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Determination of catecholamines in urine using hydrophilic interaction chromatography with electrochemical detection

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The determination of catecholamines in urine was investigated using hydrophilic interaction chromatography (HILIC) as an alternative to the commonly used reversed-phase (RP) method. A number of different approaches were explored, including per-aqueous liquid chromatography (PALC), and HILIC using bare silica, bonded amide and zwitterionic phases. The bonded phases gave superior results in terms of both peak shape and selectivity. The mechanism of the HILIC separation was investigated particularly with respect to the contribution of ion exchange to retention. The electrochemical detection of catecholamines was studied and optimised in typical HILIC mobile phases that contain high concentrations of acetonitrile. HILIC offered a number of advantages over the conventional RP approach, giving good retention of the solutes without use of ion pair reagents, the absence of which also would facilitate detection by mass spectrometry. HILIC used in conjunction with solid phase extraction based on RP also gives orthogonal separation mechanisms in the cleanup and analysis steps. Furthermore, good recoveries from the cleanup stage were obtained, as high concentrations of acetonitrile can be used as eluting solvent that are fully compatible with HILIC, and lipophilic impurities are eluted close to the void volume of the HILIC column.

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1. Introduction

Catecholamines are biological amines released mainly from the adrenal glands in response to stress. They act as neurotransmitters and hormones, playing an important role in maintaining normal physical activity of the body including heart rate, blood pressure and the reactions of the sympathetic nervous system [1]. The most abundant catecholamines are dopamine (*D*), epinephrine (*E*) and norepinephrine (*NE*). Very high levels of catecholamines in plasma and/or urine indicate tumours of the adrenal gland such as pheochromocytoma [3] or neural tumours such as neuroblastoma [4], while levels of catecholamines are also elevated during stress. Therefore there is a need for their quantitative determination in body fluids in order to identify any endocrine disorders and other physiological and pathological abnormality in the body at an early stage [2]. The most common method for analysis of catecholamines in biological fluids is RPLC in conjunction with various detection systems like mass spectrometry (MS), electrochemical detection (ECD) and fluorescence detection [2,5–9].

The analysis of some amines in RPLC gives rise to considerable problems due to their hydrophilic nature, which can result in low retention necessitating the use of ion pair agents and/or very low concentrations of organic modifier that can cause phase dewetting. Ion pair reagents can cause interference with MS detection. Furthermore, the presence of ionised silanol groups on the stationary phase may give rise to peak tailing and overloading effects that can result in poor separation [10,11]. Recent methods for catecholamine analysis typically use a C18 or C8 column at acid pH, with a low concentration of methanol (2–2.5%) and an ion pair reagent such as octanesulfonic acid [2,6]. Good resolution of standards was obtained in the latter report [6], but this RP method with ECD failed to separate *NE* from plasma constituents completely. Another study using a RP column with methanol and acetate buffer at higher pH (4.66) gave broad and tailing peaks for the catecholamines [8]. Thus, alternative methods for the analysis of catecholamines have been sought. Hydrophilic interaction chromatography (HILIC) has been shown to be a complementary method to RP for the analysis of ionised basic compounds [12,13]. Typically, HILIC uses a bare silica or polar bonded phase in conjunction with a mobile phase containing a high proportion of acetonitrile (typically >60%) together with a significant concentration of water or aqueous buffer (typically >2.5%). The mechanism of separation appears to be complex [14], but may consist of partition of hydrophilic solutes into a water layer held close to the silica column surface and/or associated with polar bonded groups, adsorption onto silanols or polar bonded groups, ion exchange and even some RP behaviour when the concentration of organic solvent is very low. The advantages of HILIC over RP include good retention of polar, hydrophilic and

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ionised solutes, alternative selectivity to RP methods with elution of solutes broadly in line with increasing polarity (the opposite to that in RP) and the low viscosity of mobile phases that leads to lower operating pressures and faster diffusion of solutes. Thus, longer columns can be used, or columns can be operated at higher flow rates without serious loss in efficiency (smaller van Deemter C terms than in RP). A further significant advantage of the use of high concentrations of organic solvent is the more efficient spraying and desolvation of eluents in ESI mass spectrometry, leading to higher sensitivity than can be obtained in RPLC–MS. Elfakir and co-workers presented a thorough survey of 12 different HILIC columns for their suitability to separate a test mixture containing 12 standard neurotransmitters, using MS or UV as detection methods [15]. However, comparative peak shape data was not reported, and the study did not present quantitative data or application of the various columns to the separation of these compounds in biological fluids. Sandra and co-workers [16] coined the term "per-aqueous liquid chromatography" (PALC) to describe a technique using a bare silica column with a mobile phase containing either 100% aqueous ammonium formate buffer pH 5 or similar buffers with very low concentrations of organic solvent. Excellent separations of standard solutions of catecholamines were shown. PALC appears very similar to the much earlier methods of amine separation on bare silica reported by Bidlingmeyer [17] and later by others including Euerby [18], which appear to be based largely on ion exchange and RP effects with the siloxane backbone of the column. However, in contrast to the findings of Sandra it is notable that peak shapes reported for bases using bare silica columns with largely aqueous buffers can be inferior to those normally found in RP separations [12,18].

