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Direct injection of plasma and urine in automated analysis of catecholamines by coupled-column liquid chromatography with post-column derivatization*

PER OLOF EDLUND† and DOUGLAS WESTERLUND‡

Department of Bioanalytical Chemistry, Research and Development Laboratories, Astra Läkemedel AB, S-151 85 Södertälje, Sweden

Abstract: Adrenaline, noradrenaline and dopamine have been quantified by direct injection of plasma and urine in a liquid chromatographic system comprising three columns, one packed with a boronic acid gel and two with reversed-phase material. The catecholamines were selectively adsorbed on the boronic acid gel and separated by ion-pair chromatography on the reversed-phase columns. Dopamine was detected by coulometry, while noradrenaline and adrenaline were detected by fluorimetry as trishydroxyindoles after post-column coulometric oxidation and alkaline rearrangement. The reaction rate constants of the post-column reactions were determined by a flow injection analysis approach.

Limits of detection were 0.04, 0.05 and 1.6 pmol for noradrenaline, adrenaline and dopamine, respectively. Endogenous plasma levels of noradrenaline and adrenaline could be quantified with a precision (RSD) of 2–4%.

Keywords: *Catecholamines; plasma; urine; post-column reaction; coulometry; fluorimetry; trishydroxyindoles; flow injection analysis.*

Introduction

The determination of catecholamines in biological fluids has been a challenge to the analytical chemist since their discovery, due to their low concentrations and their susceptibility to oxidation. There has been great interest in catecholamine analysis, especially for cardiovascular investigations and in neuroscience. A large number of methods have been described and several review articles and books are available on the subject [1–9].

The most sensitive assays have been obtained with gas chromatography–mass

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† To whom correspondence should be addressed.

‡ Present address: Department of Analytical Pharmaceutical Chemistry, University of Uppsala, Biomedical Center, Uppsala, Sweden.

spectrometry [10, 11] and with radioactive enzymatic techniques [12–15]. These methods are expensive and laborious, so that liquid chromatography with electrochemical [16–20] or fluorescence detection after post-column derivatization [21–24] have become popular alternatives. The catecholamines are usually isolated from plasma and urine by adsorption on alumina [16–20], ion exchange [25] or complexation with boronic acid gels [26–30] prior to chromatography. The different separation and detection principles have in a few cases been combined with column-switching to obtain automated methods [24, 31–33].

Electrochemical detection offers approximately equal sensitivity for all three catecholamines. Among the derivatization procedures which produce fluorescent products the ortho-phthalaldehyde method excludes adrenaline, while dopamine does not react in the trishydroxyindole method [34]. Electrochemical detection principles are often disturbed by gradient effects in the mobile phase, caused for example by column-switching procedures. Fluorimetric detection is less sensitive to such effects, while derivatization to form trishydroxyindoles is highly selective for catecholamines. When the reaction is performed in the post-column mode the conventional procedure is to use three reactors, one for each step in the reaction [21–24]. This may introduce problems attributable to excessive extra-column dispersion and interfering pulsations from the reagent pumps.

Methods for the isolation and detection of catecholamines compatible with column-switching and with efficient chromatographic systems have been studied in the present work. Suitable conditions for direct injection of plasma and urine on boronic acid gels have been developed. The coulometric oxidation and alkaline rearrangement of noradrenaline and adrenaline to trishydroxyindoles have been studied by flow injection analysis. The completed method gave quantitative yields of the trishydroxyindoles with acceptable extra-column dispersion.

Experimental

Chemicals and reagents

Adrenaline bitartrate (A), noradrenaline bitartrate (NA), dopamine hydrochloride (DA), isoproterenol hydrochloride (Isopr.) and 3,4-dihydroxyphenyl acetic acid (DOPAC) were obtained from Sigma (St Louis, MO, USA).

N-Ethylnoradrenaline hydrochloride (EtNA), adrenolutine and trisacetylated noradrenolutine were synthesized at the Department of Organic Chemistry, Astra Läkemedel AB, Södertälje, Sweden. Sodium decylsulphate was obtained from Research Plus Inc. (Bayonne & Denville, NJ, USA). Methanol, sodium hydroxide and buffer substances were all of analytical grade quality. Water was deionized in an Elgstat Spectrum water purification system (Lane End, Buckinghamshire, UK).

Apparatus

The liquid chromatograph consisted of a Kontron valve switching unit (model 670) with a programmer, model 200 (Kontron AG, Zürich, Switzerland). Three columns were coupled together in the switching unit. The first column (10 × 4.6 mm) was packed with phenylboronic acid gel (Affi-Gel 601, Bio Rad Laboratories, Richmond, CA, USA) for plasma samples, or for urine samples, a 20 × 3.8 mm column, packed either with 5-μm dihydroxyboryl silica (Serva, Heidelberg, FRG) or 5-μm Aba-silica (a gift from Magnus Glad, Dept. of Pure and Applied Biochemistry, Chemical Center, University of Lund, Sweden). The second column (20 × 4.6 mm) was a pre-packed cartridge containing 5-

μm Supelcosil LC-18 DB and the last column, 75 × 4.6 mm, was packed with 3-μm Supelcosil LC-18 DB (Supelco Inc., Bellafonte, PA, USA).

The last column was connected to a coulometric detector, Coulochem model 5100 A (Environmental Science Assoc. Inc., Bedford, Mass., USA); the outlet of the coulometric detector was connected to a tee coupling (Valco, Houston, Texas, USA). A stainless-steel capillary was connected to the same tee at an angle of 180° to the outlet of the column, for addition of the derivatization reagent (10% m/v NaOH). The third position of the tee was connected to a Shimadzu fluorescence detector (model RF-530; Shimadzu Corporation, Kyoto, Japan) via a teflon capillary for post-column reaction. The mobile phases and the reagent were pumped using two Eldex pumps, model 1004-A and 1610-A (Eldex, Menlo Park, CA, USA), a model 300 pump (Applied Chromatography Systems Ltd., Luton, Bedfordshire, UK) and a Milton Roy minipump (Laboratory Data Control, Berkeley, CA, USA) for columns one, two, three and the reagent, respectively. The output signals from the coulometric and fluorimetric detectors were connected to a two channel integrator, model PU 4810 (Pye Unicam Ltd, Cambridge, UK). A scheme of the complete system is presented in Fig. 1.

