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ROUTINE DETERMINATION OF PLASMA CATECHOLAMINES USING REVERSED-PHASE, ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A procedure is described for the determination of plasma catecholamines using reversed-phase, ion-pair high-performance liquid chromatography coupled with electrochemical detection. Optimisation of chromatographic conditions with respect to detector performance and adherence to procedures and precautions described, render the method applicable to both neurochemical research and routine clinical analysis. The limit of quantitative detection of the method was found to be approximately 30 pg per injection for individual catecholamines. A single chromatographic run, providing adequate resolution of each component, could be completed in approximately 12 min.

INTRODUCTION

High-performance liquid chromatography (HPLC) coupled with electrochemical detection (HPLC—ElCD) is a technique which has found increasing application in neurochemical research. Interest in the technique has centred on the advantages it possesses for the analysis of biogenic amines and their metabolites. Recently two state-of-art reviews of HPLC—ElCD and its applications in neurochemical research have appeared in the literature [1, 2].

Catecholamines are one of the groups of compounds amenable to analysis by HPLC—ElCD. The relatively simple procedure of sample preparation, ease of oxidation of the catecholamines to their corresponding quinones, sensitivity of electrochemical detection and the specificity of chromatographic separation means that HPLC—ElCD has distinct practical advantages over existing methods of determination where chemical modification of the catecholamine structure prior to analysis is required. Examples of established methods of catechol-

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amine analysis include gas—liquid chromatography with electron-capture detection [3, 4], gas chromatography—mass spectrometry [5—7], HPLC with ultraviolet detection [8] or fluorescence detection [9, 10], other fluorescence methods [11, 12] and radioenzymatic assay [13—15]. The low concentration of catecholamines in plasma demands that the analytical method has a high degree of both sensitivity and selectivity. Until the advent of HPLC—ElCD, radioenzymatic assay had proved to be the most effective method applicable to subjects where relatively small blood samples (< 5 ml) were available. This technique, however, suffers from the complexity of the sample preparation procedure and is both time consuming and expensive.

The first report of the application of HPLC—ElCD to the analysis of catecholamines was made by Kissinger et al. [16]. The use of the technique for the determination of plasma catecholamines was described by Hallman et al. [17] who used cation-exchange chromatography to achieve separation. More recently the use of reversed-phase ion-pair separation techniques has improved the efficiency, sensitivity and versatility of the chromatography of these compounds [18]. The analysis of picogram quantities of catecholamines that are typically encountered in plasma samples, however, poses additional difficulties due to the small currents (ca. 100—800 pA) generated at the electrode surface as a result of solute oxidation; electrical instability, with a consequent decrease in instrument performance often presents a problem to the chromatographer. The present communication describes a reversed-phase, ion-pair HPLC system, designed to overcome these difficulties. It utilises a high efficiency bonded silica analytical column specifically designed for use in the ion-pair mode and chromatographic conditions have been optimised with respect to parameters known to influence HPLC—ElCD performance. The method is shown to be suitable for the routine laboratory determination of catecholamines in plasma.

EXPERIMENTAL

Reagents and standards

Adrenaline bitartrate, noradrenaline bitartrate, dopamine hydrochloride, 3,4-dihydroxybenzylamine hydrobromide and tris(hydroxymethyl)aminomethane (Tris) were supplied by Sigma (London) (Poole, Great Britain). Methanol (HPLC grade) and sodium octane-1-sulphonate were purchased from Fisons Scientific Apparatus (Loughborough, Great Britain). Aluminium oxide, 70—230 mesh, activity grade 1, neutral washed, was supplied by E. Merck (Darmstadt, G.F.R.).

The aluminium oxide was activated by the method of Anton and Sayre [19] and stored at 37°C until required. The internal standard, 3,4-dihydroxybenzylamine, was prepared as a 0.1 μM solution in 0.1 M perchloric acid (containing 400 μM sodium metabisulphite). Stock solutions of other catecholamines were similarly prepared to the required concentration. The 0.5 M Tris—HCl buffer, pH 8.6, and a diluted solution used in the alumina washing procedure for the isolation of catecholamines, were prepared as described by Adams [20].

