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ESTIMATION OF PLASMA CATECHOLAMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION IN PATIENTS WITH SUBARACHNOID HAEMORRHAGE

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SUMMARY

Plasma catecholamine levels were determined by high-performance liquid chromatography with electrochemical detection following alumina extraction. Mean individual recoveries of 50.5, 49.9 and 48% were obtained for norepinephrine, epinephrine and dopamine, respectively, and the limits of detection for each catecholamine were 0.15, 0.34 and 0.6 pmol/ml. Total analysis time for each plasma sample was approximately 1 h. Catecholamine levels were measured in plasma from control subjects and the ranges obtained were: norepinephrine, 0.33—5.98 pmol/ml; epinephrine, 0- 4.77 pmol/ml; dopamine, 0—0.8 pmol/ml. When patients with subarachnoid haemorrhage were investigated, the ranges were found to be: norepinephrine, 0.23—7.27 pmol/ml; epinephrine, 0–4.91 pmol/ml; dopamine, 0–0.23 pmol/ml.

INTRODUCTION

Subarachnoid haemorrhage is a bleeding into the subarachnoid space of the brain with, unfortunately, a high degree of mortality amongst affected patients. Although there are criteria for the clinical assessment of the severity of subarachnoid haemorrhage in patients [1], the catagories into which patients

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are placed constitute a short ill-defined scale. Decisions on the necessity or advisability of remedial neurosurgery, therefore, are complicated by the fact that the vascular pathology, especially vasospasm, may be considerably more, or less, than the immediate clinical symptoms and hence the designated categories suggest.

Biochemical tests have been sought to improve the accuracy of the assessment scale and to provide better information on which delicate neurosurgical decisions can be made [2, 3]. Several authors [2, 4] have suggested that plasma catecholamine measurements could be of use in this context after finding that levels of the catecholamine norepinephrine were raised in patients who had suffered a subarachnoid haemorrhage. Furthermore, the degree of increase in norepinephrine concentration in the plasma was reported to give a direct correlation with the degree of severity of the subarachnoid haemorrhage [2, 4].

To investigate this problem, reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ED) was utilized to measure plasma catecholamines in patients suffering from subarachnoid haemorrhage. Factors affecting the chromatography and detection of the catecholamines were examined and the method was used in measuring catecholamine levels in patients suffering subarachnoid haemorrhage.

EXPERIMENTAL

Chemicals

The internal standard dihydroxybenzylamine and the catecholamines norepinephrine, epinephrine and dopamine were purchased from Sigma London (Poole, U.K.), while the ion-pairing agent sodium 1-octanesulphonic acid was supplied by Aldrich (Gillingham, U.K.). Helium was obtained from British Oxygen (Harlow, U.K.). All other chemicals, supplied by BDH (Poole, U.K.), were of analytical grade and were used without further purification.

Apparatus

The liquid chromatographic system employed a Pye LC-XPD high-pressure pump (Pye Unicam, Cambridge, U.K.) with an additional air-column damping system (HPLC Technology, Macclesfield, U.K.). Sample injection via a Rheodyne 7010 valve fitted with a 100-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) facilitated loading of a 30 mm × 4.6 mm I.D. Rheodyne guard cartridge containing 10-μm C_{18} reversed-phase packing (HPLC Technology), this preceding a 250 mm × 4.6 mm I.D. stainless-steel analytical column packed with 5-μm Spherisorb C_{18} (Phase Separations, Queensferry, U.K.).

Catecholamines were detected using a BAS 4A electrochemical detector with a glassy carbon electrode incorporating a 0.0127-cm gasket (Bioanalytical Systems, West Lafayette, IN, U.S.A.) at an electrode potential of +0.7 V vs. Ag—AgCl [5]. The flow cell of the detector was surrounded by a Faraday cage which, along with the pump, injection valve and columns, used a single-point earth. To minimise fouling of the electrode, a minimal buffer flow-rate of 0.5 ml/min was maintained through the system at all times. During the assay procedure, the temperature was maintained at 26°C by a constant-temperature

water circulator and this gave a pressure of 152 bar at a mobile phase flow-rate of 1.4 ml/min.

