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Simultaneous determination of catecholamines in rat brain tissue by high-performance liquid chromatography

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Abstract

A novel and highly sensitive method has been developed for the determination of catecholamines [noradrenaline (NA), dopamine (DA), serotonin (5-HT) and their metabolites 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA)] in brain tissue. The method uses isocratic reversed-phase HPLC with amperometric end-point detection. The calibration curve was linear over the range 10–150 pg on-column. The assay limits of detection for NA, DA, 5-HT, 5-HIAA and HVA were 3.8, 3.8, 6.8, 5 and 7.5 pg on-column, respectively. The mean inter- and intra-assay relative standard deviations (RSDs) over the range of the standard curve were less than 5%. The absolute recoveries averaged 99.1%, 99.5%, 97.7%, 99.5% and 98.8% for NA, DA, 5-HT, 5-HIAA and HVA, respectively. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Biogenic catecholamines play a very important role in the control and regulation of a variety of functions acting as neurotransmitters in both central and peripheral nervous systems. Measurement of brain catecholamines may be important for monitoring change in psychiatric disorders [1–3]. Also since drugs used in the control of psychiatric disorders act at catecholaminergic neurotransmitter sites it may be additionally important to determine them as this may provide valuable information in the elucidation of drug action.

The determination of catecholamines (see Fig. 1) in rat brain tissue requires a highly sensitive and selective assay procedure to measure the very low levels present. In past years a number of methods have been reported for measurement of catecholamines in both plasma and body tissues. However only a few of these papers have reported simultaneous measurement of more than two catecholamine analytes [4–12]. Shibuya et al. [13] reported a high-performance liquid chromatography (HPLC) method for the simultaneous measurement of catecholamines which utilised UV for end-point detection. In this method, the samples were chromatographed on a reversed-phase phenyl analytical column. The procedure was slow and cumbersome due to the use of a complicated liquid–liquid extraction and that each chromatographic run took more than 25 min. Additionally this method reported an assay detection limit of only 5–10 ng on-column. Alburges et al. [14] reported a relatively sensitive HPLC method which

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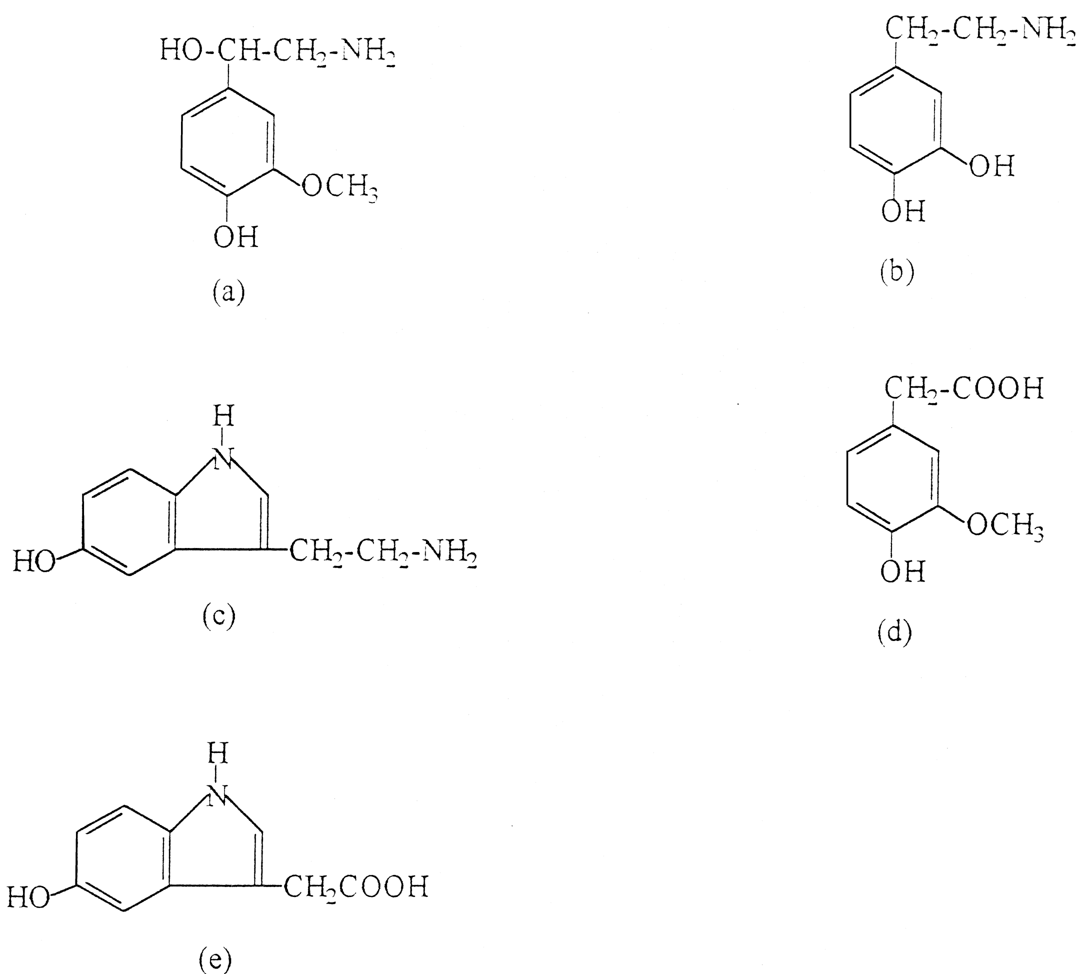

