*Journal of Chromatography*, 528 (1990) 111–121
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5220

# Determination of six indolic compounds, including melatonin, in rat pineal using high-performance liquid chromatography with serial fluorimetric–electrochemical detection

J.R. LEE CHIN

*Psychology Department, University of Newcastle, Shortland, N S W 2308 (Australia)*

(First received October 3rd, 1989; revised manuscript received January 18th, 1990)

## SUMMARY

A reversed-phase high-performance liquid chromatographic method has been developed for the simultaneous determination of 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, N-acetylserotonin, tryptophan, 5-hydroxytryptamine (serotonin) and melatonin in rat pineal using a buffered aqueous eluent containing acetonitrile and methanol as organic modifiers and an ion-pairing agent to assist in controlling the retention of compounds containing an amine group. Serial fluorimetric–electrochemical detection provided additional assurance of compound identity. Analyte preparation simply involved the sonication of pineals in dilute perchloric acid containing an antioxidant and a chelating agent, followed by centrifugation to clarify. The method simplifies the determination of this range of indolic compounds, which normally would require at least two separate runs with different eluents. Detection limits for melatonin were 60 and 135 pg for fluorimetric and electrochemical detection, respectively. (This represented the "worst case" considering the levels and detection limits of all compounds present.) Using flow programming and a flow-rate varying between 1 and 1 5 ml/min, the analysis time was 27.5 min, which made the determination of ten samples in a working day possible.

## INTRODUCTION

There are a number of publications describing methods for the determination of indoles in rat pineal, but these almost invariably involve the use of at least two and as many as four solvent systems [1–5] in order to accommodate the whole range of polar through non-polar compounds. The metabolic pathway from tryptophan to melatonin and other methoxyindoles in the pineals of vertebrates is shown in Fig. 1 [1,6].

0378-4347/90/$03.50 

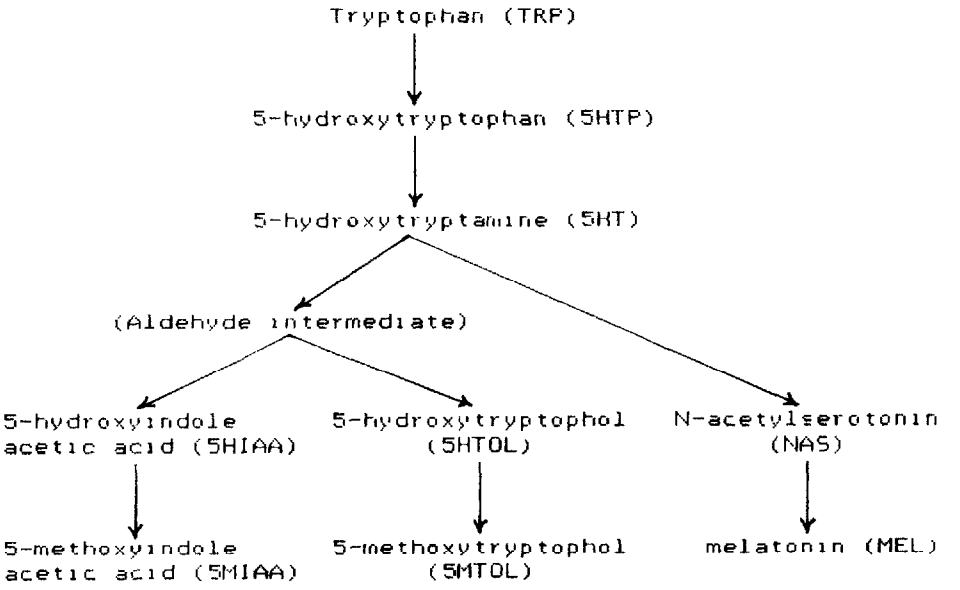


Fig. 1. Metabolic pathway of tryptophan in vertebrate pineal.