Solvents and reagents

Aliquots of 50 mg of acid-washed alumina (100–250 mesh) [6], heated at 200°C for 2 h, were viable in the extraction process for up to five days after preparation if stored desiccated under vacuum. Solutions of reducing reagent (450 μl of 1 *M* metabisulphite, 7.5 ml of water and 2.5 ml of 50 m*M* EDTA),

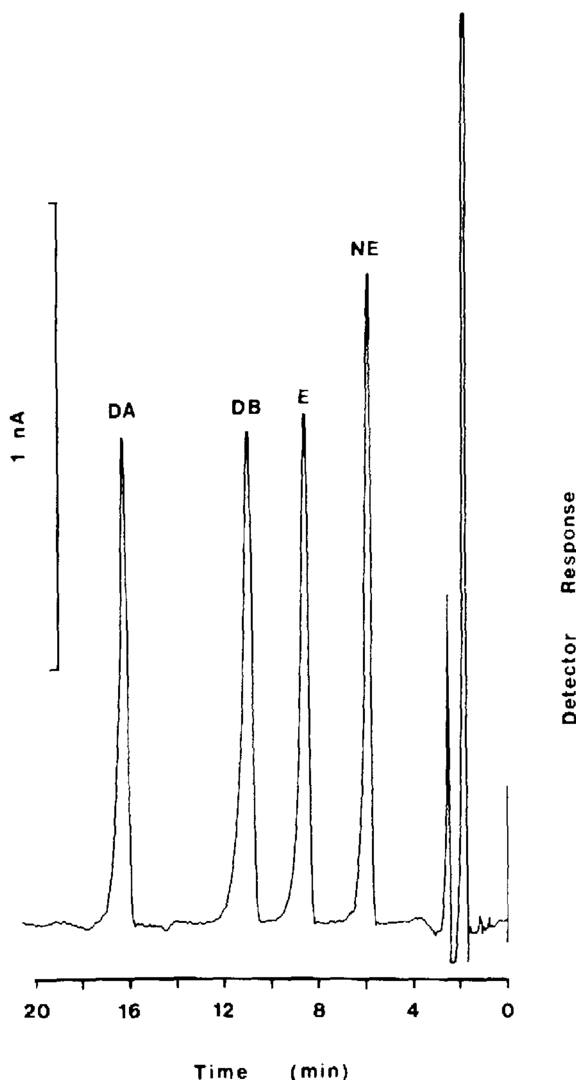

Fig. 1. Chromatogram of HPLC assay of 10 pmol/ml each of the three catecholamines and the internal standard. Solvent consisted of 85 m*M* citrate buffer (pH 4.05) containing 5% methanol and 2 m*M* octanesulphonic acid flowing at 1.4 ml/min; the column was 250 mm × 4.6 mm I.D., 5-μm Spherisorb C_{18}, with a 30 mm × 4.6 mm Rheodyne C_{18} guard cartridge. Peaks: DA = dopamine; DB = dihydroxybenzylamine (internal standard); E = epinephrine; NE = norepinephrine.

wash solution (150 μl of 1 *M* metabisulphite, 50 ml of water and 2.5 ml of 50 m*M* EDTA), Tris buffer (1 *M* Tris—HCl, pH 8.6) and acid extractant (150 μl of 1 *M* metabisulphite, 10 ml of 150 m*M* perchloric acid and 2.5 ml of 50 m*M* EDTA) were also prepared.

Mobile phase was filtered through 0.45-μm membrane filters (Millipore, London, U.K.) and degassed with helium prior to use. Recycled, the mobile phase could be used continuously for periods of up to one week. Deionised double-distilled (in glass) water was used for the preparation of the mobile phase and stock solutions.

Standards

Individual standard solutions of 100 pmol/ml catecholamines or internal standard, constituted in 10 m*M* hydrochloric acid and 0.1 m*M* EDTA, were stored at 4°C [7, 8].

Plasma extraction and chromatography

Blood was collected into prepared tubes containing 0.25 m*M* metabisulphite and 4 m*M* EDTA [8, 9]. The plasma obtained was either assayed immediately, or stored at −20°C [10, 11]. Using the reagents described, catecholamines were extracted from plasma onto alumina [12] with desorbed catecholamines being drawn through a capillary column containing lead-free glass wool to remove alumina particles prior to injection of the extract into the liquid chromatograph. Column resolution and sensitivity of the detector were checked daily by injecting a freshly prepared solution of 10 pmol/ml catecholamines and internal standard [13]. Quantification of the catecholamines was achieved using peak-height ratios [10, 13, 14] against standards processed through the extraction and assay procedure.

RESULTS

After investigation, optimal conditions for the mobile phase were chosen

TABLE I

INTER- AND INTRA-ASSAY PRECISION FOR THE DETERMINATION OF CATECHOLAMINES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

Coefficients of variation (C.V.) were calculated from the peak-height ratios given between catecholamine concentrations and internal standard. NE is norepinephrine; E is epinephrine; DA is dopamine.