Equipment

The liquid chromatograph comprised an Altex 100A pump (Altex Scientific,

Berkeley, CA, U.S.A.), a Rheodyne Model 7125 injection valve fitted with a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) and a 25 cm × 4.6 mm I.D. stainless-steel analytical column packed with 5 μm diameter Ultrasphere IP particles (Altex Scientific). The analytical column was fitted with a 5 cm × 4.6 mm I.D. precolumn packed with 30—38 μm diameter Co-Pell ODS (Whatman Lab. Sales, Maidstone, Great Britain). The detection system was a Model LC-4 amperometric detector fitted with a TL-5 glassy carbon electrode assembly (Bioanalytical Systems, West Lafayette, IN, U.S.A.). All chromatographic components were supplied by Anachem (Luton, Great Britain).

Chromatography

The mobile phase consisted of 780 ml of the acetate—citrate buffer (pH 5.2), described by Keller et al. [21], 220 ml of methanol, and sodium octane-1-sulphonate (5 m*M* final concentration). Solvent was pre-filtered through a 0.5-μm (pore diameter) Fluoropore membrane filter (Millipore, London, Great Britain) and degassed prior to use. Water used for mobile phase preparation was glass distilled and then deionised. The mobile phase flow-rate was 1.2 ml/min, and the electrode potential was set at +0.6 V vs. the Ag/AgCl reference electrode.

Optimisation of chromatographic performance

The use of the electrochemical detector to determine picomole and femtomole quantities of oxidisable (or reducable) material may be associated with increased levels of detector noise resulting in a reduction of chromatographic performance. A glassy carbon electrode cell provided improved overall performance as compared with a packed carbon paste electrode. Signal-to-noise ratio was enhanced, the detector sensitivity was stable and the electrode surface was unaffected by the passage of air bubbles occasionally formed in the mobile phase. Any surface contaminants could be washed from the electrode using water or methanol. Individual electrode cells have been found to possess a working life of greater than one year, whereas carbon-paste cells required repacking after 2—3 weeks' use.

Previous reports of catecholamine determination by HPLC—ElCD have described the use of the electrochemical detector at a number of oxidation potentials [17, 22—24]. We investigated the response of the detection system to catecholamine oxidation at values of electrode potential over the range +0.35 V to +0.85 V vs. the Ag/AgCl reference electrode in the mobile phase described. It was found that although a maximum detector response was obtained at +0.8 V (Fig. 1), an electrode potential of +0.6 V provided a response with sufficient sensitivity for the determination of picogram quantities of catecholamines, but with minimum interference from solvent effects and electrical noise.

The pH and ionic strength of the mobile phase have both been shown to affect the response of the electrochemical detector to catecholamine oxidation [18]. Optimum response of the electrode was obtained in the pH range 5.0—6.0 and ionic strength (of phosphate) of 0.07 *M* [18]. The mobile phase was composed accordingly with a pH of 5.2 and a total ionic strength of approximately 0.1 *M*.

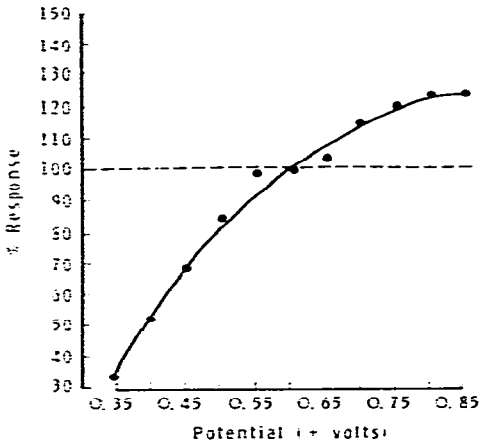

Fig. 1. Response of the electrochemical detector to catecholamine oxidation at a number of electrode potentials in the chromatographic system described. A range of electrode potentials was selected (vs. the Ag/AgCl reference electrode) and the total detector response obtained from the oxidation of individual catecholamines at each potential is represented. The broken horizontal line represents detector response at the chosen optimum oxidation potential of +0.6 V.

Long-term exposure of bonded silica columns to ion-pairing agents may significantly reduce column life [25]. In order to preserve optimum column performance, the chromatograph, when out of use (e.g. overnight), was maintained in a solvent flow of pure water of 0.2 ml/min, and with the electrode activated. The system could be quickly restored to operating conditions by equilibration of the column with mobile phase (15 column volumes approximately) and preparation of the system with two injections of a catecholamine standard mixture solution (total preparation time 1 h). Maintaining the system in this manner also enhanced detector stability.