Hence an 'ideal' method for the investigation of compounds along this pathway would include at least tryptophan (TRP), 5-hydroxytryptophan (5HTP), serotonin (5-hydroxytryptamine, 5HT), 5-hydroxyindoleacetic acid (5HIAA), 5-hydroxytryptophol (5HTOL), N-acetylserotonin (NAS), 5-methoxyindoleacetic acid (5MIAA), 5-methoxytryptophol (5MTOL) and melatonin (MEL).

We describe here a method for the simultaneous determination of six of these pineal indoles within 28 min, using reversed-phase high-performance liquid chromatography (HPLC) with serial fluorimetric–electrochemical detection.

## EXPERIMENTAL

### *Standards*

TRP, 5HTP, 5HT (as hydrochloride), 5HIAA, 5HTOL, NAS, 5MIAA, 5MTOL, MEL were purchased from Sigma (St. Louis, MO, U.S.A.) and dried over phosphorus pentoxide before use. Individual stock standard solutions were prepared at a level of 1.00 mg/ml using the following diluents: (i) for TRP, 5HTP, 5HT·HCl: acetate buffer, pH 4.0 (40 m*M* acetic acid and 154 m*M* sodium acetate) containing 0.033% cysteine hydrochloride (CYS·HCl) and 0.029% disodium EDTA; (ii) for the remaining compounds: ethanol–acetate buffer, pH 4.0 (as above, containing 0.033% CYS·HCl and 0.029% disodium EDTA) (1:9, v/v). The ethanol was added first to facilitate dissolution.

Stock standard solutions were stable for three months when stored at either −70 °C or 4 °C.

The working standard was prepared to contain 1.00 ng/μl 5HT (free base), 0.250 ng/μl TRP and 0.100 ng/μl each of the remaining compounds in 50 m*M* perchloric acid containing 0.013% CYS·HCl and 0.012% disodium EDTA. This standard was stable for at least 24 h at ambient temperature (20–25 °C) or at least seven days at 4 °C.

*HPLC eluent*

The HPLC eluent consisted of (14.4 m*M* citric acid, 10.0 m*M* sodium acetate, 4.0 m*M* sodium octylsulphonate, 1.0 m*M* disodium EDTA and 0.25 m*M* dibutylamine phosphate)–acetonitrile–methanol (8:1:1, v/v). The pH of the aqueous component was 3.25 and of the complete eluent approximately 3.5.

Chemicals used were of reagent grade or better. Dibutylamine phosphate was prepared from dibutylamine (Fluka, Buchs, Switzerland): 12.9 g of dibutylamine was slurried in 70 ml distilled water, the pH adjusted to 2.5 with concentrated phosphoric acid and, following dissolution of the dibutylamine, the solution diluted to 100 ml.

*Chromatography*

The HPLC apparatus consisted of a Waters Assoc. (Milford, MA, U.S.A.) M6000A pump, a Waters 710B WISP autosampler, a Waters Guard Pak with μBondapak $C_{18}$ or Nova-Pak $C_{18}$ inserts, a Regis (Morton Grove, IL, U.S.A.) ODS-2 (5 μm, 250 mm × 4.6 mm I.D.) stainless-steel column fitted with a column heater jacket (30 °C), a Hewlett-Packard (Analytical Division, Waldbronn, F.R.G.) HP1046A programmable fluorescence detector, a Bio-Analytical Systems (West Lafayette, IN, U.S.A.) LC-4B electrochemical detector using a BAS LC-17AT flow-cell assembly with glassy carbon electrode, RE-4 Ag/AgCl reference electrode, 51-μm gasket and preheater set at 30 °C (BAS LC-22A controller).

System control, digital data acquisition and reduction were performed using a Waters 840 chromatography control station.

The analysis was performed either isocratically at 1.00 ml/min (pressure 140 bar), which resulted in an analysis time of 37 min, or using flow programming (1.00 ml/min during the first 9 min, linearly to 1.50 ml/min during the next minute, then 1.50 ml/min), which resulted in an analysis time of 27.5 min.

The eluent was filtered through a 0.45-μm membrane filter prior to use and continuously stirred and sparged with high-purity helium during use; eluent was run to waste.

