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DETERMINATION OF INDOLES IN HUMAN AND RAT PINEAL

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SUMMARY

Tryptophan, serotonin, N-acetylserotonin, melatonin, 5-hydroxyindoleacetic acid and 5-hydroxytryptophol have been determined in rat and human pineal glands. The compounds were measured by directly injecting centrifuged tissue homogenates into a liquid chromatographic—fluorometric system. Normal ranges are reported for these compounds and upper limits established for several other indoles.

INTRODUCTION

It is well known that the pineal gland is rich in neurochemically important indoles [1—6]. Methods ranging from bioassay [7, 8] to gas chromatography—mass spectrometry [9], and including immunoassay [10], fluorometry [11], gas chromatography with electron-capture detection [12], and liquid chromatography with amperometric [13, 14] or combined amperometric and fluorometric detection [15] have been employed to determine one or more of the indoles in mammalian pineal. Liquid chromatography with fluorometric detection is well suited to the measurement of the compounds of interest. The high absorbances and quantum efficiencies of the indoles, such as melatonin and serotonin, allow concentration detection limits of $\leqslant$ 100 pg per pineal to be obtained. We present the application of such a system to the determination of several of the indoles present in rat and human pineal.

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MATERIALS AND METHODS

Chromatography

The chromatographic system consisted of an Altex 110A pump, a Rheodyne 71-25 injection valve (Rainen Instrument, Woburn, MA, U.S.A.), a 50 mm × 3.2 mm guard column with pellicular C_{18} reversed-phase packing (Whatman, Somerset, NJ, U.S.A.), and a μBondapak C_{18} reversed-phase column (300 mm × 3.9 mm I.D., 10 μm particle size) (Waters Assoc., Milford, MA, U.S.A.). A modified [15] Aminco Fluoromonitor (American Instrument, Silver Spring, MD, U.S.A.) was used with a 254-nm interference filter (Waters Assoc.). A Corning 7-51, 360-nm peak transmittance, emission filter was employed. Two flow-cell spectrophotofluorometers used with similar results were the Perkin-Elmer Model 650-10LC and Model 204: both were used with excitation and emission settings of 285 nm and 345 nm, respectively (10 nm bandpasses) (Perkin-Elmer, Norwalk, CT, U.S.A.).

An amperometric detector employed in peak-identification experiments consisted of a glassy carbon working electrode, a PTFE thin-layer cell, a Ag/AgCl reference electrode, a PTFE reference electrode compartment, and an LC-4 electrochemical controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A ca. 20-μm spacer gasket was used to define the cell thickness; the potential of the working electrode was set at +0.7 to +0.8 V versus the reference electrode. Solvent systems were prepared by mixing 0.01 *M* sodium acetate (adjusted to the desired pH with glacial acetic acid) with the proper proportion (v/v) of methanol or acetonitrile (glass-distilled grade, Burdick and Jackson Labs., Muskegon, MI, U.S.A.). Stock solutions (10 mg/100 ml) were made up in distilled water with 0.1% ascorbic acid added and were stored at ca. 4°C for up to two months. Diluted standards (0.1—10 ng/μl) were prepared daily in distilled water.

Sample preparation

Frozen pineals, stored at —80° C, were placed in 1.5-ml polypropylene microcentrifuge tubes on ice, and 200 μl of ice cold 0.1 *M* perchloric acid containing 0.1% ascorbic acid was added. The tissue was homogenized by sonicating for two 2—3 sec periods (with a 2—3 sec intervening pause for inspection) with a Polytron Sonicator (Branson Sonic Power, Danbury, CT, U.S.A.). The samples were then centrifuged for 1 min at ca. 10,000 *g* and the supernate placed in a microcentrifuge tube for temporary (on ice) or long-term (—80°C) storage. Human pineals were prepared in a similar manner, being sonicated in 0.5 ml of 0.1 *M* perchloric acid for several 5—10 sec periods.

The compounds were determined by injection of 10—100 μl of the supernates into the chromatograph. Usually, a 20-μl injection (equivalent to 0.1 rat pineal or 0.02 human pineal) was sufficient for determining the polar metabolites. A 50-μl injection was usually used when determining melatonin (MEL). The chromatographic conditions are listed in Table I. Single point standards (ca. 5—20 ng, measured by peak height) were used as a linear response was observed over the working range.