Chromatographic system

Column 1: Boronic acid affinity column. A polyacrylamide gel substituted with phenylboronic acid (Affi-Gel 601) was used for plasma samples, and a silica based

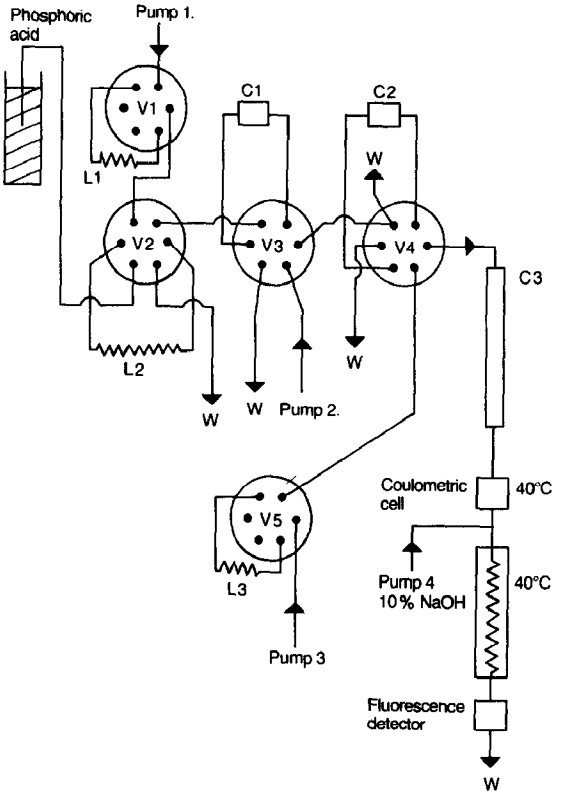

Figure 1
Column-switching, derivatization and detection system. L 1 = 1 ml; L 2 = 2 ml; L 3 = 45 μl, for direct injection. C 1 = Boronic acid affinity column; C 2 = enrichment column; and C 3 = main column.

material for urine samples. The polyacrylamide gel column was assembled from a Swagelok® union (¼″), two polymeric filters with wide pores and two low-volume connectors. The filters were prepared from the inlet filters of liquid–solid extraction columns (Bond Elut® Analytichem).

The low-volume connectors were prepared from stainless steel capillaries (0.5 mm i.d.), stainless steel tubing (¼″) and teflon spacers (see Fig. 2). The column was packed with a slurry of Affi-Gel 601 in mobile phase (phosphate buffer) by gravity flow, followed by suction with a water aspiration pump. The boronic acid silica was slurry-packed into Waters pre-columns by conventional methods (methanol–slurry, 100 bar).

During injection, the mobile phase consisted of phosphate buffer (μ = 0.1, pH 7.5) with 2 mM decylsulphate and 0.3 mM EDTA. The flow-rate was set at 0.6 ml/min by pump 1. The catecholamines were eluted with 0.2 M and 0.1 M phosphoric acid containing 2 mM decylsulphate for plasma and urine samples, respectively. A step gradient of phosphoric acid solution was generated by means of loop 2 (2 ml) by switching valve 2.

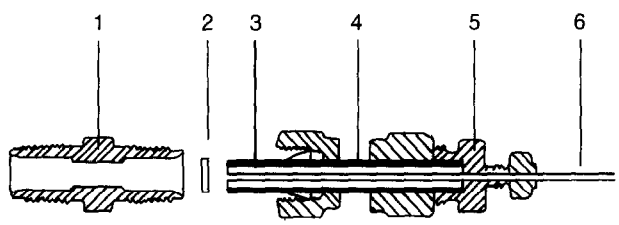

Figure 2
Boronic acid pre-column for plasma samples. The column was assembled as described in the text, utilizing: (1) ¼″ union; (2) wide-pore polymeric filter; (3) Teflon spacer; (4) ¼″ tubing; (5) ¼″ to 1/16″ reducing union; and (6) capillary.

Column 2: Enrichment column. The catecholamines were eluted with phosphoric acid from the boronic acid column, and were enriched as ion pairs with decylsulphate on a reversed-phase column. A pre-column packed with octadecylsilica (5-μm Supelcosil LC-18-DB, 20 × 4.6 mm) was used for enrichment. The mobile phase consisted of citrate buffer (μ = 0.1, pH 5.0) with 2 mM decylsulphate and 0.3 mM EDTA during conditioning. The catecholamines were eluted with a mobile phase containing 25% v/v methanol (see main column below). The flow-rate was set at 1 ml/min by pump 2.

Column 3: Main column. A reversed-phase column (3-μm Supelcosil LC-18 DB, 75 × 4.6 mm) was used for separation of the different catecholamines as ion pairs with decylsulphate. The mobile phase consisted of phosphate buffer (μ = 0.1, pH 6.65)–citrate buffer (μ = 0.1, pH 6.65)–methanol (37.5:37.5:25 v/v/v) with 2 mM decylsulphate and 0.3 mM EDTA. The flow-rate was set at 0.9 ml/min by pump 3.

Column switching

Plasma (1 ml) or urine (60 μl) were injected directly onto column 1 for 6 min after switching valve 1. The catechols were eluted onto the enrichment column by backflushing with phosphoric acid for 2.05 and 1.5 min for plasma and urine samples, respectively. The catecholamines were eluted from the enrichment column to the main column by back flushing with a buffer containing 25% v/v methanol for 2 min. These switching events are presented in Table 1.

Table 1
Sequence of column-switching procedures

Time after injection (min)		Switch of valve No.	Event
Urine	Plasma		
0.00	0.00	1	Sample is injected onto the boronic acid column with buffer pH 7.5 during 6 min
6.00	6.00	2	Catecholamines are eluted from the boronic acid column by back-flushing with phosphoric acid (0.1 and 0.2 M), during 1.5 and 2 min for urine and plasma, respectively
6.05	6.05	3	Columns 1 and 2 are connected in series, and the amines are enriched as ion pairs with decylsulphate on column 2
7.5	8.05	3 Reset	Column 1 and 2 are disconnected from each other
7.6	8.15	4	The sample is back-flushed from the enrichment column to the main column with methanol–buffer pH 6.65 (25:75, v/v)
9.0	9.5	2 Reset	The boronic acid column is conditioned with buffer pH 7.5
9.6	10.1	4 Reset	Elution from the enrichment column to the main column is completed. The catecholamines are separated as ion pairs with decylsulphate on the main column. The enrichment column is conditioned with buffer pH 5.0, containing 2 mM decylsulphate
16.6	17.2	End	The switching cycle is completed, and the next sample injected

Coulometric detection

The coulometric cell comprises two separate working electrodes coupled in series. Channels one and two were operated at +0.3 V and +0.4 V versus the reference, respectively. Channel one was used for detection and channel two was used to obtain quantitative aminochrome formation. The cell heater was switched on (40°C).

