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DETERMINATION OF KYNURENINE BY A SIMPLE GAS—LIQUID CHROMATOGRAPHIC METHOD APPLICABLE TO URINE, PLASMA, BRAIN AND CEREBROSPINAL FLUID

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SUMMARY

A simple, sensitive and specific method for the determination of kynurenine is described. This is based on alkaline cleavage of kynurenine, followed by solvent extraction, trifluoroacetylation and gas—liquid chromatography with electron capture detection. Using this method kynurenine has been determined in urine and plasma, and for the first time in brain and cerebrospinal fluid. Increases in kynurenine in brain, plasma and urine are demonstrated following tryptophan administration to man and rat.

INTRODUCTION

Kynurenine is the first major metabolite of tryptophan on the pathway initiated by the enzymes tryptophan pyrrolase (tryptophan-2,3-dioxygenase) and indoleamine-2,3-dioxygenase. The former enzyme is found in the liver and is specific for L-tryptophan, the latter has a much wider distribution in the body, and a wider substrate specificity [1, 2]. Estimation of kynurenine production, sometimes following a tryptophan load, has been used as an index of the activity of this pathway [3, 4] which has been shown to be altered in a number of pathological states [3].

We have previously presented [5] a convenient modification of the method of Tompsett [6] which could be applied to urine and to plasma, provided that interference from tryptophan was prevented during the alkaline cleavage of kynurenine to *o*-aminoacetophenone (OAAP). In these methods the product was determined colorimetrically following diazotisation and coupling with naphthylethylene diamine. OAAP derived from urinary kynurenine has previously been determined by gas—liquid chromatography (GLC) using flame ionisation detection [7]. In the present method the previous procedure [5] has been modified so that the OAAP produced can be determined after trifluoroacetylation by GLC using electron capture detection. Kynurenine can

readily be determined without prior tryptophan loading in 200 μl of human or 100 μl of rat plasma. In addition the method can be used for urine, and also to study the kynurenine pathway in brain via determinations in brain and cerebrospinal fluid (CSF) material.

MATERIALS AND METHODS

L-Kynurenine sulphate, kynuramine dihydrobromide and L-tryptophan were obtained from Sigma (London, Great Britain), *o*-aminoacetophenone from Koch-Light (Colnbrook, Great Britain, α,α′-dichloro-*p*-xylene (DCPX) from Ralph N. Emanuel (Wembley, Great Britain), N-formyl-L-kynurenine from Calbiochem (Bishops Stortford, Great Britain) and trifluoroacetic anhydride (TFAA) and other chemicals (AR grade where available) from BDH (Poole, Great Britain). Tiron was obtained from Fisons (Loughborough, Great Britain).

Procedure

Brain tissue (1—2 g) was homogenised in 5 volumes of acid butanol (0.85 ml conc. HCl per litre *n*-butanol). After centrifugation (2500 *g*, 10 min) the supernatant, together with an equal volume of *n*-heptane, was back-extracted with 1—2 ml of 0.1 *M* HCl. Plasma could be processed similarly using 10 volumes of acid butanol, or the supernatant from extraction with 10 volumes of 10% trichloroacetic acid (TCA) could be used. A suitable volume of the 0.1 *M* HCl extract, the TCA extract, or undiluted CSF or urine was made alkaline with one third of its volume of 10 *M* NaOH, and 100 m*M* Tiron (1,2-dihydroxybenzene-3,5-disulphonic acid, disodium salt) added to a final concentration of 5 m*M* [5]. After extraction with butyl acetate, the alkaline aqueous phase was heated in a boiling water-bath in a closed glass tube for 20 min and cooled to room temperature. The product was extracted into a small volume of butyl acetate (routinely 300 μl) containing 200 ng/ml DCPX as internal standard. A suitable volume (routinely 200 μl) of the organic phase was mixed briefly with an equal volume of alkali-borate buffer (0.2 *M* borax in 1.5 *M* NaOH) in a 1.5-ml Eppendorf reaction tube. After a brief interval, 1/8 volume (routinely 25 μl) of trifluoroacetic anhydride was added. The contents were mixed immediately and centrifuged to separate the phases. One μl of the supernatant organic phase was injected into a Hewlett-Packard 5713A gas chromatograph. Conditions: injection port, 250°; on-column injection onto 3 ft. × 1/4 in. coiled glass column packed with 10% OV-1 on HP Chromosorb W at 120°; exit directly into ^{63}Ni electron capture detector at 300°; carrier gas, argon—methane (95:5) at 60 ml/min.

