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Determination of the catecholamines and serotonin, their precursors tyrosine and tryptophan, and their main metabolites in rat brain using reversed-phase high-performance liquid chromatography with fluorimetric and oxidative amperometric detection in series

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ABSTRACT

A high-performance liquid chromatographic method for the determination of catecholamines and serotonin, their precursors and their main metabolites was developed applied to rat cerebellum, hypothalamus, striatum and cortex. A fluorimetric and an oxidative amperometric detector were used in series. For both detectors, detection limits (25–520 pg) were useful for this application, linearity of standards was excellent (average $r > 0.9997$), between-run precision for sample analytes was generally acceptable (coefficient of variation < 10% with appreciable concentrations present) and average recoveries of standard additions to sample analytes were better than 90%. Particular attention was paid to peak identification, including both a thorough treatment of retention time agreement of peaks in standards and sample analytes, and a comparison of results for the seven compounds amenable to quantitation by both detectors. Considerable attention was also given to determining the stability of standards and sample analytes under a wide variety of conditions, and practical recommendations were made.

INTRODUCTION

There is a considerable literature dealing with the determination of catecholamines and/or serotonin and some of their metabolites in rat or other mammalian brain tissue using high-performance liquid chromatography (HPLC). In the last decade, a number of papers [1–10] have attempted to provide methods for the concurrent determination of at least eight of these compounds. A limitation of these methods is that they rely on single-potential oxidative amperometric detection, and agreement of retention times of standard and unknown peaks only, for compound identification. Many are also merely illustrative, do not thoroughly evaluate the method performance or provide indications of longer-term reliability. As the number of compounds encompassed by a method increases, the stability and reliability of the separation, and confidence in peak identity, become increasingly important.

In the current method, an HPLC separation is presented for: tyrosine (TYR) and tryptophan (TRP), the amino acid precursors of the catecholamines and serotonin, respectively; norepinephrine (NE) and its major metabolites 4-hy-

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droxy-3-methoxyphenylethylene glycol (MHPG) and normetanephrine (NMN); epinephrine (E); dopamine (DA) and its major metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3MT); serotonin (5HT) and its major metabolite 5-hydroxyindoleacetic acid (5HIAA). This method comprehensively investigates the applicability of this separation to the determination of these compounds in rat brain. MHPG, E, NMN, DA, 5HIAA, 3MT and 5HT were determined both by fluorimetry and oxidative amperometry, enabling a much more exacting check of identity for these compounds. Agreement of the retention times of standards and unknowns was also given thorough treatment. The method has been comprehensively evaluated using four rat brain regions for between-run precision, recovery of standard additions and stability of compounds in standards and sample analytes, important aspects which many methods neglect.

EXPERIMENTAL

Reagents

MilliQ water (Millipore, Bedford, MA, USA) was used. Acetonitrile was LC 90-nm grade from Millipore, Waters Chromatography Division (Milford, MA, USA).

Dibutylamine phosphate (DBAP), 1 *M*, was prepared from dibutylamine (Fluka, Buchs, Switzerland): 12.9 g of dibutylamine were slurried in 70 ml of MilliQ water, the pH was adjusted to 2.5 with concentrated H_3PO_4 and the solution diluted to 100 ml with MilliQ water.

L-Cysteine hydrochloride (BDH, Poole, UK) was of biochemical grade. Cysteine concentrations are given as free cysteine. EDTA disodium salt (May & Baker, Dagenham, UK) was of LR grade. EDTA concentrations are given as free EDTA. Other chemicals used were of reagent grade or better.

Standards

TYR (as hydrochloride), MHPG (as hemipiperazine salt), NE (free base), E (as bitartrate salt), DOPAC, NMN (as hydrochloride), DA (as hydrochloride), 5HIAA (free acid), HVA, 3MT (as hydrochloride) and 5HT (as hydrochloride) were purchased from Sigma (St Louis, MO, USA). TRP was purchased from E. Merck (Darmstadt, Germany). All compounds were dried over phosphorus pentoxide to constant weight before use.

Individual standard stocks were prepared at a concentration of 1 mg free compound per ml using the following diluents. Diluent 1: 0.2 *M* $HClO_4$ with 0.025% each of cysteine and EDTA; diluent 2: pH 4 acetate buffer (40 m*M* sodium acetate–154 m*M* acetic acid) with 0.025% each of cysteine and EDTA. TYR, MHPG, NE, E, DOPAC, NMN, DA and 3MT used diluent 1; TRP and 5HT used diluent 2; HVA used ethanol–diluent 1 (1:9, v/v); 5HIAA used ethanol–diluent 2 (1:9, v/v). All standard stocks except MHPG were stored as 0.5- or 1-ml aliquots in 1.5-ml vials at −70°C; MHPG was stored as 0.5-ml aliquots at 4°C. MHPG and HVA stocks were discarded after one month of storage and fresh lots prepared.

Working standards used diluent 3: 60 m*M* $HClO_4$, 0.025% each of cysteine and EDTA. Working standards consisted of the following standards. Standard 1: 4 ng/μl TYR, 3 ng/μl DA and 1 ng/μl each of the remaining compounds in diluent 3; standard 2: as for standard 1 but with 3 ng/μl TYR and 2 ng/μl DA; standard 3: as for standard 1 but with 1 ng/μl TYR and 1 ng/μl DA. Working standards were stored as 4-ml aliquots in 4-ml WISP Vials (Millipore, Waters) with PTFE septa at 4°C. For the data in the tables, working standards were used for not more than one day at ambient temperature (20–25°C).