Catecholamine concentration (pmol/ml)	Intra-assay				Inter-assay			
	n	C.V. (%)			*n*	C.V. (%)		
		NE	E	DA		NE	E	DA
1	6	5.77	5.35	5.84	12	10.95	9.38	13.84
2	6	7.56	5.19	3.8	12	9.49	6.94	12.72
4	6	4.95	6.05	2.04	12	8.6	9.66	10.42
8	6	1.69	3.6	3.85	8	5.51	7.02	8.11
16	6	2.11	3.11	3.34	8	3.3	4.57	8.54

as 2 m*M* octanesulphonic acid with 5% methanol in 85 m*M* citrate buffer (pH 4.05) [7, 10, 13] giving a total analysis time of approximately 20 min (Fig. 1).

Linearity of the detection system was demonstrated by analysing known amounts of catecholamines with 10 pmol/ml dihydroxybenzylamine as internal standard. Responses to the different catecholamines were found to be linear over a range of 0—16 pmol/ml for each catecholamine investigated. Correlation coefficients (*r*) in the detection system were between 0.9993 and 0.9998 for the three catecholamines. The sensitivity of the detection system with a noise level of 3% f.s.d. and a signal-to-noise ratio of 10:1 was found to be 0.03 pmol/ml norepinephrine, 0.04 pmol/ml epinephrine and 0.08 pmol/ml dopamine.

Linearity of the extraction procedure was demonstrated by extracting and

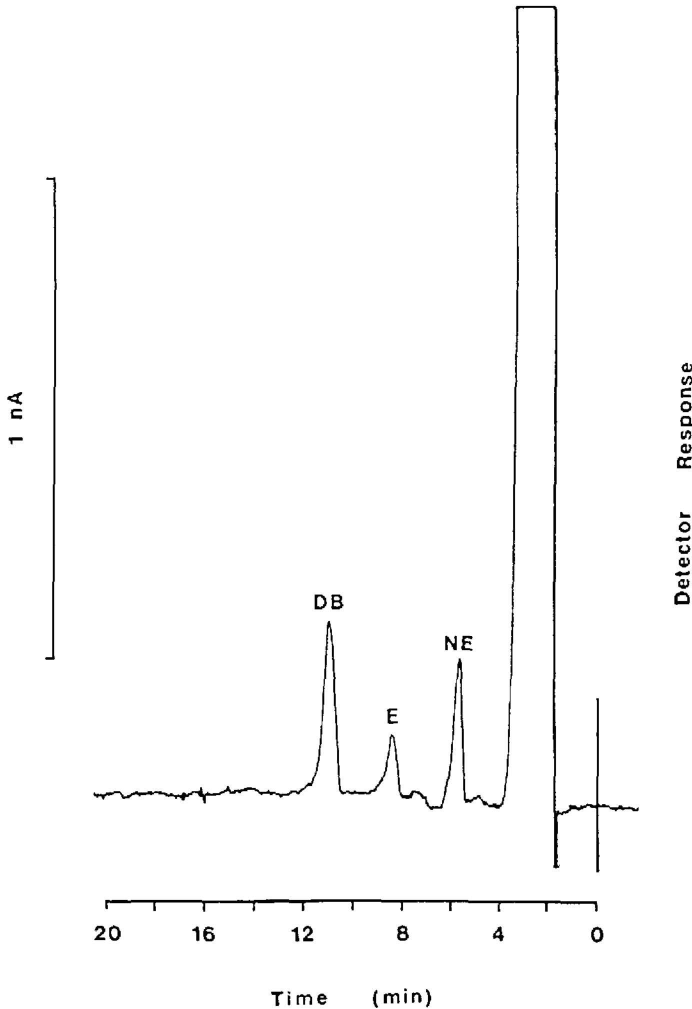

Fig. 2. Chromatogram of HPLC assay of plasma of patient suffering a subarachnoid haemorrhage showing catecholamine levels of 6.9 pmol/ml norepinephrine (NE) and 4.0 pmol/ml epinephrine (E). Conditions as in Fig. 1. DB = Dihydroxybenzylamine (internal standard).

assaying pooled plasma to which known amounts of catecholamine and internal standard had been added. Peak-height ratios of exogenous catecholamines were found to be linear over the range 0—16 pmol/ml for each catecholamine investigated. Correlation coefficients (r) in the extraction and assay systems were: norepinephrine, 0.996; epinephrine, 0.98; dopamine, 0.996. Sensitivities for the extraction and assay system were 0.15 pmol/ml norepinephrine, 0.34 pmol/ml epinephrine and 0.6 pmol/ml dopamine. Precision was evaluated for both intra- and inter-assays (Table I) as the coefficient of variation (C.V.) for the peak-height ratio at each concentration of catecholamine added.

