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A COMPARISON OF AN IMPROVED *o*-PHTHALALDEHYDE FLUOROMETRIC METHOD AND HIGH PRESSURE LIQUID CHROMATOGRAPHY IN THE DETERMINATION OF BRAIN 5-HYDROXYINDOLES OF RATS TREATED WITH L-TRYPTOPHAN AND *p*-CHLOROPHENYLALANINE

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1 The determination of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) by reaction with *o*-phthalaldehyde (OPT) in the presence of cysteine and subsequent fluorometry was compared with determination by a new high pressure liquid chromatography (h.p.l.c.) electrochemical method.

2 The methods were used to investigate a claim that the OPT method gives falsely high brain 5-HT values for rats given tryptophan (> 25 mg/kg i.p.) and as a consequence an inhibition of 5-HT synthesis by tryptophan is obscured.

3 High concentrations of tryptophan caused some increase of fluorescence when added to 5-HT solutions and carried through the OPT method, e.g. tryptophan (80 μg/ml) gave fluorescence ≡ 5-HT (0.13 μg/ml). This interference was decreased by more than half and the sensitivity of the method increased if cysteine was added at more stages and fluorescence developed at 77°C instead of 100°C.

4 The h.p.l.c. and modified OPT methods did not give significantly different mean brain 5-HT or 5-HIAA values in rats given 0,25,100,250 mg/kg L-tryptophan though values were somewhat higher by the OPT method with the highest dose. This method gave significantly higher residual brain 5-HT values (12% of control) than did the h.p.l.c. method (8% of control) after inhibiting 5-HT synthesis by *p*-chlorophenylalanine (150 mg/kg × 3).

5 There was no indication that tryptophan inhibited 5-HT synthesis even when brain tryptophan was increased about 30 fold (from 3.8 to 124 μg/g).

6 Results confirm the general reliability of the OPT method although h.p.l.c. has some advantages i.e. separation of 5-hydroxyindoles from tryptophan, greater sensitivity, ease of automation.

Introduction

5-Hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) are often determined by use of the fluorescent complexes they form with *o*-phthalaldehyde (OPT) (Maickel, Cox, Saillant & Miller, 1968; Curzon & Green, 1970). Recently, Gal, Young & Sherman (1978) claimed that tryptophan seriously interferes with the method of Curzon & Green (1970) so that previous findings of considerable increases of rat brain 5-HT and 5-HIAA after tryptophan administration (e.g. Knott & Curzon, 1974; Curzon & Marsden, 1975) may have been largely artefactual. Gal *et al.* (1978) found, using high pressure liquid chromatography with electron capture gas chromatography, that L-tryptophan (25 mg/kg) only caused small increases of rat brain 5-HT and 5-HIAA (+17% and +26% respectively) 1 h after injection, when brain tryptophan had risen about 3 fold. Larger tryptophan loads caused larger increases of brain tryptophan but apparently inhibited 5-HT synthesis.

These findings demand investigation, as they cast doubt on the utility of a commonly used analytical method. They also question a large literature on the relationship between the supply of tryptophan to the brain and 5-HT synthesis *in vivo* (reviewed; Green, 1978) which does not suggest its inhibition by tryptophan, except perhaps in rats pretreated with a monoamine oxidase inhibitor and given much larger doses of tryptophan (Grahame-Smith, 1971) than those given by Gal *et al.* (1978). The topic is of clinical as well as general interest as the beneficial effect of tryptophan in depression (Coppen, Whybrow, Noguera, Maggs & Prange, 1972; Lindberg, Ahlfors, Dencker, Fruensgaard, Hansten, Jensen, Ose & Pihkanen, 1979) is usually thought to reflect an increase of brain 5-HT synthesis.

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The present paper describes an improved version of the method of Curzon & Green (1970) and a new h.p.l.c. method for determining brain 5-hydroxyindoles. The two methods are compared using brains of rats given tryptophan, the 5-HT synthesis inhibitor *p*-chlorophenylalanine (PCPA) or both of these drugs.