The use of a polar injection solvent and a relatively non-polar HPLC eluent

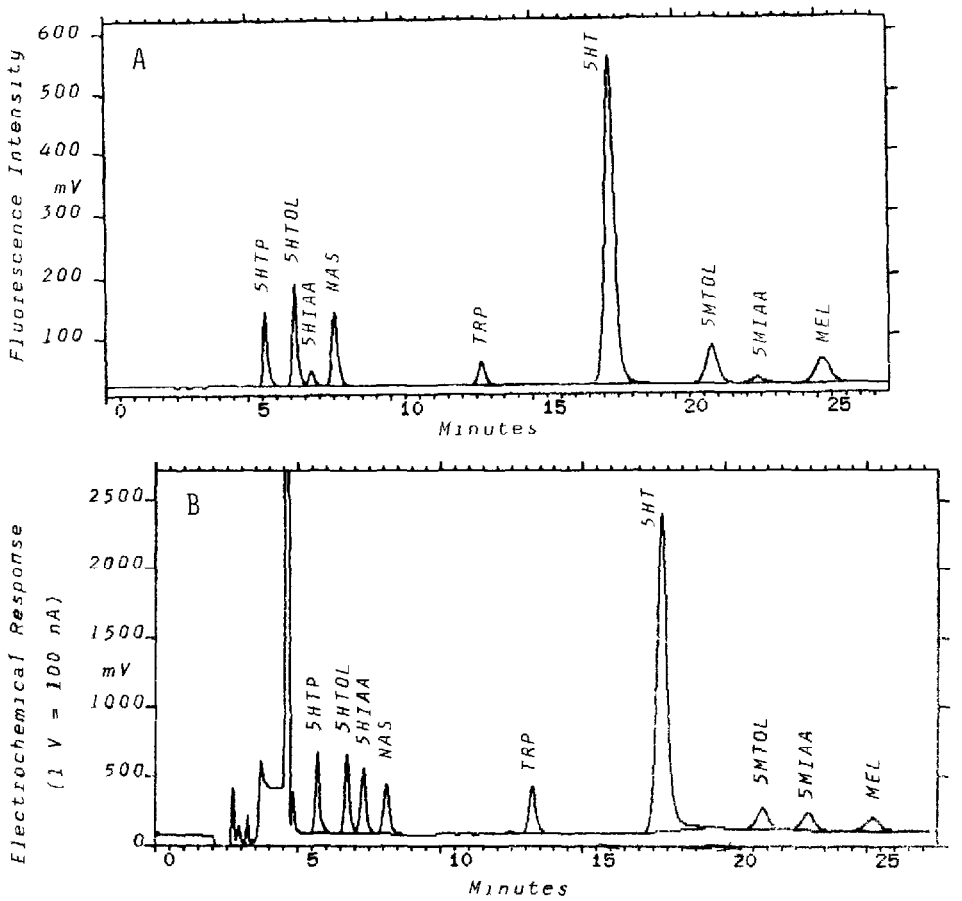


Fig. 2. Chromatograms of a 150-$\mu$l working standard using flow programming. (A) Fluorescence detection; (B) electrochemical detection. For chromatographic conditions, see text.

TABLE I

TYPICAL DETECTION LIMITS AT A SIGNAL-TO-NOISE RATIO OF 2

A 150-$\mu$l injection represents 0.75 of a pineal.

| Compound | Detection limit (pg per pineal) | |
|---|---|---|
| | Fluorescence | Electrochemical |
| 5HTP | 20 | 25 |
| 5HTOL | 15 | 20 |
| 5HIAA | 90 | 25 |
| NAS | 20 | 35 |
| TRP | 160 | 110 |
| 5HT | 45 | 55 |
| 5MTOL | 40 | 85 |
| 5MIAA | 240 | 100 |
| MEL | 60 | 135 |