Collection and storage of blood for analysis.

The stability on storage of blood samples collected for catecholamine determination was investigated in order that a protocol for routine use in a hospital laboratory, ward or clinic might be devised. Petersson et al. [26] recently reported that storage of untreated blood samples at room temperature for several hours did not result in any loss of plasma noradrenaline or adrenaline. We decided, however, to observe certain basic precautions regarding storage of samples for analysis. Blood samples (10 ml) were normally collected into lithium-heparin tubes containing 200 μl of 0.1 *M* sodium metabisulphite and stored immediately at 4°C. Under these conditions, plasma catecholamines were found to be stable for at least 24 h, the maximum time that samples would normally be stored under such conditions. The plasma was then removed and stored at —20° C until analysis.

Plasma for use in the preparation of calibration standards was obtained from a plasma pool. Aliquots of this plasma were treated with increasing amounts of catecholamines and taken through the extraction procedure. Calibration curves for individual catecholamines were constructed from the data obtained.

Isolation of catecholamines from plasma

A 2-ml sample of deproteinised plasma (protein denatured by the storage of plasma in the frozen state was separated by centrifugation at 800 *g* at 4°C), contained in a 15 ml capacity glass conical centrifuge tube, was treated with 200 μl of the internal standard solution. Then 400 μl of 0.5 *M* Tris—HCl, pH 8.6, followed by 20 mg of activated alumina, were added and the contents of the tube shaken gently for 15 min on a spiral mixer (Denley, Billingshurst, Great Britain). Following centrifugation at 600 *g* for 2 min, the supernatant was removed and the alumina washed three times with a buffer preparation [20], centrifuging each time as above. The catecholamines were eluted from the alumina into 50 μl of 0.6 *M* perchloric acid (containing 400 μ*M* sodium metabisulphite). Following centrifugation at 800 *g* for 3 min, 20 μl of the supernatant were injected onto the chromatograph.

RESULTS

Resolution and sensitivity of the chromatographic system were determined daily by the injection of a 20-μl aliquot of a catecholamine reference solution. A typical chromatogram obtained from analysis of this standard mixture is shown in Fig. 2a. A complete separation of individual components of the