HPLC eluent

This consisted of an aqueous component–acetonitrile (181:19, v/v), with the aqueous component consisting of 12.16 m*M* citric acid, 11.60 m*M* $(NH_4)_2HPO_4$, 2.54 m*M* sodium octylsulphonate, 3.32 m*M* DBAP and 1.11 m*M* disodium EDTA. The eluent pH was approximately 3.8. The eluent was filtered through a 0.45-μm filter and used at a flow-rate of 1.0 ml/min (pressure approximately 170 bar).

Chromatography

The column was a stainless-steel type (Phenomenex, Rancho Palos Verdes, CA, USA), 250 mm × 4.6 mm I.D. packed with 5-μm Spherisorb ODS-2 (Phase Separations, Deeside, UK). The column was used at 30 ± 0.2°C.

The HPLC apparatus was a modular assembly. The M6000A pump, WISP 710 B autosampler [with needle wash acetonitrile–water (1:9, v/v)], Guard-Pak guard-column (with μBondapak C_{18} or Nova-Pak C_{18} inserts) and Maxima version 3.3 software (for system control, data acquisition and reduction, operating via System Interface Module) were from Millipore (Waters). The HP 1046A programmable fluorescence detector was from Hewlett-Packard (Analytical Division, Waldbronn, Germany). The LC-4B amperometric detector with LC-17AT flow-cell assembly, glassy carbon working electrode, RE-4 Ag/AgCl reference electrode and flow-cell preheater (LC-22A controller) was from Bioanalytical Systems (West Lafayette, IN, USA). The working electrode was periodically rejuvenated by applying a drop of fresh chromic acid for 30–60 s.

Fluorescence detector settings were: excitation and emission wavelengths, 230 and 320 nm, respectively; emission filter, 280 nm; pulsed xenon lamp flash frequency, 55–220 Hz (yielding 1.25–5 W, respectively); response time, 2 s; photomultiplier setting, 13; excitation slit, 25 nm; emission slits, both 50 nm. A flash frequency of 55 Hz was employed for all data shown in the tables and for chromatograms shown in the figures. Amperometric detector settings were: applied potential, +0.70 V; range 100 nA full-scale; signal filtering 0.1 Hz; flow-cell preheater, 30°C. A 51-μm flow-cell gasket was used. All injection volumes were 25 μl.

Other items

Maxima and other software were run on a 80386DX based microcomputer using Microsoft (Redmond, WA, USA) MS-DOS version 3.3. Calculations, including correction of results for standard drift by interpolation, were performed using Lotus (Cambridge, MA, USA) 1-2-3 release 2.2 spreadsheet software. Note that 1-2-3 calculates the population, not the sample, standard deviation.

A Heat Systems-Ultrasonics (Farmingdale, NY, USA) W-225 sonicator, with microtip horn, a Beckman Instruments (Palo Alto, CA, USA) J2-21 centrifuge with JA-18.1 rotor, limited-volume inserts of 300 μl capacity (Millipore, Waters) and polypropylene vials of 1.5 ml capacity suitable for centrifugation with built-in caps were used.

Tissue and analyte preparation

Control male Wistar rats were killed by decapitation and their brains dissected into regions [11] on ice, then promptly stored at −70°C. Cortices were stored in 10-ml polypropylene capped tubes and all other regions in 1.5-ml vials.

Tissue was thawed just prior to use, then extracted/disintegrated by sonication with homogenizing agent in the ratio 1 g tissue:5 ml homogenizing agent. The sonication time was 5–10 s for all regions except the cortex (20–30 s). Rat brain was deemed to contain 80% water, giving a weight/dilution ratio of 1 g:5.8 ml.

Homogenizing agents all contained 0.025% each of cysteine and EDTA, and $HClO_4$, unless otherwise indicated. Acid concentrations varied between 0.1 and 0.5 *M*, and are given in context. Homogenizing agent 1 consisted of 0.15 *M* $HClO_4$ with 0.025% each of cysteine and EDTA.

Tissue extracts were promptly centrifuged at 12 000 *g* and 4°C for 10 min. Supernatants were taken as the sample analytes and were either promptly used for HPLC, usually in limited-volume inserts, or stored at −70, −20 or 4°C in 1.5-ml vials. Other conditions for the usage of analytes in stability trials are described in context.

Homogenizing 1 agent was used to prepare sample analytes as described above, for the relevant data in the tables. "Fresh" analytes were prepared less than 20 min before HPLC. Where data shown in Tables III–V pertain to stored analytes, these were used as follows: thaw less than 20 min before HPLC, sonicate for 5 s, recentrifuge as when originally prepared and transfer the supernatants to limited-volume inserts for HPLC.

RESULTS

In the following, the suffixes -1 and -2 mean "as determined by fluorimetry" and "as determined amperometrically", respectively. A compound name without a suffix implies that results are applicable to both detectors. Compound names in parentheses imply some question as to peak identity.

Standard chromatograms

Typical chromatograms of standards are shown in Fig. 1A and B.

Stability of standard stock solutions

Three aliquots of each stored stock were compared with fresh stocks prepared the day of the test. Working solutions contained 1 ng/μl of each compound in diluent 3, with no more than three compounds being checked on any given day.

The catechol-based compounds stored at −70°C were checked at storage periods of 37 weeks and 16 months (TYR and DA were also checked on three other occasions up to 2 years and 3 months) and showed no practical difference in concentration between stored and fresh stocks, except for HVA. HVA stored for 37 weeks showed an average 6.6% loss and after 16 months an average 13.4% loss. MHPG stocks stored at 4°C for 26 weeks showed an average loss of 8.1%, however, stocks stored at −70°C for 19 weeks showed a loss of 33%.