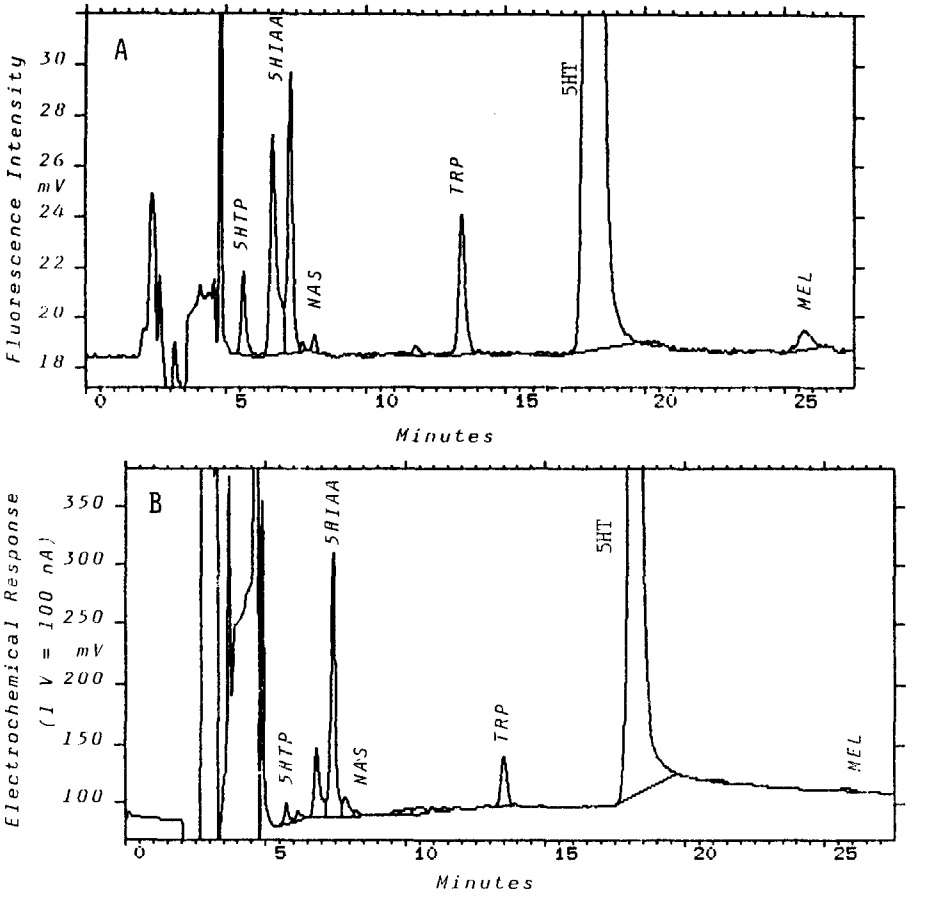


Fig. 3. Typical chromatograms of a 150-$\mu$l pineal extract from rat sacrificed during the light period (using flow programming). (A) Fluorescence detection; (B) electrochemical detection. For chromatographic conditions, see text.

resulted in on-column concentration so that injection volumes up to 200 $\mu$l could be employed without loss of resolution, though 150 $\mu$l were routinely used.

The fluorescence excitation wavelength was set at 232 nm and the emission wavelength at 353 nm (emission cut-off filter < 280 nm); the pulsed xenon lamp flash frequency was set at 220 Hz (yielding 5 W power); the response time was 4 s; photomultiplier setting, 12; excitation slit, 25 nm; emission slits, both 50 nm.

The electrochemical applied potential was set at +0.85 V; range, 100 nA full scale; signal filtering, 0.1 Hz.