Alkaline rearrangement

Adrenaline and noradrenaline were converted to adrenochrome and noradrenochrome in the coulometric cell. Trishydroxyindole (THI) derivatives were formed after basification and heating. The effluent from the coulometric cell was mixed with 10% m/v sodium hydroxide (0.6 ml/min) in a tee coupling, followed by a teflon capillary, 6.1 m × 0.4 mm, knitted according to Engelhardt [35], placed in a thermostatted water bath at 40°C. The fluorescence of the trishydroxyindoles was detected at 510 nm after excitation at 400 nm.

Determination of cyclization rate constants

The cyclization rate of noradrenaline quinone was studied by coulometric experiments in a separate chromatographic system. A short reversed-phase 3-μm ODS-column, 30 × 4.6 mm (Perkin–Elmer) was connected between a loop-valve injector (20 μl) and a coulometric guard cell (ESA model 5020), followed by a teflon capillary and a second coulometric cell (ESA model 5010). The coulometric cells were operated at +0.6 V versus the reference, and the second coulometric cell was used for detection of leuconoradrenochrome.

Different lengths of teflon capillaries were used to vary the reaction time for cyclization. The apparent rate constants were determined at ambient temperature (22–23°C) as a function of the pH of the mobile phase. The mobile phase consisted of phosphate buffer (μ = 0.1)–methanol (90:10) with 1.7 mM nonylsulphate. The first-order rate constants were determined by linear regression of the rate equation:

$$\ln \frac{A_\infty - A}{A_\infty} = - k_{obs} \cdot t \quad (1)$$

where A = peak area, A_∞ = peak area at infinite reaction time, k_{obs} = observed first-order rate constant and t = time of the reaction. A long teflon capillary (6 m × 0.5 mm) was used to obtain an estimate of A_∞.

Rearrangement rate of noradrenochrome

The rearrangement rate of noradrenochrome to noradrenolutine was studied by flow injection analysis. A short reversed-phase column was inserted between a pump and a loop-valve injector (20 μl) to obtain a pulse-free flow, followed by a coulometric guard cell, a short teflon capillary (200 × 0.5 mm), a second coulometric cell, tee for addition of sodium hydroxide, teflon capillary and fluorimetric detector. The coulometric cells were operated at +0.6 V versus the reference and the fluorimetric detector was used for detection. Different lengths of coiled teflon capillaries were used to vary the rearrangement time. The rearrangement rate constant was determined at 40°C by linear regression of rate equation (1), where k_{obs} was replaced by k_3, the rearrangement rate constant.

Noradrenaline and adrenaline were injected into the flow injection system. The trishydroxyindole derivatives (lutines) of adrenaline and noradrenaline flowed into the fluorescence detector after mixing with sodium hydroxide in a short teflon capillary (300 × 0.5 mm).

Trishydroxyindole was prepared from the acetyl derivative by hydrolysis as follows. The acetyl derivative was dissolved in methanol purged with helium. The methanol solution was diluted in 1 M sodium hydroxide containing 0.05 M sodium dithionite. The solution was acidified with acetic acid after 1 min, diluted with mobile phase and injected. Adrenolutine was dissolved in methanol, diluted with mobile phase and injected. The responses obtained after derivatization and direct injection of the corresponding lutines were used to calculate the yields in the derivatization system.

Sample preparation

Venous blood samples were collected in heparinized Venoject tubes (Leuvren, Belgium). The blood was centrifuged and the plasma stored in polypropylene tubes at

−20°C. Urine samples were acidified with hydrochloric acid to pH 2.5–3.5 and stored in polypropylene tubes at −20°C.

The samples were thawed, mixed and centrifuged and then mixed with internal standard solution (0.95 + 0.05); 1 ml of plasma or 60 μl of urine was injected directly onto the boronic acid column. *n*-Ethylnoradrenaline was used as internal standard for noradrenaline and adrenaline, and isoproterenol for dopamine.

Standard solutions for calibration

The catecholamines were dissolved and diluted with 0.1 M perchloric acid, dispensed in polypropylene tubes and frozen at −20°C. An aliquot of the stock solutions was thawed daily and diluted with nitrogen-purged phosphate buffer (μ = 0.1, pH 3.5). 50 μl was used for calibration of plasma, and 60 μl for urine samples, respectively.

Distribution of NA, DA and DOPAC on boronic acid gel

The distribution ratios of noradrenaline, dopamine and dihydroxyphenyl acetic acid to Affi-Gel 601 were determined by batch experiments. The catechols were equilibrated with the gel at different pH values, and the aqueous phases were analysed by liquid chromatography. The distribution ratios between the swollen gel and the aqueous phase were calculated.

Results and Discussion

Chromatographic system

Catecholamines are usually isolated from plasma and urine by adsorption on alumina [16–20] or by complexation with boronic acid gels [26–30] in batch operation procedures prior to chromatography. Liquid–liquid extraction with other complexation agents has also been used [36]. The separation methods mentioned have been adapted to liquid chromatography with column switching to obtain automated methods.

Goto *et al.* [31, 32] used a pre-column packed with alumina in a column-switching system for catecholamines. The alumina column was used for injection of plasma ultrafiltrates and urine, but plasma could not be injected. Yamatadani *et al.* [24] used a pre-column packed with a cation exchanger for injection of plasma samples pretreated by protein precipitation. The catecholamines were eluted from the pre-column as complexes with boric acid at neutral pH. Hansson *et al.* [33] used a pre-column with boronic acid silica, combined with reversed-phase chromatography for determination of DOPAC in urine and tissue homogenates.

The concentrations of adrenaline and dopamine in plasma are in the range of 0.05–0.7 nmol/l [9]. About 0.05–0.1 pmol of noradrenaline and adrenaline can be determined both by electrochemical detection, and by fluorimetric detection of the THI products. About 1 ml of plasma has to be analysed to obtain a signal-to-noise ratio >5 for adrenaline.