5HIAA, TRP and 5HT were checked at storage periods of 12, 20 and 31 weeks and 13, 18 and 20 months. Results showed no practical differences in concentration between stored and fresh stocks.

Stability of working standard

Aliquots (4 ml) of standard 2 stored at 20–25°C for up to 4 days showed no practical differences in concentration for all compounds when compared to working standard prepared fresh just prior to the start of the test and stored at 4°C. Beyond 4 days some degradation of MHPG and 5HIAA was apparent, but this was still only 10% at 35 days for MHPG and 8.5% at 23 days for 5HIAA. Aliquots (4 ml) of standard 2 stored at 4°C for 26, 41 and 86 days showed no practical differences in concentration for all compounds when compared to working standard prepared fresh the day of the test. Aliquots of standard 3 stored at 4°C for 152 and 170 days showed similar stability. Aliquots of standard 1 stored at −70°C for 10, 18, 23 and 33 days showed significant losses (>5%) at each of these periods for MHPG, 5HIAA and 5HT.

Regression of standards

The standard set used consisted of standard 1, with substandards containing all compounds at concentrations of 20, 40, 60 and 80% of those of standard 1 in diluent 3. Five sets of standards were run, on different days.

For all runs and for all compounds quantitated fluorimetrically, the correlation coefficients were >0.99978, with an average of 0.99994 (n = 50); all absolute values of the response axis intercept relative to the standard 1 response for the corre-

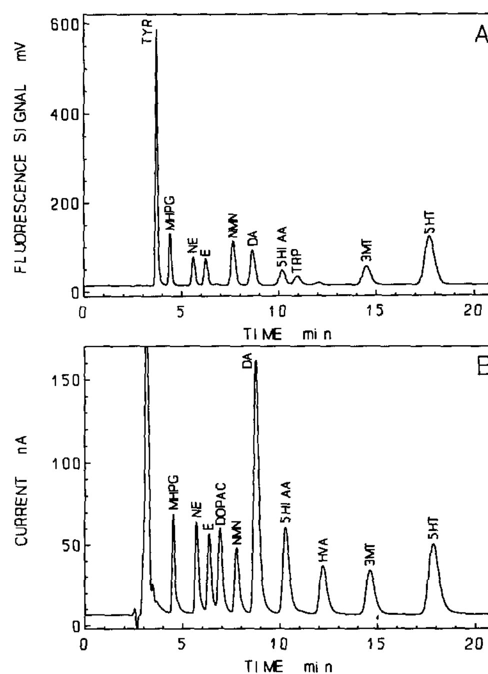

Fig. 1. Typical chromatograms of standards: 25 μl of standard 1 in diluent 3; other conditions as described in the text. (A) Fluorimetric detection; (B) amperometric detection.

sponding compound were <1.2%, with a mean of 0.35% (n = 50). For the amperometric detector, the corresponding figures were >0.99975, 0.99996 (n = 50); <0.80%, 0.30% (n = 50).

Detection limits

Detection limits were determined from nine runs involving sample analytes and are given in Table I.

Stability of compounds at 4 and 26–31°C in fresh cortex analytes prepared with spiked homogenizing agents using 0.1–0.5 M $HClO_4$

A series of homogenizing agents, designated A, B, C, D and E, were prepared containing 0.1, 0.2, 0.3, 0.4 or 0.5 M $HClO_4$, respectively, and 0.0275% each of cysteine and EDTA. Each homogenizing agent also contained 0.4 ng/μl TYR, 0.3 ng/μl DA and 0.1 ng/μl each of the remaining compounds. These were used with one cortex each to prepare analytes in the usual way. Analytes were analysed promptly on preparation and the remainder was split into two approximately 2-ml aliquots and placed in 4-ml WISP vials with self reseal septa. The remainder was stored at either 4°C or at WISP ambient temperature (25–30°C). Both sets were reanalysed after 1 and 2 days standing at their respective temperatures, while the set at 25–30°C was also analysed at 4 and 8 h.

TABLE I

TYPICAL DETECTION LIMITS

Detection limit has been defined as the amount or concentration of substance resulting in a response equal to twice the detector noise. Detection limits are the average of nine determinations from different days. Detection limits in ng/g use a weight/dilution of 1 g:5.8 ml.

Compound	Detection limit			
	Fluorimetric detector[a]		Amperometric detector[b]	
	pg	ng/g	pg	ng/g
TYR	75	17	–	–
MHPG	90	21	30	7
NE	160	40	25	6
E	170	40	30	7
DOPAC	–	–	25	6
NMN	100	24	40	9
DA	380	90	25	6
5HIAA	310	70	30	7
TRP	520	120	–	–
HVA	–	–	50	12
3MT	240	55	50	12
5HT	95	22	35	8

[a] Noise = 0.20 mV.

[b] Noise = 0.275 mV (1 V represents 100 nA).

At 25–30°C, the catechol-based compounds showed acceptable stability (concentrations 91–109%, and mostly 95–105%, of those of fresh analytes) in analytes from all homogenizing agents over 2 days for all compounds except MHPG-2 and NE-2. MHPG-2 showed good stability up to 8 h in analytes from all homogenizing agents, or from homogenizing agents A and B over 2 days, but from homogenizing agents C through E MHPG-2 showed significant apparent increases in concentration (8–44%) over 1 and 2 days. NE-2 showed a similar pattern, but with smaller increases (8–18%). TRP showed significantly better stability than the other two indole-based compounds with losses of <16%. In analytes from homogenizing agents A and B over 2 days, TRP yielded concentrations >95% of those of fresh analytes. 5HIAA and 5HT in analytes from all homogenizing agents showed clear trends of losses (10–55%), increasing with both time and $HClO_4$ concentration, except for homogenizing agent B where losses were less than, or equal to, those from homogenizing agent A.