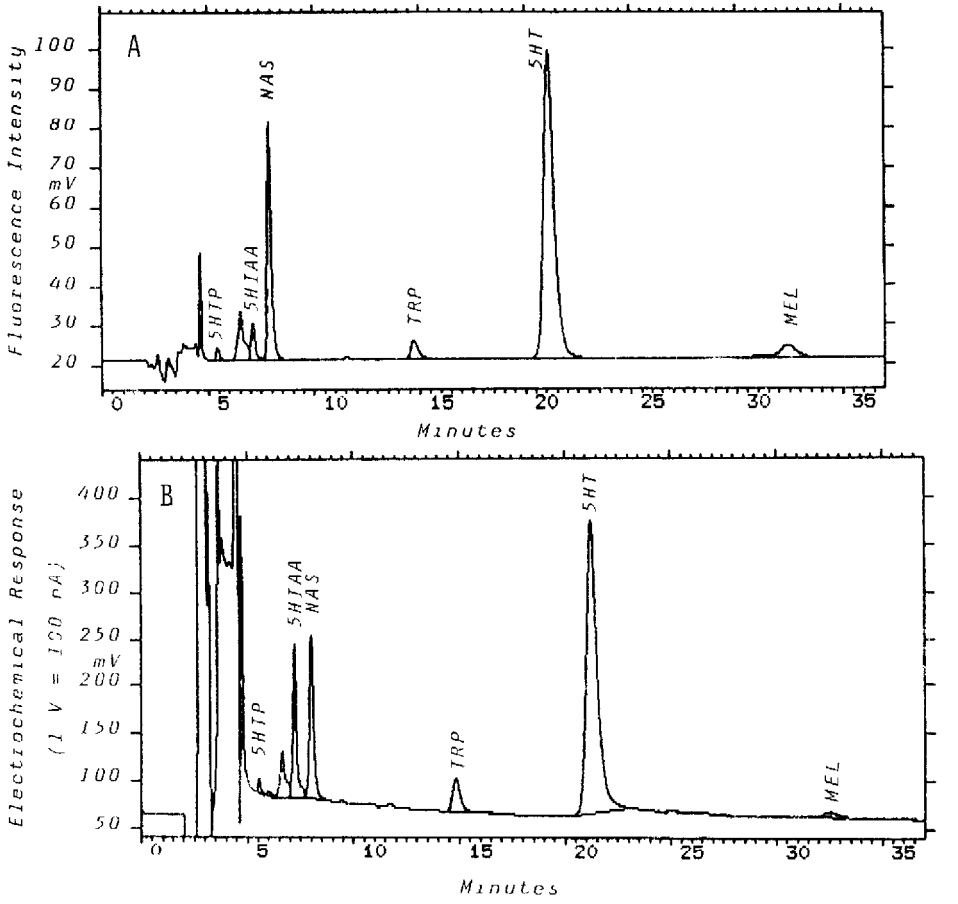


Fig. 4. Typical chromatograms of a 150-$\mu$l pineal extract from rat sacrificed during the dark period (isocratic conditions). (A) Fluorescence detection; (B) electrochemical detection. For chromatographic conditions, see text.

TABLE II

COMPOSITION OF SAMPLES

| | |
|---|---|
| Lt1 | Five light-killed pineals |
| Lt2 | Two light-killed pineals |
| Lt3 | Two light-killed pineals |
| Lt4 | Two light-killed pineals |
| Dk1 | Five dark-killed pineals |
| Dk2 | Two dark-killed pineals |
| Dk3 | Two dark-killed pineals |
| Dk4 | Two dark-killed pineals |