TABLE I

CHROMATOGRAPHIC DATA

Compound	Chromatographic condition*	Retention time (min)	Detection limit** (pg)
Serotonin (5-HT)	1	4.28	
	2	3.18	10
	3	2.88	
Tryptophan (TRP)	1	5.48	
	2	4.40	40
	3	4.14	
5-Hydroxyindoleacetic acid (5-HIAA)	1	7.65	
	2	5.39	40
	3	5.30	
5-Hydroxytryptophol (5-HTOL)	1	9.59	
	2	6.89	20
	3	6.36	
N-Acetylserotonin (NAS)	1	17.4	
	2	—	40
	3	9.74	
Melatonin (MEL)	4	6.27	30
Indoleacetic acid (IAA)	4	5.51	30
5-Methoxyindoleacetic acid (5-MIAA)	4	4.13	30
Tryptamine (TAM)	4	2.60	20
5-Methoxytryptophol (5-MTOL)	4	5.71	20

*Chromatographic conditions: (1) 0.01 *M* sodium acetate (pH 4.50)—methanol (92:8); (2) 0.01 *M* sodium acetate (pH 4.50)—methanol (88:12); (3) 0.01 *M* sodium acetate (pH 4.50)—methanol (85:15); (4) 0.01 *M* sodium acetate (pH 4.25)—methanol (65:35), all delivered at 2.0 ml/min.

**Injected amount giving a signal equal to twice the peak-to-peak noise.

RESULTS AND DISCUSSION

The compounds determined are listed in Table I with their retention times and fluorometric detection limits. Either an 8% or 12% methanol solvent system was used for the polar metabolites (5-HT, TRP, 5-HIAA, 5-HTOL, and NAS)*, while a 35% methanol system was used for the more lipophilic species (5-MTOL, IAA, TAM, and MEL). The detection limits observed were in the 5—40 pg range, allowing the compounds to be determined at concentrations of ca. 100 pg per pineal if necessary. Standards were determined with within-day coefficients of variation (C.V.) of 2—10%, with a 5% value being typical. Fig. 1 is a chromatogram of the determination of 5-HT, TRP, 5-HIAA, and 5-HTOL in three different rat pineals, along with an injection of the appropriate standards. The peaks were identified on the basis of their retention

*For abbreviations see Table I.

TABLE II

RAT PINEAL INDOLE CONCENTRATIONS

Compound	Previously reported means or ranges		Present study*	
			Diurnal maximum	Diurnal minimum
5-HT	45 μg/g [16] 85, 102 ng/pineal [13] 82.9 ng/pineal [14]	34–72 ng/pineal [20] 54 μg/g [9] 10–90 ng/pineal [22]	90.9 ± 28.0	29.3 ± 19.8
TRP	16.1 ng/pineal [14] 20–40 μg/g [11]		7.97 ± 2.76	3.54 ± 1.83
5-HIAA	7.64 μg/pineal [14]		17.0 ± 6.61	3.22 ± 1.74
5-HTOL	ca. 2 ng/pineal [18]		3.39 ± 2.32	0.52 ± 0.36
NAS	7.8 μg/g [16] 0.9 ng/pineal [17] 0.13 μg/g [9]		4.78 ± 2.55	<0.3
MEL	0.20–0.61 ng/pineal [17] 0.48 μg/g [16] 0.2–3 ng/pineal [18]	0.5–6.8 ng/pineal [19] 1.35 ng/pineal [14] 3.9 μg/g [9]	2.06 ± 1.08	0.31 ± 0.09
5-MTOL	1–6 ng/pineal [18]		<0.3	<0.1
5-MIAA	0.98 ng/pineal [24]		<0.1	—
IAA	—		<0.1	—
TAM	0.3 ng/g [21]		<0.1	—

*Values are ng per pineal, reported as means ± S.D. (n = 8). From a study using 48 rats on a 12-h light/12-h dark cycle with groups of eight sacrificed every 6 h.

