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Determination of melatonin and monoamines in rat pineal using reversed-phase ion-interaction chromatography with fluorescence detection

MALCOLM H. MILLS and DAVID C. FINLAY

Department of Psychology, University of Newcastle, Ranklin Drive, Shortland, N.S.W. 2308 (Australia)

and

PAUL R. HADDAD*

Department of Analytical Chemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033 (Australia)

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ABSTRACT

A method is reported for the ion-interaction, reversed-phase separation of 24 compounds (chiefly monoamines) arising from the metabolism of tyrosine and tryptophan. These compounds were separated as two groups. The first group comprised 3,4-dihydroxyphenylethylene glycol, tyrosine, 3-methoxy-4-hydroxyphenyl glycol, 5-hydroxytryptophan, norepinephrine, 3,4-dihydroxyphenylacetic acid, epinephrine, 5-hydroxyindole-3-acetic acid, homovanillic acid, 5-hydroxytryptophol, dopamine, tryptophan, N-acetylserotonin, N-acetyltryptophan, 5-methoxytryptophan and serotonin. The mobile phase consisted of a 6.8:93.2 (v/v) mixture of acetonitrile and an aqueous solution containing 0.16 *M* ammonium phosphate, 0.06 *M* citric acid, 0.15 m*M* disodium EDTA, 10 m*M* dibutylamine and 6 m*M* sodium 1-octanesulphonate at pH 4.50. The second group of compounds comprised 6-hydroxymelatonin, 5-methoxyindole-3-acetic acid, indole-3-acetic acid, 5-methoxytryptamine, tryptamine, 5-methoxytryptophol, melatonin and tryptophol. The mobile phase consisted of a 16:84 (v/v) mixture of acetonitrile and an aqueous solution containing 0.05 *M* ammonium phosphate, 0.05 *M* citric acid, 0.15 m*M* disodium EDTA, 25 m*M* dibutylamine and 5 m*M* sodium 1-octanesulphonate at pH 5.30. Detection was by fluorescence measurement (λ_{ex} = 280 nm, λ_{em} = 340 nm). The proposed method exhibited linear calibration over the biochemically significant concentration range, with detection limits in the 10–200 pg range. Excellent precision for peak areas and retention times was observed, even over a period of 24 h. The applicability of amperometric detection (at 0.72V) is also demonstrated. The method is applied to the determination of monoamines in individual rat pineals. Low nanogram levels of tyrosine, norepinephrine, 5-hydroxyindole-3-acetic acid, tryptophan, serotonin and 6-hydroxymelatonin, and picogram levels of 5-hydroxytryptophan, 5-hydroxytryptophol, 5-methoxyindole-3-acetic acid, indole 3-acetic acid, 5-methoxytryptophol and melatonin were indicated in most of the samples.

INTRODUCTION

Pineal melatonin (MEL) levels normally rise at night after stimulation by the pineal's (noradrenergic) sympathetic innervation from the superior cervical gan-

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glion, which activates the pineal enzyme hydroxyindolemethyltransferase. This, in turn, converts N-acetylserotonin (NAS) to MEL at the expense of its precursor, serotonin (5HT) and 5HT's other (catabolic) product, 5-hydroxyindoleacetic acid (5HIAA). Thus, at night, MEL, NAS and norepinephrine (NA) levels are elevated, whilst those of 5HT and 5-HIAA are depressed in comparison to levels occurring during day (or artificial) light [1].

Changes in pineal melatonin levels have been reported previously in stressed rats using a bioassay [2,3] or a radioimmunoassay [4]. An ongoing research project in this laboratory aims to determine whether the reported melatonin elevations can be confirmed by high-performance liquid chromatographic (HPLC) analysis, and to determine what other changes take place during its metabolism. To elucidate the changes in pineal monoamine levels in these studies, it was essential to develop a suitable chromatographic separation in which as many of the monoamines possibly present in pineal are resolved. These include all of the major compounds of indoleamine and catecholamine metabolism. We have adapted previously published chromatographic methods [1,5–7] to permit the separation of 24 compounds, using two mobile phases. The utility of the developed separations is illustrated by their application to the analysis of rat pineal. Not only are the methods shown to be suitable for direct measurement of brain monoamines, using well established extraction techniques [1,7,8], but they also permit the quantification of compounds not usually detectable in normal metabolism, but pertinent to *in vivo* and *in vitro* enzyme kinetic studies. The purity of manufactured compounds may also be assessed using this approach. Fig. 1 shows the interrelationship of the monoamine compounds examined in this study.