TABLE III

LEVELS OF INDOLES IN COMBINED RAT PINEAL SAMPLES

| Sample[a] | Detection[b] | Concentration (ng per pineal) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 5HTP | 5HIAA | NAS | TRP | 5HT | MEL |
| Lt1 | FL | 0 48 | 12.58 | 0.17 | 2.38 | 49.71 | 0.31 |
| | ED | 0.47 | 11.72 | 0.14 | 2.31 | 52.51 | 0.40 |
| Average | | 0.48 | 12.15 | 0.16 | 2.35 | 51.11 | 0.35 |
| Diff.[c] (%) | | 2.1 | 7.1 | 19.4 | 3.0 | −5.5 | −25.4 |
| Lt2 | FL | 0.48 | 13.23 | 0.26 | 4.08 | 54.57 | 0.43 |
| | ED | 0.48 | 12.61 | 0.30 | 3 75 | 56.98 | 0 42 |
| Average | | 0.48 | 12.92 | 0.28 | 3.92 | 55.78 | 0.43 |
| Diff. (%) | | 0.0 | 4.8 | 14.3 | 8.4 | −4.3 | 2.4 |
| Lt3 | FL | 0.43 | 13.36 | 0.23 | 2.71 | 55.40 | 0.49 |
| | ED | 0.42 | 11.31 | 0.26 | 2.56 | 58.73 | 0.54 |
| Average | | 0.43 | 12.34 | 0.25 | 2.64 | 57.07 | 0.52 |
| Diff. (%) | | 2.4 | 16.6 | 12.2 | 5.7 | −5.8 | −9.7 |
| Lt4 | FL | 0.46 | 9.44 | 0.13 | 2.53 | 50.15 | 0.29 |
| | ED | 0.42 | 8 64 | 0.12 | 2.30 | 53.97 | 0.36 |
| Average | | 0.44 | 9.04 | 0.13 | 2.42 | 52.06 | 0.33 |
| Diff (%) | | 9.1 | 8.9 | 8.0 | 9.5 | −7.3 | −21.5 |
| Dk1 | FL | 0.53 | 7.38 | 8.87 | 6.47 | 27.81 | 1.29 |
| | ED | 0.57 | 6.63 | 9.38 | 6.37 | 29.69 | 1.48 |
| Average | | 0.55 | 7.01 | 9.13 | 6.42 | 28.75 | 1.39 |
| Diff. (%) | | −7.3 | 10 7 | −5.6 | 1.6 | −6.5 | −13.7 |
| Dk2 | FL | 0.51 | 12.05 | 2.62 | 4.79 | 57.13 | 0.92 |
| | ED | 0.49 | 10.92 | 2.63 | 4.16 | 59.26 | 0.99 |
| Average | | 0.50 | 11.49 | 2.63 | 4.48 | 58.20 | 0.96 |
| Diff. (%) | | 4.0 | 9.8 | −0.4 | 14 1 | −3.7 | −7.3 |
| Dk3 | FL | 0.46 | 5.86 | 6.65 | 5.44 | 11 03 | 1.57 |
| | ED | 0.49 | 5.37 | 6.67 | 5.17 | 12.18 | 1.64 |
| Average | | 0.48 | 5.62 | 6.66 | 5.31 | 11.61 | 1.61 |
| Diff. (%) | | −6.3 | 8.7 | −0.3 | 5.1 | −9.9 | −4.4 |
| Dk4 | FL | 0.37 | 7.87 | 12.20 | 5.21 | 9.99 | 1.57 |
| | ED | 0.39 | 7.20 | 12.33 | 4.98 | 10.74 | 1.64 |
| Average | | 0.38 | 7.54 | 12.27 | 5.10 | 10.37 | 1.61 |
| Diff (%) | | −5.3 | 8.9 | −1.1 | 4.5 | −7.2 | −4.4 |

[a]For the composition of each combined sample, see Table II.

[b]FL = fluorescence detection; ED = electrochemical detection.

[c]Diff. is FL result minus ED result expressed as percentage of the average result.

TABLE IV

SUMMARY OF LIGHT AND DARK RESULTS FOR EACH DETECTOR

Averages and coefficients of variation (C.V.) have been derived from the results of all four combined samples (i.e. all eleven rats) for each detector.

| Compound | Fluorescence detection | | Electrochemical detection | | Mean average concentration (ng per pineal) | Difference[a] (%) |
|---|---|---|---|---|---|---|
| | Concentration (average) (ng per pineal) | C.V. (%) | Concentration (average) (ng per pineal) | C.V. (%) | | |
| *Light* | | | | | | |
| 5HTP | 0.46 | 5.1 | 0.45 | 7 2 | 0 46 | 2 2 |
| 5HIAA | 12.15 | 15.1 | 11.07 | 15.4 | 11.61 | 9.3 |
| NAS | 0.20 | 29.6 | 0.21 | 43.2 | 0.21 | −4.9 |
| TRP | 2.93 | 26.7 | 2.73 | 25.3 | 2.83 | 7.1 |
| 5HT | 52.46 | 5.6 | 55.55 | 5.1 | 54.01 | −5.7 |
| MEL | 0.38 | 25.2 | 0.43 | 18.0 | 0 41 | −12.3 |
| *Dark* | | | | | | |
| 5HTP | 0.47 | 15.3 | 0.49 | 15.2 | 0.48 | −4.2 |
| 5HIAA | 8.29 | 32.0 | 7.53 | 31.7 | 7.91 | 9.6 |
| NAS | 7.59 | 53.0 | 7.75 | 53 2 | 7.67 | −2.1 |
| TRP | 5.48 | 13.0 | 5.17 | 17.6 | 5.33 | 5.8 |
| 5HT | 26.49 | 83.0 | 27.97 | 80.7 | 27.23 | −5.4 |
| MEL | 1.34 | 23.0 | 1.44 | 21.4 | 1.39 | −7.2 |

