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Short Communication

Determination of 3-hydroxykynurenine in human brain and plasma by high-performance liquid chromatography with electrochemical detection

Increased concentrations in hepatic encephalopathy

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ABSTRACT

A simple and specific method was developed for the determination of 3-hydroxykynurenine in brain tissue and blood plasma using high-performance liquid chromatography with electrochemical detection. This involved an extraction procedure using strong cation-exchange columns and also permitted the determination of 3-hydroxyanthranilic acid in brain tissue. The method was applied to the investigation of post mortem brain tissue from patients with hepatic encephalopathy. Cortical 3-hydroxykynurenine concentrations were substantially increased in such patients above control values, providing evidence for a dysfunction of tryptophan metabolism in this disease.

INTRODUCTION

The metabolism of tryptophan via the kynurenine pathway has provoked much interest, especially over recent years, as many of these compounds have been demonstrated to be neuroactive (*e.g.* quinolinic and kynurenic acids) [1]. A further example is 3-hydroxykynurenine (3HK), which is known to induce seizures after acute intracerebroventricular injection [2] and is neurotoxic when applied to neuronal cells in culture [3]. Increased concentrations of 3HK have been demonstrated in vitamin B_6 deficiency in both neonatal rat brain [4] and in adult urine (human and rodent) [5]. These findings have led to the suggestion that 3HK has a possible aetiological role in various neurotoxic states including glutaric aciduria, febrile convulsions and the neuropathology of AIDS [6]. Furthermore, the involvement with vitamin B_6 deficiency implicates this compound in hepatic encephalopathy.

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One consequence of vitamin B_6 deficiency is a loss of striatal neurons containing γ-aminobutyric acid [7]. Along with other evidence indicating abnormal kynurenine metabolism in Huntington's disease, this observation indicated to us the possibility of an involvement of 3HK in this disorder; our own recent report [8] of increased 3HK in the brains of patients with Huntington's disease provides support for this hypothesis.

Previous methods have enabled the measurement of 3HK in urine [5], rat plasma [9] and animal brain tissue [10], initially using ion-exchange with colorimetric detection and latterly high-performance liquid chromatography with electrochemical detection (HPLC–ED). Analysis is often preceded by an extraction process to remove any contaminants (*e.g.* uric acid). Here we describe a simple HPLC–ED method used for the determination of 3HK in human post mortem brain tissue, and involving a straightforward extraction of human plasma (which is also applicable to brain if necessary). We have applied it to the measurement of 3HK in brain tissue from patients with hepatic encephalopathy and control subjects.

EXPERIMENTAL

Direct method

Brain tissue (50–100 mg) was homogenised in 0.5 ml of 0.1 *M* perchloric acid containing 100 μM ascorbic acid. Tissue was obtained post mortem from patients pathologically confirmed as having hepatic cirrhosis and from control subjects; preparation and storage at -70°C was as described previously [11]. Refrigerated stock solutions of 3HK (1 mg/ml in 0.1 *M* hydrochloric acid with 100 μM ascorbic acid) were diluted daily to 20 ng/ml with perchloric acid. Aliquots (20 μl) of standards or sample supernatant were injected directly into the HPLC system.

Extraction method

This was used for measurement of 3HK in plasma (and brain tissue if 3-hydroxyanthranilic acid determination was required). Equal volumes of plasma and perchloric acid were centrifuged at 12 000 *g* for 3 min to produce a supernatant for extraction. Alternatively, supernatant from brain tissue (prepared as described above for the direct method) was extracted in the same way as plasma supernatant. Thus, 200 μl supernatant were mixed with 50 μl of perchloric acid and 250 μl of 0.1 *M* phosphate buffer (pH 4.0), resulting in a pH 2.3 solution. This was then added to a strong cation-exchange (1 cc Bond Elut SCX) column which had been pre-washed with 1 ml of methanol, 1 ml of 0.1 *M* phosphate buffer (pH 7.0), 1 ml of 50% methanol–pH 2.3 solution, 4 ml of water and 1 ml of pH 2.3 solution. Aliquots (0.5 ml) of 50% methanol–pH 2.3 solution and water washed the column prior to elution with 1 ml of 0.1 *M* phosphate buffer (pH 7). All solutions contained 100 μM ascorbic acid. Standard solutions (0.5 ml of 10 ng/ml at pH 2.3) were extracted in the same way as the samples.

HPLC system

Aliquots (20 μl) of the unextracted standard solutions and prepared extracts of standards and samples were injected onto the column. Isocratic reversed-phase separation was achieved at 40°C using a Spherisorb ODS-2 5-μm (250 mm × 4.6 mm I.D.) column, with 0.1 *M* phosphate–acetate buffer (pH 3.2) containing 2.5 m*M* octylsulphonate and 0.05 m*M* EDTA flowing at 1.0 ml/min. Quantification used an electrochemical detector (BAS) containing a glassy carbon electrode set at +0.60 V (*vs.* Ag/AgCl).

