Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 4/5, pp. 323–333, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

The determination of 5-hydroxytryptamine, related indolealkylamines and 5-hydroxyindoleacetic acid in the bovine eye by gas chromatography–negative ion chemical ionization mass spectrometry

S.A. BEST, J.M. MIDGLEY,* W. HUANG and D.G. WATSON

Department of Pharmaceutical Sciences, University of Strathclyde, Royal College, 204 George Street, Glasgow G1 1XW, UK

Abstract: Methods were developed for the analysis of 5-hydroxytryptamine, related indolealkylamines (tryptamine, melatonin, 5-methoxytryptamine, *N*-acetyl-5-hydroxytryptamine and 6-hydroxymelatonin) and 5-hydroxyindole-3-acetic acid (5HIAA) in bovine retina, aqueous and vitreous humours. 5-Hydroxytryptamine and related indolealkylamines were extracted and derivatized to form their corresponding pentafluoropropionyl spirocyclic derivatives. 5HIAA was extracted and derivatized to the corresponding pentafluoropropionamide-trifluoroethyl derivative. Identification and quantitation by gas chromatography–negative ion chemical ionization mass spectrometry was made with reference to deuteriated internal standards. 5-Hydroxytryptamine was present in all (n = 34) retinal samples analysed (20.53 ± 1.64 ng) while *N*-acetyl-5-hydroxytryptamine was detected in half of the samples of retina (0.06 ± 0.02 ng). Melatonin (0.15 ± 0.06 ng) and tryptamine (0.78 ± 0.34 ng) were detected in only a small number of retinas. 5-Methoxytryptamine was not present in retina. 5-Hydroxytryptamine was also present in aqueous (0.76 ± 0.17 ng ml^{-1}) and vitreous (0.35 ± 0.05 ng ml^{-1}) humours from bovine eye. Tryptamine, melatonin, 5-methoxytryptamine and *N*-acetyl-5-hydroxytryptamine were not detected in bovine aqueous and vitreous humours. 5HIAA was found in both bovine aqueous (2.03 ± 0.38 ng ml^{-1}) and vitreous (0.65 ± 0.06 ng ml^{-1}) humours, but its consistent determination in retina was obviated by interference from spurious peaks.

Keywords: *Bovine eye; 5-hydroxytryptamine; indolealkylamines; 5-hydroxyindole-3-acetic acid; GC–NICIMS.*

Introduction

Indolealkylamines, particularly 5-hydroxytryptamine (5HT, serotonin), have been implicated in retinal function for the last 20 years [1]. Despite this there there has been considerable debate on whether or not 5HT is a neurotransmitter in the retina; the presence of serotonergic neurones has not been demonstrated in the retina, although neurones which accumulate exogenous 5HT are present. This prompted the suggestion that the transmitter of these 5HT-accumulating neurones was a substance related to 5HT [2, 3].

Tryptophan hydroxylase (EC 1.14.16.4), the rate-limiting enzyme of the biosynthesis of 5HT (see Fig. 1), has not been identified in the retina [2]. In part this may be due to the susceptibility of the enzyme to inactivation by molecular oxygen [4]. Evidence for the presence of this enzyme in retina has been furnished by the following observations *in vitro*: that retinal tissue converted radioactive tryptophan to 5-hydroxytryptophan, 5HT and 5-hydroxyindoleacetic acid (5HIAA; the major metabolite of 5HT) and that the addition of *p*-chlorophenylalanine (an inhibitor of tryptophan hydroxylase) resulted in a large reduction in these conversions. In addition, it has been reported that the enzymes, 5-hydroxytryptophan decarboxylase (EC 4.1.1.28) and monoamine oxidase (EC 1.4.3.4), were present in the retina [2].

The determination of 5HT in retina (see Table 1) has been achieved by fluorimetry, HPLC [2] and gas chromatography–negative ion chemical ionization mass spectrometry (GC–NICIMS) [5], although the determination of 5HT in some mammalian retinas has not been successful [1]. The analysis of 5HT has been reported in the retinas of chicks [6], amphibia, reptiles [7], cows and rabbits [2, 3, 5].

Binding sites of 5HT have been localized in crude membrane fractions of rabbit and bovine retinas [1, 3]. It has been reported that the uptake of 5HT into retinal neurones was of high affinity, selectivity and similar to that

* Author to whom correspondence should be addressed.