Tryptophan in plasma or brain was determined as previously described [5] on a further aliquot of the 0.1 *M* HCl phase from the above procedure.

RESULTS

Development of the method

In the previously described method [5] OAAP formed by heating kynurenine in strong alkali was extracted into amyl alcohol. This was clearly not a

suitable solvent for trifluoroacetylation since it could react with TFAA. Chloroform, as used by Naruse et al. [7], is not compatible with electron capture detection. Ethyl acetate appeared suitable, but was hydrolysed to some extent by the strong alkali used. Butyl acetate was not hydrolysed, and extracted the OAAP efficiently.

Since removal of excess TFAA by evaporation under a stream of gas might lead to loss of the rather volatile trifluoroacetyl derivative of OAAP, it was removed by hydrolysis with an alkaline buffer. NaOH (2.5 moles/l) alone resulted in some loss of the derivative, but the amount of alkali-borate buffer used resulted in a maximal yield, while keeping the aqueous phase alkaline so that the trifluoroacetic acid produced remained in the aqueous phase. Since the addition of the highly volatile TFAA was likely to be subject to some error the adequacy of the buffering capacity of the alkali-borate was checked by varying the amount of TFAA added. 15 μl TFAA resulted in a lower yield, but the yield was essentially the same for 20 to 30 μl TFAA (Table I). The reaction of TFAA with OAAP in butyl acetate appeared to be almost instantaneous, and no advantage was obtained by incubating with TFAA before addition of the alkali. Prior addition of the alkali resulted in equally high yields and was adopted because of its convenience. The final volume of butyl acetate used for extraction was kept low in order to maximise the final concentration of OAAP while avoiding evaporation. Under these circumstances a reduction in the volume of the aqueous phase to be extracted with butyl acetate was sought. This was achieved by the use of acid butanol with subsequent back extraction into 0.1 *M* HCl, and resulted in a ten fold reduction in the volume of aqueous phase compared with the perchloride acid extraction used in the previous method [5]. The recovery of kynurenine with acid butanol at the initial extraction is lower, but the improved recovery at the butyl acetate extraction step more than compensates for this, resulting overall in higher peaks for a given amount of OAAP or kynurenine. In addition acid butanol extraction enables tryptophan, 5-hydroxytryptamine, 5-hydroxytryptophan and 5-hydroxyindole acetic acid [8, 9] to be determined concurrently on a single sample. Another advantage of the acid butanol technique is the reduction in interference (see below).

TABLE I

VARIATION IN PEAK HEIGHT RATIO OF OAAP—TFA TO INTERNAL STANDARD WITH VOLUME OF TFAA USED IN ASSAY

Methods: as described in text using butyl acetate containing 200 ng DCPX and 244 ng standard OAAP per ml.

TFAA volume (μl)	Peak height ratio
15	68.4
20	112.5
25	118.6
30	119.2

Evaluation of the method

DCPX internal standard carried through this method alone yielded a single sharp symmetrical peak with a retention time of 6.4 min (Fig. 1). Standard OAAP carried through the final stage of derivative formation, or standard kynurenine carried through the whole method yielded a similar peak with a retention time of 7.2 min (Fig. 1). Quantitation was achieved by expressing the height of the OAAP peak as a ratio of the internal standard peak height for each chromatographic run. The method was simple and convenient in practice, the use of differential extraction, before and after heating, resulting in very clean GLC traces. Blanks were normally less than 1% of internal standard peak, corresponding to 5 ng kynurenine added initially (a peak height of 20% internal standard corresponded to 100 ng kynurenine). The linear yield of product with varying amounts of kynurenine is shown in Fig. 2. Reproducibility of duplicate determinations was 5% of their mean.

