolet (UV) [14,15] or fluorescence detection [16–22], but electrochemical detection has also been utilized [23]. UV detection is generally simple, but does not provide an adequate specificity and limit of detection (LOD) in biological matrices. Fluorescence detection can be a good choice, but HPLC coupled with mass spec-

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trometry (MS) detection [24–27] is the most powerful tool for the specific and sensitive qualification and quantification of different compounds in biological matrices. The lower LOD and unambiguous identification are the most important reasons why LC–MS (or LC–MS/MS) is nowadays applied more frequently for KYNA determination. Besides HPLC-based methods, capillary electrophoresis coupled with MS detection was recently applied for the determination of tryptophan metabolites [24,28].

The aim of the present study was to examine the serum pharmacokinetics of the above novel KYNA amide. Special attention was paid to the possible metabolism of this analog to KYNA, and a method applicable for the simultaneous detection of KYNA and the analog has been developed.

2. Materials and methods

2.1. Materials

N-(2-*N*,*N*-Dimethylaminoethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride was synthesized from KYNA. The synthetic procedure involved amidation between KYNA and *N*,*N*-dimethylethylenediamine in dimethylformamide in the presence of 1-hydroxybenzotriazole, with *N*,*N*′-diisopropylcarbodiimide as coupling reagent, followed by chromatographic purification. The structure and purity of the product were demonstrated by ^{1}H and ^{13}C NMR spectroscopy [9].

KYNA and other reagents of analytical grade were purchased from Sigma–Aldrich (Saint Louis, MO, USA). The Milli-Q water was further purified by filtration on a 0.45 μm filter (HV, Millipore, Molsheim, France).

2.2. Animals

The 5 month-old C57B/6 mice used were housed in cages (at most 5 per cage), with free access to food and water, under standard conditions. Male and female mice were distributed equally between the experimental groups. All animal experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) to minimize animal suffering and were approved in advance by the local animal care committee.

2.3. Treatment

A total of 36 mice (n = 4 in each group) were treated with single intraperitoneal injections of the KYNA amide (100 mg kg^{-1}, in a volume of 5 ml kg^{-1}, dissolved in distilled water; the pH of the solution was adjusted to 6.5 with NaOH).

2.4. Sample preparation

At set time points (5, 15, 30, 60, 90, 120, 150, 180 and 300 min) following the intraperitoneal injection with the KYNA amide, mice were deeply anesthetized with isoflurane (Forane®; Abott Laboratories Hungary Ltd., Budapest, Hungary). After thoracotomy, 0.3–0.7 ml venous blood was obtained from the right ventricle by intracardial puncture. To determine the basal serum level of KYNA, the same procedure was performed on untreated control mice (n = 4). The blood samples were left to coagulate for at least 30 min, and were then centrifuged for 5 min at 10,000 × g. The supernatant sera were pipetted into polypropylene Eppendorf tubes and were stored at −80 °C until further sample handling. Before analysis, the sera were thawed and centrifuged for 5 min at 10,000 × g, and 100 μl amounts were 'shot' onto a mixture of 675 μl acetonitrile (ACN) and 225 μl distilled water for protein precipitation. The mixture was centrifuged at 10,000 × g for 15 min, and 900 μl supernatant was pipetted into an Eppendorf tube and placed into a speed vac for 3 h.

2.5. Chromatographic conditions

The KYNA concentrations of the samples were quantitated with two HPLC systems, both based on the Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA), consisting of a binary pump, a micro vacuum degasser, a thermostated column compartment, an automatic liquid sampler, and ChemStation data managing software.

One of the systems was equipped with fluorescence detection, and the other with a MS detector (LCMSD VL). The former was applied for the determination of KYNA, and the latter for the simultaneous determination of KYNA and KYNA amide. In this way, the selectivity and reliability of the HPLC–MS method applied for the quantification of KYNA could be checked with a completely independent system.

Chromatographic separations with the HPLC system with fluorescence detection were performed on a Hypersil ODS C18 column, 150 mm × 4 mm I.D., 5 μm particle size (Thermo Fisher Scientific, Waltham, MA, USA) and a Hypersil ODS C18 guard column, 20 mm × 4 mm I.D., 5 μm particle size, with a mobile phase composition of 0.2 M zinc acetate/ACN = 95/5 (v/v), applying isocratic elution. The flow rate and the injection volume were 1 ml min^{-1} and 50 μl, respectively. The fluorescence detector was set at excitation and emission wavelengths of 344 and 398 nm, respectively.