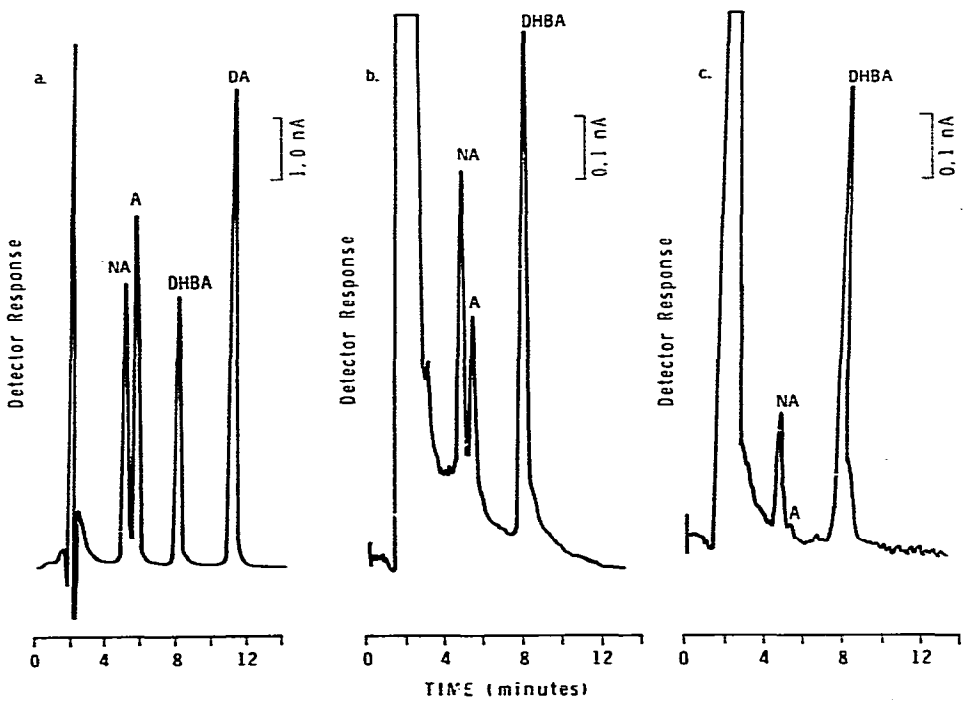

Fig. 2. Chromatograms of HPLC assay of plasma catecholamines. (a) Standard mixture containing 7 ng each of noradrenaline (NA) and adrenaline (A), 6.6 ng of 3,4-dihydroxybenzylamine (DHBA), 9.5 ng of dopamine (DA) injected; (b) plasma extract from a patient on admission to hospital following a road traffic accident; NA = 3.2 pmol/ml, A = 1.6 pmol/ml; (c) plasma extract from the same patient after 24 h treatment; NA = 1.6 pmol/ml, A = < 0.2 pmol/ml.

TABLE I

CHROMATOGRAPHIC PARAMETERS OF THE HPLC SYSTEM FOR THE SEPARATION OF CATECHOLAMINES

Compound	Retention time (min)	Capacity ratio (k')	Resolution factor (R_s)			
			NA	A	DHBA	DA
NA	4.84	1.69	—	1.2	5.3	8.6
A	5.38	1.99	1.2	—	8.1	8.1
DHBA	7.75	3.31	5.3	4.5	—	5.2
DA	10.92	5.07	8.6	8.1	5.2	—

mixture was obtained and a total sample running time of approximately 12 min recorded. Values of column capacity ratios (k') and resolution factors (R_s) are shown in Table I.

The linearity of both the extraction procedure and detector response (determined from peak area) was verified for each catecholamine over the anticipated range of assay. The former was investigated by assaying pooled plasma to which known amounts of noradrenaline (NA), adrenaline (A) and dopamine (DA) had been added and determining the peak area ratios (sample vs. internal standard), obtained for each compound. Calibration curves were constructed for each compound (Fig. 3); in each case a linear relationship between catecholamine concentration and peak area ratio was observed over the concentration ranges studied. The equations for the calibration curves obtained were as follows:

NA: $y = 0.12x - 0.012; r = 0.990$
A: $y = 0.20x - 0.009; r = 0.995$
DA: $y = 0.14x - 0.006; r = 0.998$

Each point on the calibration curve was established from the mean of five determinations.

The endogenous catecholamine concentrations of the pooled plasma were determined to be NA, 1.5 pmol/ml; A, none detected; DA, none detected.

The linearity of detector response was confirmed by the injection of known amounts of catecholamine standards directly onto the chromatograph. Response for each compound was found to be linear over the range investigated (NA, 0—8.4 ng, $r = 1.0$; A, 0—3.5 ng, $r = 1.0$; DA, 0—4.0 ng, $r = 1.0$). For the routine application described, the amplifier was operated at a sensitivity of either 1.0 nA/V or 2.0 nA/V full scale deflection (f.s.d.). At the former level of sensitivity a noise level of ± 1.0% f.s.d. was observed, which enabled a quantitative detection limit for each catecholamine of about 30 pg per injection to be achieved. This is similar to detection limits reported by other authors [17, 27, 28].

The precision of the extraction procedure and chromatography was evaluated by processing aliquots of pooled plasma containing known amounts of NA, A and DA. Values of inter- and intra-assay are shown in Table II. The recovery from the extraction procedure was determined by comparing the yields from a series of extractions of plasma containing known amounts of