EXPERIMENTAL

Instrumentation

The chromatographic system consisted of a Millipore Waters (Milford, MA, U.S.A.) Model M 510 pump and Model U6K injector, used with a Perkin-Elmer (Norwalk, CT, U.S.A.) Model MPF4 spectrofluorimeter equipped with a 40-μl flow-through cell. The faces of this flow-cell opposite to the excitation and emission paths were coated with a mirror-finish of aluminium, thereby conferring a four-fold increase in signal. The detector was operated at an excitation wavelength of 280 nm and an emission wavelength of 340 nm, with slit widths set at 20 nm. A Millipore Waters Model 740 data module was used to record and process chromatograms. Other detectors used during the study included a Hitachi (Tokyo, Japan) Model HPLC F 1050 fluorescence detector and Millipore Waters Model 460 and 410 electrochemical detectors. With each of the fluorescence detectors used, great care was exercised in the alignment of the optics in order to maximize sensitivity.

The column used was a 250 mm $\times$ 4.6 mm I.D., 5-μm Spherisorb ODS II reversible column (Regis, Morton Grove, IL, U.S.A.), which was housed in an aluminium block through which water at controlled temperature was circulated.

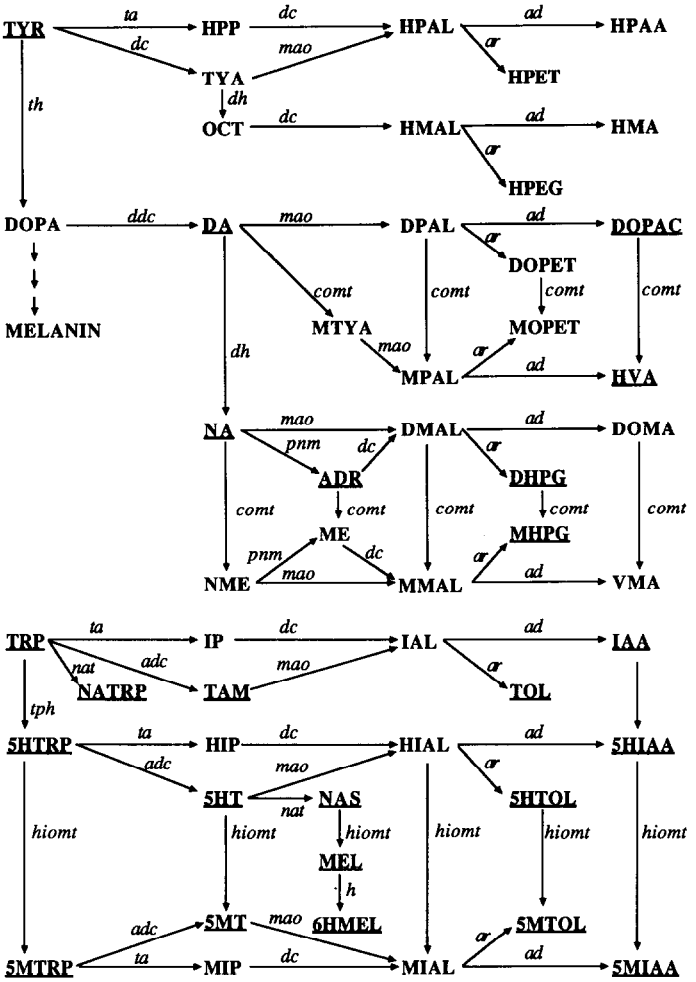

Fig. 1. Biochemical relationships between the compounds considered in this study. Enzymes are lower case and italicised: *ad* = aldehyde dehydrogenase; *ar* = aldehyde reductase; *adc* = L-aromatic amino acid decarboxylase; *dc* = decarboxylase; *ddc* = dopa decarboxylase; *dh* = dopamine β-hydroxylase; *comt* = catecholamine-O-methyltransferase; *h* = hydroxylase; *hiomt* = hydroxyindole-O-methyltransferase; *mao* = monoamine oxidase; *nat* = N-acyltransferase; *ta* = transaminase; *tph* = tryptophan hydroxylase; *pnm* = phenylethanolamine N-methyltransferase. The compounds are shown in upper case and are identified as follows: 3,4-dihydroxyphenylethylene glycol (DHPG), tyrosine (TYR), 3-methoxy-4-hydroxyphenyl glycol (MHPG), 5-hydroxytryptophan (5HTRP), norepinephrine (NA), 3,4-dihydroxyphenylacetic acid (DOPAC), epinephrine (ADR), 5-hydroxyindole-3-acetic acid (5HIAA), homovanillic acid (HVA), 5-hydroxytryptophol, (5HTOL), dopamine (DA), tryptophan (TRP), N-acetylserotonin (NAS), N-acetyltryptophan (NATRP), 5-methoxytryptophan (5MTRP), serotonin (5HT), 6-hydroxymelatonin (6HMEL), 5-methoxyindole-3-acetic acid (5MIAA), indole-3-acetic acid (IAA), 5-methoxytryptamine (5MT), tryptamine (TAM), 5-methoxytryptophol (5MTOL), melatonin (MEL), tryptophol (TOL), 3,4-dihydroxymandelic acid (DMAL), 3,4-dihydroxyphenyl acetaldehyde (DPAL), 3,4-dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenyl alanine (DOPA), dihydroxyphenyl ethanol (DOPET), 5-hydroxyindole-3-acetaldehyde (HIAL), 5-hydroxyindole-3-pyruvic acid (HIP), *p*-hydroxymandelic acid (HMA), *p*-hydroxymandelic aldehyde (HMAL), *p*-hydroxyphenylacetic acid (HPAA), *p*-hydroxyphenyl acetaldehyde (HPAL), *p*-hydroxyphenylethylene glycol (HPEG), *p*-hydroxyphenyl ethanol (HPET), *p*-hydroxyphenylpyruvic acid (HPP), indole-3-acetaldehyde (IAL), indole-3-pyruvic acid (IP), metanephrine (ME), 5-methoxyindole-3-acetaldehyde (MMAL), 3-methoxy-4-hydroxymandelic aldehyde (MIAL), 3-methoxy-4-hydroxyphenyl ethanol (MOPET), 3-methoxy-4-hydroxyphenylacetaldehyde (MPAL), 5-methoxyindole-3-pyruvic acid (MIP), octopamine (OCT), tyramine (TYA), 3-methoxytyramine (MTYA), normetanephrine (NME) and vanillylmandelic acid (VMA). The underlined species are those separated in Fig. 2.

Reagents and mobile phases

3,4-Dihydroxyphenylethylene glycol (DHPG), tyrosine (TYR), 3-methoxy-4-hydroxyphenyl glycol (MHPG), 5-hydroxytryptophan (5HTRP), norepinephrine (NA), 3,4-dihydroxyphenylacetic acid (DOPAC), epinephrine (ADR), 5-hydroxyindole-3-acetic acid (5HIAA), homovanillic acid (HVA), 5-hydroxytryptophol, (5HTOL), dopamine (DA), tryptophan (TRP), N-acetylserotonin (NAS), N-acetyltryptophan (NATRP), 5-methoxytryptophan (5MTRP), serotonin (5HT), 6-hydroxymelatonin (6HMEL), 5-methoxyindole-3-acetic acid (5MIAA), indole-3-acetic acid (IAA), 5-methoxytryptamine (5MT), tryptamine (TAM), 5-methoxytryptophol (5MTOL), melatonin (MEL) and tryptophol (TOL) were obtained from Sigma (St. Louis, MO, U.S.A.). Stock solutions of these compounds were prepared by dissolving 1 mg in either 1 ml of 0.1% ascorbic acid for polar compounds (group A, see below) or 1 ml of ethanol for lipophilic compounds (group B, see below), and these stock solutions were further diluted to prepare standard solutions, as required. The stock solutions were stored at −20°C for periods of up to a month, whilst the more dilute standard solutions were stored at 4°C for up to three days.