Methods

Sample preparation

Tissue was homogenized in a 1LX 1020 homogenizer (Scientific Instrument Co., London) in 10 volumes of 1-butanol containing 850 μl conc HCl per litre. The homogenates were centrifuged at 1500 *g* for 10 min and the supernatants used for determinations as follows.

Fluorometric determinations

The method was largely that of Curzon & Green (1970) except that incubation was not at 100°C but at 77°C as this led to increased sensitivities (Atack & Lindqvist 1973). In addition cysteine was used as an antioxidant at more stages of the method. This was found to improve recoveries, especially when large numbers of determinations were made. A 2.5 ml portion of the supernatant was shaken for 5 min with 5.0 ml *n*-heptane and 600 μl 100 mM HCl containing 0.1% cysteine. After brief centrifugation to separate the phases, 5.0 ml of the upper organic phase was withdrawn for later extraction of 5-HIAA. The remainder of the organic phase was removed by aspiration and two 200 μl portions of the aqueous phase taken for assay of tryptophan and 5-HT. Tryptophan was determined by the method of Denckla & Dewey (1967) as modified by Bloxam & Warren (1974). For the assay of 5-HT, 200 μl of the aqueous phase was heated for 15 min at 77°C with 20 μl 1% cysteine and 800 μl conc HCl containing 0.004% OPT. After cooling, fluorescence at 480 nm was read at an activation wavelength of 370 nm using an Aminco Bowman spectrophotofluorometer. For the determination of 5-HIAA, the organic phase was shaken for 5 min with 600 μl 500 mM Na_2HPO_4/NaH_2PO_4, pH 7.0 containing 0.1% cysteine. After centrifugation to separate the phases, the organic phase was removed by aspiration and 400 μl of the aqueous phase was heated for 15 min at 77°C with 40 μl 1% cysteine solution and 800 μl conc HCl containing 0.004% OPT. Fluorescence was read as for 5-HT. Reagent blanks were used throughout as they were found not to differ from tissue blanks.

Sensitivities (amount of substance/g brain for a total fluorescence of twice the blank) were 25 ng/g (5-HT) and 41 ng/g (5-HIAA). Calibration curves were linear up to at least 160 ng 5-HT or 5-HIAA/ml butanol carried through the procedure. Recoveries of standards (5 to 30 ng/ml butanol homogenate of brain) were also linear and averaged 87% (5-HT) and 95% (5-HIAA) for 5 determinations. 5-HT and 5-HIAA duplicates varied by $3.1 \pm 1.6\%$ and $4.1 \pm 3.0\%$ respectively (means ± s.d., 12 determinations). Recoveries were slightly higher and sensitivities considerably higher (5-HT, three fold; 5-HIAA, two fold) than with the unmodified method of Curzon & Green (1970). Values are given uncorrected for losses.

High pressure liquid chromatography system

The system consisted of a reciprocating pump (Model 750/05; Applied Chromatography Systems Ltd., Luton) with a pulse damper (HETP, Macclesfield) a WISP 710A automatic sample injector (Waters Associates Ltd., Northwich) and C_{18} reverse phase column (3.9×300 mm, μ Bondapak, Waters). The column was protected by a 4 cm precolumn packed with C_{18}/Corasil (Waters). An Aminco Bowman fluoromonitor with a 70 μl flow cell modified as described by Anderson & Purdy (1978) to take a 254 nm interference filter (Waters) in the exciting beam and a mercury-argon pen lamp (Oriel Scientific Ltd., Kingston-Upon-Thames) were used to measure tryptophan. 5-HT and 5-HIAA were also detectable by fluorometry but electrochemical detection was more sensitive. A model LC2A detector with a carbon paste-oil electrode (Bioanalytical Systems Inc. West Lafayette, IN. U.S.A.) placed down stream from the fluorometer was used. At the potential used (+0.5V), tryptophan did not give a detectable electrochemical signal.