[a]Difference of averages: average concentration obtained by fluorescence detection minus the average concentration obtained by electrochemical detection expressed as percentage of the mean average concentration.

*Other apparatus*

A Heat Systems-Ultrasonics (Farmingdale, NY, U.S.A.) W-225 sonicator fitted with Microtip and operated on setting 7 (microtip limit) was used.

*Tissue and analyte preparation*

Twenty-two male Wistar rats were used. Eleven rats were maintained on a 'normal' 12 h–12 h (06:00–12:00 'day') light–dark cycle and the other eleven rats on a 'reverse cycle' (light and dark interchanged) for at least two weeks prior to being killed. The animals were 127–128 days old when killed, which was done between 10:30 and 16:30 h at regular intervals on two consecutive days (normal-cycle animals were killed during their light period and reverse-cycle animals during their dark period).

Pineals were placed in 1.5-ml polypropylene microcentrifuge tubes, promptly frozen at −70 °C after dissection and used after seven to eight weeks of storage.

Pineals were sonicated for 5–10 s collectively using 200 μl homogenate (50

m*M* perchloric acid containing 0.013% CYS·HCl and 0.012% disodium EDTA) per pineal, then centrifuged at 12 000 *g* for at least 1 min. The supernatant was either injected immediately or stored in a clean tube at 4°C or −70°C for stability trials.

## RESULTS

### *Standards*

Using 150-μl injections of a set of five standards (spanning in equal intervals the ranges 0.200–1.00 ng/μl 5HT, 0.050–0.250 ng/μl TRP and 0.020–0.100 ng/μl of each remaining compound) the response of all compounds was linear for both detectors with correlation coefficients greater than 0.996.

Typical chromatograms of standards are shown in Fig. 2. Detection limits are shown in Table I.

### *Samples*

Typical chromatograms of pineal extracts are shown in Fig. 3 (light-killed) and Fig. 4 (dark-killed). Combined samples were constituted as shown in Table II. Results are shown in Tables III and IV.

## DISCUSSION

The results obtained are in good agreement with those of other workers [1–5, 7–13]. In particular, the increase in NAS and MEL and the decrease in 5HT levels in dark-killed animals relative to light-killed animals is well documented [2,3,5,12].

Table III shows that the results obtained with fluorimetric and electrochemical detection are in good agreement (difference generally less than 10%); however, consistently large relative differences in the results may indicate peak impurity or, alternatively, relatively low levels of the compound present. Combined with retention time agreement with standard peaks (the difference between sample and standard retention times was less than 2% of the standard retention time), the use of serial fluorimetric–electrochemical detection offers a convenient way of increasing confidence in compound identity and purity.

Table IV also shows a good agreement between fluorimetric and electrochemical detection. The substantial variation within Lt and Dk groups is not unexpected as there may be considerable variation among animals (hence the common practice of pooling results from several animals to even out individual differences). Even with the large coefficients of variation for the NAS results, there is little doubt of a pronounced elevation of Dk over Lt animals. The large coefficients of variation for Dk 5HT results are due to the Dk2 results being abnormally high (the NAS and MEL results for the same sample are lower

than expected), so it would seem that pineals of animals from this sample have responded more sluggishly than expected.