Chromatographic separations with the HPLC–MS system were performed on a Kinetex C18 column, 100 mm × 4.6 mm 2.6 μm particle size (Phenomenex, Torrance, CA, USA) with a mobile phase composition of 0.05% aqueous HCOOH/ACN = 90/10 (v/v), applying isocratic elution. The flow rate and the injection volume were 1 ml min^{-1} and 20 μl, respectively.

The mass spectrometer was used in positive electrospray ionization mode. The drying gas temperature, drying gas flow rate, nebulizer pressure and capillary voltage were 350 °C, 13 l min^{-1}, 60 psi and 3500 V, respectively. Both KYNA and the amide were measured in the single ion measurement mode.

2.6. Preparation of calibration standards

Stock solutions of KYNA and the KYNA amide were freshly prepared in Milli-Q water. To improve the solubility of KYNA 1 ml of 0.1 M NaOH was added to 49 ml of Milli-Q water. Calibration standards were obtained by dilution of the stock solutions with the mobile phase.

2.7. Method validation

2.7.1. Calibration curve and linearity

Calibrants were prepared at 8 and 10 different concentration levels, from 1 to 4000 nM and from 10 to 25,000 nM, for KYNA and the KYNA amide, respectively. Three parallel injections of each solution were made under the chromatographic conditions described above. The peak area responses were plotted against the corresponding concentration, and the linear regression computations were carried out by the least square method with the Microsoft Excel program.

2.8. Data evaluation

The pharmacokinetic data were evaluated with PKSolver, a freely available menu-driven add-in program for Microsoft Excel [29].

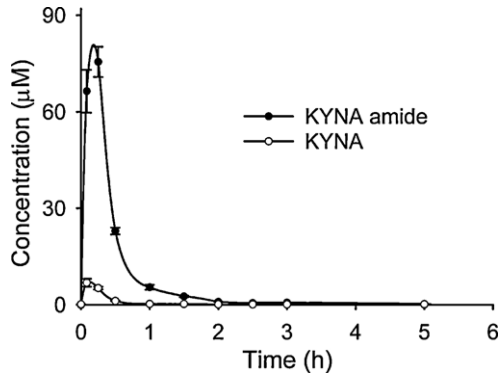

Fig. 1. Time-course profiles of the concentrations of the KYNA amide and KYNA in mouse serum after intraperitoneal administration of the KYNA amide (100 mg kg^{-1}; $n = 4$). Data are presented as means ± S.E.M.

Table 1
Pharmacokinetic parameters of the KYNA amide and KYNA in mouse serum after intraperitoneal administration of KYNA amide.

Parameter	KYNA amide	KYNA
C_{max} (μM)	78 ± 5	6.8 ± 1
T_{max} (h)	0.21 ± 0.04	0.13 ± 0.04
$AUC_{0-5\,h}$ (μM h)	38.3 ± 0.7	2.5 ± 0.2
$t_{1/2}$ (h)	0.96 ± 0.16	0.61 ± 0.3
CL_{tot} (l h^{-1} kg^{-1})	8.8 ± 0.2	–
V_d (l kg^{-1})	12.2 ± 2	–

The KYNA amide was applied in a dose of 100 mg kg^{-1} ($n = 4$). Data are presented as means ± S.E.M.