High pressure liquid chromatography determinations

A 250 μl portion of the homogenate supernatant was shaken for 5 min in a 1.5 ml plastic capped conical tube (W. Sarstedt Ltd., Leicester), with 500 μl *n*-heptane and 60 μl, 500 mM Na_2HPO_4/NaH_2PO_4, pH 7.0 containing 0.2% cysteine. After centrifugation, the upper organic phase was removed by aspiration and 40 to 50 μl of the aqueous phase transferred to 300 μl limited volume inserts (Waters) from which 20 μl was automatically injected on to the column by the WISP 710A.

The mobile phase was 50 mM sodium acetate, 50 μM EDTA, pH 3.75 to 4.0 plus either 5 or 10% methanol. The pH and methanol concentrations were varied to maintain optimal retention times and sensitivity as the column aged and plate counts decreased. Flow rate was maintained at 1.5 ml/min. The mobile phase was passed under vacuum through a FHUP 04700, 0.5 μm filter (Millipore Ltd., London) and further degassed by helium before use.

Figure 1 shows a chromatogram of an extract of

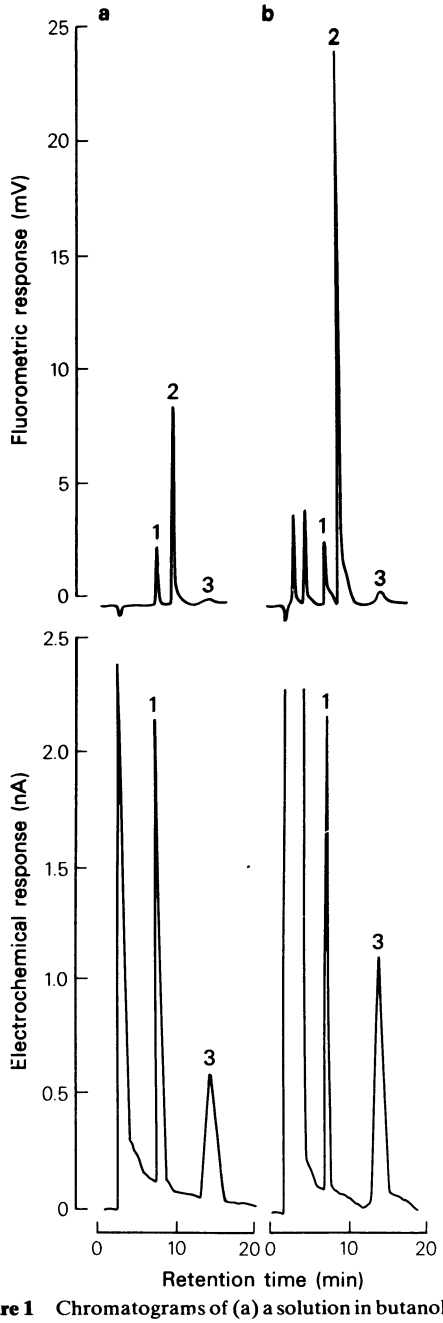

Figure 1 Chromatograms of (a) a solution in butanol of (1) 5-hydroxytryptamine (5-HT) (64 ng/ml), (2) tryptophan (185 ng/ml), (3) 5-hydroxyindoleacetic acid (5-HIAA) (37 ng/ml) and (b) butanol extract of brain (see Methods). Electrochemical and fluorometric detection were used. The sensitivity setting on the fluorometer was 10 (full scale deflection = 50 mV). Indole concentrations in brain were 0.656 μg/g (5-HT), 5.429 μg/g (tryptophan) and 0.714 μg/g (5-HIAA). The mobile phase was 50 mM sodium acetate pH 4.0, 50 μM EDTA, 5% methanol with a flow rate of 1.5 ml/min.