Two mobile phases were used. The first (mobile phase A) comprised a 6.8:93.2 (v/v) mixture of acetonitrile and an aqueous solution containing 0.16 *M* ammonium phosphate, 0.06 *M* citric acid, 0.15 m*M* disodium EDTA, 10 m*M* dibutylamine and 6 m*M* sodium 1-octanesulphonate (obtained from Aldrich, Milwaukee, WI, U.S.A.). The pH was adjusted to 4.50 after acetonitrile addition. The flow-rate was 1.3 ml/min and the column temperature was 45 ± 1°C. Mobile phase A was used for the separation of DHPG, TYR, MHPG, 5HTRP, NA DOPAC, ADR, 5 HIAA, HVA, 5HTOL, DA, TRP, NAS, NATRP, 5MTRP and 5HT, which will be referred to as group A compounds.

The second mobile phase (mobile phase B), which was used in a separate chromatographic run, comprised a 16:84 (v/v) mixture of acetonitrile and an aqueous solution containing 0.05 *M* ammonium phosphate, 0.05 *M* citric acid, 0.15 m*M* disodium EDTA, 25 m*M* dibutylamine and 5 m*M* sodium 1-octanesulphonate. The pH was adjusted to 5.30 after acetonitrile addition. The flow-rate was 1.3 ml/min and the column temperature was 30 ± 1°C. Mobile phase B was used for the separation of 6HMEL, 5MIAA, IAA, 5MT, TAM, 5MTOL, MEL and TOL, which will be referred to as Group B compounds.

Animals

Experimental and control rats were initially housed under similar conditions, in individual wire cages in air-conditioned rooms and fed the usual laboratory dry pellets with water on demand. Rats used in the experiments were stressed prior to sacrifice by restraint in cylindrical cages, according to the method of Lynch and co-workers [2–4], after 2 h of darkness. Pineals were taken, extracted by sonication using about fifteen 1-s pulses, centrifuged at 10 000 *g* for 20 min and stored at −80°C for eighteen months prior to analysis. Pineal tissue was extracted into a

solution of 0.1 *M* perchloric acid and 0.1% ascorbic acid [7] containing 0.01% EDTA [1]. For analysis, pineal extracts were thawed, vortexed ten times, centrifuged at 1000 *g* for 1 min and injected manually.

RESULTS AND DISCUSSION

Selection of optimal mobile phase composition

Our attempts to separate simultaneously all 24 compounds listed in the Experimental section were unsuccessful and we have found it necessary to divide them into two groups, according to their approximate lipophilicity. The first group (group A) comprises the less lipophilic species, whilst the second group (group B) comprises the more lipophilic species. A separate mobile phase is required for the separation of the component species of each group.

The separation of group A (less lipophilic) compounds was approached as follows. All components were eluted in an acceptable time using 6.8% (v/v) aqueous acetonitrile as the mobile phase, but resolution was inadequate. Sodium octanesulfonate was then added as an ion-interaction reagent and dibutylamine was included in the mobile phase to minimize the occurrence of silanophilic interactions, especially with 5HT. The mobile phase concentrations of these species were optimized empirically. Citrate was used to provide buffering capacity and the pH was varied over the range 3.5–5.0 until the resolution of the 16 components of group A was maximized. Increased pH caused a general decrease in the retention times for acidic solutes and a general increase for basic solutes. pH effects were particularly pronounced for 5HT, 5HTOL and 5HIAA. Ammonium phosphate served to increase the ionic strength of the mobile phase and to provide a competing cation in the ion-interaction retention mechanism. The addition of EDTA was necessary to scavenge metal ions in the eluent and sample, which led to increased baseline noise when an amperometric detector was used.

Raising the temperature to 45°C was found to produce a number of effects. First, the retention of all compounds was reduced without loss of resolution, but with more effect on basic compounds than other species. Second, back-pressure was reduced considerably, allowing higher flow-rates and shorter analysis times. Third, peak shape was improved considerably due to improved mass transfer characteristics at the elevated temperatures. A fourth, and unexpected, effect of increased temperature was a reduction in the occurrence of peak splitting, which was prevalent at lower temperatures. Further increases in temperature were found to have no further benefit, but caused peak co-elution, required frequent supplementation of the water bath, and could cause leaks in the MPF4 fluorescence flow-cell seal.

A chromatogram showing the separation of group A compounds under optimal mobile phase conditions is shown in Fig. 2A. Whilst it is recognized that the mobile phase composition is complex, each component has been found to be essential in achieving the desired separation.