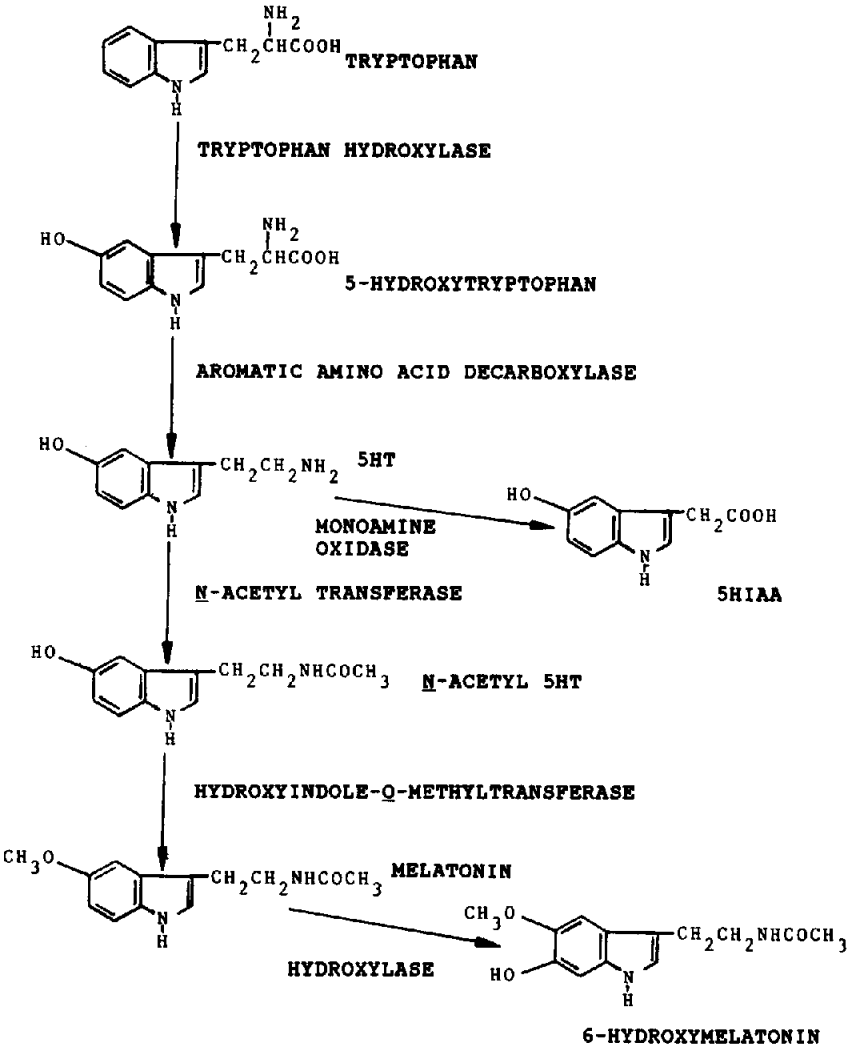

Figure 1
Biosynthetic and metabolic pathways of 5HT and melatonin.

Table 1
Concentration of 5HT in retina

Species	Method	Concentration of 5HT ng g^{-1} wet tissue	Reference
Cow	Fluorimetry	100.3 ± 10.1	[2]
Cow	HPLC	26.2 ± 7.0	[2]
Cow	HPLC	39.0 ± 4.0	[2]
Cow	GC-NICIMS	25.0 ± 12.0	[5]
Chick	HPLC	127.0 ± 15.0	[6]
Rat	HPLC	37.0 ± 8.0	[10]

reported in the brain. In addition it has been postulated that the receptors for 5HT in the brain and retina were similar [8]. Therefore it has been proposed that the retina could serve as a model of specific neuronal systems because the retina and brain have many similar characteristics. Embryological, morphological and functional information indicate that the retina, in conjunction with the optic nerve, can be considered as an extension of the brain [2, 8].

The retina and pineal gland have also been

compared in terms of their ability to synthesize melatonin, the methoxy-*N*-acetylated metabolite of 5HT, which is present in the retina; albeit in much lower concentrations than 5HT. The retina and pineal gland both contain the enzymes involved in the biosynthesis of melatonin, namely; *N*-acetyl transferase and hydroxyindole-*O*-methyltransferase (Fig. 1). It has been reported that, in general, the regulation of the biosynthesis and release of melatonin, in both the retina and pineal gland, are similarly influenced by light–dark exposure. Interestingly, the concentration of melatonin in the retina exhibited a diurnal pattern that was in phase with the concentrations of melatonin in the pineal gland and in plasma [2, 8–10].

Our laboratory has previously reported the determination of 5HT in three bovine retinas. These determinations were made with reference to an analogue of 5HT (4,6-difluoro-5-hydroxytryptamine), as we did not have an appropriate deuterium-labelled isotopomer at the time of the analysis [5]. We now report the determination of 5HT and melatonin in bovine retina against deuteriated standards synthesized in our laboratory. In addition tryptamine, 5-methoxytryptamine, *N*-acetyl 5HT and 6-hydroxymelatonin were also determined. *N*-Acetyl 5HT is the precursor and 6-hydroxymelatonin the metabolite of melatonin [2, 11]. It has been suggested that 5-methoxytryptamine is a possible intermediate in the biosynthesis of melatonin since it was observed that both melatonin and 5-methoxytryptamine were present in the hypothalamus, but *N*-acetyl 5HT was not [12]. Tryptamine has been proposed to be a trace amine which may be involved in neuromodulatory functions [13].

Experimental

Chemicals

All solvents used in the procedure were HPLC grade (Rathburn Chemicals, Peebleshire, UK). Chemicals were obtained from the following sources: 2,2,2-trifluoroethanol (TFE), 5-hydroxyindole-3-acetic acid (5HIAA), propionic anhydride, pyridine, tryptamine, *N*-acetyl-5-hydroxytryptamine, serotinin hydrochloride hemi-hydrate, melatonin, deuterium oxide (D_2O) and trifluoroacetic anhydride (Aldrich Chemical Company Ltd, Dorset, UK); 5-methoxytryptamine, [$^2H_4$]-5-methoxytryptamine, 6-hydroxymelatonin and ethylenediaminetetraacetic acid trisodium salt (Sigma Chemical Company Ltd, Poole, Dorset, UK); pentafluoropropionic anhydride (Fluorochem Ltd, Glossop, Derbyshire, UK); percholoric acid 60% (Fisons, Loughborough, UK); anhydrous sodium carbonate, di-potassium hydrogen orthophosphate anhydrous, orthophosphoric acid and acetic anhydride (BDH Ltd, Poole, Dorset, UK).

Synthesis of deuteriated standards

(1) [$^2H_3$]-5HIAA. 5HIAA (22 mg) was reacted with a solution of trifluoro[^{2}H]acetic acid–D_2O prepared by the dropwise addition of trifluoroacetic anhydride (1.356 ml) to D_2O (0.644 ml) cooled in an ice bath. The solution was protected from light and heated at 65°C for 23 h. Reagents were removed by rotary evaporation under reduced pressure and the residue dissolved in ethyl acetate and used without further purification. GC–NICIMS analysis of the pentafluoropropionamide–trifluoroethyl (PFP–TFE) derivative (see 'sample preparation') revealed that 2–68% of the unlabelled species was present relative to the base peak in the mass spectrum.