whole brain and of similarly extracted aqueous standards. Peaks in the brain extract were identified by comparing their retention times with those of standards at various concentrations of methanol in the mobile phase. Concentrations were determined by measuring peak heights. Sensitivities (defined as the amount injected on to the column giving a signal to noise ratio of 2) for 5-HT, 5-HIAA and tryptophan were 70 pg, 120 pg and 700 pg respectively corresponding to brain concentrations of 8, 15 and 84 ng/g). These are typical values for a column though which approx. 300 samples had passed and for an electrode, u.v. lamp and interference filter about half way through their useful lives. Peak heights for 5-HT and 5-HIAA increased linearly over the range 2 to 160 ng/ml butanol and for tryptophan over the range 10 to 10,000 ng/ml butanol. Recoveries of standards (5 to 30 ng/ml) added to butanol homogenates were also linear and averaged 86%, 100% and 97% for 5-HT, 5-HIAA and tryptophan respectively (5 determinations). Duplicates varied by $2.5 \pm 2.8\%$, $1.7 \pm 2.6\%$ and $2.8 \pm 1.8\%$ (mean ± s.d., 6 duplicate determinations) for 5-HT, 5-HIAA and tryptophan respectively. Values are given uncorrected for losses.

Reagents

L-Tryptophan, 5-hydroxytryptamine creatinine sulphate complex, 5-hydroxyindoleacetic acid, *p*-chlorophenylalanine (as methylester) and *o*-phthalaldehyde were purchased from Sigma (London) Chemical Co., Poole, Dorset, Sodium acetate, methanol, butan-l-ol, *n*-heptane, trichloracetic acid, formaldehyde, ferric chloride, ethylenediaminetetracetic acid disodium salt, L-cysteine, sodium dihydrogen phosphate and disodium hydrogen phosphate were Analar grade (except *n*-heptane, which was to I.P. specification) and purchased from BDH Chemicals Ltd., Poole, Dorset.

Results

Tryptophan-o-phthalaldehyde fluorescence

Tryptophan forms a fluorescent product (peak at 420 nm) on heating with OPT in conc HCl under the conditions used for development of the 5-HT fluorophor. At very high tryptophan concentrations this appreciably increased fluorescence at 480 nm, the wavelength at which 5-HT is measured (Table 1). Thus, when fluorescence was developed at 100°C, as in the method of Curzon & Green (1970), normal brain tryptophan concentrations (1 μg/ml=approx. 1 μg/g brain) had negligible effect but 10 times, 20 times, and 100 times normal concentrations gave fluorescences corresponding to 0.08, 0.14 and

Table 1 Interference of tryptophan with the determination of 5-hydroxytryptamine (5-HT) by the *o*-phthalaldehyde (OPT) method

5-HT (μg/ml)	*Tryptophan* (μg/ml)	*Fluorescence units* 77°C	100°C
0.4	0	58.5 ± 0	45.5 ± 0.9
0.4	4	59.8 ± 1.2	47.2 ± 1.4
0.4	40	63.7 ± 1.3	54.5 ± 1.0
0.4	80	66.5 ± 0.5	60.8 ± 1.0
0.4	400	88.7 ± 2.4	86.8 ± 1.1

The amounts of 5-HT and tryptophan shown were in 1 ml solution which was then taken through the OPT method. Fluorescences were developed at 77°C or 100°C (and shown as means ± s.d. ($n = 3$).

0.36 μg 5-HT/g brain respectively. This effect was less marked if fluorescence was developed at 77°C (as in the modified procedure) when the above tryptophan concentrations were associated with fluorescences corresponding to 0.04, 0.06 and 0.20 μg 5-HT/g brain.

Comparison of the improved OPT and h.p.l.c. methods for determination of brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

Tryptophan-treated rats Results in Table 2 show that rats injected with L-tryptophan (25 to 250 mg/kg) and killed 1 h later had increased brain concentrations of tryptophan, 5-HT and 5-HIAA. The 5-hydroxyindole values by the OPT method were within about 10% of corresponding values by h.p.l.c. and did not differ significantly from them. However, values by the OPT method tended to be slightly lower at lower tryptophan doses and slightly higher at higher doses than were those obtained by h.p.l.c. Therefore, according to the OPT method, tryptophan (250 mg/kg) increased brain 5-HT and 5-HIAA by 0.26 and 0.42 μg/g respectively (i.e. by 60% and 88%) but corresponding increases by h.p.l.c. were somewhat smaller being 0.14 and 0.32 μg/g (i.e. by 29% and 62%). Both methods showed 5-HT synthesis, as indicated by increase in 5-HT and 5-HIAA, to rise as brain tryptophan increased to 69 μg/g. A further increase to 124 μg/g (Table 3) did not cause additional rises of 5-HT or 5-HIAA (as indicated by h.p.l.c.) but there was no suggestion of any inhibition of 5-HT synthesis.