3. Results and discussion

3.1. Pharmacokinetic parameters

Besides setting up a possibility for the simultaneous measurement of the KYNA amide and KYNA from serum samples after intraperitoneal injections of the KYNA amide, the main aim of this study was to examine the *in vivo* stability of this novel KYNA amide. The time-course profile of the KYNA amide revealed that after a steep increase in the concentration, a subsequent steep decrease occurred in the first hour, followed by a prolonged further gradual decrease (Fig. 1). In the fifth hour, the KYNA amide was still present at 95 ± 25 nM in the serum. The formation of KYNA after administration of its direct (i.e. L-kynurenine) [22] or indirect (i.e. L-tryptophan) [30] precursors has rarely been reported. The study with tryptophan enantiomers at a dose of 100 mg kg^{-1} in the form of a single intraperitoneal injection [30], demonstrated a marked elevation in KYNA level, with $C_{max} \sim 300–800$ nM, involving prolonged increase and subsequent decrease in concentration. In contrast with those findings, we observed a higher (~45-fold), steep increase in KYNA concentration from the basal serum level of 150 ± 30 nM, following the intraperitoneal administration of the KYNA amide. Although this elevation is noteworthy, it is considerably less than the initial increase in the serum level of the KYNA amide itself. Furthermore, the serum level of KYNA had returned to the basal level by 2 h. Table 1 shows various calculated pharmacokinetic parameters (C_{max}, T_{max}, the area under the curve ($AUC_{0-5\,h}$) and the serum half-life ($t_{1/2}$)) for both the KYNA amide and KYNA, and additionally the total clearance (CL_{tot}) and distribution volume (V_d) for the KYNA amide. To avoid the influence of the basal serum KYNA level on the calculated pharmacokinetic parameters, these basal concentrations were subtracted from the corresponding subsequent concentrations. The pharmacokinetic data therefore reflect only the KYNA amide-induced changes in KYNA concentrations. The calculated values of $AUC_{0-5\,h}$ indicate that, although the initial elevation of KYNA level is convincing, only a small proportion of the KYNA amide decays into KYNA. Accordingly, the KYNA amide exhibits good *in vivo* stability.

3.2. Selectivity

The selectivity of the method was checked by comparing the single ion monitoring chromatograms of KYNA and the KYNA amide for a blank serum sample and those for a spiked serum sample. Both compounds could be detected in their own selected ion chromatograms without any significant interference.

3.3. Linearity

Very good linearity was observed throughout the investigated concentration range for KYNA when either fluorescence or MS detection was applied (Table 2). At higher concentrations of the KYNA amide, deviation from linearity was observed. For determination of the LOD and the limit of quantitation (LOQ) of the KYNA amide, the concentration range 10–400 nM was applied, where quite good linearity was observed (Table 2).

3.4. LOD and LOQ

LOD and LOQ were determined via the calibration curve according to the ICH guidelines [31] based on regression statistics, using the Microsoft Excel program. The LOD was 200 fmol and 160 fmol, while LOQ was 600 fmol and 460 fmol for KYNA and the KYNA amide, respectively. (The HPLC systems involving fluorescence or MS detection afforded the same LOD and LOQ in the determination of KYNA. The difference between the KYNA concentrations measured on identical samples, never exceeded 5%.)

3.5. Precision

Replicate HPLC-MS analysis showed that the relative standard deviation was ≤ 10% for the peak area response and < 5% for the retention time.

3.6. Recovery

The relative recoveries were estimated by measuring spiked samples of KYNA and the KYNA amide at 2 different concentrations with 3 replicates for each. No significant difference was observed for the lower and higher concentrations. The recoveries ranged from 73 to 85% and from 71 to 87% for KYNA and the KYNA amide, respectively.

Table 2
Validation parameters for the determination of KYNA and KYNA amide by HPLC methods, applying fluorescence or MS detection.

Compound	Method	Concentration range [nM]	Linearity [nM]	Regression equation	R^2	LOD [fmol]	LOQ [fmol]
KYNA	Fluorescence	1–1000	1–1000	$y = 18.1x + 30$	0.9999	200	600
KYNA	MS	10–4000	10–4000	$y = 186x - 1780$	0.9999	200	600
KYNA amide	MS	10–25,000	10–400	$y = 276x - 977$	0.9991	160	460

4. Conclusions

The serum pharmacokinetics of the novel KYNA amide was studied with special attention to the possible metabolism of this analog to KYNA, so as to characterize its *in vivo* stability. For this purpose, a sample preparation procedure based on protein precipitation and an HPLC method applicable for the simultaneous detection of KYNA and KYNA amide was developed. The recoveries (>70%) on spiked samples of KYNA and the KYNA amide demonstrated that the sample pretreatment applied is applicable for the reliable analysis of KYNA and the KYNA amide. The analytical performance characteristics for the HPLC methods applied indicated that the MS detection can offer the same sensitivity as the more frequent fluorescence detection, permitting a simple simultaneous determination of other KYNA analogs. The results point to the good *in vivo* stability of the novel KYNA amide.

Conflicts of interest

The authors declare no conflicts of interest.

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