(2) Synthesis of [$^2H_4$]-tryptamine. [$^2H_4$]-Tryptamine was prepared by a procedure similar to that employed in the synthesis of [$^2H_3$]-5HIAA (see also ref. 14).

Trifluoroacetic anhydride (3.12 ml) was added in a dropwise manner to deuterium oxide (1.48 ml) cooled in an ice bath. Tryptamine (1 g) was dissolved in the resultant solution and the mixture heated (90°C, 48 h). The reagents were then removed by rotary evaporation under reduced pressure and a residue of deuteriated trifluoroacetate salt of tryptamine obtained. This residue was shaken with sodium hydroxide (1 M) to liberate the deuteriated tryptamine, which was then extracted into ethyl acetate. The ethyl acetate was removed by rotary evaporation under reduced pressure and the residue was crystallized from hexane as leaflets (22.1%, m.p. 108–111°C).

Analysis of the spirocyclic derivative (see 'sample preparation') of the deuteriated tryptamine by GC–NICIMS revealed that less than 0.8% of undeuteriated tryptamine was present relative to the base peak of PFP [$^2H_4$]-tryptamine.

(3) Synthesis of α,α,β,β-[$^2H_4$]-melatonin. A solution of [$^2H_4$]-5-methoxytryptamine (1 mg) in acetic anhydride (200 μl) was heated (60°C, 30 min) and the excess acetic anhydride evaporated under a stream of nitrogen. The resultant residue was dissolved in methanol and used as an internal standard without further purification.

Analysis of the PFP (see 'Sample preparation') derivative of the deuteriated melatonin by GC–NICIMS, revealed that the amount of undeuteriated melatonin present was less than 0.3% of the base peak of PFP [$^2H_4$]-melatonin. The ions (see 'Results') corresponding to the PFP derivative of [$^2H_4$]-5-methoxytryptamine were not detected in the derivatized product by GC–NICIMS.

(4) Synthesis of α,α,β,β-[$^2H_4$]-5HT. The synthesis of [$^2H_4$]-5HT picrate in our laboratory was based on a literature procedure [15]. Briefly, 5-(benzyloxy)indole-3-glyoxyloyl chloride was prepared from the reaction of oxalyl chloride with 5-benzyloxyindole and reacted with ammónia to give 5-(benzyloxy)-indole-3-glyoxylamide. Reduction with lithium aluminium deuteride resulted in *O*-benzyl-[$^2H_4$]-5HT hydrochloride. The *O*-benzyl-[$^2H_4$]-5HT hydrochloride salt was hydrogenolysed and the product converted to the picrate salt. Analysis of the Pr-PFP (see 'Sample preparation') derivative of the deuteriated 5HT by GC–NICIMS, revealed that the concentration of undeuteriated 5HT was below the limit of detection.

Bovine eye samples

Bovine eyes from freshly slaughtered animals were collected from the local abattoir. They were dissected within 2 h of their collection and the retina, aqueous and vitreous humours stored at −20°C until required for analysis.

Sample extraction and derivatization

(1) Determination of the concentrations of 5HT and related indolealkylamines in retina. The method used for the identification and quantitation of 5HT was based on that reported by Markey *et al.* [16].

Separate stock solutions (1 μg μl^{-1}) of tryptamine, melatonin, 5-methoxytryptamine, *N*-acetyl 5HT, 5HT, 6-hydroxymelatonin, [$^2H_4$]-tryptamine, [$^2H_4$]-melatonin, [$^2H_4$]-5-methoxytryptamine and [$^2H_4$]-5HT were prepared in methanol. Perchloric acid (0.3 M, 1 ml; containing 1 mg ml^{-1} EDTA) and 5–20 ng of [$^2H_4$]-tryptamine, [$^2H_4$]-melatonin, [$^2H_4$]-5-methoxytryptamine and [$^2H_4$]-5HT was added to each retina which was homogenized manually in a ground glass homogenizer.

The sample was centrifuged (800*g*, 15 min) and the supernatant collected. Saturated sodium carbonate (0.3 ml), propionic anhydride (0.15 ml) and pyridine (30 μl) were added and the mixture vortexed (1 min) vigorously. Extraction into ethyl acetate (2 ml × 1, 1 ml × 1) was achieved by vigorous vortexing (1 min) and the organic layers collected; any emulsification being overcome by centrifugation (800*g*, 5 min). The ethyl acetate was removed by evaporation under a stream of nitrogen and the residue derivatized by reaction (15 min, 60°C) with pentafluoropropionic anhydride (100 μl), in an open-topped vessel. The excess reagent was evaporated, under a stream of nitrogen; toluene (0.6 ml) and phosphate buffer (0.05 M, 0.3 ml, pH 7.5–8.3) were added to the residue. The sample was vortexed vigorously and the toluene layer collected. The toluene was evaporated under a stream of nitrogen and the residue dissolved in ethyl acetate for subsequent GC–NICIMS analysis.

(2) Determination of the concentrations of 5HT and related indolealkylamines in bovine vitreous and aqueous humours. The procedure employed for the determination of 5HT in vitreous and aqueous humours was very similar to that used for 5HT in retina. The only difference was that the centrifugation step was omitted. The determinations used aliquots (1 ml) of vitreous humour or aqueous humour.