The aim of this study was to investigate the potential applicability of a HILIC-based procedure in conjunction with a simple electrochemical detector for the analysis of catecholamines in urine, exploring also if such a method was compatible with current sample preparation procedures that are mainly based on solid phase extraction (SPE) in the RP mode. There are few if any reports of the use of electrochemical detection with HILIC, although Flanagan and Jane showed the analysis of basic drugs using an amperometric detector with high concentrations of methanol in the mobile phase in separations which appear to be based on ion-exchange [19]. Furthermore, the use of HILIC as the final separation method, in conjunction with sample preparation by RP might prove a useful orthogonal pair of separation methods, giving rise to improved selectivity, although problems might also arise in that elution of the SPE cartridge with largely aqueous solutions (as used typically in conventional procedures for catecholamines [20]) is detrimental to HILIC, as water is a strong solvent in this technique. Additionally, we wished also to study the mechanism of separation, in order to contribute to the understanding of the processes that occur in HILIC, which have not been well investigated.

2. Experimental

Dopamine (pK_a 8.9), epinephrine (pK_a 8.6), norepinephrine (pK_a 8.4) were obtained from Sigma Aldrich, Poole, UK; stock solutions were prepared separately at a concentration of 5 mg/mL in 0.1% formic acid and further diluted with mobile phase to obtain the desired concentrations. HPLC grade water was obtained from an 18 MΩ water purifier (Millipore, Watford U.K.). An Agilent 1100 HPLC system (Waldbronn, Germany) with high pressure mixing and Chemstation version 10.01 was used, with either the Agilent UV detector or a Coulochem II electrochemical detector (Dionex, Sunnyvale, USA). Cyclic voltammetry was performed with a μAutolab Type II potentiostat (Windsor Scientific, Slough UK), connected to a three electrode cell comprising a 6 mm diameter glassy carbon working electrode, an Ag/AgCl reference electrode, and a platinum counter electrode. The columns used were Atlantis silica (Waters, Milford, USA), Zorbax Rx-SIL (Agilent), silica-based zwitterionic phase ZIC–HILIC (Merck, Darmstadt, Germany) all of dimensions 25 cm × 0.46 cm, and 5 μm particle size); polymer-based zwitterionic phase ZIC–pHILIC (Merck) 15 cm × 0.46 cm 5 μm particle size; and BEH amide (Waters, Milford, USA) 15 cm × 0.46 cm 3 μm particle size. Phenylboronic acid cartridges (PBA, 100 mg) were obtained from Varian (now Agilent). Mobile phases were prepared from ammonium formate buffers and ACN (far UV grade, Fisher, Loughborough U.K.) and filtered through 0.2 μm filters (Millipore, Watford U.K.). The final scheme for extraction of the catecholamines from urine was as follows:

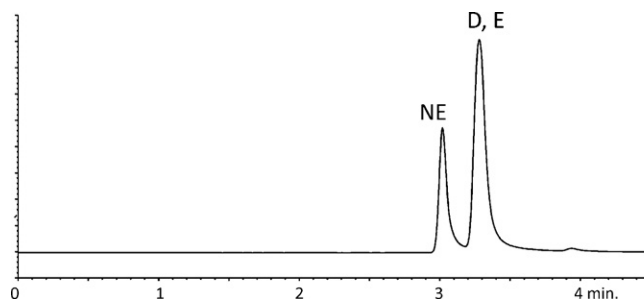

Fig. 1. Analysis of catecholamines using 5 μm Zorbax Rx-SIL silica column 250 mm × 4.6 mm in the PALC mode with mobile phase 50 mM ammonium formate buffer (pH 5) at 30 °C using 280 nm UV detection. 5 μL injection. Peak identities: *NE* = norepinephrine, *D* = dopamine, *E* = epinephrine.