Incubation of standards or tissue samples in strong alkali for 20 min at room temperature in place of the boiling water-bath (see Materials and Methods) resulted in a reduction of the OAAP detected to 3%. Fig. 3 shows a mass spectrum of the GLC peak obtained from rat plasma carried through the method compared with that from standard OAAP, which identifies the substance produced from rat plasma as OAAP. However, the possibility that the OAAP produced by heating in strong alkali comes from sources other than kynurenine must be considered. Other possible sources include tryptophan, acetyl-kynurenine, kynuramine and formyl-kynurenine. In the presence of Tiron, tryptophan does not interfere detectably at 3 times its normal level in brain, and interferes only to a small extent (Table II) at 30 times its normal level (500-fold molar excess over kynurenine). The other three substances will interfere, in that when heated in alkali they do yield OAAP, kynuramine and formyl kynurenine on approximately a mole for mole basis, and acetyl

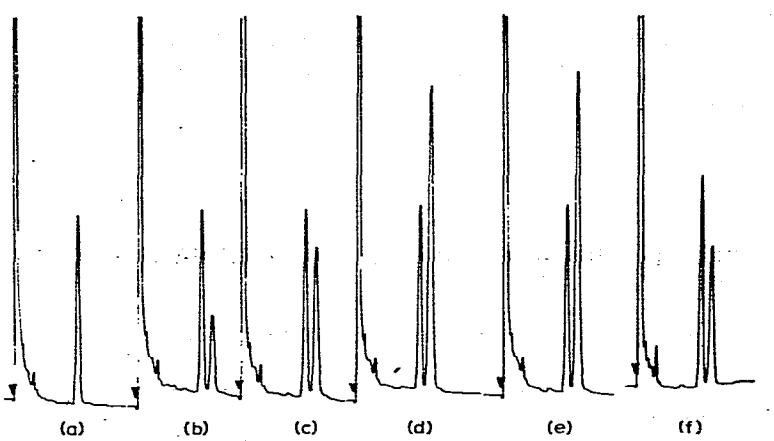

Fig. 1. GLC records from kynurenine analysis. (a) Blank, (b) 150 ng, (c) 300 ng, (d) 600 ng added kynurenine standard, (e) autopsied human brain cortex and (f) rat brain. Injection is indicated by arrow; the first major peak is DCPX internal standard; the second is the OAAP derivative.

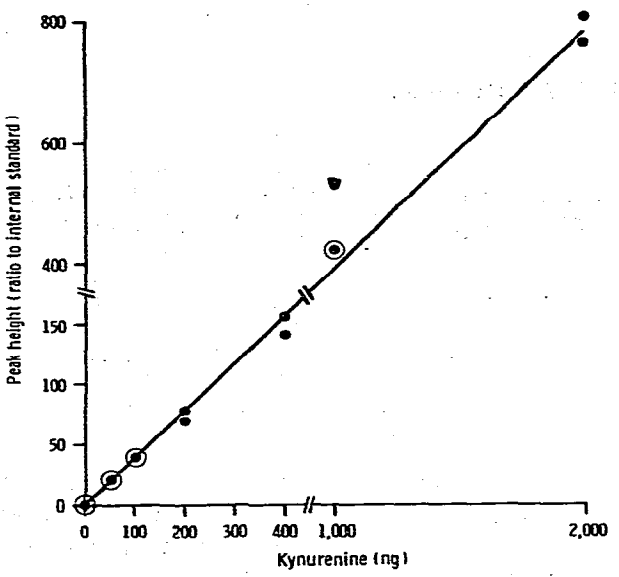

Fig. 2. Standard curve for kynurenine assay. Duplicate determinations at each point; ⊖ indicates co-incident results.

