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Note

High-performance liquid chromatography of tryptophan metabolites

Applications in biosynthesis and kinetics

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In 1958 Lerner et al. [1] isolated melatonin from the pineal gland. Shortly afterwards Axelrod and Weissbach [2] described its biosynthesis showing that hydroxytryptamine was first acetylated by means of N-acetyltransferase and then methylated by hydroxyindole-O-methyltransferase. Lerner et al. [1] also showed that methoxyindole acetic acid was present in the gland and McIsaac et al. [3] showed that methoxytryptophol was present. Since that time many workers have incubated pineal glands with radioactive tryptophan, methyl (S-adenosylmethionine) and acetyl (acetyl coenzyme A) in order to study various aspects of the biosynthesis and biology of melatonin (for reviews see ref. 4). Originally it was assumed that melatonin was the main if not the sole radioactive product when labelled S-adenosylmethionine was used and the radioactive material extractable with chloroform or isoamyl alcohol—toluene was counted and equated with melatonin synthesis. Subsequently one dimensional thin-layer chromatography was used to separate the components of the chloroform extract and radioactivity running coincident or in parallel with synthetic melatonin was, again, equated with melatonin. Although various workers had noted other unidentified radioactive spots on the chromatograms they were entirely ignored until Smith and co-workers [5,6] showed that one such compound was the tryptophol analogue of melatonin, namely O-acetyl, 5-methoxytryptophol. They postulated a biosynthetic pathway also starting from hydroxytryptamine through hydroxytryptophol, O-acetylhydroxytryptophol to O-acetyl, 5-methyltryptophol. Balemans et al. [7] have independently suggested the existence of the same pathway. Subsequently Balemans et al. [8] have shown there is an O-acetyl transferase circadian rhythm in the rat. The existence of both melatonin and acetylmethoxytryptophol must indicate

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that all their precursors are present in the pineal to a greater or lesser degree although only N-acetyl, hydroxytryptamine has been granted any significance. Studies on the circadian rhythm of melatonin in the pineal have been carried out by many (for reviews see ref. 4) but the most comprehensive have been by Balemans et al. [7]. The growing realisation that a number of tryptophan metabolites were present in the pineal suggested to us that it would be well worthwhile to develop high-performance liquid chrromatographic (HPLC) methods suitable for separating the various metabolites.

A limited number of previous studies on indole HPLC have been carried out. As these were designed either for urinary compounds (Richards [9]; Graffeo and Karger [10]) or indole amine metabolites formed in the lung (Crooks et al. [11]), there is relatively little overlap between that work and ours.

The acyltryptophols are all hydrolysed by choline esterase (see Fig. 4). Concurrent with the above studies we have developed a much simpler isocratic HPLC method for studying the kinetics of these reactions.

MATERIALS AND METHODS

In all, tryptophan and eighteen indole metabolites have been examined. The compounds are listed together with their abbreviations shown in brackets; the abbreviations have been fully elaborated and discussed elsewhere [4]. Tryptophan (W), N-acetyltryptophan (aW), 5-hydroxytryptophan (HW), 5-methoxytryptophan (MW), N-acetyl-5-hydroxytryptamine (aHT, acetylserotonin), melatonin (aMT), tryptophol (L), 5-hydroxytryptophol (HL), 5-methoxytryptophol (ML), 5-hydroxyindole acetic acid (HA) and 5-methoxyindole acetic acid (MA) were all obtained from Sigma. N-Acetyl-5-methoxytryptophan (aMW), O-acetyl-5-methoxytryptophol (aML), N-acetyl-5-hydroxytryptophan (aHW), O-acetyltryptophol (aL), O-acetyl-5-hydroxytryptophol (aHL), N-acetyltryptamine (aT), 5-hydroxyskatole (HS) and 5-methoxyskatole (MS) were prepared as previous described [12].