Step 1: A 100 mg, 1 mL Bond Elut™ PBA cartridge was equilibrated with 1 mL 80:20 ACN–water (v/v) containing 1% formic acid (to remove any interfering impurities that would be washed off in the final elution stage) and then with 1 mL 50 mM phosphate buffer, pH 10.
Step 2: The buffered urine sample (1 mL urine + 2 mL phosphate buffer pH 8.5) was applied.
Step 3: The cartridge was washed with 1 mL ACN–10 mM phosphate buffer pH 8.5 (50:50 v/v).
Step 4: Finally the cartridge was eluted with 1 mL ACN–water (80:20 v/v) containing 1% formic acid.

3. Results and discussion

3.1. Per aqueous liquid chromatography (PALC)

In view of the excellent results achieved previously [16] for the separation of a standard mixture of *E*, *NE* and *D*, we first investigated this procedure using the same bare silica column (Zorbax Rx-SIL) using ammonium formate buffer pH 5. However, Fig. 1 shows disappointing results, with poor selectivity of the separation and tailing peaks for each catecholamine. The separation appears very different from the good selectivity and excellent peak shape reported previously. While it appears that there may not be large differences in the performance of modern Type B bare silica HILIC columns with ionised bases, we substituted instead an Atlantis silica column, which previously gave excellent results for basic drugs [12]. However, the separation as indicated in Fig. 2 remained poor. In an attempt to improve the resolution of the peaks, the effect of the buffer pH and concentration were studied on the Atlantis column. Over the pH range 3.2–5.0 using 50 mM ammonium formate buffers, the selectivity of the separation was hardly changed, although a slight increase in retention times was observed at higher pH. We did not explore the use of pH > 5.0, as the solubility of silica can be problematic in highly aqueous mobile phases at neutral or alkaline pH. The surface is not protected by the presence of

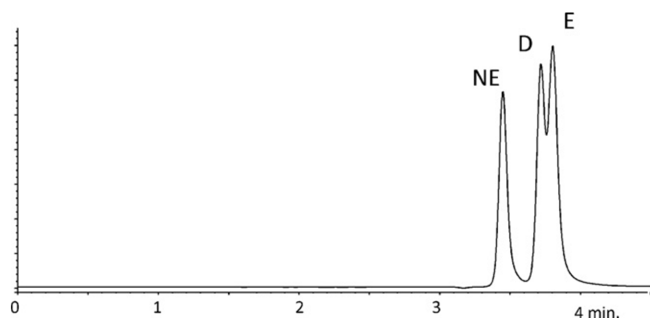

Fig. 2. Analysis of catecholamines using 5 μm Atlantis silica column 250 mm × 4.6 mm with mobile phase 50 mM ammonium formate buffer pH 3.2. Other conditions as Fig. 1.

organic ligands as in RP columns. Nevertheless, peak shapes were improved compared with the previous phase, and column efficiencies over the range 14,000–18,000 plates in the 25 cm column with asymmetry factors 1.3–1.8 were obtained. Change in the buffer concentration over the range 20–100 mM gave little improvement in the separation. We cannot at present explain the discrepancy between the present and the previously reported results [16]. However, in subsequent papers, Gritti and Guiochon (together with the original authors) showed that the surface of silica columns in the presence of PALC mobile phases was seriously heterogeneous, with up to 5 different adsorption sites including a small number of very strong sites [21,22]. They concluded from theoretical and practical considerations that better sensitivity, higher efficiency and better resolution could be obtained in the conventional HILIC mode where the adsorption mechanism was found to be much more homogeneous. A problem with the PALC mode was found to be serious overloading of the few strong column sites even with very small amounts of solute, giving poor peak shape. PALC separations with moderate solute k gave the worst column efficiencies, and only addition of ACN, resulting in very small solute k, and apparent blocking of these strong sites by this solvent, gave reasonable column efficiency. These later results appear to contradict the earlier findings [16], and are much more in line both with our previous results for PALC type separations [12] and those of other workers [18].