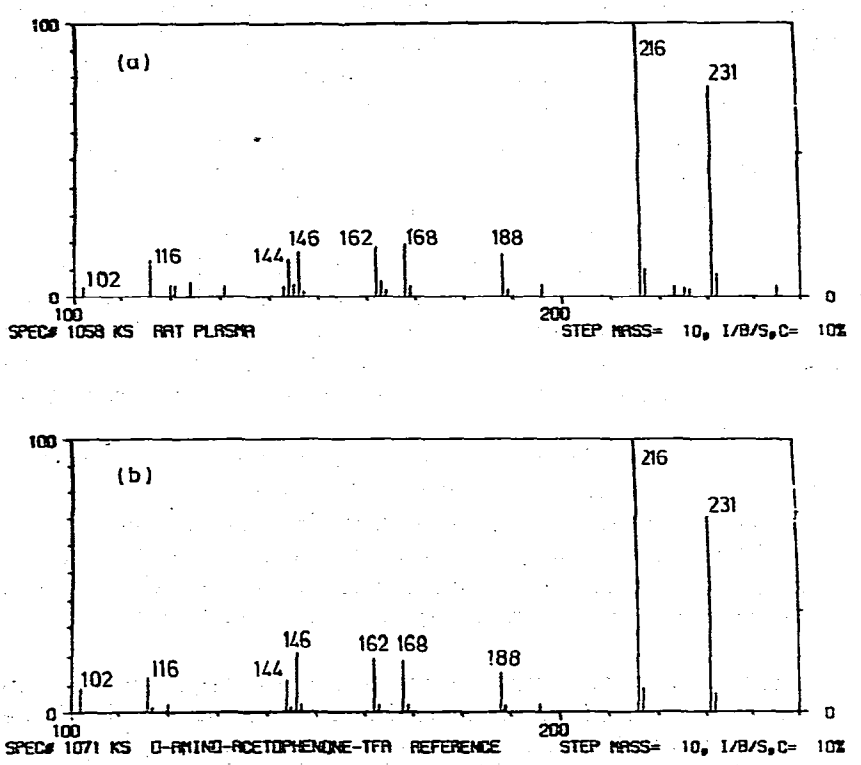

Fig. 3. Mass spectra of material derived from (a) rat plasma and (b) OAAP standard. Mass spectra were obtained using the Varian MAT-112 mass spectrometer and 100 MS Spectro-System.

TABLE II

INTERFERENCES AND RECOVERIES OF STANDARDS

All figures are percentages.

Compound	Interference* (added to alkali before heating)	Recovery through		Overall interference*
		Butyl acetate	Acid butanol	
L-Tryptophan				0.03
Acetyl-L-kynurenine	21.1	91.6	54.4	11.8
Formyl-L-kynurenine	86.6	90.5	32.6	28.6
Kynuramine	92.2	14.3	75.5	11.2
L-Kynurenine	(100)	83.7	106.6	(100)

*Expressed as per cent of kynurenine on an equimolar basis.

kynurenine at about one fifth on a molar basis (Table II), a result similar to that reported previously [5]. However, when carried through the whole method kynuramine interference is reduced to 11% since it is efficiently extracted by the butyl acetate wash prior to heating (Table II). If present, kynuramine can readily be detected, and indeed quantified, by adding an equal volume of heptane to the butyl acetate and back-extracting with 0.1 *N* HCl. If the HCl phase is now heated with alkaline Tiron, as in the kynurenine method described, OAAP is produced, which can be quantified by the same procedure. The recovery through this procedure of kynuramine added to brain is 90.1%, and endogenous kynuramine has not been detected in any rat or human brain samples analysed to date (limit of detection about 5 ng/g).

When acetyl-kynurenine and formyl-kynurenine are carried through the whole method their interferences are reduced as shown in Table II since they are not so well recovered from the acid butanol phase. The residual interferences are of little importance where kynurenine formation is being studied, since both are likely to be produced in parallel with kynurenine. Indeed, in using the method to assess pyrrolase activity this could be a positive advantage since formyl-kynurenine and kynurenine will both be measured if the acid butanol stage is omitted, and thus the assay will be independent of the presence of excess formamidase. This would be particularly useful in purification studies on tryptophan pyrrolase.

Should it be necessary to unequivocally identify kynurenine as the source of OAAP a small column of Dowex 50W produces distinctive elution profiles for kynurenine and acetyl kynurenine [10, 11] and also for formyl kynurenine and tryptophan [12]. In this way we have established [11] that the OAAP produced from rat or human brain tissue carried through the method is derived from kynurenine and not from acetyl-kynurenine, formyl-kynurenine or kynuramine.

The levels of kynurenine and recovery values found by applying the described methods to tissues and fluids from several species are shown in Table III. The values for rat brain confirm those given in preliminary reports from this laboratory [13] and from another [14]. The values for human urine and human and rat plasma agree reasonably well with those previously published from this laboratory and others [7, 15, 16]. The results for rat plasma

TABLE III

CONCENTRATIONS OF KYNURENINE FOUND IN VARIOUS TISSUES AND FLUIDS

Concentrations are expressed in ng kynurenine per ml or g wet weight and represent means ±standard deviation. The number of observations is given in parentheses.

Tissue	Species	Specification	Concentration	Recovery (%)
Urine	Human	Early morning sample	573±99(6)	85.3
Plasma	Human	Fasting sample at 09.00—10.00 h	404±158(6)	—
	Rat		807±111(10)	76.3
	Goat		143	—
CSF	Human	Lumbar	7.1 ±1.7(5)	71.4
	Pig	Cisternal	38.7	68.5
	Goat	Cisternal	13.4	—
	Beagle	Cisternal	7.2	—
Brain	Human	Putamen	317±176(8)	—
		Temporal cortex-autopsy	216±176(8)	—
		Temporal cortex-biopsy*	114±20(3)	—
	Rat	Whole brain	161±16(7)	80.3

*Frozen immediately after removal at therapeutic neurosurgical operation.