(3) Determination of the concentration of 5HIAA in retina. Separate stock solutions (1 μg μl^{-1}) of 5HIAA and [$^2H_3$]-5HIAA were prepared in ethyl acetate. Water (1 ml) and [$^2H_3$]-5HIAA (5 ng) were added to each retina and the sample homogenized manually in a ground glass homogenizer. Water (1 ml) was also added to a blank (a sample containing only [$^2H_3$]-5HIAA) and standard (1:1, v/v) mixture. The suspension was centrifuged (800*g*, 15 min) and the supernatant collected. After acidification with HCl (1 M, 0.3 ml), the 5HIAA was extracted into ethyl acetate (2 ml) by vigorous vortexing (1 min). The organic layer was collected, any emulsification was overcome by centrifugation (800*g*, 5 min), and the solvent

evaporated to dryness under a stream of nitrogen. Derivatization was achieved by the reaction (80–90°C, 1 h) with pentafluoropropionic anhydride (100 μl) and trifluoroethanol (40 μl). Excess reagents were evaporated under a stream of nitrogen and the residue dissolved in ethyl acetate for GC–NICIMS analysis.

(4) Determination of the concentration of 5HIAA in bovine vitreous humour. [$^2H_3$]-5HIAA (5 ng) was added to aliquots (1 ml) of vitreous humour. Water (1 ml) and [$^2H_3$]-5HIAA were added to a blank and standard (1:1, v/v) mixture. Samples were acidified with HCl (1 M, 0.3 ml) and extracted into ethyl acetate (2 ml) by vigorous vortexing (1 min). The samples were derivatized as described above.

(5) Determination of the concentration of 5HIAA in bovine aqueous humour. The procedure was identical to that described for the determination of 5HIAA in bovine vitreous humour and used 1 ml aliquots of bovine aqueous humour.

Validation

Instrumental response was linear over the following concentration ranges: tryptamine/[$^2H_4$]-tryptamine (0.02–5 ng, $y = 0.0132 + 0.0535x$, $r = 0.9980$); melatonin/[$^2H_4$]-melatonin (0.04–5 ng, $y = -0.0140 + 0.2987x$, $r = 0.9999$); *N*-acetyl 5HT/[$^2H_4$]-5HT (0.005–5 ng, $y = -0.0631 + 2.679$, $r = 0.9958$); 5HT/[$^2H_4$]-5HT (0.03–30 ng, $y = 0.0904 + 0.0698x$, $r = 0.9945$); 5HIAA/[$^2H_3$]-5HIAA (0.1–30 ng, $y = 0.01439 + 0.230x$, $r = 0.9969$).

The limit of detection for the spirocyclic derivatives of 5HT and related indolealkylamines and the PFP–TFE derivative of 5HIAA were: tryptamine (0.02 ng sample^{-1}); melatonin (0.04 ng sample^{-1}); *N*-acetyl 5HT (0.005 ng sample^{-1}); 5HT (0.03 ng sample^{-1}); 5HIAA (0.1 ng sample^{-1}).

The reproducibility of the methods employed was measured by the repeated determination of the ratio of the concentration of each substance to that of its respective deuteriated standard in the standard 1:1 mixture. The mean and SEM of this ratio for each substance are as follows: 5HT (0.527 ± 0.011); melatonin (0.397 ± 0.013); *N*-acetyl 5HT (0.040 ± 0.003); tryptamine (0.541 ± 0.011); 5HIAA (0.776 ± 0.001).

GC–MS analysis

GC–MS in the NICI mode was carried out as described previously [5, 17, 18]. The gas chromatograph was fitted with a Restek Corporation Rtx-1 crossbonded 100% dimethyl polysiloxane capillary column (15 m × 0.25 mm i.d.); helium carrier gas was used with a head pressure of 5 psi. The GC conditions were as follows: injector temperature, 250°C; transfer line temperature, 280°C; oven temperature maintained at 100°C for 1 min, then programmed to increase at 10°C min^{-1} to 300°C. Injections were made using a Grob splitless injection system.

Results and Discussion

The propionyl-pentafluoropropionyl spirocyclic (Pr-PFP) derivatives of *N*-acetyl 5HT, 5HT, and 6-hydroxymelatonin and the pentafluoropropionyl spirocyclic (PFP) derivatives of melatonin, tryptamine and 5-methoxytryptamine, were formed consistently in good yield. The mass spectra of these derivatives under NICI conditions were in general dominated by ions resulting from the loss of one or two HF residues from the molecular ion. Table 2 indicates the molecular ion, base peak, and other major ions in the mass spectra of the derivatives of the deuteriated and undeuteriated indolealkylamines. As reported previously [19] the derivatives where the alkyl side chain nitrogen is propionylated, are formed as two geometric isomers so that, apart from *N*-acetyl 5HT, melatonin, and 6-hydroxymelatonin, each compound yields two closely eluting peaks with very similar mass spectra. For the compounds in which derivatization results in the formation of geometrical isomers, Table 2 records the mass spectrum of the later eluting larger peak.

The pentafluoropropionamide-trifluoroethyl (PFP-TFE) derivatives of 5HIAA and [$^2H_3$]-5HIAA afforded two peaks due to the species with and without an acylated indole nitrogen. It was not readily possible to obtain complete acylation of the indole nitrogen; this observation has been previously reported in the analysis of 5-hydroxytryptophol; a metabolite of 5HT [20]. The spectra of both the fully acylated compound and the species lacking an acylated indole nitrogen were dominated by ions resulting from the loss of PFPH from the molecular ion (Table 2). The highest degree of reproducibility in quantitative analysis was

Table 2
Mass spectral characteristics of the spirocyclic derivatives of 5HT and related indolealkylamines and the PFP-TFE derivative of 5HIAA