The direct injection of large volumes of plasma samples into a liquid chromatographic system normally causes two major problems. Firstly, plasma proteins and other endogenous macromolecules may precipitate onto the support, at least if it is silica based, and this may result in a continuous pressure increase. Secondly, there must be adequate margins in the retention of the analytes on the first column in order to create enrichment effects and to prevent break-through of the analytes.

Boronic acid columns

Immobilized boronic acid is commercially available bonded to gels as well as to silica. The preparation of boronic acid-substituted silica has recently been described [33, 37]. The gels have high affinity for *cis*-diols, e.g. catechols, carbohydrates, ribonucleosides and ribonucleotides. Affi-Gel 601 consists of phenyl boronic acid bound to Bio-Gel P6, a polyacrylamide gel with a cut off at 6000 daltons. The distribution of noradrenaline, dopamine and DOPAC between Affi-Gel 601 and phosphate buffers of different pH was studied by batch experiments (Table 2). The increase in distribution with increasing pH demonstrates that the anionic form of phenylboronic acid (pK_a = 8.8) is responsible for the complex formation with catechols. The distribution of the amines was about three times higher compared with DOPAC, which can probably be explained by a difference in net charge of the catecholboronic acid complex. The amines are mainly present in their protonated form in the pH-range studied (pK_a = 8.9 for NA), so that they will form an uncharged complex with phenylboronic acid. At pH >7.5 a very high degree of adsorption to the gel was obtained. However, even at low pH values ($D \approx 1.4$) an adsorption of nonspecific character remains.

Table 2
Distribution on boronic acid gel*

pH	Distribution ratio		
	NA	DA	DOPAC
1.20	1.34	1.42	1.50
6.08	11.9	7.27	2.59
6.58	27.5	17.8	6.42
7.16	101	68.6	18.9
8.04	115	115	37.5
8.82	249	216	76

* Affi-Gel 601 (0.17 g) was washed with methanol (5 ml), water (5 ml), 0.1 M sodium hydroxide (5 ml) and five times with phosphate buffer (pH 7.0, μ = 0.1; 5 ml). The swollen gel (1 ml) was equilibrated with phosphate buffer (μ = 0.1; 5 ml) containing EDTA (0.25 mM), sodium sulphite (0.04 mM), NA (4.78 μM), DA (4.5 μM) and DOPAC (5.1 μM). The samples were shaken for 20 min at 25°C, and 20 μl of the aqueous phase was analysed by liquid chromatography.

When the gel is packed into a column, the retention volume will be given by the equation:

$$V_R = V_i + D \cdot V_g \quad (2)$$

where V_R = retention volume, V_i = interstitial volume, D = distribution ratio between the gel and aqueous phases, respectively, and V_g = gel volume. Table 2 and equation (2) show that a very small column can be used if the pH of the mobile phase is >7.5. A 10 × 4.6 mm column will give a retention volume of about 10 ml, but breakthrough will occur at a lower volume due to band broadening. Since the distribution decreases substantially with decreasing pH, the catecholamines can be eluted onto the next column in a small volume of acidic buffer.

Enrichment column

Amines can be separated as ion pairs on reversed-phase columns. In general, retention increases with the lipophilicity and concentration of the counter ion, and decreases with increasing concentrations of organic modifier in the mobile phase. Thus amines can be enriched on the top of the column if they are injected with an aqueous buffer containing a lipophilic counter ion; they can be desorbed by applying a buffer containing a certain amount of organic modifier.

Alkylsulphates with different chain lengths were tested as counter ions for enrichment of noradrenaline on small reversed-phase columns. The break-through and retention volumes were measured (see Table 3). Very lipophilic counter ions were required to obtain sufficient retention for enrichment. However, equilibration times were excessively long for the most lipophilic alkylsulphates (decylsulphate ≈10 h and laurylsulphate ≈20 h); furthermore, the chromatographic efficiency decreased when the chain length was increased from ten to twelve carbons. Decylsulphate was therefore chosen as showing the best compromise between equilibration time and enrichment effect.

Table 3
Break-through volumes of NA on enrichment columns

Column	Mobile phase	V_b* (ml)	V_r† (ml)
ODS, 5 μm 30 × 2.1 mm i.d.	3 mM octylsulphate phosphate buffer pH 3.7	0.55	0.62
PRP-1, 10 μm 30 × 2.1 mm i.d.	3 mM octylsulphate phosphate buffer pH 3.8	0.80	1.00
ODS, 5 μm 30 × 2.1 mm i.d.	1.5 mM laurylsulphate phosphate buffer pH 3.8	0.96	1.08
ODS, 5 μm 30 × 4.6 mm i.d.	1.5 mM laurylsulphate phosphate buffer pH 3.8	4.60	5.35
ODS, 3 μm 30 × 4.6 mm i.d.	2mM decylsuphate phosphate buffer pH 4	7.30 (5.1‡)	7.60 (5.4‡)
ODS, 5 μm 20 × 3.8 mm i.d.	1.5 mM laurylsulphate phosphate buffer pH 6.65	8.2	10.4
ODS, 5 μm 20 × 4.6 mm i.d.	2 mM decylsulphate phosphate buffer pH 5.0	6.0	6.8

* V_b = break-through volume.
† V_r = retention volume.
‡ Break-through and elution volumes were determined after a step gradient of mobile phase from the main column (10 ml; phosphate buffer (pH 6.65)–methanol (75:25, v/v) with 2 mM decylsulphate).

Due to the long equilibration times involved, the pre-column had to be reconditioned between injections for 10–15 min with a buffer containing decylsulphate (see Table 3).

Main column

Catecholamines are usually separated on reversed-phase columns as ion pairs with alkyl sulphates or sulphonates at an acidic pH [15, 18–20]. In this work the chromatographic separation was followed by coulometric oxidation of adrenaline and

noradrenaline to adrenochrome and noradrenochrome, respectively. A pH of 6.65 was required to obtain a quantitative yield of the product.

