Journal of Chromatography, 377 (1986) 350—355
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3027

Note

Melatonin determination in human urine by high-performance liquid chromatography with fluorescence detection

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(First received August 16th, 1985; revised manuscript received December 16th, 1985)

Melatonin and 5-methoxytryptophol are implicated in the physiology of circadian rhythms, reproductive development, and behaviour [1]: the compounds being chiefly of pineal origin [2, 3].

After its isolation [4], a bioassay [5], a fluorometric assay [6], a radioimmunoassay [7], a gas chromatographic—mass spectrometric technique [8] and a high-performance liquid chromatographic (HPLC) method [9] for pineal glands have successively been developed to measure melatonin.

For human studies it is necessary to measure melatonin in body fluids, and a system combining the selectively sensitive HPLC and fluorometric techniques invites this approach. A selective extraction method to partially purify and concentrate the samples prior to their analysis was required, as these compounds exist in trace amounts in the presence of much higher levels of impurities. Mass spectrometric analyses also were employed to indicate the presence of melatonin in our samples.

0378-4347/86/$03.50

EXPERIMENTAL

Materials and reagents

All the indole compounds were obtained from Sigma (St. Louis, MO, U.S.A.); acetonitrile, HPLC grade, was obtained from Waters Assoc. (Milford, MA, U.S.A.); dichloromethane, spectrofluorometric grade, was obtained from BDH (Poole, U.K.). All other reagents were analytical grade and all water used was purified by a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.).

Standards used routinely consisted of a mixture of 5-methoxyindole-3-acetic acid, indole-3-acetic acid, 5-methoxytryptophol, tryptophol and melatonin. For each compound 1 mg was dissolved in 1 ml of ethanol and diluted to 100 μg/ml in 0.1% ascorbic acid to form the stock solution. This could be stored for at least six months at —20°C before diluting in 0.1% ascorbic acid on the day required. The other indoles were prepared in 0.1% ascorbic acid.

Chromatography

The system consisted of a Waters M6000A pump, a Waters WISP 710B automatic injector, a Regis 25 cm × 4.6 mm I.D., 5-μm Spherisorb ODS II column (Regis, Morton Grove, IL, U.S.A.) and a Perkin-Elmer MPF4 spectrofluorimeter, with excitation at 283.5 nm and emission at 333 nm, a Corning 9863 excitation filter, a UV31 emission filter and slit widths set at 20 nm. The mobile phase, 0.05 *M* ammonium phosphate–acetonitrile (82:18), pH 3.95, was delivered at 1 ml/min and the column was heated in a water bath to 60°C.

Sample preparation

Urine samples were saturated with boric acid and stored at 4°C for up to seven days or at —20°C for longer periods. Aliquots (8 ml) were extracted with 8 ml dichloromethane by agitation for 30 min in 16-ml borosilicate glass culture tubes with PTFE caps (Pyrex), centrifuged for 5 min at 1000 *g*, and the aqueous phase was aspirated. The samples were washed with 8 ml of 0.1 *M* sodium hydroxide and water sequentially by agitation (5 min), centrifugation and aspiration as before. The organic layer was decanted into a centrifuge tube, evaporated under a stream of nitrogen at 40°C, the walls were rinsed with 0.5 ml dichloromethane, followed by reevaporation. These could be stored, or redissolved in 100 μl of 0.1% ascorbic acid followed by centrifugation for 1 min at 1000 *g* and 50 or 75 μl could be injected or also stored, in both cases at —20°C for up to a week.

The melatonin eluted was collected in a 3-ml fraction, and the eluates before and after this (15 ml) were frozen and lyophilized before mass spectrometric analysis.

Mass spectrometry

Mass spectrometry was performed with an AEI MS-30 mass spectrometer, utilizing an AEI direct-introduction probe at 60°C, with the source at 200°C and vacuum pressure at $5 \cdot 10^{-6}$ Torr. The data were processed via a Kratos computing interface with a Data General Nova 4 computer and a Kratos DS:55 data system.

The lyophilized eluates were dissolved in 10 μl of dichloromethane, transferred to mass spectrometer sample tubes, and the solvent evaporated. The tubes were inserted in the electron-impact source of the MS-30 mass spectrometer on a direct-introduction probe which was evacuated. The evaporation of melatonin from the sample tube was followed using selected-ion monitoring at *m/e* 232 units.

RESULTS AND DISCUSSION

The compounds analyzed are listed in Table I, with their retention times, detection limits and coefficients of variation, calculated from replicates of two experiments performed on different days. The apparent levels were quantified by peak-height measurement, and the detection limits (peak height twice that of noise) were 30 pg/ml for standards and 7 pg/ml for samples (adjusted for extraction and recovery). A three-fold increase in sensitivity can be achieved by increasing the detector response time, but the flow-rate must be reduced to 0.4 ml/min.