Derivative	Base peak *m/z*	Molecular ion *m/z*	Other major ions *m/z*
Tryptamine PFP	324	344 (0.9%)	304 (53.8%)
[$^2H_4$]-Tryptamine PFP	328	348 (0.5%)	308 (48.1%), 329 (61.1%), 327 (62.2%)
Melatonin PFP	320	360 (0.4%)	340 (34.4%)
[$^2H_4$]-Melatonin PFP	323	364 (0.5%)	343 (37.6%)
5-Methoxytryptamine PFP	354	374 (0.6%)	334 (42.0%)
[$^2H_4$]-5-Methoxytryptamine PFP	357	378 (0.4%)	337 (46.6%)
N-Acetyl 5HT Pr-PFP	362	402 (1.1%)	382 (49.1%)
5HT Pr-PFP	396	416 (1.69%)	340 (56.0%), 376 (42.9%)
[$^2H_4$]-5HT Pr-PFP	344	420 (3.8%)	379 (28.3%), 399 (69.4%)
6-Hydroxymelatonin Pr-PFP	392	—	412 (35.5%)
5HIAA PFP-TFE			
Peak 1	417	565 (0%)	
Peak 2	271	419 (0%)	
[$^2H_3$]-5HIAA PFP-TFE			
Peak 1	420	568 (0%)	
Peak 2	274	422 (0%)	

obtained by combining the peak areas of the selected ions of both species. This procedure was carried out both for the deuteriated internal standard and for undeuteriated 5HIAA.

The indolealkylamines were quantified against deuteriated isotopomers for all compounds except *N*-acetyl 5HT which was quantified with reference to [$^2H_4$]-5HT. Figure 2 shows in the upper trace a retina extracted and derivatized to form the PFP derivatives in comparison with the lower trace of a standard mixture containing 5 ng of each unlabelled and labelled species similarly extracted and derivatized. Figure 3 shows the individual ion currents for the PFP derivatives of endogenous amines extracted from bovine retina in comparison with the ion currents for derivatized deuteriated standards, except for *N*-acetyl 5HT which is compared against the corresponding ion current of the standard mixture. Table 3 shows the mean concentration and range of 5HT and related compounds from the retinas analysed ($n = 34$). The only compound consistently within the limits of detection was 5HT, although *N*-acetyl 5HT was quantified in 17 retinas. The mean concentration of 5HT was comparable to the preliminary determination (25.0 ± 12 ng g^{-1} wet weight) carried out in this laboratory [5]. 5HT has been determined in the retina of various species (Table 1); fluorometric methods are non-specific and tend to give high values, but the values obtained by GC–MS and HPLC analyses are comparable; the sensitivity of GC–MS procedures enables the determination of related indolealkylamines such as melatonin and tryptamine. Thus a wide range of components in the 5HT biosynthetic/metabolic pathway may be simultaneously determined using GC–MS as in the present case.

It has been previously reported [2] that melatonin occurs in the eye at low concentrations and it has been shown that it effects the aggregation of melanosomes in the pigmented epithelium and the release of dopamine from amacrine and/or interplexiform cells. The biosynthesis of melatonin and its subsequent release are reportedly associated with the photoreceptors of the retina. It has been suggested that receptors for melatonin are present also in the retina [8, 9]. Melatonin was detected in 7 out of 34 retinas analysed (see Fig. 3B and Table 3) and its concentration ranged from 0.08 to 1.52 ng g^{-1} wet tissue: the variability perhaps reflecting its diurnal variation.

N-acetyl 5HT, an intermediate in the biosynthesis of melatonin, was detected in half of the retinas analysed (see Fig. 3E and Table 3). The presence of *N*-acetyl 5HT in the bovine retina, together with our failure to detect 5-methoxytryptamine (see Table 3), suggests that the biosynthesis of melatonin occurs through the *N*-acetyl 5HT intermediate. This is further supported by the observation that *N*-acetyl 5HT and melatonin were normally found together in retinal samples (see Table 3). The concentration of *N*-acetyl 5HT in the bovine retina was found to be mostly low (or below the limit of detection) and similarly the concentration of melatonin was also usually