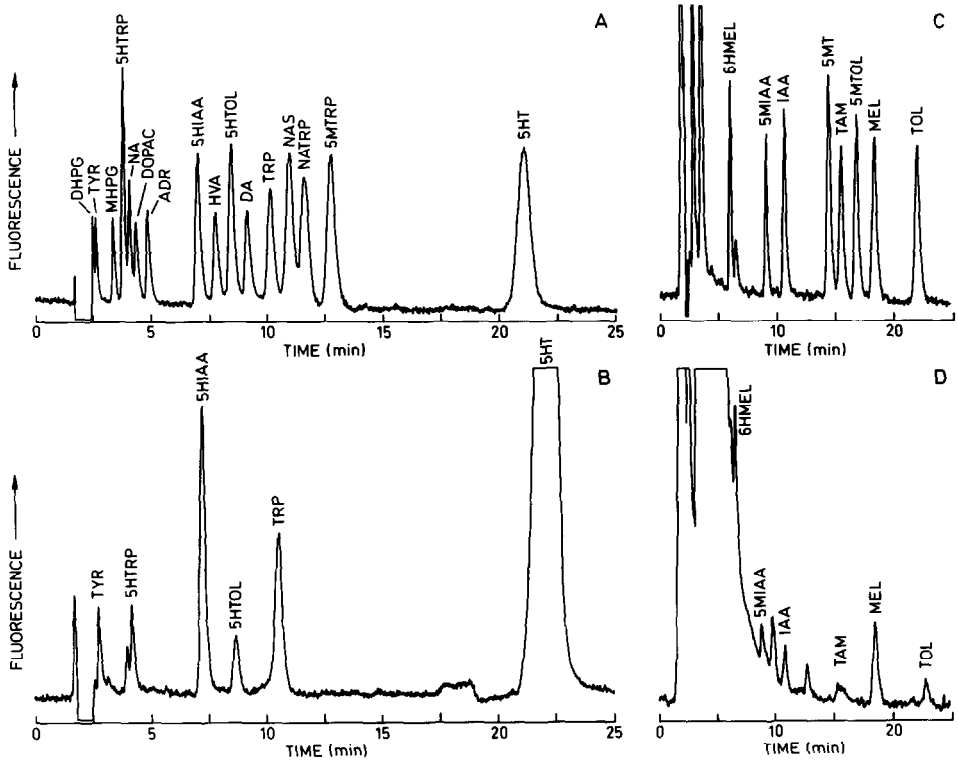

Fig. 2. Chromatograms obtained using fluorescence detection (excitation wavelength 280 nm, emission wavelength 340 nm). (A) Separation of group A compounds using mobile phase A (see text). A 50-μl injection containing DHPG (2 ng), TYR (4 ng), MHPG (2 ng), 5HTRP (0.4 ng), NA (2 ng), DOPAC (4 ng), ADR (2 ng), 5HIAA (0.8 ng), HVA (8 ng), 5HTOL (0.4 ng), DA (4 ng), TRP (0.8 ng), NAS (0.8 ng), NATRP (0.8 ng), 5MTRP (0.8 ng) and 5HT (0.8 ng) was used. (B) Analysis of 50 μl of rat pineal under the same chromatographic conditions. (C) Separation of group B compounds using mobile phase B (see text). A 200-μl injection containing 6HMEL (2 ng), 5MIAA (200 pg), IAA (200 pg), 5MT (200 pg), TAM (200 pg), 5MTOL (200 pg), MEL (200 pg) and TOL (200 pg) was used. (D) Analysis of 50 μl of rat pineal under the same chromatographic conditions.

The separation of the components of group B was approached in a similar fashion to group A. An appropriate percentage of organic modifier (16%) was determined on the basis of the retention of all solutes, and resolution was then manipulated by the progressive addition of further mobile phase components. At 30°C, it was possible to resolve TOL from MEL and MTOL. Sodium hexanesulfonate, sodium heptanesulfonate and, finally, sodium octanesulfonate were examined as ion-interaction reagents suitable for the retention of 5MT and TAM and their resolution from 6HMEL. The pH of the mobile phase was then varied over the range 3.5–5.8 to achieve resolution of 5MIAA from IAA. The surface amine modifier, dibutylamine, was used to reduce tailing and improve the resolu-

tion of 5MT and TAM; a concentration of 25 m*M* was found to be suitable for this purpose. Again, ammonium phosphate was used as an ionic strength modifier and EDTA was utilized so that the mobile phase was suitable for amperometric detection. Fig. 2C shows the separation of group B compounds using the optimal mobile phase composition.