Results at 4°C for catechol-based compounds were similar to those at 25–30°C, though the increases observed then with MHPG-2 and NE-2 were not seen. TRP showed acceptable stability in analytes from all homogenizing agents over 2 days, with concentrations being mostly >95% of those of fresh analytes. Stability of 5HIAA and 5HT was significantly better than at 25–30°C, with the worst loss being <27% and many losses being <10%. Interpolation of results for 5HIAA and 5HT to yield an 8-h result suggests that concentrations would be >95% of those of fresh analytes, for all homogenizing agents.

Stability of compounds at 25–30°C in cortex analytes prepared with spiked homogenizing agents using 0.1–0.5 M $HClO_4$ and stored at −70 or −20°C for 20 days

Five analytes were prepared as in the preceding section, again a series of spiked homogenizing agents A through E (though these contained 0.025% rather than 0.0275% each of cysteine and EDTA). Concentrations were determined in the fresh analytes, and the remainder was apportioned equally for −70 or −20°C storage.

A preliminary trial indicated the necessity of sonicating the analytes after thawing, rather than merely vortex-mixing them for 10 s, to obviate apparent incomplete dissolution of some compounds.

Analytes stored at −20°C were analysed at thawing only. All compounds except MHPG, 5HIAA and 5HT for all homogenizing agents showed concentrations of 85–105% of those of fresh analytes; however, MHPG, 5HIAA and 5HT showed losses of 75, 63 and 61%, respectively, averaged over all homogenizing agents, with a large and unpredictable variation between homogenizing agents.

Analytes stored at −70°C were analysed at thawing, and after 6, 12 and 24 h standing at 25–30°C. All compounds except MHPG, 5HIAA and 5HT for all homogenizing agents showed concentrations 85–111% of those of fresh analytes with most results being within 90–105%. MHPG in the analyte from homogenizing agent A showed better stability and higher concentrations (82–91%) than those from the other homogenizing agents, which all showed significant apparent increases with time from depressed concentrations (57–80% at thawing). 5HIAA and 5HT both tended to show significant losses (<28 and 32%, respectively, at thawing) proportional to $HClO_4$ concentration and increasing with time.

Effect of $HClO_4$ concentration on response

No significant effect was indicated from any mismatch in acid concentration between standards and sample analytes. Standards were prepared as for standard 1 but with 3, 10, 20, 50, 100 or 200 mM $HClO_4$, and their responses compared with those of standard 1. For the fluorimetric detector, the average (n = 60) of all relative responses was 101.1%, with a coefficient of variation (C.V.) of 1.8%; for the amperometric detector, the corresponding figures were: n = 60, 100.7% and 1.2%.

Sample concentrations

Analytes from three samples each of cerebellum, hypothalamus and striatum were prepared singly and analysed within 20 min of preparation (these analytes are referred to in the following three sections as analytes-1). The remainder from each analyte was promptly apportioned to eight aliquots for −70°C storage and subsequent reanalysis. Sample concentrations as determined from fresh analytes are given in Table II.

Chromatograms of each sample type are shown in Figs. 2–4.

Peak identification in samples

A detailed comparison of retention times (t_R) of peaks in standards with those of peaks assigned the same identity in sample analytes was made for data from analytes-1 over nine runs (one run on fresh analytes, eight runs on analytes stored at −70°C). Relative ΔT was defined as:

$$\text{Relative } \Delta T = 100\left(\frac{\text{sample } t_R - \text{standard } t_R}{\text{standard } t_R}\right)\%$$

Average relative ΔT values for each compound and sample type for the nine runs was calculated and found to lie within −0.5 to 0.5% with the following exceptions: E-1 (cerebellum), −0.8%; NMN-1 (all sample types), −1.1 to −1.3%; NMN-2 (all sample types), −1.9 to −2.1%; DA-1 (cerebellum), −1.7%; DA-2 (cerebellum), −2.0%.

MHPG, E, NMN, DA, 5HIAA, 3MT and 5HT were determined using both detectors. A comparison of the concentrations obtained over the same nine runs is given in Table III.

Between-run precision

This was determined over eight runs (except striatum 3 analyte, seven runs) on 8 different

TABLE II

CONCENTRATIONS IN CEREBELLUM, HYPOTHALAMUS AND STRIATUM AS DETERMINED ON FRESH ANALYTES

All concentrations in ng/g of tissue. Rel. R (%) = 100 (maximum − minium)/average.

Compound	Cerebellum (n = 2)		Hypothalamus(n = 3)		Striatum (n = 3)	
	Average	Rel. R (%)	Average	Rel. R (%)	Average	Rel. R (%)
TYR	9958	12	9361	22	9298	13
MHPG-1	85	8	34	65	44	64
MHPG-2	36	2	9	27	8	57
NE-2	236	3	1012	8	373	50
E-1	12	200	23	31	n/d	
E-2	10	60	29	36	22	33
DOPAC	20	12	208	92	1012	9
NMN-1	40	6	47	50	32	17
NMN-2	154	8	115	29	87	7
DA-1	59	26	723	121	3837	28
DA-2	40	41	708	108	3796	32
5HIAA-1	73	2	616	12	502	38
5HIAA-2	85	5	626	11	457	30
TRP	4427	4	3941	14	3990	19
HVA	20	43	91	104	420	10
3MT-1	0		70	101	275	33
3MT-2	9	28	62	129	280	33
5HT-1	65	1	725	6	387	47
5HT-2	60	0	715	6	379	50