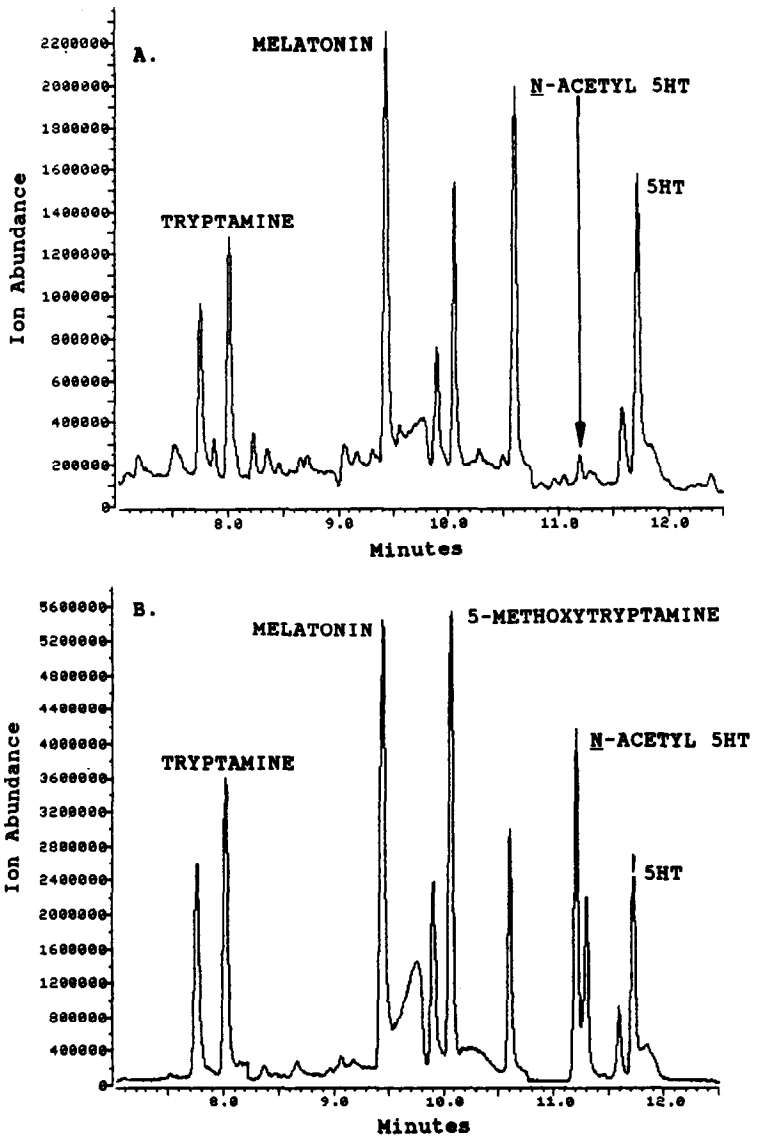

Figure 2
(A) Total ion current trace of tryptamine, melatonin, *N*-acetyl 5HT and 5HT extracted from retina as their PFP spirocyclic derivatives. (B) Total ion current trace of a standard mixture (5 ng) of tryptamine, melatonin, 5-methoxytryptamine, *N*-acetyl 5HT and 5HT extracted as their PFP spirocyclic derivatives.

below the limit of detection. In addition, only for a small number of retinas was melatonin detected in the absence of *N*-acetyl 5HT. It has been reported previously that 5-methoxytryptamine occurs in brain tissue [2] and this may be an intermediate in the biosynthesis of melatonin [12].

Tryptamine, the decarboxylation product of tryptophan [21] has been classified as a trce amine [13] and it has been suggested that trace amines may act as possible neurotransmitters and/or neuromodulators [13]. Tryptamine may also be involved in neuroregulation and it has specific binding sites which arc different to those of 5HT and other neutotransmitters [22]. Tryptamine was identified in eight our of 34 samples of retina (0.78 ± 0.34 ng g^{-1} wet tissue).

Difficulties were experienced in the analysis of the Pr-PFP derivative of 6-hydroxymelatonin and its recovery after derivatization was very variable. This substance has been reported to be acid labile [11] and this may account for poor recovery. Addition of ascorbic acid to samples prior to derivatization gave some improvement. Despite this 6-

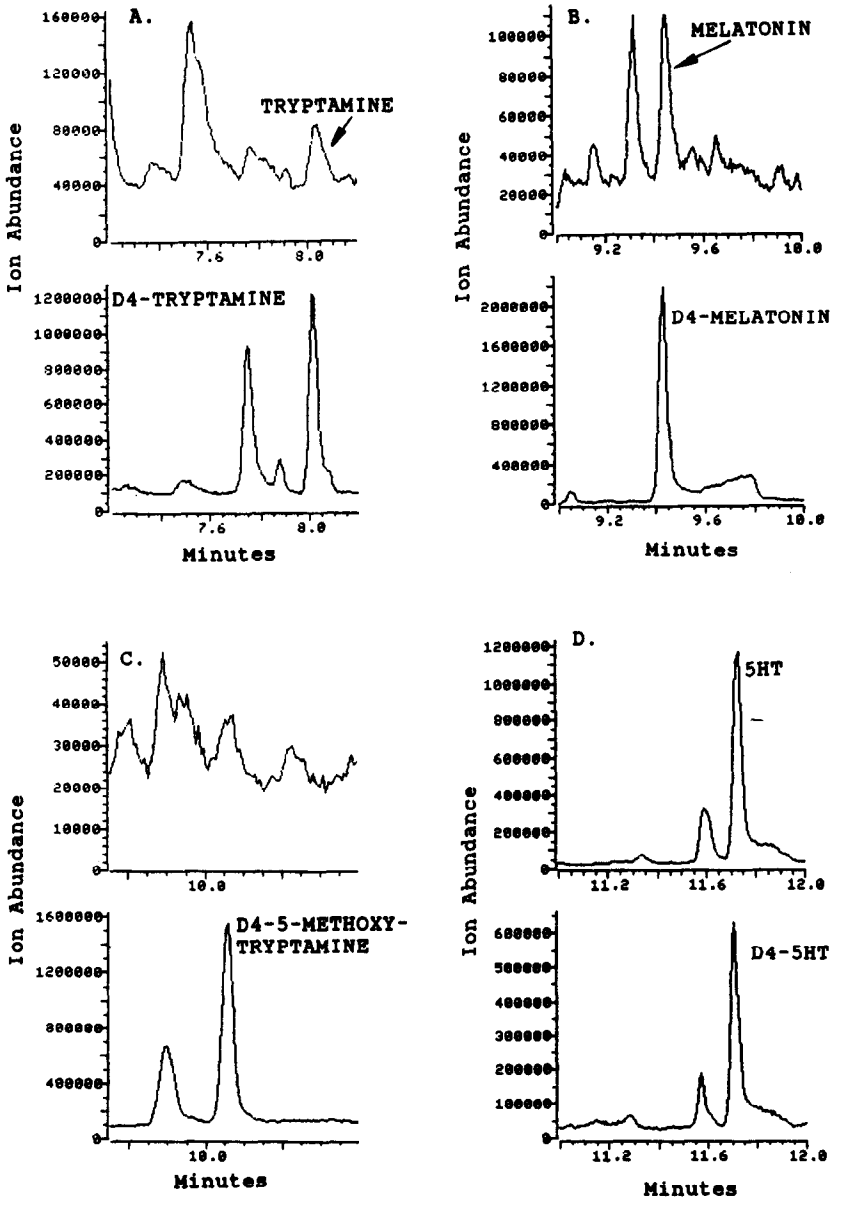

Figure 3
Selected ion monitoring traces of indolealkylamines and their deuteriated standards extracted from retina by the PFP spirocyclic procedure. (A) Tryptamine (upper) and [$^2H_4$]-tryptamine (5 ng) (lower). (B) Melatonin (upper) and [$^2H_4$]-melatonin (5 ng) (lower). (C) Ions corresponding to 5-methoxytryptamine (ions *m/z* 334 and 354) (upper) and [$^2H_4$]-5-methoxytryptamine (5 ng) (lower), showing the absence of 5-methoxytryptamine. (D) 5HT (upper) and [$^2H_4$]-5HT (5 ng) (lower). (E) *N*-Acetyl 5HT extracted from retina (upper) and a standard mixture (5 ng) (lower).

hydroxymelatonin could not be detected in bovine retina although the results are not definitive because of the difficulties in analysis.