Different reversed-phase columns were tested for the separation of catecholamines at neutral pH (Ultrasphere ODS 3-μm particles, Beckman; Microsphere ODS 3-μm particles, Cp-spher ODS 8-μm particles, Chrompack; Supelcosil LC-18-DB 3-μm particles, Supelco). The catecholamines were not eluted at all from some columns, while other columns gave symmetrical peaks and appeared to be inert. The tailing of the catecholamines decreased when complexation agents (citrate, EDTA) were added to the mobile phase, but it was not possible to obtain symmetrical peaks on the most active columns. The best results were obtained with Supelcosil columns (see Figs 3 and 4). The tailing of catecholamines on reversed-phase columns may depend on complex formation

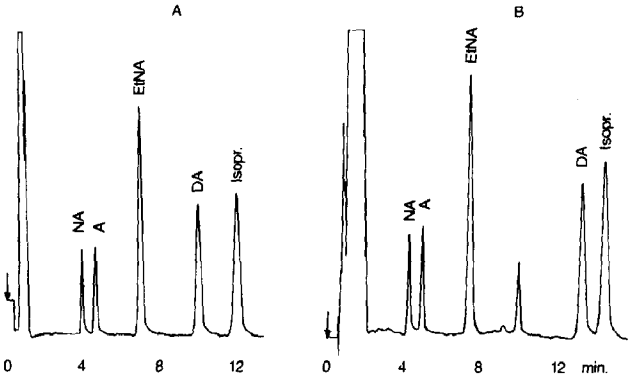

Figure 3
Comparison between chromatograms obtained by injection of a standard solution for urine assays into: A, the main column (45 μl); and B, the switching system (60 μl). The conditions were as described in the text for urine samples, with coulometric detection. NA, 149 nM; A, 194 nM; and DA, 1180 nM.

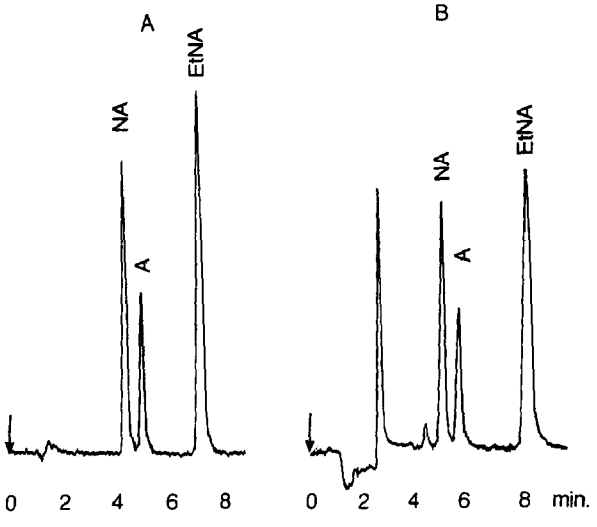

Figure 4
Comparison between chromatograms obtained by injection of a standard solution for plasma assays into: A, the main column (45 μl); and B, the switching system (50 μl). The conditions were as described in the text for plasma samples with fluorimetric detection. NA, 1.87 nM; and A, 1.34 nM.

between the catechol structure and metal ions on the silica surface, and/or on interactions between the amine and free silanol groups.

The complexation of catechols with metal ions would be expected to increase with increasing pH and to decrease when competing complexation agents are present in the mobile phase. It has been shown that reversed-phase columns give low chromatographic efficiencies for the separation of chelating agents, such as acetylacetone [38] and dihydroxynaphthalenes [39].

Column-switching

The recoveries and band-broadening of the switching system were determined by comparison with direct injection. A separate loop-valve injector for direct injection onto the main column was included in the instrument (see Fig. 1). A stock solution of the catecholamines was injected onto the boronic acid column (Affi-Gel 601). The eluent was collected in small fractions during injection with buffer pH 7.5 and during elution with 0.2 M phosphoric acid. The fractions were injected onto the main column for the determination of breakthrough and elution volumes. The break-through volume was larger than 6 ml, 1.5 ml of phosphoric acid was needed for elution in the normal direction and 1.2 ml by back-flushing. The elution volume was lower (0.9 ml) for the silica-based columns used for urine samples.

The sample capacity of the boronic acid gel column (10 × 4.6 mm i.d.) was studied by injecting different volumes of plasma. The switching events were adjusted according to the volume injected. Proteins and other matrix components were washed out with 2.6 ml phosphate buffer pH 7.5 prior to elution with acid. The peak heights of the catecholamines were directly proportional to the volume injected (cf. Table 4).

Table 4
Sample capacity*

	Noradrenaline		Adrenaline		Ethylnoradrenaline	
Sample volume	Peak height	Response factor	Peak height	Response factor	Peak height	Response factor
1 ml	514	1.00	142	1.00	282	1.00
2 ml	1014	0.99	284	1.00	572	1.01
2 ml	1027	1.00	282	1.00	564	1.00
3 ml	1426	0.96	456	1.07	926	1.10

* Peak heights were measured after injection of the specified volumes of a plasma sample with noradrenaline (2.18 nM), adrenaline (0.13 nM) and ethylnoradrenaline (nM).

Band-broadening in the enrichment column was independent of the elution mode (normal elution or back-flushing) for small volumes (30 × 2.1 mm i.d.). Larger columns (20 × 4.6 mm i.d.), however, gave best results by back-flushing. The band-broadening in the enrichment columns was similar for 30 × 2.1 mm i.d. and 20 × 4.6 mm i.d. columns when eluted by back-flushing. The large diameter precolumn (Supelcosil) was chosen because it gave a safe margin towards break-through and a lower flow-resistance compared with 2.1 mm i.d. columns. The low back-pressure of the enrichment column made it possible to use Teflon loops in the system.

Band-broadening in the system was independent of the sample volume in the range studied (1–3 ml). The influence of matrix on the absolute and relative recoveries of the

switching system was also studied by injection of 1-ml samples of spiked catecholamine-free plasma and injection of 50 μl of standard solution pH 3.5 onto the main column and the switching system respectively. Catecholamine-free plasma was prepared by column extraction of endogenous catecholamines on Affi-Gel 601. The peak heights were equal after injection of 50 μl buffer and 1 ml of plasma, respectively (cf. Tables 5 and 6), which implies that the relative recovery was 100%. The relative recoveries of urine samples were also 100% (Table 7).

Table 5
Relative and absolute recoveries

Sample	Relative peak heights* (±S.D.)			Absolute recovery† (±S.D.)		
	n	Noradrenaline	Adrenaline	Noradrenaline	Adrenaline	Ethylnoradrenaline
50 μl buffer pH 3.5	4	1.412 ± 0.072	0.621 ± 0.019	86 ± 3	84 ± 3	80 ± 2
1 ml spiked plasma	3	1.440 ± 0.032	0.619 ± 0.007	83 ± 3	81 ± 7	81 ± 1

* Relative peak height of analyte:internal standard.
† Determined by comparison of peak areas after injection onto the main column and switching system respectively.