Analysis of plasma catecholamine levels from 30 patients who had entered hospital for non-sympathetically related disorders yielded mean (± S.D.) control values of 3.25 ± 1.21 S.D. pmol/ml norepinephrine, 1.36 ± 0.83 S.D. pmol/ml epinephrine and 0.09 ± 0.08 S.D. pmol/ml dopamine. However, while norepinephrine was determined in all the patient plasmas, epinephrine and more often dopamine were not detectable in some patient plasmas, ranges being 0.33—5.98 pmol/ml norepinephrine, 0—4.77 pmol/ml epinephrine and 0—0.8 pmol/ml dopamine. These subjects were compared with 42 patients suffering from subarachnoid haemorrhage (Fig. 2), drawn from a large clinical trial which excluded any patients on anti-hypertensive medication. Patients entered the clinical trial up to three days post-ictus and were removed from the trial at surgery. Few patients were admitted within 48 h of ictus and most patients were operated upon within twelve days of ictus. Consequently, the

TABLE II

PLASMA CATECHOLAMINE LEVELS IN PATIENTS SUFFERING SUBARACHNOID HAEMORRHAGE AT DAYS POST-ICTUS

Days post-ictus	Sample number	Plasma catecholamine levels (mean ± S.D.) (pmol/ml)	
		Norepinephrine	Epinephrine
1	2	6.1 ± 1.64	2.48 ± 1.66
2	2	2.97 ± 2.82	1.06 ± 0.16
3	5	1.34 ± 0.76	1.12 ± 1.46
4	5	1.35 ± 1.15	0.52 ± 0.65
5	8	1.12 ± 1.06	0.23 ± 0.27
6	5	1.08 ± 0.75	0.33 ± 0.32
7	4	0.89 ± 0.52	0.14 ± 0.17
8	6	1.66 ± 1.17	1.38 ± 1.82
9	2	1.1 ± 0.75	0
10	2	0.89 ± 0.81	0.25 ± 0.27
11	7	1.01 ± 0.91	1.01 ± 1.76
12	2	3.01 ± 2.61	0.37 ± 0.52
13	2	0.94 ± 0.61	0.99 ± 1.39
14	1	2.26	0.66
15	2	2.77 ± 1.57	0.14 ± 0.2
16	2	1.54 ± 2.18	0
18	3	1.11 ± 0.23	0.58 ± 0.38
20	1	2.21	1.36
24	3	2.29 ± 0.33	1.85 ± 1.45
Means derived from ranges		0.23—7.27	0—4.91

number of subarachnoid haemorrhage patients available for investigation at each post-ictal day varied (Table II). The number of measurements made on each patient differed and, of the 64 observations made, approximately half were single measurements of individual patients. Plasma catecholamine levels in patients who had suffered subarachnoid haemorrhage showed ranges of 0.23—7.27 pmol/ml norepinephrine, 0—4.91 pmol/ml epinephrine and 0—0.23 pmol/ml dopamine though, as with Table II and Fig. 3, three sample values on post-ictal days two, six and four are not tabulated because these contained 17.32 and 16.32 pmol/ml norepinephrine and 13.05 pmol/ml epinephrine, respectively, values that did not fulfil Chauvenet's criteria for a relationship with the mean levels.

DISCUSSION

Watson [11] reported an interfering non-catecholamine peak co-eluting with norepinephrine that appeared when plasma was allowed to stand for more than 1 h at room temperature but not at —5°C or below. Similarly, Eriksson and Persson [10] found light-induced peaks interfering with the chromatogram, especially with epinephrine. Such light-induced problems were not experienced during this assay though small artifact peaks, eluting with k' values of 6.8 and 9.1, appeared with 5 m*M* octanesulphonic acid. As these peaks appeared in chromatograms of standards after injection of catecholamines in water, acid or plasma, but only at the higher concentration of octanesulphonic acid, it was considered that they were an artifact arising from an interaction between the high ion-pairing agent concentration, water in the injection medium and residues on the reversed-phase column, possibly uncapped silanol residues.