Fig. 1. Structures of (a) NA, (b) DA, (c) 5-HT, (d) HVA and (e) 5-HIAA.

used electrochemical detection. The detection limits for this procedure were 12, 6, 12, 18 and 12 pg for noradrenaline (NA), dopamine (DA), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA), respectively. The method used very a complicated mobile phase in terms of its composition whilst the low pH of 3.1 used, might jeopardise the chemical stability of the column. Additionally each chromatographic run took more than 30 min. Recently Duine et al. [15] reported a HPLC method which utilised amperometric end-point detection. The sensitivities of this assay for NA, DA, 5-HT, 5-HIAA and HVA were 15, 20, 11, 25 and 50 pg, respectively. Each chromatographic run took approximately 30 min. The poor chromatographic resolution and long retention time for 5-HT may have been a contributing factor to the variability in the intra- and inter-assay relative standard deviations (RSDs) (9 and 21%, respectively) for this analyte.

Our aim was to set up an assay procedure that was quick, simple, inexpensive to run, robust and would give a high degree of selectivity. We describe a method which we feel satisfies all the criteria. The method utilises isocratic reversed-phase HPLC with amperometric end-point detection.

2. Experimental

2.1. Materials

NA, DA, 5-HT, 5-HIAA and HVA were obtained from Sigma (Poole, UK). All other reagents used for the assay were of the highest grade available. Water was deionised and glass distilled prior to use.

Stock standard solutions of NA, DA, 5-HT, 5-HIAA and HVA were prepared at concentrations of 100 μg/ml in phosphate buffer (pH 5) and stored at −25°C. They were stable for at least one week when stored under these conditions. The working standards were prepared freshly for each assay from the stock solutions.

2.2. Apparatus

The HPLC system consisted of a Constametric 3000 pump (LDC, Stone, UK), a manual Rheodyne 7125 injection valve equipped with a 50-μl loop, a 3 μm particle size (150×4.6 mm, I.D.) C_{18} analytical column (Capital HPLC, Edinburgh, UK). End-point detection was achieved with an Intro amperometric detector (Antec, Leiden, The Netherlands). A Model 4400 integrator (Varian, Northants, UK) was used to analyse the chromatographic data. The operating potential was 0.75 V. This was chosen to be that potential at which the maximum response was given with the least amount of background noise for all analytes of interest (see Fig. 2).

The mobile phase consisted of 0.1 *M* KH_2PO_4–acetonitrile (91:9, v/v) and octane sulphonic acid (100 mg/l) adjusted to pH 4.75 (with 0.5 *M* K_2HPO_4). The mobile phase was filtered and degassed prior to use. The flow-rate was 1.0 ml/min. The analytical column was kept at 30°C.

Peak heights rather than areas in the chromatography were normally measured. Concentrations of all analytes were calculated by interpolation of their respective standard curves.