The complete chromatography system (Anachem, Luton, Great Britain) consisted of an Altex Model 332 programmable gradient system, a Model 210 Universal sample injector fitted with a 20-μl loop and a Model 115-40 variable-wavelength UV—VIS spectrophotometer. A 50 mm × 2.1 mm I.D. LiChroprep RP-18 pre-column was inserted between the injection valve and the 150 mm × 4.5 mm 5-μm Hypersil ODS analytical column (HPLC Technology Ltd.). The primary solvent was unbuffered (pH ca. 6) and the secondary solvent was methanol (Rathburn Chemicals, Walkerburn, Great Britain). Gradient from 0—100% methanol were used as shown in the figures. Both solvents were filtered through a SintAGlass Filter, porosity 5 and degassed under vacuum. A 10-μl volume of a standard solution of indoles was injected onto the column. The eluent was monitored at 280 nm at 0.1 a.u.f.s. Chromatography was carried out at room temperature and at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

We first devised a method for separation of the methoxyindoles as these are obtained radioactive after incubation of the gland with radioactive S-adenosylmethionine (see Fig. 1). The rationale for this is that non-radioactive standards

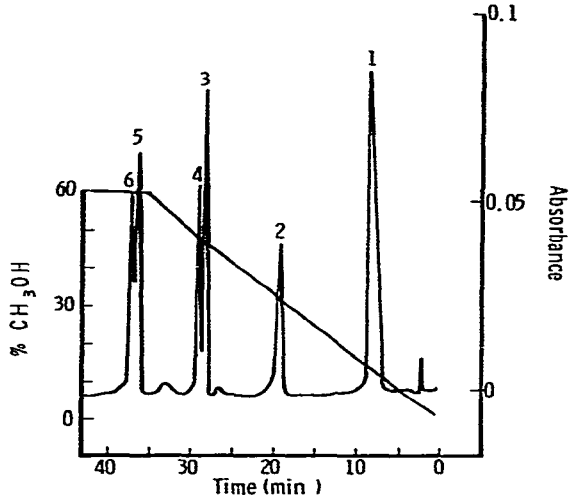

Fig. 1. The separation of methoxyindoles. General chromatographic conditions are described in the text and the methanol gradient is shown in the figures. Peaks: 1 = aMW +MA, 2 = MW, 3 =ML, 4 = aMT, 5 =MS, 6 = aML. MS has not yet been found to occur naturally but was included because HS has been found in urine. Specific conditions were: flow-rate 1 ml/min; pressure 83 bar (1250 p.s.i.); chart speed 0.2 cm/min; detection 280 nm UV at 0.2 a.u.f.s. Sample: 10 μl of standard mixture containing 100 μg/ml of each compound.

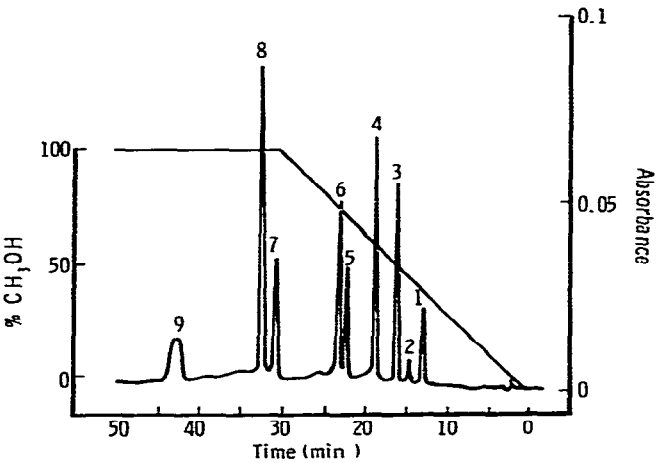

Fig. 2. The separation of acetylindoles. Peaks: 1 = aW, 2 = aHW, 3 = aHT, 4 = aMW, 5 = aMT, 6 = aT, 7 = aML, 8 = aL, 9 = aHL. Specific conditions as in Fig. 1.