The methodology employed to determine 5HT and related indolealkylamines in bovine aqueous and vitreous humour was very similar to that used for the retinal analysis and included the addition of perchloric acid since a deproteinization agent had been employed in a previous analysis of human vitreous humour [23]. However, we observed little evidence of any precipitation of protein. Table 4 shows the concentrations of 5HT determined in bovine aqueous and vitreous humours. Tryptamine (with the exception of three samples), melatonin, 5-methoxytryptamine, *N*-acetyl 5HT and 6-hydroxymelatonin were not detected. Presumably the 5HT in the vitreous humour

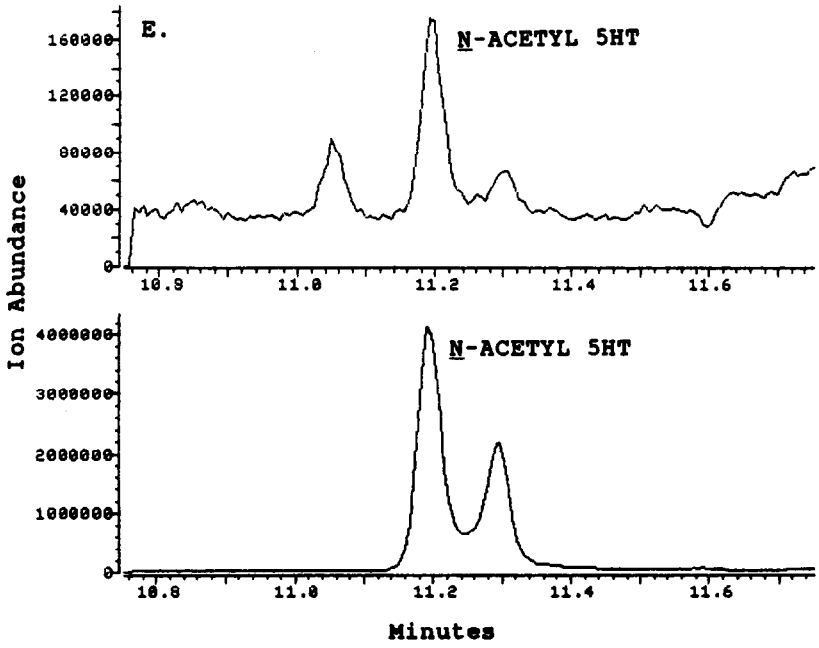

Figure 3(E)

Table 3
The concentrations of 5HT, *N*-acetyl 5HT, melatonin and tryptamine in the bovine retina (n = 34)

Substance	Mean ± SEM (ng g^{-1} wet weight)	Range (ng g^{-1} wet weight)
5HT	20.53 ± 1.64 (n = 34)	6.53–46.01
N-Acetyl 5HT	0.06 ± 0.02 (n = 17)	ND–0.52
Melatonin	0.15 ± 0.06 (n = 7)	ND–1.52
Tryptamine	0.78 ± 0.34 (n = 8)	ND–8.18

5-Methoxytryptamine and 6-hydroxymelatonin were not detected in bovine retina.

ND = not detected.

Limit of Detection (per sample): tryptamine (0.02 ng); melatonin (0.04 ng); *N*-acetyl 5HT (0.005 ng); 5HT (0.03 ng).

Table 4
The concentration of 5HT in bovine aqueous and vitreous humour

	Conc. of 5HT (ng ml^{-1}) (Mean ± SEM)
Aqueous humour (n = 15)	0.76 ± 0.17 (0.12–2.94)
Vitreous humour (n = 17)	0.35 ± 0.05 (0.14–1.36)

Tryptamine was detected in three samples of aqueous humour (1.27, 0.83 and 1.2 ng ml^{-1}), but was not detected in bovine vitreous humour. *N*-Acetyl 5HT, melatonin, 5-methoxytryptamine and 6-hydroxymelatonin were not detected.

originates from the retina; however, the origin of the 5HT in the bovine aqueous humour may be from blood.

5HIAA is the major metabolite of 5HT and

Table 5
The concentration of 5HIAA in bovine retina, bovine vitreous and bovine aqueous humours

	Conc. of 5HIAA (ng ml^{-1} or ng g^{-1} wet weight) (Mean ± SEM)
Aqueous humour (n = 16)	2.03 ± 0.38 (0.52–5.5)
Vitreous humour (n = 24)	0.65 ± 0.06 (0.35–1.69)
Retina (n = 2)	4.22, 10.81

is formed by the oxidative deamination of the parent amine by monoamine oxidase to give an unstable intermediate aldehyde product, which affords 5HIAA under the influence of aldehyde dehydrogenase [12]. The concentration of 5HIAA was determined in two retina samples only (see Table 5) because of interference by spurious peaks. Interestingly, the mean concentration of 5HIAA in these two samples was considerably lower than the concentrations of 5HT determined in retinas. It has been reported that the metabolism of 5HT into 5HIAA occurs slowly in the retina and that reuptake of 5HT into the neurone may be the method for the termination of its action in the retina [2].