p-*Chlorophenylalanine-treated rats* Results in Table 4 show that PCPA caused comparable percentage decreases of brain 5-HT and 5-HIAA as determined by either method. Thus, rats killed 24 h after a single injection of PCPA (150 mg/kg) had 5-HT and 5-HIAA values which were decreased by 41% and 45% (OPT method) and by 40% and 51% (h.p.l.c. method). This dosage given on 3 successive days decreased 5-HT and 5-HIAA by 88% and 81% (OPT method) and by 92% and 89% (h.p.l.c. method). However, with this dose schedule the small residual 5-HT and 5-HIAA concentrations were significantly higher by the OPT than by the h.p.l.c. method. When tryptophan (150 mg/kg) was injected

Table 2 Effect of L-tryptophan on brain tryptophan, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) as determined by the *o*-phthalaldehyde (OPT) and high pressure liquid chromatography (h.p.l.c.) methods

Dose	*Tryptophan*	*Brain concentration* (μg/g)	*5-HT*	*5-HIAA*
0	4.14 ± 0.36	OPT	0.434 ± 0.039	0.502 ± 0.035
		h.p.l.c.	0.492 ± 0.039	0.522 ± 0.044
25 mg/kg	11.16 ± 2.07	OPT	0.531 ± 0.035**	0.660 ± 0.039***
		h.p.l.c.	0.581 ± 0.066*	0.685 ± 0.039***
100 mg/kg	39.07 ± 8.10	OPT	0.594 ± 0.038***	0.828 ± 0.059***
		h.p.l.c.	0.622 ± 0.062**	0.780 ± 0.070***
250 mg/kg	68.86 ± 15.88	OPT	0.695 ± 0.068***	0.943 ± 0.080***
		h.p.l.c.	0.635 ± 0.071**	0.842 ± 0.082***

Rats (6 per group) were killed 1 h after giving L-tryptophan in saline i.p. Tryptophan values were determined by the method of Bloxam & Warren (1974). Values are given as means ± s.d. Differences from control values: * $P < 0.02$; ** $P < 0.01$; *** $P < 0.001$ (unpaired t test).

Table 3 Effect of L-tryptophan at high dosage on brain tryptophan, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) as determined by high pressure liquid chromatography (h.p.l.c.)

	Brain concentration (μg/g)		
Dose	*Tryptophan*	*5-HT*	*5-HIAA*
0	3.81 ± 0.35	0.486 ± 0.037	0.591 ± 0.025
250 mg/kg	77.18 ± 16.33	0.819 ± 0.101	1.162 ± 0.185
500 mg/kg	124.00 ± 18.64	0.805 ± 0.073	1.203 ± 0.101

Rats (6 per group) were killed 1 h after giving L-tryptophan in saline i.p. Values are given as means ± s.d. All differ significantly from 0 dose group ($P < 0.001$, unpaired t test).

1 h before killing, the reduction of 5-HT by PCPA (150 mg/kg) was completely reversed and 5-HIAA was increased to 50% above the value for saline (0.9% w/v NaCl solution)-treated rats. Results by both methods were essentially identical. However, discrepancies occurred when rats were given PCPA (150 mg/kg × 3) followed by tryptophan (250 mg/kg) as 5-HT and 5-HIAA increased to 28% and 55% of values for saline-injected rats with the OPT method but only to 14% and 33% of the corresponding values with h.p.l.c. In agreement with findings when this dose of PCPA was given without tryptophan, 5-HT and 5-HIAA concentrations were significantly higher by the OPT than by the h.p.l.c. method.