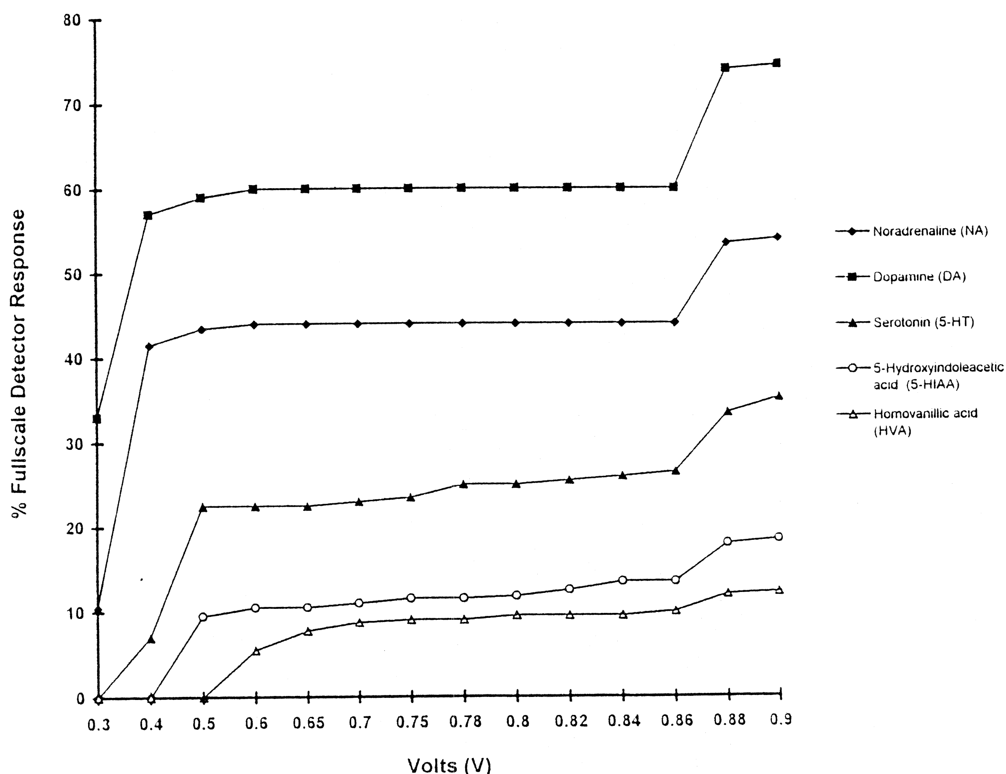

Fig. 2. Voltammograms for NA, DA, 5-HT, 5-HIAA and HVA at various oxidation potentials as measured on the Intro amperometric detector.

2.3. Procedure

Rat brain samples were collected on dry-ice and stored at −20°C until required for assay.

Working standards for the assay were prepared using the mobile phase as the diluent and consisted of five concentration points over the range 2–30 ng/ml.

Approximately 50 mg of rat brain tissue was weighed out, it was then homogenised for 1 min in 0.8 ml 0.1 *M* phosphate buffer (pH 5). The whole procedure was carried out on ice. The homogenate was centrifuged at 12 000 *g* for 4 min at 4°C, the resultant supernatant was transferred into a 12K cut-off filtration unit (Whatman, Maidstone, UK). The unit was centrifuged at 12 000 *g* for 10 min at 4°C. Normally 1 to 10 μl of the filtrate solution was injected into the HPLC system.

3. Results

Resolution and sensitivity were determined by injection of standard solutions (Fig. 3). The retention times of NA, DA, 5-HT, 5-HIAA and HVA were 2.8, 6.6, 15.4, 9.6 and 11.2 min, respectively. The linearities were verified over the assay range (10–150 pg on-column). Calibration curves were calcu-