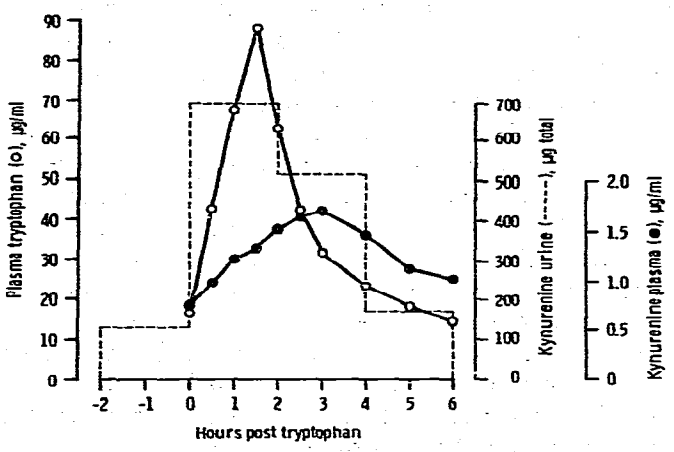

Fig. 4. Time course of plasma tryptophan and kynurenine and of urinary kynurenine following a tryptophan load in man. Tryptophan (3 g) suspended in orange juice was administered at time 0. Nicotinamide (100 mg) had been administered four times the previous day, and at 2 h before tryptophan.

reported here are a little lower than those we reported previously [4], but the present method is more specific than the method used in that study.

Tryptophan administration to man (3 g orally) results in increased kynurenine in plasma and urine (Fig. 4), the plasma results agreeing broadly with those previously presented from this laboratory [5]. The high sensitivity of this method allows a much more detailed study of the dynamics of kynurenine formation and excretion in man than was previously possible, and we

TABLE IV

TRYPTOPHAN AND KYNURENINE IN BRAIN AND PLASMA FOLLOWING TRYPTOPI ADMINISTERED TO RATS

Tryptophan was dissolved in 0.33 *M* HCl and neutralised with 0.155 *M* sodium carbonate to yi neutral solution in isotonic saline. It was administered i.p in a volume of 1 ml per 200 g rat. trols were injected with vehicle alone. Animals were decapitated 2 h later.

Administration	Number of observations	Brain (mean±S.D.)		Plasma (mean ±S.D.)	
		Tryptophan (μg/g)	Kynurenine (ng/g)	Tryptophan (μg/ml)	Kynurenin (ng/ml)
Control	6	1.90±0.35	115±38	20.8±3.0	1064±237
Tryptophan (50 mg/kg)	8	4.00±1.06*	201±35**	34.4±9.3*	1334±261
Tryptophan (100 mg/kg)	6	5.02±1.50**	228±94	37.3±5.3**	2481±347

*Different from control $p<0.01$.
**Different from control $p<0.001$.

are currently applying this approach in studies on psychiatric patients and normal controls.

Tryptophan administration to rats (50 or 100 mg/kg i.p. to male Sprague Dawley rats, 200—250 g) leads to an increase in kynurenine in plasma and in brain (Table IV). The demonstration of kynurenine in rat and human brain [11, 13, 14], together with the demonstration of the necessary enzyme in rat brain [2, 17] and the synthesis in rat brain of labelled kynurenine from labelled tryptophan [14] make it likely that at least a part of the kynurenine normally found in brain is synthesised there, and that at least part of the increase in rat brain kynurenine following tryptophan loading is synthesised within the brain. Since the amount of kynurenine found in rat brain is about half and the amount in human brain about double that of the amounts of 5HT found in the same brain samples [12] the physiological significance of the kynurenine pathway in brain may be considerable.

In summary the use of this method offers the possibility of detailed studies on the kynurenine pathway in man and other species in a variety of tissues including the brain. It also offers the possibility of sensitive and direct assays of tryptophan pyrrolase and indoleamine-2,3-dioxygenase.

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NOTE ADDED IN PROOF

A fuller account of this work has now appeared [18]. The kynurenine levels reported in whole rat brain agree with the present report, but these authors report a lower level of kynurenine in rat plasma, and also report kynuramine in rat brain and plasma, which could not be detected in the present study.

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