In this experiment, tryptophan was determined by both the fluorometric method of Bloxam & Warren (1974) and by h.p.l.c. Values by the latter method were about 15 to 20% lower, except for the group of rats given PCPA (150 mg/kg × 3).

Regression analysis of brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid values obtained by OPT and h.p.l.c. methods Figure 2 shows the regressions of 5-HT and 5-HIAA values by h.p.l.c. (y) on corresponding values by OPT (x). Results obtained by the two methods correlate high significantly. Both regression lines intercept the y axis at small but significant negative values.

Discussion

Although Gal *et al.* (1978) reported that tryptophan loading led to falsely high brain 5-HT values as determined by the OPT method of Curzon & Green (1970), *in vitro* work (Table 1) suggests only slight errors at the brain tryptophan concentrations they found after giving the highest dose of tryptophan they used (100 mg/kg). Both in the present work and previously (Curzon & Marsden, 1975) brain tryptophan values after giving this dose were comparable with those obtained by Gal *et al.* Interference with the 5-HT determination was greater at much higher tryptophan concentrations but these corresponded to

Table 4 Effect of *p*-chlorophenylalanine (PCPA) and L-tryptophan on brain tryptophan, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) as determined by different methods

			Brain concentration (μg/g)	
Drug	*Tryptophan*		*5-HT*	*5-HIAA*
Saline	(5.20 ± 0.40)	OPT	0.549 ± 0.018	0.475 ± 0.019
	4.33 ± 0.48‡	h.p.l.c.	0.568 ± 0.064	0.455 ± 0.047
PCPA (150 mg/kg)	(5.04 ± 1.01)	OPT	0.323 ± 0.055***	0.264 ± 0.030***
	4.21 ± 1.07	h.p.l.c.	0.342 ± 0.060***	0.222 ± 0.036***
PCPA (150 mg/kg) + L-tryptophan (150 mg/kg)	(22.62 ± 12.26)	OPT	0.564 ± 0.111	0.713 ± 0.156**
	18.17 ± 10.34	h.p.l.c.	0.581 ± 0.098	0.684 ± 0.186*
PCPA (150 mg/kg×3)	(3.58 ± 0.27)	OPT	0.067 ± 0.006***	0.092 ± 0.009***
	5.49 ± 0.91‡	h.p.l.c.	0.043 ± 0.011***,‡‡	0.052 ± 0.021***,‡
PCPA (150 mg/kg ×3)+L-tryptophan (250 mg/kg)	(45.14 ± 8.07)	OPT	0.152 ± 0.018***,††	0.260 ± 0.022***,††
	38.92 ± 8.12	h.p.l.c.	0.078 ± 0.037***,†,‡	0.148 ± 0.026***,††,‡‡

PCPA was given i.p. either once or daily for 3 days and animals (5–6 per group) killed 24 h later and 1 h after giving L-tryptophan in saline. Tryptophan was determined by the method of Bloxam & Warren (results in parentheses) and by h.p.l.c. Values are given as means ± s.d. Differences from values for rats given saline: * $P < 0.02$; ** $P < 0.01$; *** $P < 0.001$. Differences from values for rats given PCPA (150 mg/kg×3): † $P < 0.05$; †† $P < 0.001$. Differences from values determined by h.p.l.c. ‡ $P < 0.01$; ‡‡ $P < 0.001$ (unpaired t test).