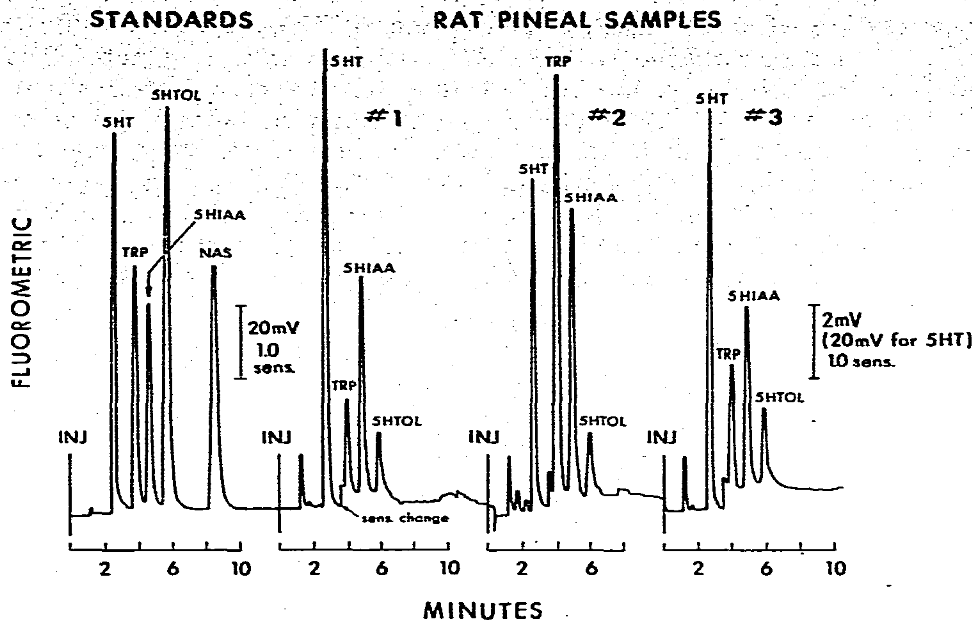

Fig. 1. Determination of indoles in three different rat pineal glands. The pineals were homogenized in 200 µl of 0.1 *M* perchloric acid and 20 µl were injected. The samples shown had concentrations per pineal of 52.0—108 ng 5-HT, 3.68—17.2 ng TRP, 8.55—13.5 ng 5-HIAA, and 1.45—1.89 ng 5-HTOL. Amounts of standards injected were 5 ng 5-HT, 10 ng TRP, 5-HIAA, and 5-HTOL. Chromatographic condition 3 (see Table I).

times and by comparing their response ratio (fluorometric/amperometric) to that of standards (except for TRP). The compounds were determined with usual within-day C.V. values of $\leqslant 5\%$; a quality control sample composed of pooled rat pineal homogenates was determined with day-to-day C.V. values of 7—12%. Standard addition studies for the compounds measured gave recoveries of > 95%. Correlations between amperometric and fluorometric detection were excellent ($r \geqslant 0.99$; $n = 10$—15) for 5-HT, 5-HIAA, 5-HTOL, and NAS. The identity of 5-HTOL in rat pineal, measured here for the first time in single rat pineals, is based on co-chromatography in different solvent systems [pH 3.5, 4.0, and 4.5; 0.1 *M* sodium acetate—methanol (88:12)] and close agreement ($r = 0.996$) between amperometric and fluorometric measurements. N-Acetylserotonin (NAS) was also identified in the rat pineal on the basis of retention times and relative response; however, it was often below the level measurable with a 20-µl injection (ca. 300 pg per pineal). The maximum and minimum concentrations of 5-HT, TRP, 5-HIAA, 5-HTOL, and NAS observed in rat pineal over a 24-h period are presented in Table II.

In general, the values are in excellent agreement with previous reports (see Table II). However, the mean of our observed diurnal TRP values was approximately five times lower than found in a recent study [11]. Our method should be more specific than the fluorometric method used in that study, and, although we have not compared the procedures directly, we believe our values to be a more accurate estimation of TRP levels and fluctuations in rat pineal.

The determination of 5-HT, TRP, 5-HIAA, and 5-HTOL in human pineal, shown in Fig. 2, is accomplished with an 8% methanol solvent system. This was necessary in order to determine 5-HTOL (and NAS) without interference. The other compounds (5-HT, TRP, and 5-HIAA) could be determined more quickly using the same system (15% methanol) employed for their measurement in rat pineal. Using the 8% methanol system, fluorometric and amperometric values for 5-HTOL were well correlated ($r = 0.985$, $n = 16$) in human pineal. Values obtained for 5-HT and 5-HIAA were well correlated ($r > 0.98$) using either of the solvent systems. The ranges of concentrations observed for