Recoveries and their coefficients of variation (between parentheses) of melatonin (1 ng and 500 pg), tryptophol (1 ng and 500 pg), and 5-methoxytryptophol (500 and 250 pg) were: 68.8% (1.9%), $n = 6$, 67.4% (5.0%), $n = 6$, and 77.0% (5.2%), $n = 6$, respectively. Three of both concentrations were used. Recoveries were determined for each run by standard addition to boric acid blanks and to all samples, which also confirmed peak identity. Samples with no detectable analyte gave equivalent recoveries when spiked.

5-Methoxytryptophol, tryptophol and melatonin standards were stable with coefficients of variation less than 4.1% from groups ($n \geqslant 6$) containing both

TABLE I

CHROMATOGRAPHIC DATA

Mobile phase: 0.05 *M* ammonium phosphate–acetonitrile (82:18), pH 3.95. Flow-rate: 1 ml/min. Column temperature: 60°C. Coefficients of variation (%) were derived from triplicate and duplicate samples from two different experiments. Detection limit of a peak was defined as peak height twice that of peak-to-peak noise.

Standard compound	Retention time (min)	Detection limit (pg/ml)	Coefficient of variation (%)	*n*
Melatonin	20.2	30	2.8	6
5-Methoxytryptophol	18.2	30	4.1	8
Tryptophol	16.8	30	2.9	7
Indole-3-acetic acid	13.8	60		
5-Methoxyindole-3-acetic acid	13.4	60		

Sample compound	Apparent concentration (pg/ml)	Retention time (min)	Detection limit (pg/ml)	Coefficient of variation (%)	*n*
Melatonin	41	20.2	7	8.1	5
Tryptophol	30	18.2	7	8.2	5