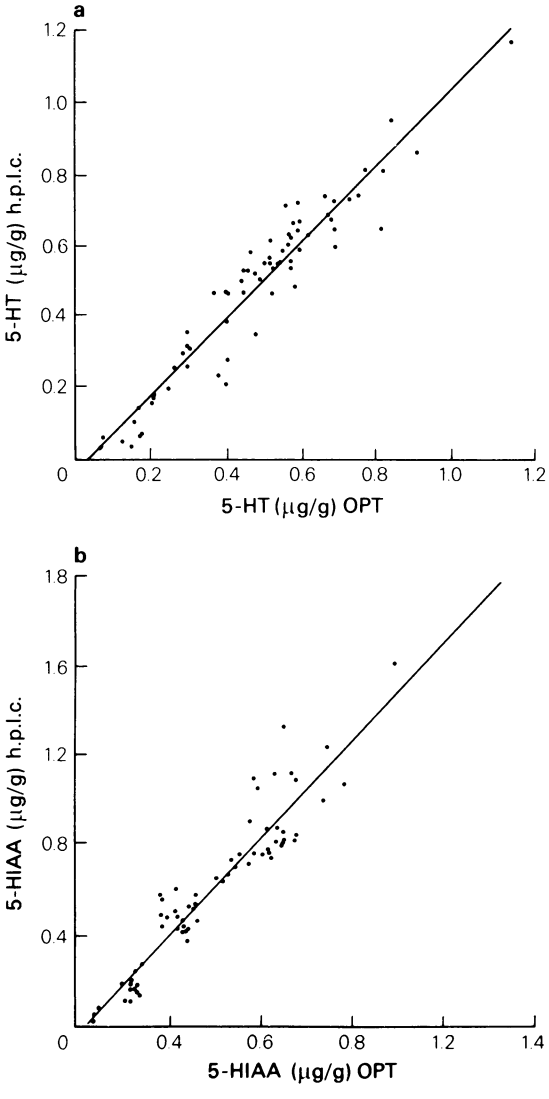

Figure 2 Regression lines of brain 5-hydroxyindole concentrations as determined by *o*-phthalaldehyde (OPT) (x) and high pressure liquid chromatography (h.p.l.c.) (y) methods. All results in Tables 2 and 4 and some additional data are included: (a) 5-hydroxytryptamine (5-HT). $y = -0.030 + 1.04x$, ($r = 0.96$, $n = 70$, $P < 0.001$); (b) 5-hydroxyindoleacetic acid (5-HIAA). $y = -0.048 + 1.09x$, ($r = 0.95$, $n = 70$, $P < 0.001$). The intercepts on the x axes are significantly different from 0: (a) $P < 0.05$; (b) $P < 0.02$.

brain values only to be expected after enormous doses of tryptophan e.g. 1600 mg/kg (Eccleston, Ashcroft & Crawford, 1965). Furthermore, interference was less marked with the slightly modified OPT method described than with the earlier procedure of Curzon & Green.

In agreement with these *in vitro* results, no significant differences were found between the effects of injecting L-tryptophan (25, 100, 250 mg/kg) on brain 5-HT and 5-HIAA values, whether determinations were made by the modified OPT or the h.p.l.c. method. However, the small differences between the mean values obtained by the two methods are consistent with the slight interference of tryptophan at high concentrations with the OPT method shown *in vitro*. Interference of tryptophan with the h.p.l.c. method is most unlikely as the amino acid is well separated from the 5-hydroxyindoles and also is not oxidized at the voltage used for their electrochemical determination.

Gal *et al.* (1978) using h.p.l.c. found that tryptophan (100 mg/kg) increased brain tryptophan concentration from 5.7 μg/g to 36 μg/g but 5-HT and 5-HIAA concentrations fell below their control values. In the present study, on the contrary, the 5-hydroxyindoles were shown to rise gradually as the tryptophan dose increased to 250 mg/kg. Furthermore, in an experiment in which only the h.p.l.c. method was used, there was no indication of inhibition of 5-HT synthesis even when tryptophan (500 mg/kg) was given so that its concentration in the brain rose from 3.8 to 124 μg/g.

Results confirm the usefulness of the OPT method for determining brain 5-HT and 5-HIAA as it shows only minor discrepancies with the h.p.l.c. method even under extreme conditions, i.e. when very high doses of tryptophan are given or when 5-HT is grossly depleted by PCPA. These discrepancies are compounded in rats given both drugs (Table 4). The h.p.l.c. procedure has additional advantages of higher sensitivity, the separation and concurrent determination of all three brain indoles and (using the sample injector) of automation. Nevertheless, results indicate the overall reliability of the OPT method especially in the modified version described. Certainly, they do not suggest that its use has been responsible for any serious misconceptions about brain 5-HT metabolism.

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