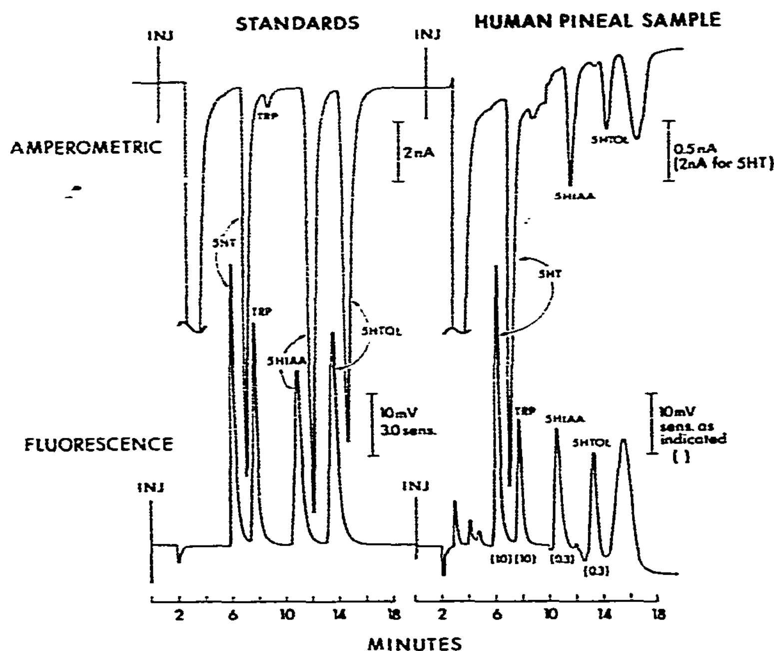

Fig. 2. Chromatogram of standards (10 ng 5-HT, 20 ng TRP, 5-HIAA, and 5-HTOL) and a human pineal sample. The pineal was homogenized in a total volume of 745 µl and 5 µl were injected. Concentrations per pineal for the sample were 210 ng 5-HT, 1540 ng TRP, 180 ng 5-HIAA, and 144 ng 5-HTOL. The sensitivity is inversely related to the sensitivity number (sens.). Chromatographic condition 1 (see Table I).

TABLE III

RANGE OF INDOLE CONCENTRATIONS IN HUMAN PINEAL

Values are ng per pineal; human pineals (n = 28) were obtained between 4 and 24 h after death.

Compound	Maximum value	Minimum value	Previous reports (range)
5-HT	5160	58.5	120—4500 [23]
TRP	28,100	686	—
5-HIAA	930	71.6	—
5-HTOL	153	<1.0	—
MEL	79.6	1.12	0—71 [12]
NAS	<2.0	—	—

the compounds in human pineal are given in Table III. This is the first report of the levels of the compounds (with the exception of MEL and 5HT) in human pineal. Considering that the human pineal weights (not given) were generally 100—200 times the average rat pineal weight of ca. 1 mg, the values are similar, on a per weight basis, to levels seen in rats.

The determination of MEL in a series of rat pineals is shown in Fig. 3. Standards were determined with a C.V. of 4% at the 5-ng level; a pooled sample was determined with a within-day C.V. of 5% and a day-to-day C.V. ($n = 4$) of 7%. The method is extremely rapid and simple compared to existing procedures for determining MEL in pineal. Values obtained for MEL in rat pineal are given in Table II, as well as the upper limits established for several other lipophilic indoles.

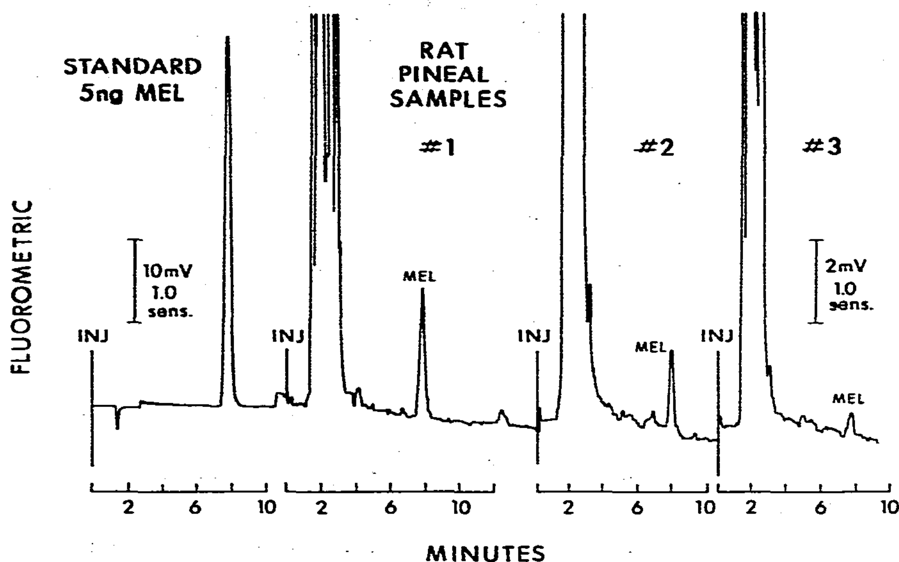

Fig. 3. Melatonin (MEL) determined in three different rat pineals, 75 µl of a 200-µl homogenate were injected. The MEL concentrations per pineal were: 0.93 ng, 0.58 ng, and 0.17 ng, for samples 1, 2, and 3, respectively. These levels are in the low normal range (see Table II). Chromatographic condition 4 (see Table I).