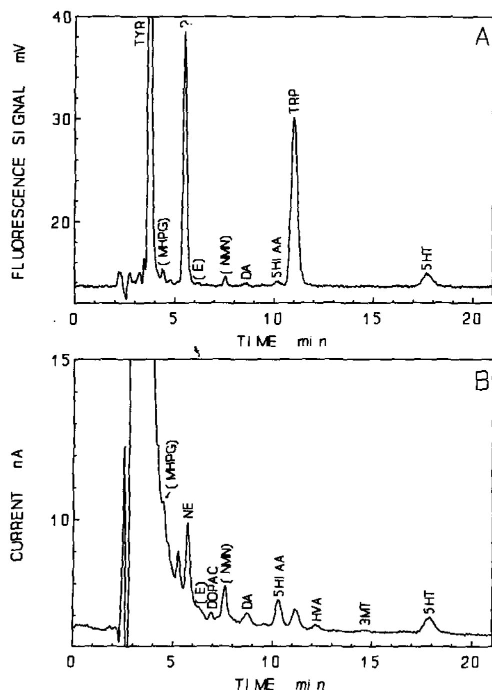

Fig. 2 Typical chromatograms of fresh cerebellum analyte: 25 μl analyte from W g of cerebellum extracted with 5W ml of homogenizing agent 1; other conditions as described in the text. (A) Fluorimetric detection; (B) amperometric detection.

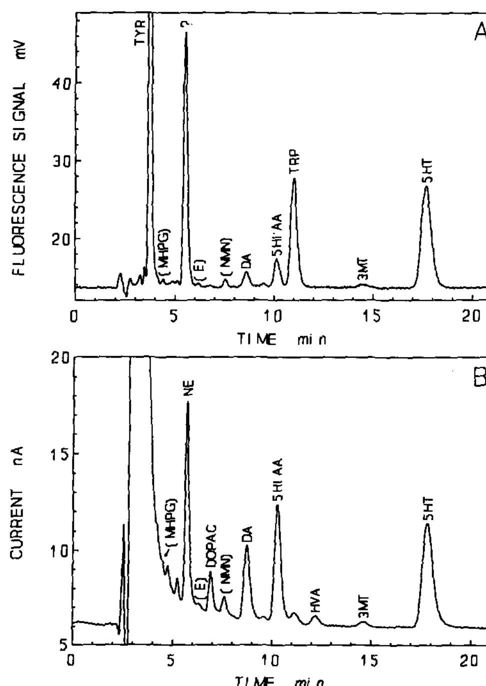

Fig. 3. Typical chromatograms of fresh hypothalamus analyte: 25 μl analyte from W g of hypothalamus extracted with 5W ml of homogenizing agent 1; other conditions as described in the text. (A) Fluorimetric detection; (B) amperometric detection.

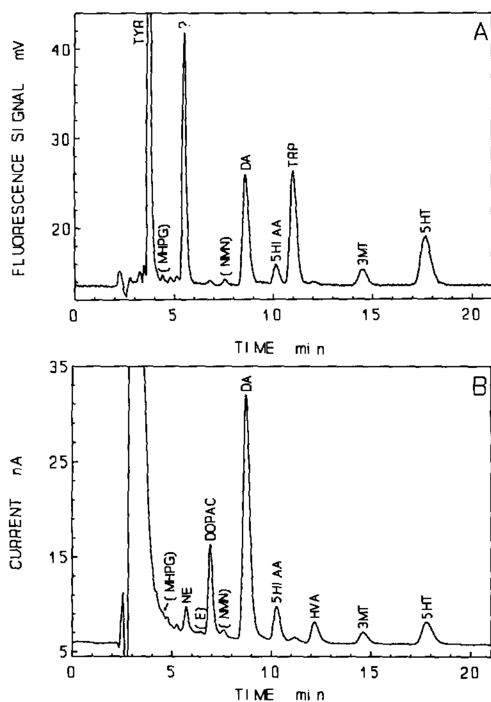

Fig. 4. Typical chromatograms of fresh striatum analyte: 25 μl analyte from W g of striatum extracted with $5W$ ml of homogenizing agent 1; other conditions as described in the text. (A) Fluorimetric detection; (B) amperometric detection.

days, on analytes-1 stored at −70°C. The results are summarised in Table IV.

Stability of compounds in sample analytes stored at −70°C for 5–14 days

Concentrations obtained over eight runs from analytes-1 stored at −70°C were compared with each other and with those from the fresh analytes. Results referred to in the following are expressed as percentage remaining relative to fresh analyte concentrations, averaged for each sample over the eight runs. Unless otherwise indicated, changes in results were free of any particular trends.

TYR, TRP and NE-2 results were within 99.5–101.5, 98–100 and 96–102%, respectively, for all sample types; DOPAC and HVA results (hypothalamus and striatum only) were within 91–99 and 95–107%, respectively; DA results (hypothalamus and striatum only) were within 100–115%, or within 100–104% if hypothalamus 2 was excluded; 3MT (hypothalamus and striatum only) results were within 74–105%, or within 90–105% if hypothalamus 2 was excluded. 5HIAA and 5HT results from all sample types (except cerebellum for 5HIAA-1) appeared to show a decrease of < 18% due to being frozen, but during actual storage at −70°C stayed quite constant at this diminished level. Cerebellum 5HIAA-1 concentrations showed apparent increases with time, averaging 26 and 11% for cerebellum 1 and cerebellum 2, respectively. (NMN) gave results between 76 and 126% for all sample types and in many cases showed a significant trend to increase. No reliable conclusions regarding (MHPG) and (E) stability could be drawn for any sample types.