Table 6
Calibration curves in buffer and plasma: linear regression of the relative peak height* (Y) versus the amount injected (X)

NA† $Y = 1.243 \cdot 10^{-3} + 0.528\,X$ ($r = 0.9993$; $n = 6$)
NA‡ $Y = 5.69 \cdot 10^{-3} + 0.529\,X$ ($r = 0.9999$; $n = 6$)
A§ $Y = -1.48 \cdot 10^{-2} + 0.374\,X$ ($r = 0.9999$; $n = 6$)
A‖ $Y = -2.64 \cdot 10^{-2} + 0.382\,X$ ($r = 0.9992$; $n = 6$)

* Relative peak height of analyte: internal standard.
† Concentration range 0.467–3.74 pmol in 50 μl buffer pH 3.5.
‡ Concentration range 0.467–3.74 pmol in 1 ml spiked plasma.
§ Concentration range 0.336–2.69 pmol in 50 μl buffer pH 3.5.
‡ Concentration range 0.336–269 pmol in 1 ml spiked plasma.

The absolute recoveries were 83% and >95% for Affi-Gel 601 and dihydroxyboryl silica, respectively. A small amount was lost (5–10%) in the switching system during elution from Affi-Gel 601, but the remaining *ca* 10% loss remained unexplained. The loss may depend on decomposition by oxidation; however, the addition of antioxidant and nitrogen-purging of the mobile phase had no beneficial effect.

The pH of the buffer solution injected (50 or 60 μl, pH 3.5) was adjusted by dispersion in the sample loop (1 ml) before the sample zone reached the boronic acid column.

It is an advantage to use acidic buffers for calibration due to the instability of the catechols at neutral pH. The catecholamines are remarkably stable in plasma, and permit

Table 7
Recoveries by coulometric and fluorimetric detection after standard additions to urine

Sample	*n*	Noradrenaline (nM)			Adrenaline (nM)			Dopamine (nM)	
		Added	Found* C†	F‡	Added	Found* C	F	Added	Found* C
1	2	299	293	307	389	382	380	2360	2410
2	3	299	286	303	389	375	372	2360	2380

* Found values = concentration after standard addition — endogenous concentration.
† C = coulometric detection.
‡ F = fluorimetric detection.

the use of an automatic injector. No decomposition was observed after storage at ambient temperature (22–23°C) overnight [C.S. 40].

Fast and efficient separations of catecholamines in plasma (Fig. 5) and urine (Fig. 6) were obtained; it was found that up to 2 ml of plasma could be injected without unacceptable band-broadening (Fig. 5B). The boronic acid columns were used for 50 samples (1 ml) without any sign of deterioration. The possibility of direct injection onto the main column was very useful in controlling the function of the various parts of the instrument.

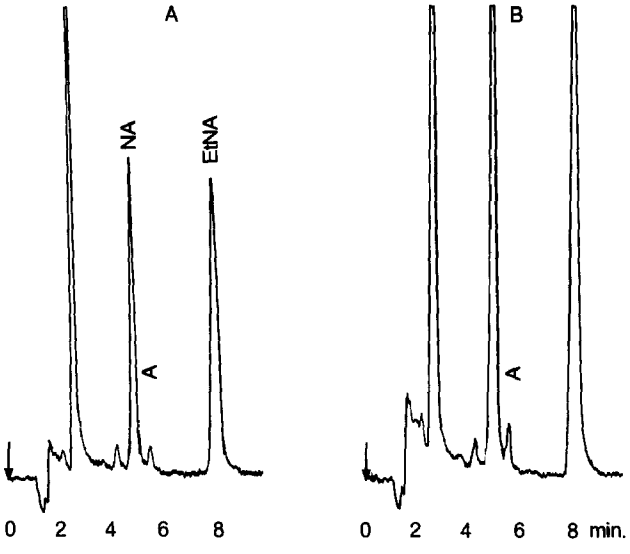

Figure 5
Chromatograms obtained from a plasma sample by injection of: A, 1 ml; and B, 2 ml. The conditions were as described in the text for plasma samples with fluorimetric detection. Noradrenaline, 2.41 nM; and adrenaline, 0.23 nM.

Post-column derivatization and detection

The ease of oxidation of catecholamines has been recognized since the time of their discovery. The colour reactions resulting from the oxidation of adrenaline and related catecholamines formed the basis of early assay procedures for adrenaline. A yellow–green fluorescence develops when alkaline solutions of adrenaline are allowed to stand in air, and this has become the basis of the so-called 'lutin' or 'trishydroxyindole'

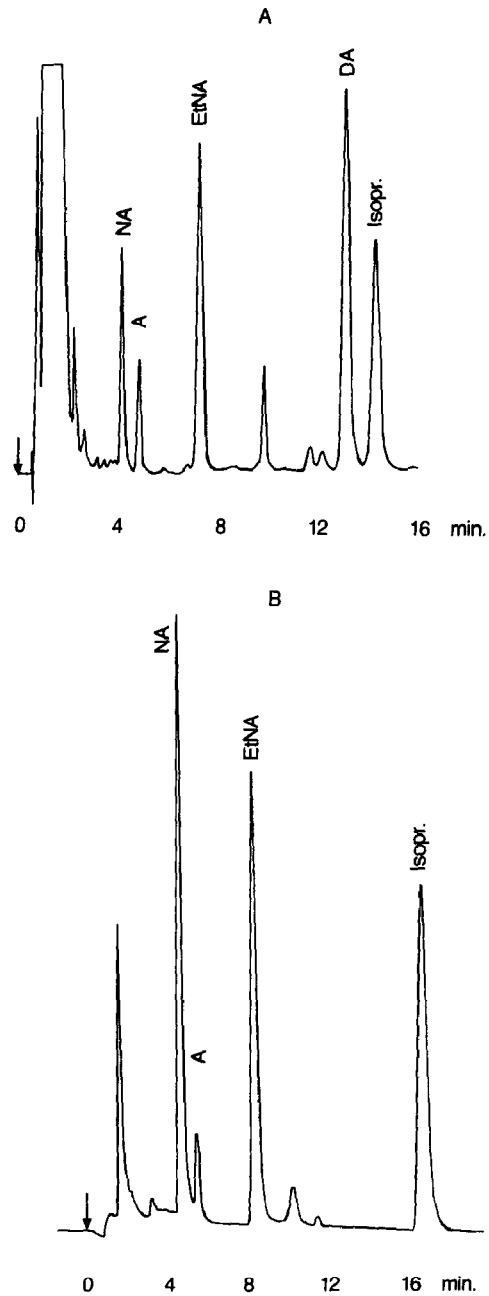

Figure 6
Chromatograms obtained by injection of a urine sample. Injection volume: 60 µl. The conditions were as described in the test for urine samples with coulometric detection (A) and fluorimetric detection (B). Noradrenaline 227 nM; adrenaline 63.9 nM; and dopamine 2340 nM. Internal standards used were ethylnoradrenaline (EtNA) and isoproterenol (Isopr.).