Fluorescence was suitable for the detection of the monoamines DHPG, MHPG, 5HTRP, NA, ADR, 5HIAA, 5HTOL, TRP, NAS, NATRP, 5MTRP, 5HT, 5MIAA, IAA, 5MT, TAM, 5MTOL, MEL and TOL, but was less appropriate for TYR, DOPAC, HVA, DA and 6HMEL. Amperometric detection is better suited for solutes with active functional groups (*e.g.* hydroxyl), such as DHPG, MHPG, 5HTRP, NA, ADR, 5HIAA and 5HTOL, irrespective of their backbone structure. Maximum fluorescence was observed for MEL using excitation and emission wavelengths of 295 and 345 nm, respectively, but wavelengths of 280 and 340 nm, respectively, gave roughly equivalent fluorescence intensities for all the lipophilic indoles, whilst still retaining about 80% of the maximum signal intensity for melatonin.

In preliminary studies, amperometric detection at 0.72 V was applied to 500 ng of 5HMEL and 50 ng of 5 MIAA, IAA, (5MT and TAM), 5MTOL, MEL and TOL, as shown in Fig. 3. However, the principal analyte MEL was not detectable in rat pineal extracts using this approach. For this reason, fluorescence detection was preferred in this study.

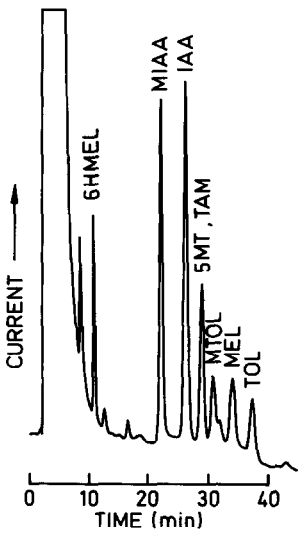

Fig. 3. Chromatogram obtained using amperometric detection at 0.72 V. Mobile phase B was used (see text) and the injected amounts were 500 ng for 5HMEL, and 50 ng each for 5MIAA, IAA, (5MT and TAM), 5MTOL, MEL and TOL.

TABLE I

ANALYTICAL PARAMETERS FOR THE DEVELOPED SEPARATIONS

Compound	Retention time		Linear calibration range	Correlation coefficient for	Precision of peak area at two levels of concentration						Detection limit (pg)	Amount per pineal		
	Min	%R.S.D.[a]	(ng or pg)	calibration	%R.S.D.	ng or pg	*n*	%R.S.D.	ng or pg	*n*		ng or pg	S.E.[b]	*n*
Group A (ng)														
DHPG	2.55	0.06	2–200	0.9998	4.39	5	5	1.14	50	5	50	6.61	5.9	2
TYR	2.70	0.06	4–400	0.9998	13.63	10	5	3.04	100	5	100	8.01	1.5	6
MHPG	3.42	0.15	2–200	0.9996	7.66	5	5	1.80	50	5	50	0.165	0.15	3
5HTRP	3.84	0.19	0.4–40	0.9996	1.66	1	5	1.56	10	5	10	0.061	0.024	6
NA	4.13	0.20	2–200	0.9995	3.89	5	5	4.22	50	5	50	3.14	0.77	8
DOPAC	4.40	0.13	4–400	0.9999	5.16	10	5	1.88	100	5	100	–		
ADR	4.92	0.12	2–200	0.9999	8.03	5	5	2.52	50	5	50	0.046	0.029	4
5HIAA	7.01	0.27	0.8–80	0.9990	4.60	2	5	1.42	20	5	20	1.44	0.236	6
HVA	7.77	0.27	8–800	0.9999	8.91	20	5	1.94	200	5	200	–		
5HTOL	8.44	0.25	0.4–40	0.9970	6.62	1	5	1.35	10	5	10	0.129	0.029	6
DA	9.20	0.14	4–400	0.9996	13.48	10	5	1.32	100	5	100	–		
TRP	10.17	0.12	0.8–80	0.9998	7.64	2	5	1.31	20	5	20	2.24	0.25	8
NAS	11.00	0.37	0.8–80	0.9895	7.29	2	5	1.27	20	5	20	–		
NATRP	11.62	0.22	0.8–80	0.9985	2.95	2	5	1.41	20	5	20	–		
5MTRP	12.78	0.32	0.8–80	0.9998	0.95	2	5	0.87	20	5	20	–		
5HT	21.01	0.27	0.8–80	0.9982	3.83	2	5	1.07	20	5	20	8.62	0.84	8
Group B (pg)														
6HMEL	6.10	0.36	100–5000	0.9933	16.78	500	3	6.96	5000	3	100	3128	478	5
5MIAA	9.18	0.11	10–500	0.9990	16.09	50	3	2.38	500	3	10	129	46	8
IAA	10.73	0.13	10–500	0.9991	1.76	50	3	2.49	500	3	10	159	85	7
5MT	14.49	0.16	10–500	0.9994	12.69	50	3	1.99	500	3	10	85	41	2
TAM	15.55	0.17	10–500	0.9990	16.82	50	3	2.34	500	3	10	48	–	1
5MTOL	16.95	0.25	10–500	0.9995	5.55	50	3	1.61	500	3	10	123	38	4
MEL	18.47	0.06	10–500	0.9994	11.12	50	3	1.85	500	3	10	219	30	8
TOL	22.10	0.14	10–500	0.9986	18.17	50	3	2.54	500	3	10	109	68	2