Recovery of standard additions to cortex analytes

Analytes from five cortices were prepared and stored at −70°C; 6 days later, supernatants from these were prepared and 180 μl of each supernatant were spiked with 20 μl of a standard addition solution containing 18 ng/μl TYR, 8 ng/μl TRP and 1 ng/μl each of the remaining compounds. The addition was such that it approximately equalled TYR and TRP concentrations naturally present, or contributed 0.1 ng/μl each of the remaining compounds to the composite solution. Recoveries are given in Table V.

DISCUSSION

The choice of compounds for inclusion in the separation was dictated by several considerations. NE, DA and 5HT were of obvious importance as were their major metabolites DOPAC, HVA and 5HIAA. The use of fluorimetric detection and the high concentrations of TYR and TRP in rat brain made these compounds an obvious choice for inclusion. While TRP (and to a lesser extent TYR) is commonly regarded as not electroactive at the potentials used, it is sufficiently so that at TRP concentrations present, a substantial peak was observed (see the unidentified peak between 5HIAA and HVA in Figs. 2B, 3B and 4B). Failure to locate TRP in methods using

TABLE III

AGREEMENT BETWEEN DETECTORS FOR MHPG, E, NMN, DA, 5HIAA, 3MT, 5HT CONCENTRATIONS OVER NINE RUNS[a]

Ratio = concentration determined fluorimetrically/concentration determined amperometrically. Rel. R (%) = 100 (concentration determined fluorimetrically − concentration determined amperometrically/average concentration. *n* for each compound is the number of pairs of results where non-zero concentrations were obtained for each detector.

	Cerebellum	Hypothalamus	Striatum
MHPG			
n	26	27	26
Av. ratio	2.7	4.6	3.3
Min. ratio	1.9	2.0	1.6
Max. ratio	4.3	8.6	7.0
E			
n	6	26	16
Av. rel. R (%)	102	−10	−27
Min. rel. R (%)	−56	−146	−88
Max. rel. R (%)	188	41	52
NMN			
n	26	27	26
Av. ratio	0.24	0.45	0.39
Min. ratio	0.18	0.32	0.27
Max. ratio	0.32	0.59	0.60
DA			
n	22	27	26
Av. rel. R (%)	81	2.7	0.7
Min. rel. R (%)	7.7	−12	−1.1
Max. rel. R (%)	154	13	3.4
5HIAA			
n	26	27	26
Av. rel. R (%)	6.0	−0.8	4.3
Min. rel. R (%)	−37	−8.7	−3.0
Max. rel. R (%)	40	9.9	15.4
3MT			
n	0	27	26
Av. rel. R (%)		4.0	−1.4
Min. rel. R (%)		−20	−9.7
Max. rel. R (%)		52	7.6
5HT			
n	26	27	26
Av. rel. R (%)	1.4	0.1	0.3
Min. rel. R (%)	−13	−1.7	−3.6
Max. rel. R (%)	11	2.6	5.1

[a] Except cerebellum 3, eight runs.

amperometric detection only coud result in large errors for some compounds from undetected co-elution. MHPG is of importance as it is the only common metabolite of NE, though in many brain regions it occurs mainly as the sulphate ester [12], and only small amounts of free MHPG

TABLE IV

BETWEEN-RUN PRECISION

DLt × is the concentration found expressed as the number of times the relevant detection limit. DLt × and C.V. were calculated for each sample for the non-zero concentrations obtained over eight runs. These were averaged over the samples in each sample type to give Av. DLt × and Av. C.V. (%). n is the number of non-zero concentrations represented in the averages.

	TYR	(MHPG)	(E)	(NMN)	DA	5HIAA	TRP	3MT	5HT
Fluorimeter									
Cerebellum									
n	24	24	8	24	21	24	24	0	24
Av. DLt ×	553	3.4	0.3	1.3	0.4	1.1	34		2.2
Av. C.V. (%)	0.4	10.4	42	8.7	27	10.7	0.7		5.5
Hypothalamus									
n	24	24	23	24	24	24	24	24	24
Av. DLt ×	546	1.2	0.6	1.9	8.4	7.4	33	1.1	28
Av. C.V. (%)	0.9	22	33	7.6	5.5	4.8	1.6	12	1.2
Striatum									
n	23	23	16	23	23	23	23	23	23
Av. DLt ×	541	1.7	0.4	1.5	43	6.2	33	4.9	16
Av. C.V. (%)	0.7	16	26	20	1.3	3.3	1.4	3.4	1.9

	(MHPG)	NE	(E)	DOPAC	(NMN)	DA	5HIAA	HVA	3MT	5HT
Amperometer										
Cerebellum										
n	24	24	15	24	24	22	24	24	0	24
Av. DLt ×	3.9	36	0.4	1.4	15	2.3	10.6	1.6		6.2
Av. C.V. (%)	15	2.9	59	17	3.9	41	8.3	20		3.9
Hypothalamus										
n	24	24	24	24	24	24	24	24	24	24
Av. DLt ×	0.9	174	3.4	31	12	113	79	7.6	5.3	79
Av. C.V. (%)	28	0.8	17	2.8	4.8	1.1	1.4	4.7	5.8	1.2
Striatum										
n	23	23	23	23	23	23	23	23	23	23
Av. DLt ×	1.9	65	3.3	160	10.1	605	63	36	23	44
Av. C.V. (1%)	21	1.7	16	1.1	6.0	0.5	2.0	1.6	1.5	3.9

would be expected to be found with this method. NMN and 3MT were included as it has been shown [12–14] that these can be rapidly produced by enzymatic breakdown of NE and DA, respectively, at ambient temperatures, following death by decapitation. E was included for completeness. The large unknown fluorescent peak which coelutes with NE has been shown to be not electroactive at +0.7 V and somewhat higher potentials, in separations (not shown here) which achieve baseline separation of it and NE. It has been possible to achieve separation of all twelve compounds of interest plus the unknown compound but only with eluents having large (around 200 m*M*) buffer salt concentrations.