method. The electrochemistry of the catecholamines has been extensively studied by polarography, cyclic voltammetry and related techniques. The chemistry of amino-chrome [41] and the electrochemistry of catecholamines [42] have been reviewed.

Catecholamines are oxidized in acidic solution by a reversible 2 electron process to form quinones (Fig. 7). Methoxylated catecholamines eliminate methanol after oxidation to form the corresponding quinones [41]. Quinones with a β-hydroxyl group cyclize by an intra-molecular 1,4-Michael addition to give leucoaminochromes [42]. Only the unprotonated amine acts as a nucleophile in the reaction and the apparent cyclization

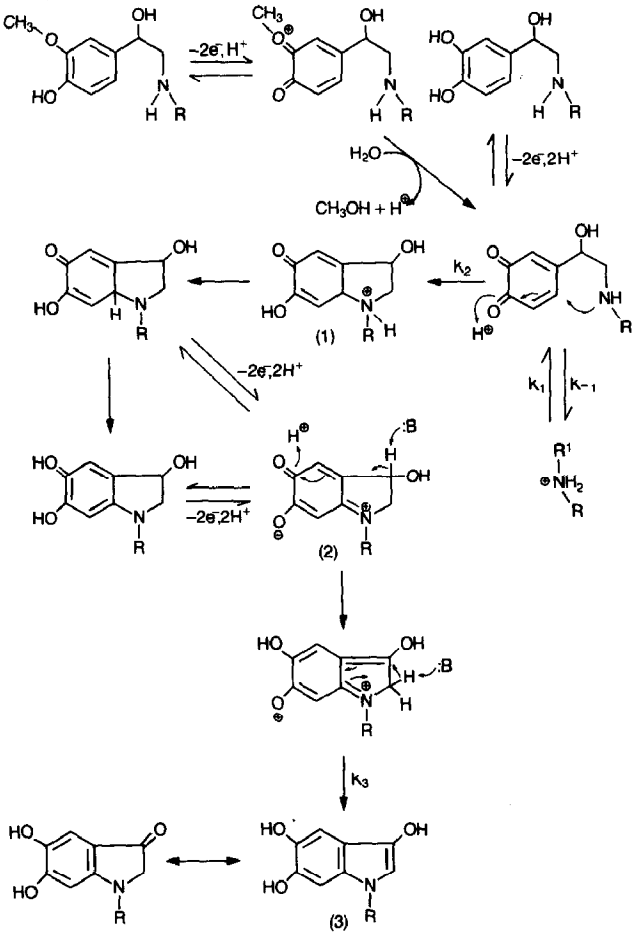

Figure 7
Reaction sequence for post-column derivatization. (1) Leucoaminochrome; (2) aminochrome; and (3) lutine.

rate depends on the solution pH. Leucoaminochromes are further oxidized to adrenochromes, which can be rearranged in strongly alkaline solution to trishydroxyindoles. The trishydroxyindoles oxidize further to the corresponding quinones in the presence of oxidation agents.

The intramolecular cyclization reaction (k_2) is the rate-limiting step for aminochrome formation. The cyclization rate of noradrenaline was studied by flow injection analysis at different pH values as described above. The rate constants determined are presented in Table 8. The relationship between k_{obs} and the various rate constants shown in Fig. 7 is given by the equation:

$$k_{obs} = \frac{k_1 \cdot k_2}{k_{-1}[H^+] + k_1} \tag{2}$$

At low pH the rate equation may be reduced to:

$$k_{obs} = \frac{k_2 \cdot K_a}{[H^+]} \tag{3}$$

Table 8
Cyclization rate constants* for noradrenaline quinone

pH	5.65	6.15	6.65	7.00
$k_{obs}(s^{-1})$	0.113	0.281	0.564	0.882

*k_2 = 61 according to equation (3) (see text).

where K_a (the acid dissociation constant) = k_1/k_{-1}; k_2 can be estimated from a plot of $K_a/[H^+]$. The acid dissociation constant of noradrenaline (pK_a = 8.9) was used to estimate for K_a of the quinone. The estimated value of k_2 was 61 (Table 8), which is close to that determined by chronoamperometry (k_2 = 50) [42]. The cyclization rate constants for secondary amines such as adrenaline and isoproterenol are much higher compared with those for primary amines [42].

When coulometric oxidation is combined with reversed-phase chromatography, the choice of pH is restricted by the stability both of the packing material and of the compounds to be separated. A pH of 6.65 was chosen as a compromise between the stability of the chromatographic system and the cyclization rate. A reaction time of 5.3 s was required to obtain a 95% yield of noradrenochrome at this pH and 22°C. The residence time of the eluting solutes in the coulometric cell was <1 s, so that two separate coulometric cells had to be coupled together by a capillary in order to increase the reaction time to 6 s.

The thermostat was preset to 40°C in order to increase the reaction rate. The signal on channel two was *ca* 1.5% of the total, which indicated that the yield of noradrenochrome was quantitative (>99%) with one cell unit operated at 40°C. Both channels were used for oxidation, while channel one was used for detection.

The rearrangement rate of noradrenochrome to trishydroxyindole was studied by flow injection as described above. The rearrangement rate constant was measured to 0.100 s^{-1} at 40°C; a reaction time of 30 s was required to obtain 95% rearrangement of noradrenochrome. One experiment was performed with constant reaction time (21 s) at different temperatures to establish whether the reaction rate could be increased. The peak heights decreased at temperatures higher than 50°C, probably because of degradation of the unstable products; thus 40°C was chosen for the rearrangement step. The total yield in the derivatization system was checked by flow injection analysis as described above. The yields of adrenolutine and noradrenolutine were >90%.