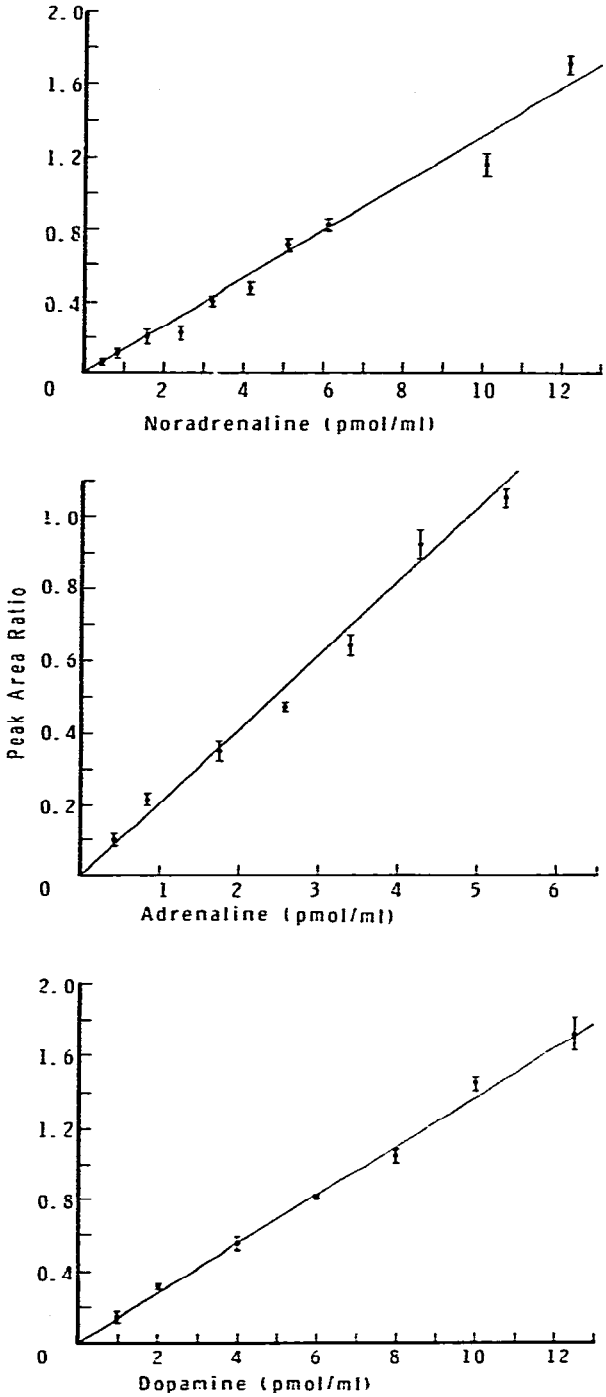

Fig. 3. Calibration curves for the determination of noradrenaline (NA), adrenaline (A) and dopamine (DA) in plasma by the assay procedure described.

TABLE II

INTER- AND INTRA-ASSAY PRECISION FOR THE HPLC DETERMINATION OF PLASMA CATECHOLAMINES

	NA			A			DA		
	Concn. added (pmol/ml)	*n*	C.V. (%)	Concn. added (pmol/ml)	*n*	C.V. (%)	Concn. added (pmol/ml)	*n*	C.V. (%)
Inter-day	0.73	5	10.2	0.5	5	9.2	0.9	5	13.1
	1.45	5	9.7	1.5	5	2.6	1.8	5	10.0
	11.6	5	4.4	6.0	5	5.4	14.4	5	4.7
Intra-day	0.73	9	12.1	0.5	9	7.7	0.9	9	13.7
	1.45	9	11.9	1.5	9	6.9	1.8	9	14.1
	11.6	8	5.8	6.0	8	6.3	14.4	8	5.5

TABLE III

PLASMA NORADRENALINE AND ADRENALINE LEVELS IN ACCIDENT CASUALTIES OF VARYING INJURY SEVERITY

Blood samples were taken from casualties as soon as possible following admission to hospital (Day 0). Further samples were taken at 24-h intervals following admission (Day 1, 2, etc.). Values = mean ± S.D.

Day	Admitted ward		Admitted intensive therapy	
	NA (pmol/ml)	A (pmol/ml)	NA (pmol/ml)	A (pmol/ml)
0	2.96 ± 1.4	1.37 ± 1.2	11.98 ± 7.8	7.52 ± 5.2
	n = 56		*n* = 10	
1	2.77 ± 1.7	0.58 ± 0.49	6.0 ± 3.4	0.82 ± 0.36
	n = 52		*n* = 7	
2	2.98 ± 2.1	0.45 ± 0.6	5.1 ± 4.2	0.96 ± 1.03
	n = 48		*n* = 7	
3	2.67 ± 1.5	0.35 ± 0.28	4.1 ± 3.2	0.60 ± 0.23
	n = 42		*n* = 8	

Discharged following treatment (Day 0; *n* = 15), NA = 2.75 ± 0.8 pmol/ml, A = 0.59 ± 0.38 pmol/ml.

catecholamines with standard solutions. Individual recoveries of 48.7% (NA), 50% (A), 47.5% [3,4-dihydroxybenzylamine (DHBA)], 41.2% (DA) were calculated, representing an overall recovery of 47%. This was lower than has been reported by other workers [17, 27, 29]. However, as was shown in Fig. 3, the procedure was linear over the assay ranges required for each catecholamine.