Stability of stock standard solutions at −70°C is often taken for granted but the instability of MHPG and HVA in the given diluents when stored under these conditions has been observed several times by this author. The better stability of MHPG stocks at 4°C compared to −70°C is notable. The results with spiked cortex analytes stored at −20°C were consistent with work with standards stored at −20°C (not shown here) and reinforce convincingly the suggestion that stability of MHPG, 5HIAA and 5HT solutions is unacceptable at −20°C.

TABLE V

RECOVERIES OF STANDARD ADDITIONS TO CORTEX ANALYTES

For each compound, n = 5. Rel. R (%) = 100 (maximum − minimum)/average.

Compound	Average (%)	C.V. (%)	Rel. R (%)
Fluorimeter			
TYR	100.7	0.8	1.8
MHPG	100.9	1.7	5.2
NE	96.3	2.4	7.3
E	100.5	3.7	9.2
NMN	101.3	0.9	2.6
DA	102.5	2.6	6.9
5HIAA	101.4	1.8	5.3
TRP	101.9	2.6	7.0
3MT	99.5	1.5	4.2
5HT	101.0	0.7	1.8
Average (%)	100.6	1.9	5.1
Minimum (%)	96.3	0.7	1.8
Maximum (%)	102.5	3.7	9.2
Amperometer			
MHPG	90.8	2.8	6.9
NE	97.5	1.3	3.7
E	91.8	1.3	3.8
DOPAC	91.2	1.1	3.2
NMN	94.6	1.1	3.0
DA	99.1	1.7	4.7
5HIAA	102.3	1.3	3.8
HVA	107.7	3.0	7.7
3MT	102.0	0.7	2.2
5HT	102.8	1.2	3.6
Average (%)	98.0	1.6	4.3
Minimum (%)	90.8	0.7	2.2
Maximum (%)	107.7	3.0	7.7

The excellent stability of working standards in diluent 3 led to the expectation that all compounds of interest in sample analytes using this or slight modifications thereof as homogenizing agent might be equally stable. This has not been demonstrated, though in general the stability of catechol-based compounds in sample analytes under a variety of conditions was reasonable. Degradation of some compounds might have been due to incomplete enzyme deactivation resulting from the relatively low $HClO_4$ concentrations used, but as the stability trials have shown, increasing acid concentrations tend to increase indole-based compound degradation without particularly improving the stability of other compounds. Experience with brain tissue and other homogenizing agents similar to homogenizing agent 1 but containing lower concentrations of $HClO_4$ suggests that insufficient acid results in inadequate protein denaturation and premature failure of the guard column by irreversible protein adsorption. With sample analytes prepared as described using homogenizing agent 1, guard columns each sustained at least fifty 25-μl sample analyte injections plus numerous standard injections. Most were replaced at this point but were still functioning adequately. It is possible that analytes stored at −70°C may have had some damaging contaminants removed due to freezing–thawing.

Overall, the stability work suggests that with homogenizing agent 1, for minimal losses of compounds each analyte should be prepared as close to the actual analysis time as possible. An acceptable alternative may be to prepare analytes as a group, then store them at 4°C until just before analysis the same working day. If analytes must be prepared days before analysis, they should be stored at −70°C in the interim, though it should be expected that significant losses of MHPG, 5HIAA and 5HT will occur. Storage at −20°C is not recommended.

Standard regression was excellent. Though the standard concentrations for some compounds were much in excess of those found in some analytes, this difficulty was obviated by digital data processing where peaks could be scrutinised to a degree limited only by detector noise. While the integration software was capable of automatic integration, all integration was either performed or at least checked manually. It is the experience of this author that virtually all algorithm-based integrators are prone to large errors unless signal levels greatly exceed detector noise; devices employing heuristic "expert-system" principles may eventually achieve truly reliable, unattended integration under all conditions.

Amperometric detection limits were clearly lower than those of the fluorimetric, detector as

shown in Table I; however, fluorimetric detection was still useful for many of the concentrations present, though the precision of the results near the detection limit obviously suffers. The advantages of having two (independent) detection methods rather than one is obvious and has been illustrated previously [15–20]. Some degradation of the guard columns employed was observed during these runs, resulting in reduction of peak height; also, the glassy carbon electrode normally suffers some reduction in sensitivity during usage for some of the compounds (the electrode was rejuvenated just prior to the start of, but not during, this set of particular runs), thus the detection limits reported should be fairly typical for routine analysis conditions.

Sample concentrations (Table II) as determined from fresh analytes were in agreement with expectations for these sample types [2–4, 21–23]. Where there is clearly a systematic major disagreement between results from the two detectors, the ratio rather than the relative range of results has been given.

Those instances where standard and sample retention times disagreed significantly (average ΔT outside -1 to 1%) coincided with significant disagreement between results for both detectors, except for (MHPG). With (MHPG), MHPG-2 peaks occurred on a rapidly descending baseline and while digital data processing still permitted such peaks to be adequately defined, the results may have been to underestimate the peak area. Some underestimation certainly occurred from the inability of the integration software to interpolate the concave baseline, approximating it by a line segment instead, though it is doubtful that this accounts for all of the disagreement. It was noted from MHPG-2 recoveries (Table V) that a depression of response (average 9%, $n = 5$) appears to occur though the standard addition results in a concentration much larger than that apparently naturally present. Literature values for MHPG in these rat brain regions [4,12] are much closer to results obtained for MHPG-2 than for MHPG-1, though such results have been amperometrically determined. This case illustrates the general problem of the large baseline disturbance which occurs at the solvent front with oxidative amperometry, and the implications for the quantitation of early-eluting peaks. Fluorimetric detection by contrast shows only a small disturbance there and the MHPG-1 peak appears quite "clean" (though could still contain a coeluting interferent). It is difficult therefore to be certain as to the true MHPG concentration present but it probably lies within the range of MHPG-1 and MHPG-2 results.