The teflon capillary was knitted [35] to reduce the dispersion in the alkaline rearrangement step. The band-broadening contributions of the different parts of the total system were determined by injection of noradrenaline onto the main column, into the column-switching system for urine samples, and by flow injection into the teflon capillary (see Table 9). The main column gave 78 700 theoretical plates m^{-1} for noradrenaline (capacity factor, k' = 5). Column-switching and post-column derivatization decreased the efficiency to 41 300 plates m^{-1}. The other catecholamines were retained more strongly on the main column, and less affected by external band-broadening.

The major sources of external band-broadening were: the teflon capillary, the tee for addition of the derivatization reagent and the connections. The column-switching device involving pre-columns, and the coulometric cell itself, contributed little to the total band-broadening in the system.

Fluorescence was detected in the alkaline effluent with excitation at 400 nm and

Table 9
Sources of band-broadening*

	Columns C1 + C2	Main column C3	Teflon capillary	Residual† (C+F+T)	Total
δ^2	520	2880	1300	1100	5610
δ^2%	6	51	23	20	100
δ (μl)	23	54	36	33	75

* The peak variances (δ) were determined after injection of noradrenaline into different parts of the system:

$$\psi^2_{Total} = \delta^2_{C1+C2} + \delta^2_{C3} + \delta^2_{TC} + \delta^2_R$$

† Residual (R) (C+F+T) = coulometric detector + fluorimetric detector + tee for addition of reagent and connections.

emission at 510 nm. Neutralization of the effluent in a second teflon capillary prior to fluorimetric detection increased the fluorescence intensity by a factor of two. However, the signal-to-noise ratio remained unchanged due to dilution and increased detector noise.

The step-gradients formed during column-switching disturbed the background current in the coulometric detector, whereas the fluorescence response was unaffected. The thermostat in the heater of the coulometric cell created low frequency noise (*ca* 0.01 Hz), which increased the detection limit of dopamine from 0.3 to 1.6 pmol. Dopamine levels in normal plasma samples were below the detection limit, but the concentrations in urine were much higher, so that coulometric detection could be used.

Accuracy and precision

The methodology described yields an unusually high selectivity for catecholamines, since several of the procedures included are individually selective for such compounds: isolation on the boronic acid pre-column; the ion-pairing chromatographic system; and finally the post-column derivatization to trishydroxy-indoles, coupled with fluorimetric detection.

The precision (relative standard deviation; RSD) for the determination of catecholamines in plasma and urine was usually in the range of 2–4% (Table 10). The quantifiable limit for 1 ml of plasma was 0.10 and 0.07 nmol/l for adrenaline and noradrenaline, respectively. Manual measurements of peak heights gave better precision at low concentrations, compared with electronic integration, due to detector noise.

A comparison between coulometric and fluorimetric detection of noradrenaline and adrenaline in nine different urine samples is presented in Table 11. There was no significant difference between coulometric and fluorimetric detection of noradrenaline, but coulometric detection gave about 40% higher values for adrenaline compared with the fluorimetric method. The difference cannot be explained by fluorescence quenching, since accurate results were obtained by standard addition to urine (see Table 7). The difference may be attributable to interference by a biogenic compound with the same retention volume as adrenaline. This example illustrates the importance of method correlation in the validation of assays for biogenic compounds.

Conclusions

Column-switching has proved to be a suitable technique for the automated analysis of catecholamines in biological fluids. Boronic acid pre-columns gave the high sample

Table 10
Intra-assay precision*

Sample	Substance	Concentration (nM)	RSD (%)	*n*
1 ml plasma	NA	2.41	2.6 (2.1)†	9
1 ml plasma	NA	2.39	3.2	7
1 ml plasma	NA	2.18	1.9	9
1 ml plasma	A	0.46	4.2	7
1 ml plasma	A	0.25	10 (4.5)†	9
1 ml plasma	A	0.13	14 (4.3)†	9
60 µl urine	NA	105	3.6	9
60 µl urine	A	47.9	7.1	9
60 µl urine	DA	1470	0.9	9

* The absolute recoveries of the internal standards in urine samples were found to be 94 ± 0.7% and 103 ± 6% for ethylnoradrenaline and isoproterenol, respectively.

† Determined by manual measurement of peak heights. Other values were obtained by electronic integration.

Table 11
Comparison between coulometric (C) and fluorimetric (F) detection for quantification in urine

	Noradrenaline (nM)			Adrenaline (nM)		
Sample No.	Coulometric	Fluorimetric	(C–F)	Coulometric	Fluorimetric	(C–F)
1	113	122	−8	202	144	+58
2	280	277	+2	174	110	+64
3	132	125	+7	95.0	71.5	+23.5
4	101	105	−4	57.3	47.9	+9.4
5	141	143	−2	62.8	29.5	+33.3
6	196	196	0	84.6	57.3	+27.3
7	—	—	—	57.9	39.9	+18.0
8	47.3	49.6	−2.3	48.6	33.3	+15.3
9	169	171	−2	191	129	+62
10	94.0	96.3	−2.3	62.2	32.8	+29.4
Mean ±S.D.			−1.3 ± 4.1			+34 ± 20

capacity and selectivity for direct injection of plasma and urine. The combination of two pre-columns, the first for selective isolation and the second for enrichment, made it possible to inject large sample volumes with a minimum of band-broadening. Furthermore, post-column derivatization by coulometric oxidation can be performed with low dispersion, which makes it possible to obtain fast separations with adequate resolution between noradrenaline and adrenaline.

Fluorimetric detection of noradrenaline and adrenaline gave better baseline stability and higher selectivity compared with coulometric detection. However, coulometric detection of adrenaline in urine gave about 40% higher values compared with post-column derivatization to trishydroxyindole and fluorimetric detection. The method can be expected to give accurate quantitation because the responses were shown to be unaffected by the biological matrices; moreover, both isolation and detection procedures were selective for catecholamines. The instrument gave the sensitivity and stability required for routine determinations of noradrenaline and adrenaline in plasma, and of

noradrenaline, adrenaline and dopamine in urine. Analytical results were obtained within 17 min; 50–60 samples/day could be analysed if an automatic injector was used.

Normal dopamine levels in plasma were found to be below the detection limit of the instrument. The instrument was safe in operation, and a simple control procedure was developed to check the function of the different parts of the system during set-up and to maintain its overall performance.

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