The present method has been applied to routine clinical analysis and a number of research studies. One such study is an investigation of the report that plasma catecholamines are elevated in response to trauma following injury.

Unless the injuries are life-threatening, a return to normal levels is seen within 72 h [30]. Figs. 2b and c are chromatograms obtained from the analysis of plasma samples of a 59-year-old male injured in a road traffic accident. Blood samples were taken on admission to hospital and following 24 h treatment. Plasma NA and A were found to be 3.2 pmol/ml and 1.6 pmol/ml respectively on admission (Fig. 2b), but following 24 h treatment (Fig. 2c) had fallen to 1.6 pmol/ml and 0.2 pmol/ml (limit of quantitation). Control levels for plasma NA and A of 1.4 ± 0.7 pmol/ml and 0.19 ± 0.08 pmol/ml respectively were calculated from data compilations from two sources [27, 31].

Analysis of the data obtained from 81 casualties has suggested that elevation of plasma NA and A following injury is related to the severity of injury sustained. Table III shows mean plasma noradrenaline and adrenaline levels from casualties whose injury severity has been assessed by the degree of medical treatment required. Patients were grouped into those who could be treated and discharged the same day, those who required admission to a routine surgical ward and those requiring intensive therapy. These increases in plasma catecholamines also correlated with the numerical evaluation of injury severity. Validation of these observations and assessment of their clinical relevance is currently being undertaken. However, the requirement for a reliable, sensitive method for the determination of plasma catecholamines in this and other biomedical applications is of paramount importance.

DISCUSSION

The HPLC—ElCD system described in this paper has been shown to possess the sensitivity, selectivity and reproducibility required for the determination of picogram quantities of catecholamines from 1—2 ml plasma samples. Improvement in chromatographic performance has been achieved through the use of a high-efficiency bonded silica analytical column specifically designed for use in the ion-pair mode and the problem of electrical instability sometimes encountered in the use of electrochemical detection at high sensitivity was overcome through adherence to the procedures and precautions described above. Column life was extended by eliminating unnecessary exposure of the analytical column to mobile phase containing ion-pairing agent (typical column life ca. 2000 injections).

Previous studies have proved the validity of HPLC—ElCD for the measurement of biogenic amines [28, 32—36]. Comparison of the method with some recent reports on the application of HPLC—ElCD to the determination of plasma catecholamines suggests an improvement in sensitivity of up to four-fold. Jenner et al. [22] required up to 4 ml of plasma for their assay of plasma catecholamines; half of their final eluate was subjected to analysis at a sensitivity of 0.5 nA/V f.s.d. The method of Goldstein et al. [27] was also performed at a similar level of detector response, using half of the total eluate from a 1-ml plasma extraction. In the present assay, basal levels of NA (0.5—1.5 pmol/ml) could be easily determined using 1 ml plasma. However, for basal levels of A (0.1—0.3 pmol/ml), 2 ml were required with a limit of quantitative detection set at 0.2 pmol/ml plasma equivalent. In our experience we have found that the response characteristics of individual glassy-carbon

electrode cells may vary considerably. Caution should therefore be exercised in the selection of electrodes to ensure optimum performance is achieved. However, once installed into an HPLC—ElCD system, their stability of performance renders them more suitable for high-sensitivity work than carbon paste electrodes.

In the traces shown in Figs. 2b and c no chromatographic evidence for the presence of DA was found. The definition of a normal DA level in plasma has been somewhat controversial [36]. Hallman et al. [17] reported a range of resting plasma dopamine levels in healthy volunteers of between < 0.05 and 0.23 pmol/ml, whilst Fenn et al. [37] put the level as high as 0.75 pmol/ml. Since response to stress may not provide significant changes in plasma DA levels [28], normal levels within the lower range quoted above would fall below the limit of detection of dopamine.

HPLC—ElCD possesses the significant practical advantages of speed of sample processing (individual samples may be processed in less than 30 min) and low cost as compared with other methods for the measurement of biogenic amines. In addition to the improvements in technique reported above, these render the present method suitable for research application and routine clinical analysis.

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