Disagreement between detectors for (E) results is probably due to the low concentrations apparently present, which appeared generally lower than the E-1 detection limit. Non-zero E-1 results were generally lower than those for E-2, and the E-2 result is thought to be an upper limit of any true E present. It seems clear that whatever concentrations are present are small.

The agreement of retention times of NMN in standards with standard-added NMN was similar (ΔT within -1.4 to -0.9%, $n = 10$) to that of NMN in standards with (NMN) in unspiked analytes (ΔT within -1.7 to -1.1%, $n = 10$), which suggests that the analyte matrix tends to shift NMN retention times to values significantly earlier than they would be in standards. Hence the relatively "early" NMN retention times consistently obtained for all sample types do not necessarily mean that NMN-1 and NMN-2 are artifacts, rather than true NMN. NMN-1 results were consistently lower than those for NMN-2 and this, coupled with the recovery data indicating that the analyte matrix does not bias NMN-1 results away from true NMN concentrations, suggests a true NMN concentration no greater than the NMN-1 result and considerably lower than that for NMN-2, which by implication appears to be coeluting with interferences. As indicated before, NMN concentrations in brain tissue from decapitated animals will be elevated by enzymatic degradation of NE, unless suitable procedures are adopted. They can thus serve as an indicator of technique.

For cerebellum DA results, DA-1 results were consistently higher than those for DA-2. For this sample type, retention times of DA-1 and DA-2 were shifted to significantly earlier values than

those for DA in standards or in hypothalamus and striatum, perhaps indicating coeluting interferences. Recovery data indicate that the analyte matrix does not bias results from either detector away from true DA concentrations, and true cerebellum DA concentrations are thus thought to be no higher than DA-2 results.

Results from both detectors for all sample types for 5HIAA, 3MT and 5HT and for DA (hypothalamus and striatum only) were in quite reasonable agreement, with average relative ranges in all cases over the nine runs being $<5.4\%$ (except cerebellum 1 5HIAA, average of 10.2%). The average of results from both detectors in these cases should thus be a good estimate of actual concentrations present. It was noted that these compounds all occur in the latter part of the separation.

As precision depends not merely on the absolute concentration present but on how this relates to detection limit, average results for between-run precision (Table IV) have been expressed in terms of detection limits and this given together with C.V. results. (MHPG) and (E) compose most of the cases having C.V. much greater than 10%. For E-1 and cerebellum E-2 results, this is probably because the concentrations obtained are less than the average detection limit. This may also be the case with (MHPG) where results for both detectors are mostly only slightly above the respective detection limits. With hypothalamus and striatum E, C.V.s of 17 and 16%, respectively, with results substantially above the detection limit may provide additional evidence of interference. With cerebellum DA-2 where the average C.V. was 41% (individual C.V.s within 31–47%) and results were 2.1–2.4 times detection limit, some type of interference is certainly suggested. With these and the few other low concentration cases excepted, precision for the analysis appears quite reasonable.

Recoveries (Table V) are generally good. Results between samples for a particular compound show good consistency with all C.V.s (n = 5) being $<3.7\%$. For fluorimetric detection, average recoveries are within 99.5–102.5% except for NE-1 with an average of 96.3%. For amperometric detection, MHPG-2, E-2, DOPAC and NMN-2 appear to show consistently depressed recoveries (though still greater than 90%) while the recovery of HVA is consistently elevated. MHPG, E and NMN are particularly interesting cases in that the corresponding recoveries for fluorimetric detection were very close to 100%, indicating no error in procedure and therefore strongly suggesting a real depression of amperometric response due to analyte matrix effects. By implication, confidence can also be expressed in the observed depression and elevation of DOPAC and HVA recoveries, respectively. The remaining average recoveries for compounds detected amperometrically lie within 97.5–102.8%.

The HPLC separation has proven quite stable over a period of at least a year with only minor adjustments required in the eluent to compensate for the effects of column ageing. In between uses the column is stored, as recommended, in acetonitrile–water (3:1, v/v) and it appears that eluent adjustments are necessary mostly after a storage period. If it were practical to keep the column always in equilibrium with the eluent, it is likely that even fewer changes would be necessary.

Both detectors performed without particular incident for the duration of the trials. However, during any given run the fluorimetric detector showed a continually decreasing response at least partly due to the lamp's output being attenuated by ozone accumulation in the excitation compartment (apparently a design oversight), ozone being a strong absorber in the ultraviolet. A catalytic mat supplied by the manufacturer and placed in the chamber obviated this effect for a short time, but appears to have deactivated for some unknown reason (the compartment is sealed; the mat is supposed to last indefinitely). This necessitated a stabilisation period of several hours before each run, though this was usually achieved before the day's work by using a timer. The behaviour of each detector signal at the solvent front has already been mentioned and is clearly an advantage for the fluorimetric detector.

Newly acquired facilities will provide the capability for dual-potential amperometric detection

as has been demonstrated [24] for a similar application, and it is planned to use this to investigate the purity of the peaks identified here as NE, DOPAC and HVA. While this approach does not use two truly independent detection methods, it should supply valuable suggestive data regarding peak purity otherwise unavailable.

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