can be added to the solution to be chromatographed and the peaks located and separated by linking the UV output to a Gilson CPR fraction collector so that each peak is run into a different collector tube. We then devised a method suitable for the separation of acetylindoles derived from radioactive acetyl coenzyme A (see Fig. 2). Finally we devised a method which is suitable for the separation of radioactive metabolites derived from tryptophan or hydroxytryptamine and, although all the possible metabolites are not completely resolved, separation is sufficient for fractions to be collected for scintil-

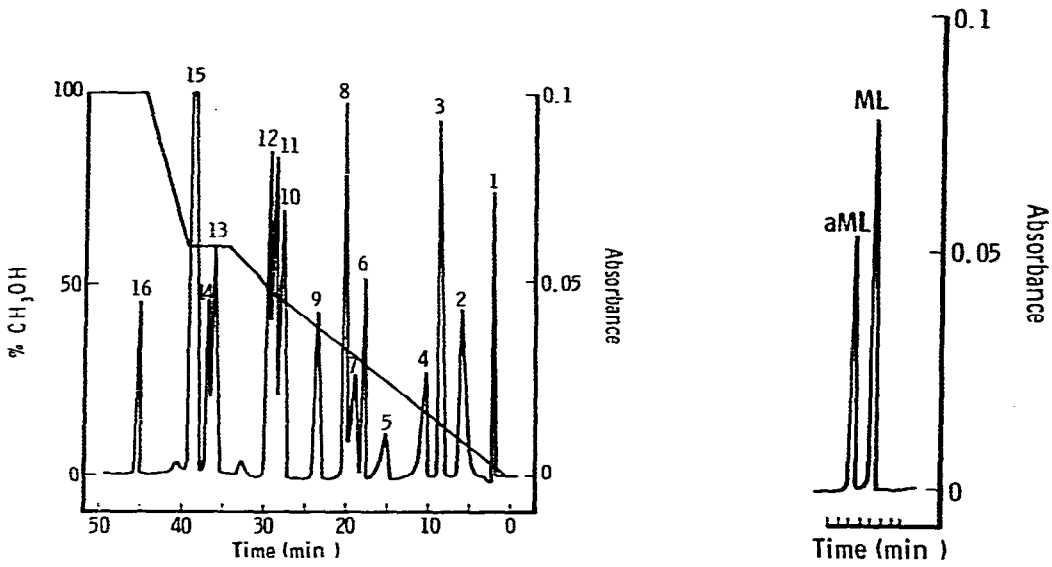

Fig. 3. Separation of nineteen indoles. Peaks: 1 =HA, 2 = aW + aMW +MA, 3 = HW, 4 = aHW, 5 =W, 6 = HL, 7 = MW, 8 = aHT, 9 = HS, 10 = ML, 11 = aMT + L, 12 = aT, 13 = MS, 14 = aML, 15 = aL, 16 = aHL. Specific conditions as in Fig. 1.

Fig. 4. Rapid isocratic separation of acyltryptophols from the parent compound. In this example, aML was incubated in buffer with choline esterase when ML is formed. Both compounds were extracted with chloroform and an aliquot was applied to the column. Separation was effected with 60% methanol. Relative peak heights plus molar extinctions or, alternatively, calibration curves with known amounts of aMl and ML, can be used to determine the degree of hydrolysis for each timed sample.

lation counting or further separation of individual pairs of compounds (see Fig. 3). Clearly resolution could be improved by selective solvent extraction prior to the HPLC. Thus, from neutral aqueous solution, chloroform [1] and ethyl acetate [3,5] extracts 90—100% of melatonin, methoxytryptophol, acetylmethoxytryptophol and other neutral compounds but isoamyl alcohol—toluene [13] extracts a much wider range of indoles [14].

Choline esterase hydrolysis of the acyltryptophols yields the parent tryptophol itself. Hence, it is necessary to separate only two compounds for each kinetic study. One example is illustrated in Fig. 4 which shows the separation of acetylmetroxytryptophol from methoxytryptophol and, allowing for the different extinction coefficients, the peak heights enable the degree of hydrolysis to be calculated for each time of hydrolysis.

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