RESULTS AND DISCUSSION

Fig. 1 illustrates typical chromatograms from a directly injected standard and an extracted brain sample. In the samples, a single peak was demonstrated for

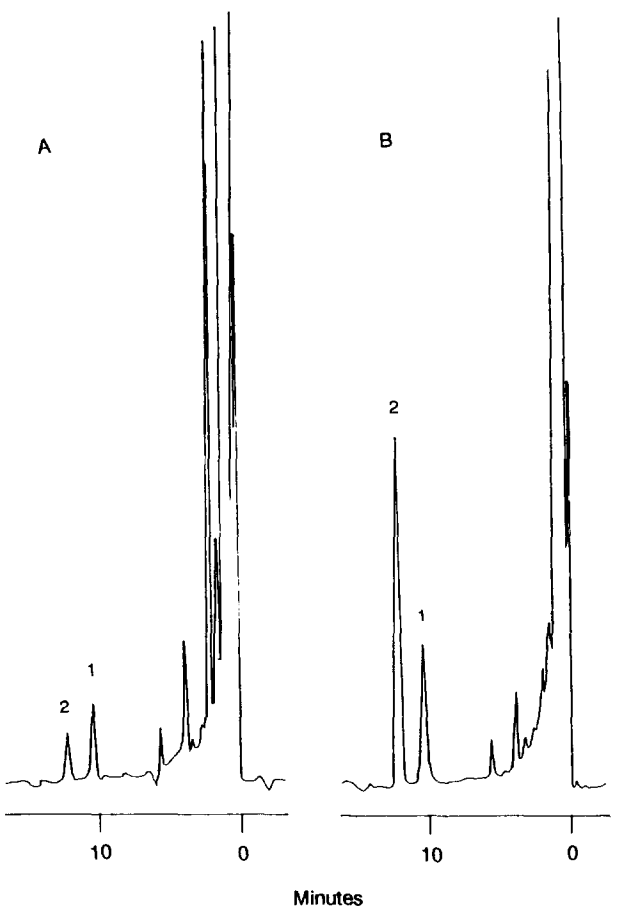

Fig. 1. Chromatogram of extracted 3HK and 3HA (full scale deflection = 1 nA). (A) Typical brain sample; (B) typical brain standard sample (10 ng/ml). Peaks: 1 = 3HK; 2 = 3HA.

3HK (retention time = 10.4 min) which was verified by comparison with the retention time of authentic standard peaks, under varying chromatographic conditions. Recovery from standards added to samples was 97 ± 2% (coefficient of variation, $n = 5$) for unextracted samples and 100 ± 4% ($n = 5$) for extracted samples. Also, oxidation curves were performed by measuring the peak height at varying voltages (0.4–0.9 V). These proved similar for the standard, sample and sample with added standard, with +0.60 V providing optimal conditions. Linear standard curves for concentration (0–20 ng/ml) *versus* response were demonstrated for both unextracted and extracted 3HK. The limit of detection was approximately 40 fmol 3HK on the column. Replicate injections ($n = 4$) of 20 μl gave a 1.0% coefficient of variation. 3HK samples and standards were shown to be stable over 18 h if kept at 4°C, but only when ascorbic acid was present. The presence of ascorbic acid at all stages was found to be a prerequisite for good recovery of extracted 3HK, preventing degradation on the SCX column.

A related kynurenine, 3-hydroxyanthranilic acid (3HA), could also be determined using this system. However, measurement of 3HA without extraction was impossible due to coeluting peaks, and although the extraction process was applicable to 3HA in the brain, plasma 3HA still contained interfering peaks even after extraction. Similar validation to the above confirmed the identity of the peak in the brain with a retention time of 12.2 min (see Fig. 1A and B).

Concentrations of 3HK were calculated from comparison of sample peak height with standard peak height, corrected for dilution. Table I demonstrates a typical value for human cortex 3HK, whereas human plasma ($n = 7$) had a concentration of 7.2 ± 4.1 ng/ml (mean ± S.D.).

The method described here has been applied successfully to the identification of increased concentrations of 3HK in brain tissue from patients with Huntington's disease [8]. Table I shows concentrations of 3HK and 3HA obtained using this method, in post mortem cortical tissue from patients with hepatic encephalopathy and control subjects. The results show a significant increase in the

TABLE I

CONCENTRATIONS OF 3HK AND 3HA IN THE FRONTAL CORTEX

Group	n	Concentration (ng/g of tissue)			
		3HK		3HA	
		Mean ± S.D.	Range	Mean ± S.D.	Range
Controls	13	42 ± 35	16–151	12 ± 7.7	3–29
Hepatic encephalopathy	5	279 ± 256[a]	72–720	16 ± 7.1	8–22

[a] $p < 0.002$ (using Mann-Witney U-test).

concentration of 3HK, but not 3HA in the frontal cortex. There is no overlap of cortical 3HK ranges between the control and the hepatic encephalopathy groups, except for one control value. 3HK metabolism is vitamin B_6-dependent and impaired utilization of vitamin B_6 has been reported to occur in alcoholism [12]. As one result of vitamin B_6 deficiency is increased brain 3HK in neonatal rats [4], our results are consistent with these observations. Furthermore, a dysfunction of tryptophan metabolism has been implicated in hepatic encephalopathy [13]. In addition, quinolinic acid (another kynurenine metabolite) has been shown to be significantly increased in the frontal cortex and cerebrospinal fluid in patients with hepatic encephalopathy [14].

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