5HIAA was also determined in both aqueous and vitreous humours of the bovine eye. Figure 4 (upper) shows the chromatogram of a sample of vitreous humour extracted and

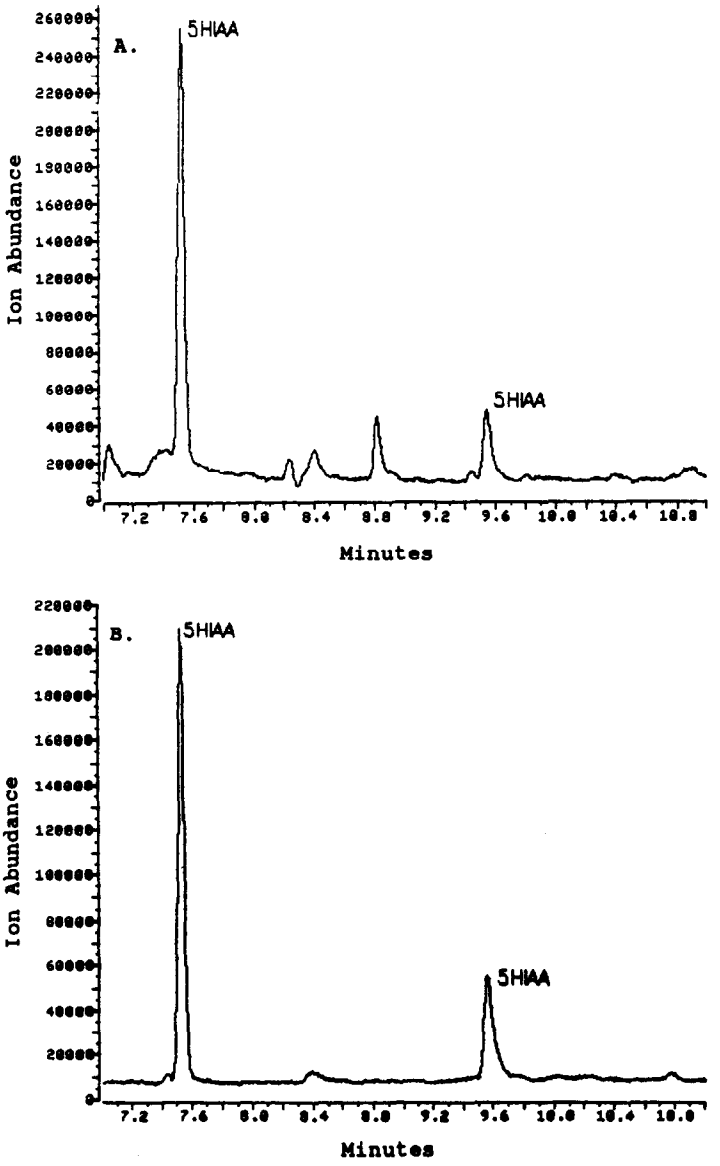

Figure 4
(A) Total ion current trace of 5HIAA extracted from bovine vitreous humour (1 ml) as its PFP-TFE derivative. (B) Total ion current of a standard mixture (5 ng) of 5HIAA extracted as its PFP-TFE derivative.

derivatized by this technique whilst the lower chromatogram is that of a standard mixture (5 ng) of [$^2H_3$]-5HIAA similarly extracted and derivatized. The concentrations of 5HIAA in bovine aqueous and vitreous humours are shown in Table 5. The mean concentrations of 5HT in aqueous and vitreous humours are less than those of 5HIAA in the same fluid (see Tables 4 and 5). The 5HIAA present in vitreous humour could have resulted from enzymatic degradation of 5HT in vitreous humour or retinal 5HT. Similar considerations apply to the 5HIAA in aqueous humour which may be the result of the enzymatic degradation of 5HT in the humour or may be of blood origin.

It has been suggested that 5HT in the retina may originate from blood within retinal blood vessels. However, studies looking at the intra-retinal distribution of 5HT have strongly indicated that it is an endogenous substance [2]. The question remains whether a small quantity of blood could significantly add to the endogenous 5HT of the retina. If we consider human blood, the concentration of 5HT circulating free in plasma is low (0.578–0.277 ng

ml^{-1}) [24]. However, the majority of 5HT in blood is localized within the platelets and it has been reported that the total quantity of 5HT in blood is 100–200 ng ml^{-1} [24, 25] and therefore even a few microlitres of blood could contain significant quantities of 5HT. We have applied the procedure described in this paper to the determination of 5HT in human blood, plasma, and serum. The determination of 5HT in serum and plasma was straightforward but when 5HT was determined in whole blood, that had been frozen and thawed, 5HT could not be recovered. This was despite a quantity of 1 ml of whole blood, possibly containing 100–200 ng of 5HT, being analysed and a limit of detection of 50 pcg for 5HT. It has been reported that the presence of iron (liberated from erythrocytes by haemolysis during freezing) and oxygen in blood severely affects the recovery of 5HT during the deproteinization step [24]. Consequently the loss of 100–200 ng of 5HT from blood, during deproteinization with perchloric acid, would have suggested that the 5HT in the very small quantity of blood remaining in the blood vessels of the retina would be lost during the homogenization step. In addition it has been reported that the neurones in the retina which possess the biosynthetic enzymes for the synthesis of 5HT and contain high affinity uptake systems for exogenous 5HT, do use 5HT as their neurotransmitter [7]. The presence of such high affinity uptake systems and 5HT receptors which are similar to those reported in the brain has been demonstrated in the retina [1–3, 8, 26]. It has also been reported that the release of 5HT from neurones in the retina is dependent on calcium, as is the case in the release of well-established neurotransmitters [2].

Conclusions

The present study gives a clearer picture of the biosynthesis and metabolism of 5HT in the bovine eye. The occurrence of 5HT in aqueous humour is of interest in relation to its possible effects on intraocular pressure. We have previously carried out analysis of catecholamines and their metabolites in the glaucomatous and non-glaucomatous eye without finding definitive differences which might account for an elevation in intraocular pressure [27].

Acknowledgements — One of us (S.A.B.) thanks the SERC for a Quota Studentship support. We would also like to thank Z. Iqbal and R. Andrew for their assistance in the dissection of the eyes.

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[*Received for review 2 September 1992*]