It is obvious from the HPLC profiles that some unknown compounds interfere with 5HIAA in both detection modes. However, this is less pronounced with electrochemical detection because of the much greater sensitivity of 5HIAA to electrochemical detection than to fluorescence detection and because the electrochemical sensitivity of the interferents has not increased proportionally. Hence, the electrochemical results are consistently lower than the fluorescence results. Nevertheless, there is still a reasonable agreement between the two detection methods, probably because the 5HIAA response for both detectors is considerably greater than that of the interferents.

Although some information on the levels of 5HTOL can be obtained, this information is not consistently reliable because of the lack of agreement between fluorescence and electrochemical results. Using digital processing of data, the peak shape can be scrutinised to a degree limited only by the detector noise, and it is obvious from distortions in the peak shape that 5HTOL is coeluting with interferences. The detection limits for 5MTOL and 5MIAA are too high for the levels present to be quantitated (usually less than 0.1 ng per pineal).

The stability of all compounds at various temperatures could not be demonstrated. 5HTP, NAS and TRP showed variable stability in pineal analytes stored at ambient temperature (20–25 °C) for 24 h or at 4 or −70 °C for several days. Surprisingly, storage at −70 °C did not improve the stability of the compounds compared to storage at 4 °C. It is, therefore, recommended to analyse the pineal analytes immediately after preparation . Obviously, the possibility exists that compounds in intact pineals could degrade even while stored at −70 °C, although it has been shown with this group of pineals, as well as with two other lots of 24 and 30 samples analysed after three months or within ten days of storage at −70 °C, that typical results are still obtained. While analyte preparation destroys degradative enzyme activity through protein denaturation by the acid homogenate, the resultant 200-fold dilution of compound levels combined with the solution environment may well be worse conditions for compound stability than −70 °C storage of intact pineals, where enzyme activity should be reduced to negligible levels.

Using flow programming, which, as opposed to solvent programming, does not disturb column equilibrium, and the reproducibility of which is limited only by the pump and controller performance, ten samples can be analysed in an ordinary working day. The use of a 5-$\mu$m column packing material results in an almost negligible loss of peak height at higher flow-rates.

While detection limits for MEL in particular are higher than for methods optimised for non-polar indoles, they are still adequate to determine the levels of interest (0.1–3 ng per pineal) usually present and the convenience of being able to determine this range of indoles by a single method is of value.

ACKNOWLEDGEMENT

Thanks are due to Mrs. Diane Bull for performing the killing of the animals and the dissections of the brains.

REFERENCES

1 I.N. Mefford and J.D. Barchas, J. Chromatogr., 181 (1980) 187.
2 I.N. Mefford, P. Chang, D.C. Klein, M.A.A. Namboodiri, D. Sugden and J. Barchas, Endocrinology, 113 (1983) 1582.
3 J.A. McNulty, M.M. Prechel, T.K. Audhya, D. Taylor, L. Fox, T A. Dombrowski and W H. Simmons, Endocrinology, 117 (1985) 1035.
4 P.B. Foley and K.D. Cairncross, J Pineal Res., 4 (1987) 107.
5 J.A. McNulty, L.M. Fox and S.J. Lisco, J Pineal Res., 4 (1987) 45.
6 J.A. Kappers and P. Pevet (Editors), Progress in Brain Research, Vol. 52. The Pineal Gland of Vertebrates Including Man, Elsevier/North-Holland Biomedical Press, Amsterdam, 1979, p. 222.
7 V.S. Vassilief, T.L. Sourkes and G.M. Anderson, J. Neural Transm., 55 (1982) 45.
8 S.M. Young and G M Anderson, Neuroendocrinology, 35 (1982) 464.
9 T.H. Champney, A.P. Holtorf, R.W. Seger and R J. Reiter, J Neurosci. Res , 11 (1984) 59.
10 H. Wakabayashi, K. Shimada and Y. Aizawa, Chem. Pharm. Bull., 33 (1985) 3875.
11 F. Tang, M. Hadjiconstaninou and S F. Pang, Neuroendocrinology, 40 (1985) 160.
12 J.A. McNulty, M.M. Prechel and W.H. Simmons, Life Sci., 39 (1986) 1.
13 H. Wakabayashi and K. Shimada, J. Chromatogr., 381 (1986) 21.