The MEL concentrations are similar to previous observations (see Table II). The upper limit established for the 5-methoxy compound, 5-MTOL, is, however, well below previously reported levels.

In Fig. 4, MEL is determined in human pineal. As opposed to the rat pineal sample, here the MEL peak is surrounded by other fluorescent species. The MEL peak, while well formed, was often on a sloping baseline. Extraneous peaks were a greater problem with amperometric detection and precluded its use on a regular basis. The identity of the MEL peak was checked by collecting the peak and rechromatographing in the same solvent system, and with an 0.01 *M* sodium acetate (pH 4.0)—acetonitrile (80:20) mobile phase, using both amperometric and fluorometric detection. One of the human pineal samples so studied is shown in Fig. 5. The retention time was identical to authentic MEL,

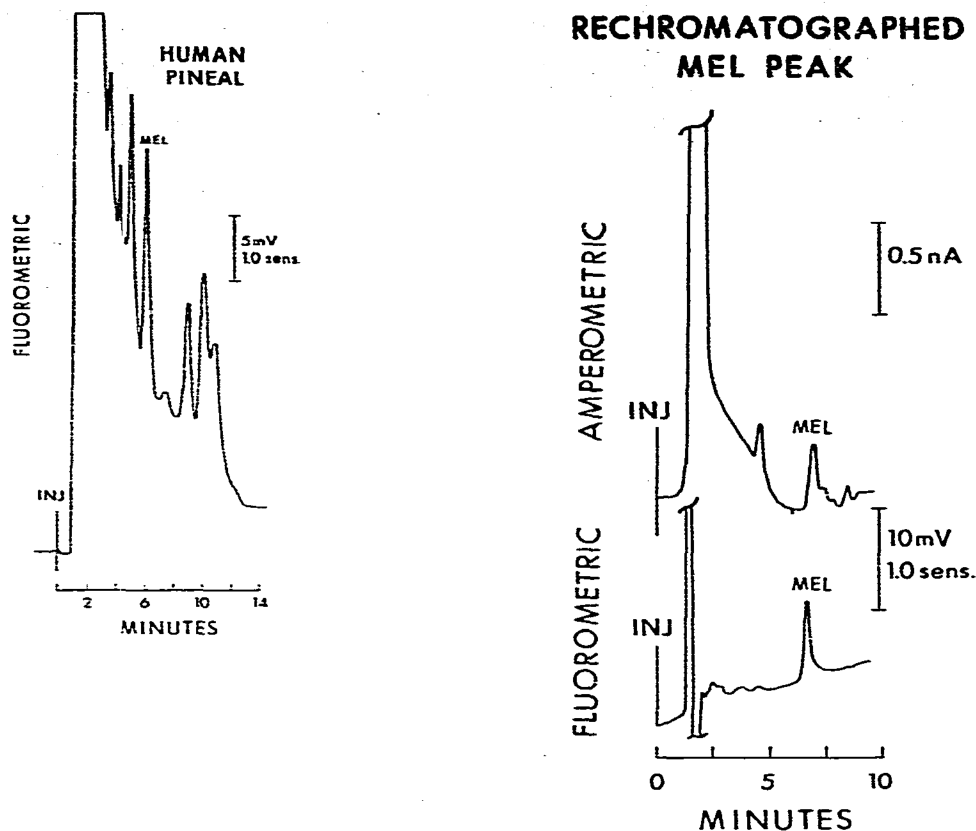

Fig. 4. Melatonin (MEL) determined in a human pineal sample. The sample was homogenized in a total volume of 0.720 μl, and 50 μl were injected. The concentration for the sample was 20.3 ng MEL per pineal.

Fig. 5. Dual detection of a collected, rechromatographed melatonin peak. The sample was first separated using 0.1 *M* sodium acetate (pH 4.25)—methanol (65:35) (chromatographic condition 4) and the melatonin peak collected (peak volume 1.0 ml). A 100-μl aliquot (10%) of the collected volume was reinjected using a solvent system of 0.01 *M* sodium acetate (pH 4.0)—acetonitrile (80:20). The peak observed in the 80:20 system had the correct retention time and amperometric/fluorometric response ratio. When quantitated, after allowing for dilution, the concentrations agreed with the original value obtained using the 65:35 mobile phase.

as was the amperometric/fluorometric response ratio. The range of concentrations (see Table III) seen for MEL in human pineal is similar to that reported [12]. While the great number and apparently large concentrations of the fluorescent compounds present are a hindrance to the facile measurement of MEL in human pineal (as compared to rat pineal), it also suggests that much remains to be learned of indoles in human pineals.

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