Prolonged exposure of reversed-phase columns to ion-pairing agents is claimed to reduce the working life of the column. It was thus suggested to wash the column overnight in a stream of water with the glassy carbon electrode activated [13, 15]. Following this procedure, however, reproducible chromatograms of catecholamine standards could only be obtained after running a new buffer for several hours. Such an observation supports the deduction of Crombeen et al. [16] that the ion-pairing agent adsorbs onto the column to give an ion-exchange-like effect. Instead, the advice of Michaud et al. [7] was employed whereby the system was run at a reduced flow-rate during standby, a steady baseline and reproducible results being rapidly obtained when required.

Control levels of norepinephrine and epinephrine were comparable with those reported by other authors using electrochemical detection following liquid chromatographic separation [13, 17]. A concensus on dopamine levels, however, is somewhat more uncertain [13], with our findings agreeing with the lower end of a wide range (0.05—0.75 pmol/ml) of reported values [17, 18]. Measurement of plasma catecholamine levels in patients suffering subarachnoid haemorrhage (Fig. 3) showed an expected elevation of norepinephrine and epinephrine levels during the first two days following ictus [2, 4]. The subsequent fall over the following four to seven days is more surprising as, interestingly, this corrsponds to a period, acknowledged by many authors [2, 19, 20] of increased susceptibility to vasospasm. Certainly, it has not been possible to

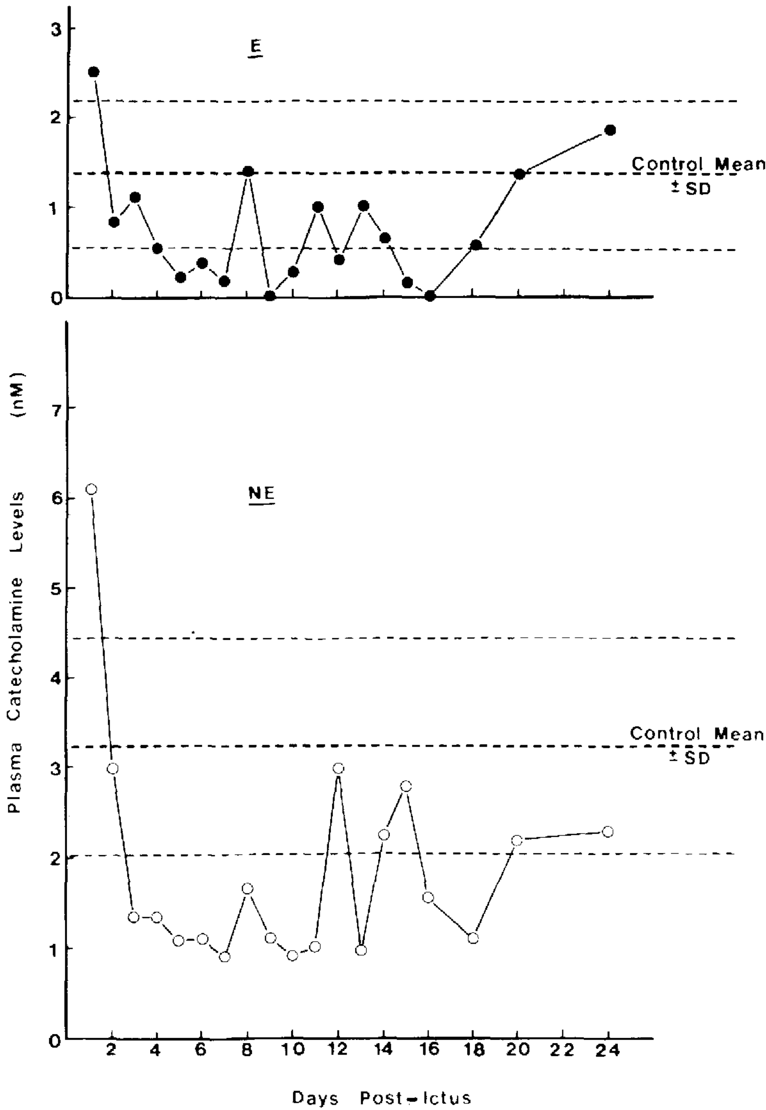

Fig. 3. Mean plasma levels of epinephrine (E) and norepinephrine (NE) in patients at each day following subarachnoid haemorrhage compared with control values. Numbers of patients per day varied (Table II).

correlate changes in catecholamine levels with any post-ictal clinical or surgical changes. The observation, however, may deserve further investigation, and as such, the sensitive and reliable method reported here should be of further value in the estimation of catecholamines when applied to groups of subarachnoid haemorrhage patients.

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