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Letter to the Editor

Melatonin and other indoles in rat pineal

Sir,

A number of recent publications [1–7] have centered on the presence of melatonin and other indole-based compounds in the pineal gland.

We present a high-performance liquid chromatographic (HPLC) separation of five lipophilic indole-based compounds (5-methoxyindole-3-acetic acid, 5MOI3AA; indole-3-acetic acid, I3AA; 5-methoxytryptophol, 5MOTOL; melatonin, MEL; tryptophol, TOL) and its application to the analysis of rat pineal extracts. Previous work by others [8] applied a similar separation to human urine. However, incomplete resolution of all compounds and unstable retention times afforded by that method [8] led to the current separation. The developed method shows advantages over the earlier work [8] with respect to baseline separation of all compounds (in the earlier work, the two acids showed marked overlap), shorter analysis time (14 min versus 23 min) and lower column operating temperature (35°C versus 60°C, the higher temperature being necessary in the earlier work to effect separation of MEL and TOL).

EXPERIMENTAL

The HPLC apparatus consisted of a Waters Assoc. (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.) M6000A pump, Waters 710B WISP autosampler, Waters Gard-Pak with 10-μm μBondapak C_{18} insert, Regis (Morton Grove, IL, U.S.A.) ODS-2 5-μm 25 cm $\times$ 4.6 mm I.D. stainless-steel column fitted with a column-heater jacket, Perkin-Elmer MPF-4 dual-monochromator scanning fluorimeter.

Data acquisition, reduction and system control was carried out with a Waters 840 data and chromatography control station.

All chemicals were reagent grade or better. Standards of each compound were obtained from Sigma (St. Louis, MO, U.S.A.) and dried over phosphorus pentoxide before use. Individual stock standards were prepared at a concentration of 1.00 μg/μl in a 1:9 (v/v) mixture of ethanol and "diluent". Diluent consisted of 0.4 *M* perchloric acid containing 0.1% sodium metabisulphite and 0.064% diso-

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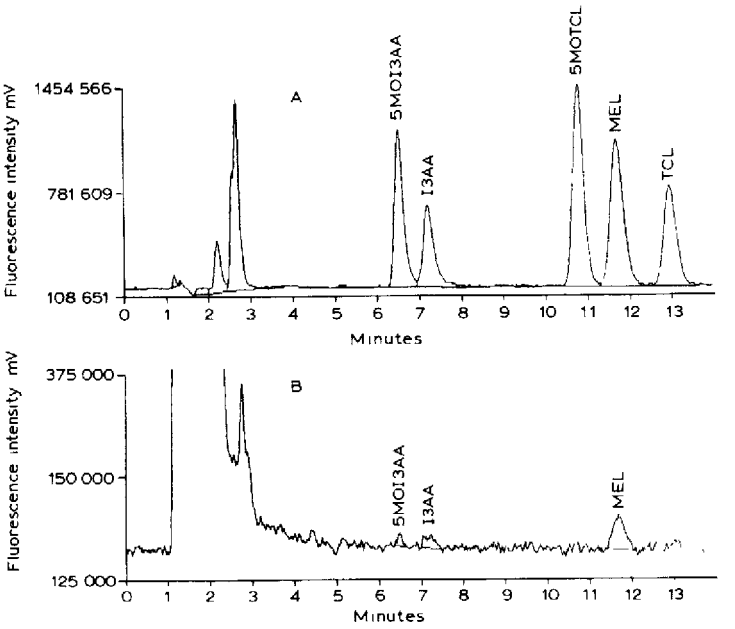

Fig 1 (A) Typical chromatogram of a mixture of reference compounds (see text for conditions; also column temperature 35°C, flow-rate 2.0 ml/min); 100 μl of working standard containing 30 pg/μl of each compound in diluent were injected. (B) Typical chromatogram of a rat pineal extract.

dium edetate. Working standards were prepared by diluting stock standards with diluent to required levels.

The HPLC eluent consisted of 50 m*M* diammonium hydrogen orthophosphate, 50 m*M* phosphoric acid–acetonitrile (83:17, v/v) of which the apparent pH was adjusted to 5.0 with 1 *M* ammonia.

Fresh rat pineals were sonicated for 1 min in 200 μl of diluent, centrifuged, and the clear supernatants stored in closed 1.5-ml polypropylene tubes at −80°C until analysis.

RESULTS AND DISCUSSION

The method of extraction (brief sonication in dilute perchloric acid with antioxidant/chelating agents, followed by centrifugation to remove precipitated proteins) has been used previously by several workers [2,3,5–7] and appears to be a method of choice because of its simplicity and efficiency.

The choice of extractant (diluent) was dictated by the work of Kotake et al. [9] with indole (and catechol) compounds, which has been used quite successfully in this laboratory, and by a recent study on the stability of indoles in solution [10]. Nevertheless, as a precaution, extracts were promptly frozen at −80°C, stored for no longer than a week, and thawed just before injection.

Calibration curves were linear over the range 10–50 pg/μl (25-μl injections) with correlation coefficients ($n=5$) of greater than 0.993. Wavelength maxima

for the five compounds in the HPLC eluent were: excitation 283–297 nm and emission 339–358 nm. The maxima for melatonin (excitation 297 nm, emission 341 nm) were used.

The use of a polar injection solvent with a much less polar HPLC eluent effected good on-column concentration, with no significant peak broadening up to 200-μl injection volume. Detection limits in pg absolute were: 5MOI3AA 40 pg, I3AA 70 pg, 5MOTOL 25 pg, MEL 35 pg, TOL 50 pg. These are also the detection limits per pineal provided the whole 200-μl pineal extract was injected.

Fig. 1A shows a typical separation of reference compounds. Fig. 1B shows a typical sample chromatogram. The levels of indoles present per pineal were: 5MOI3AA 90 pg, I3AA 185 pg, 5MOTOL not detected, MEL 270 pg, TOL not detected.

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