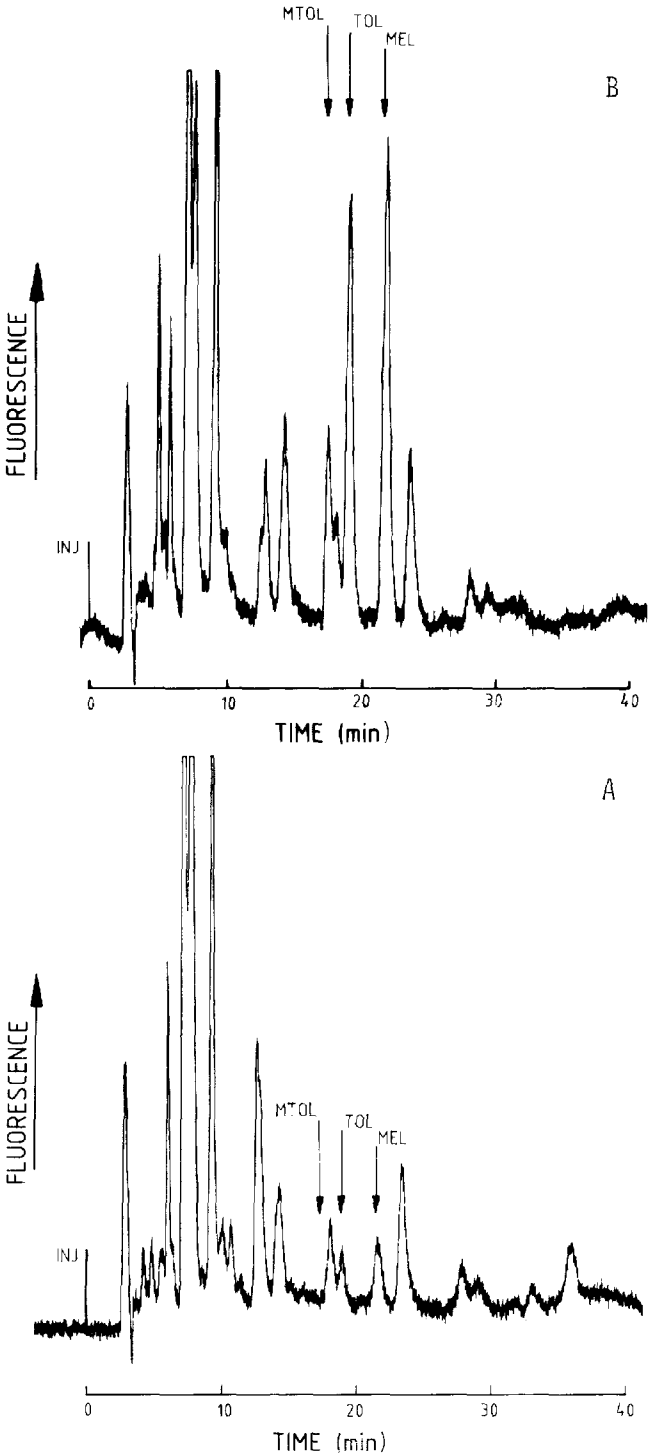

Fig. 1. (A) Chromatogram of a urine sample collected at 07.00 a.m. Arrows indicate retention time of corresponding standards: MTOL = 5-methoxytryptophol; TOL = tryptophol; MEL = melatonin. Mean levels (n = 5) were 41 pg/ml of melatonin and 30 pg/ml of tryptophol. Mobile phase was 0.05 M ammonium phosphate—acetonitrile (82:18), pH 3.95, column temperature 60°C, flow-rate 1 ml/min. (B) Chromatogram of the same urine sample spiked prior to the extraction with 500 pg MTOL, 1 ng TOL and 1 ng MEL.

1-ng and 500-pg standards over a period of 12 h (Table I). Linearity was determined from 1000-, 500-, 200-, 100- and 50-pg duplicate standards, resulting in correlation coefficients of 0.999 (y = 2.3 mm) for 5-methoxytryptophol, 1.000 (y = 1.9 mm) for tryptophol and 0.999 (y = 1.9 mm) for melatonin. Standards of 1 ng in blanks had removed sufficient 5-methoxyindole-3-acetic acid and indole-3-acetic acid to be below their detection limits, i.e. at least 99%. However, these two (fused) peaks showed baseline resolution from the adjacent 5-methoxytryptophol. 5-Methoxytryptophol, tryptophol and melatonin were resolved to the baseline.

Hydroxytryptophan, 5-hydroxytryptamine, L-tryptophan, 5-methoxytryptophan, 5-hydroxyindole-3-acetic acid, 5-hydroxytryptophol, N-acetylserotonin, tryptamine, 5-methoxytryptamine, indole-3-acetaldehyde, N-acetyl-L-tryptophan and N-methyltryptamine, which fluoresce at the same wavelength settings, all had retention times shorter than that of 5-methoxyindole-3-acetic acid at 60°C and at ambient temperature. However, most of these would be removed by the extraction procedure.

Fig. 1 is a chromatogram of a sample from a female subject collected at 07.00 a.m. Peaks corresponding to melatonin and tryptophol indicated apparent levels of 41 and 30 pg/ml, respectively, with coefficients of variation of 8.1 and 8.2% (n = 5).

Peak identity was confirmed by mass spectral analysis, monitoring at m/e 232 units as in a previous study [10]. A histogram of the normalized data is shown in Fig. 2 from a urine sample of a male subject collected at 08.00 a.m. Samples were extracted and chromatogaphed with acetonitrile–water (20:80) at ambient temperature, and the fraction corresponding to that of authentic melatonin showed a maximum of 232 ion counts. This example was one of four analyses taken from two samples at 08.00 a.m. and 05.00 p.m. from the same subject on the same day.

Urinary melatonin levels are generally reported as a secretion rate. Our results would approximate to 17 pg/min for melatonin and 12 pg/min for tryptophol, falling within the ranges previously reported: 3–15 ng per 8 h

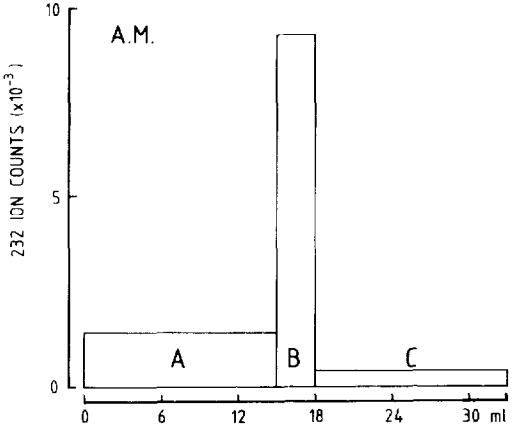

Fig. 2. Mass spectral analysis of chromatographic fractions (A, B and C) by monitoring 232 ion counts. Melatonin standard eluted in fraction corresponding to B. This sample was from a male subject collected at 08.00 a.m. For full details, see Experimental.

[11], 0.3–0.4 pmol/min [12] and 11–28 pmol/h [13]. It was necessary to raise the column temperature to separate melatonin and tryptophol.

No 5-methoxytryptophol was measured above our detection limits. However, plasma levels reported (0–14 pg/ml) [14] are about five times lower than plasma melatonin. 5-Methoxytryptophol, also detectable at *m/e* 232 units by mass spectroscopy, would occur in the fraction preceding melatonin.

The findings for tryptophol are tentative at this stage and will require further verification of peak identity. Further studies, with more subjects in controlled conditions, are in progress.

ACKNOWLEDGEMENTS

Many thanks are due to Mrs. E. Brown for her patient technical assistance and the staff of Waters and Perkin-Elmer Ptys. Ltd. (Australia) for their constant support and advice.

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