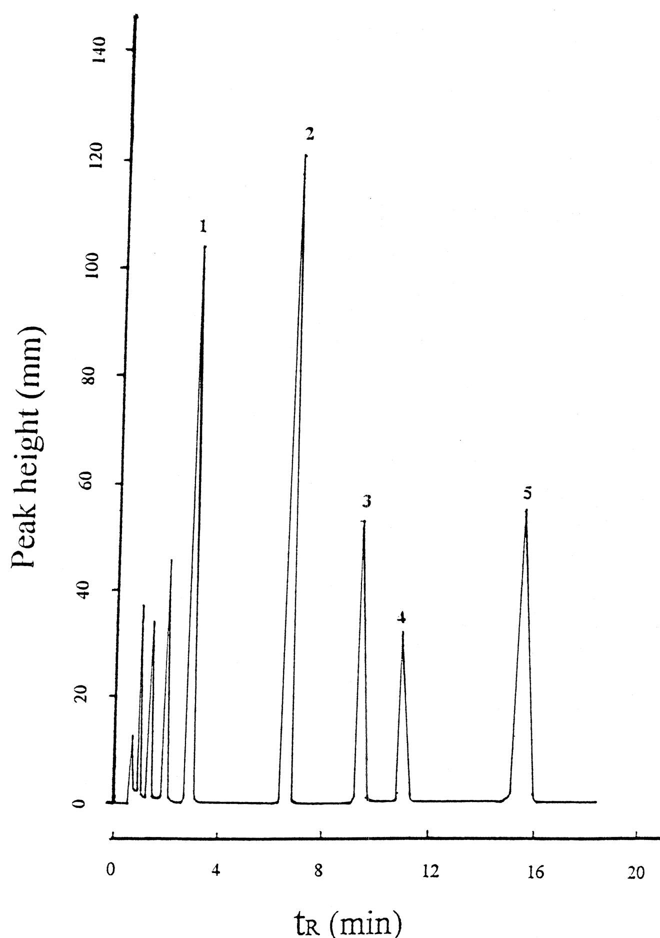

Fig. 3. Chromatogram of five catecholamine standards. Peaks 1, 2, 3, 4 and 5 represent NA, DA, 5-HIAA, HVA and 5-HT, respectively. The retention time for peaks 1, 2, 3, 4 and 5 were 2.8, 6.6, 9.6, 11.2 and 15.4 min, respectively, with a total run time of 16 min.

Table 1
Intra-assay precision and accuracy for the determination of NA, DA, 5-HT, 5-HIAA and HVA from rat brain tissue (n=6 replicates)

Actual value (pg)	Mean observed value±SEM (pg)					RSD (%)[a]				
	NA	DA	5-HT	5-HIAA	HVA	NA	DA	5-HT	5-HIAA	HVA
50	49.2±0.4	50.3±0.6	50.7±1.0	49.4±0.9	48.5±0.7	2.2	2.8	4.7	4.2	3.7
100	101.7±0.7	98.7±1.1	101.0±1.9	97.9±1.2	96.4±1.3	1.8	2.6	4.5	3.0	3.4

[a] The RSD of the method was calculated from rat brain tissue (20 mg) spiked with known amounts of NA, DA, 5-HT, 5-HIAA and HVA.

lated using the peak height versus catecholamine concentration.

The mean equations for NA, DA, 5-HT, 5-HIAA and HVA calibration curves were y=296.8x−0.0185 (r=0.9999, n=6), y=382.2x+0.078 (r=0.9999, n=6), y=162.0x+0.0655 (r=0.9997, n=6), y=157.6x−0.086 (r=0.9999, n=6) and y=168.4x+0.302 (r=0.9998, n=6), respectively. The intra-assay RSDs are given in Table 1. The mean inter-assay RSDs over the range of the respective method curves for NA, DA, 5-HT, 5-HIAA and HVA were 2.7, 3.1, 3.8, 2.9 and 4.4%, respectively (n=12 assays). The extraction recoveries for the analytes are given in Table 2. Sample extracts were stable for one week when stored out of light at −20°C.

Fig. 4 shows a profile of various endogenous catecholamines detected from rat brain following administration (intraperitoneal, i.p.) of 200 mg/kg of Hypericum perforatum extract (LI160, Lichtwer Pharma, Berlin, Germany). The animal was killed 2 h after dosing.

4. Discussion

Described here is a quick simple, and highly selective HPLC assay procedure with amperometric end-point detection. The detection limits (i.e., peak height equal to three-times baseline noise) were 3.8, 3.8, 6.8, 5 and 7.5 pg for NA, DA, 5-HT, 5-HIAA and HVA, respectively. This allows for the measurement of the very low levels of catecholamines present in rat brain tissue. Several methods of sample deproteinisation and preparation were tried, the majority of which gave rise to wide solvent fronts on the chromatographic trace, this included the saturation of the NA peak. However, homogenisation in 0.1 M phosphate buffer (pH 5.0) followed by homogenate filtration allowed for highly satisfactory chromatographic separation of the solvent front and analytes of interest.