[a] Relative standard deviation.

[b] Standard error.

Analytical parameters

The linearity of calibration, reproducibility of retention times, precision of peak areas and detection limits (calculated at a signal-to-noise ratio of 2:1) for each of the 24 compounds examined are listed in Table I. The linearity studies used injection volumes in the range 20–100 μl, whilst precision values are based on three to five replicate 50-μl injections at two different sample concentrations. It can be seen that the proposed methods offer excellent sensitivity and precision.

Determination of monoamines in rat pineal

The developed separations were applied to the determination of monoamines in rat pineal. Typical chromatograms obtained for group A and group B compounds are shown in Fig. 2B and D, respectively. Recovery experiments were not performed on these samples because of the very small volumes available. Nevertheless, the chromatograms of pineal show that TYR, 5HTRP, 5HIAA, NA, 5HTOL, TRP, 5MIAA and MEL were found in significant quantities in most samples. Stressed rats, with significantly increased plasma corticosterone levels ($p < 0.05$), showed no significant changes in MEL or other metabolites, except for an increase ($p < 0.05$) in 5HTRP. NAS, which has been reported previously [1,7], does not appear to survive the extended storage time, although low levels are expected.

The extractant used for the pineal samples was found to keep MEL, 5HT and 5HIAA stable for at least 16 h at room temperature [1]. Interestingly, during our efforts to achieve stability of the samples for automation, we found that after extraction in buffer which did not contain EDTA, 5HTOL and MTOL levels rose on standing at room temperature for 12 h, whilst MEL levels halved. When EDTA was included in the homogenizing fluid, no change in the levels of these species was apparent for the same period. One possible action of EDTA is the chelation of ferrous ions, which in the presence of oxygen and ascorbic acid, will catalyse the conversion of TAM to 5-hydroxytryptamine [9], and presumably the formation of 5HTOL would also occur from TOL. It is unlikely that EDTA confers complete long-term stability on the samples, as evidenced by the very low levels of 5HTOL and MTOL observed in the pineal samples after the lengthy storage period used. Thus the composition, and particularly the pH and redox potential, of the extraction buffer can determine the levels of indole and catechol metabolites found.

CONCLUSION

The chromatographic method reported in this paper has the advantage of providing resolution of some of the stress compounds which cross-react in bioassays (*e.g.* ADR and NA [10]) and in radioimmunoassays (*e.g.* 5HTRP, 5HTOL, 5HIAA and 5HT [11]; 5MT, 6HMEL, 5HTOL and 5HT [12]; and DA and NA [13]). A full account of the results of the stress studies is to be reported elsewhere,

but it would appear that the advantage of increased analytical selectivity has the potential to permit redefinition of some current psychoneuroendocrine theories.

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