The method demonstrates significant improvements over other published procedures. Higher recoveries, a reduced sample tissue requirement (50

Table 2
Recoveries of NA, DA, 5-HT, 5-HIAA and HVA from rat brain tissue (n=6 replicates)

Amount (pg)[b]	Recovery±S.E.M. (%)[a]				
	NA	DA	5-HT	5-HIAA	HVA
50	98.6±1.2	98.4±1.3	96.9±1.5	99.2±1.1	99.6±1.8
100	99.6±1.0	100.6±1.2	98.5±1.5	99.9±1.3	98.0±1.7

[a] For each result two 20-mg pieces of brain tissue from the same cortical area were weighed out and homogenised as described in the text. The first homogenate was treated as a blank and to the second was added known amounts of the analytes. Both homogenates were then deproteinised by filtration as described. The final recovery was calculated from the difference between the determined results of the spiked and the blank homogenates and was expressed as a percentage recovery. Results were calculated against non-extracted standards in mobile phase.

[b] Amounts of each catecholamine added to brain tissue.

S.E.M.=Standard error of the mean.

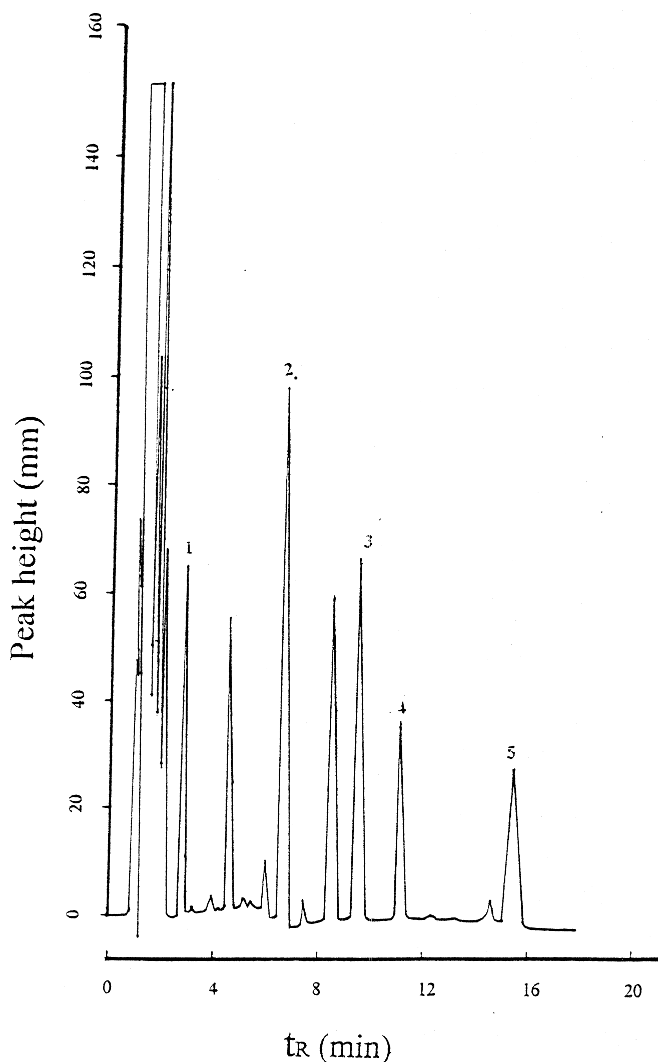

Fig. 4. Chromatogram of a prepared brain tissue sample (50 mg) from a rat after administration of 200 mg/kg of LI160. Peaks 1, 2, 3, 4 and 5 represent NA (194.3 ng/g), DA (230.6 ng/g), 5-HIAA (381.4 ng/g), HVA (186.5 ng/g) and 5-HT (159.5 ng/g), respectively. Unlabelled peaks are due to extraneous substances from the brain tissue and are of unknown origin (but possibly represent other extracted brain neurotransmitters).

mg), a simple sample preparation procedure and good chromatographic separation of all analytes have all been attained. In addition chromatography took only 16 min for each assay run.

5. Conclusion

A novel, rapid, simple and highly specific HPLC method has been described for the measurement of catecholamines in rat brain tissue. The assay is inexpensive to run and may easily be set up in the laboratory. The method has clear advantages over previously described procedures.

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