3.2. Hydrophilic interaction chromatography (HILIC)

3.2.1. Separation using bare silica

The behaviour of the same bare silica column (Atlantis) was subsequently studied in the conventional HILIC mode (using a high concentration of ACN, 90% v/v) in combination with ammonium formate buffer (pH 3.0) of concentration 5–100 mM (see Fig. 3). It is interesting that the peaks are eluted in the order *D*, *E*, *NE* which is different from that shown on the same column but in the PALC mode (*NE*, *D*, *E*). This difference results from the different contribution of various retention mechanisms in either technique. For example, there is likely to be a negligible contribution from the RP mechanism caused by the siloxane groups in PALC when using the high concentrations of ACN typical of HILIC. Instead, HILIC is likely to involve partition and adsorption mechanisms, in addition to ion exchange, which is involved in both techniques. However, the HILIC separations were still inadequate, with tailing peaks and poor resolution of the solutes using any buffer concentration. Previous results show in contrast excellent peak shapes with some strongly basic drugs on the same bare silica phase [12]. Retention appears to decrease slightly and then increase with increasing buffer concentration (Fig. 3), which could be a combination of decreased ionic interactions with the stationary phase as the buffer concentration increases, together with increase in the extent of the aqueous layer

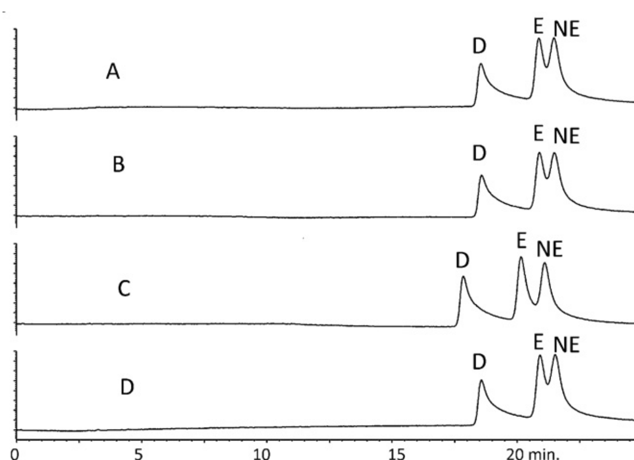

Fig. 3. Effect of buffer concentration on the retention of catecholamines on Atlantis column with mobile phase ammonium formate pH 3.0 (various concentrations) in 90% ACN v/v. *A* = 0.5 mM, *B* = 1 mM, *C* = 5 mM, *D* = 10 mM ammonium formate. Peak identities as Fig. 1.

close to the column surface, producing increased partition into the stationary phase layer [14,23]. An increase in the concentration of water in the mobile phase (10 mM ammonium formate pH 3.0 in 75% ACN) produced a decrease in retention of the solutes as expected, together with an improvement in peak shape. However, the resolution of *E* and *NE* was lost (results not shown).

3.2.2. Separation using bonded HILIC phases

Change of the stationary phase in HILIC can produce large changes in selectivity [14,24]. Thus, analysis was attempted using a 5 μm zwitterionic phase or an amide phase as alternatives to bare silica. Fig. 4(A) indicates a good separation of the analytes was obtained on the 25 cm 5 μm zwitterionic phase in around 10 min using 25 mM ammonium formate pH 3.0 in 75% ACN; the peak at ~2.5 min is toluene, used as an unretained t_0 marker compound. Fig. 4(B) shows that a good separation was also obtained on the 15 cm 3.5 μm amide column which yielded approximately the same column efficiency, although a slightly inferior resolution of *D* and *E*. The water content of the mobile phase was reduced in an attempt to improve resolution of these peaks on the amide phase, however, some loss of efficiency resulted, so further investigations were made using the zwitterionic phase. The mobile phase conditions were varied, in order to optimise but also to elucidate factors that influence the separation.

Firstly the effect of pH was investigated at constant buffer concentration; retention and peak shape data are shown in Table 1. The increase in the retention time with increase in the pH from 3.0 to 4.6 for all solutes provides evidence for increased ionic interactions at the higher pH. Due to the presence of a high concentration of ACN in the mobile phase, pH values (measured in the aqueous–organic mobile phase with the pH meter calibrated with aqueous buffers)

Table 1
Retention and peak shape data for catecholamines using ZIC–HILIC column with mobile phase 6.25 mM ammonium formate pH 3.0 and pH 4.6 in 75% ACN at 1 mL/min, T = 40 °C. Standard concentrations: 200 mg/L.

	t_R (min)	N	A_s	USP T_f
pH 3.0				
Dopamine	7.1	15,000	1.0	1.0
Epinephrine	7.9	13,650	1.1	1.0
Norepinephrine	10.8	14,750	1.0	1.0
pH 4.6				
Dopamine	9.1	10,300	0.40	0.90
Epinephrine	10.3	8,500	1.7	1.5
Norepinephrine	14.1	8,300	2.1	1.8

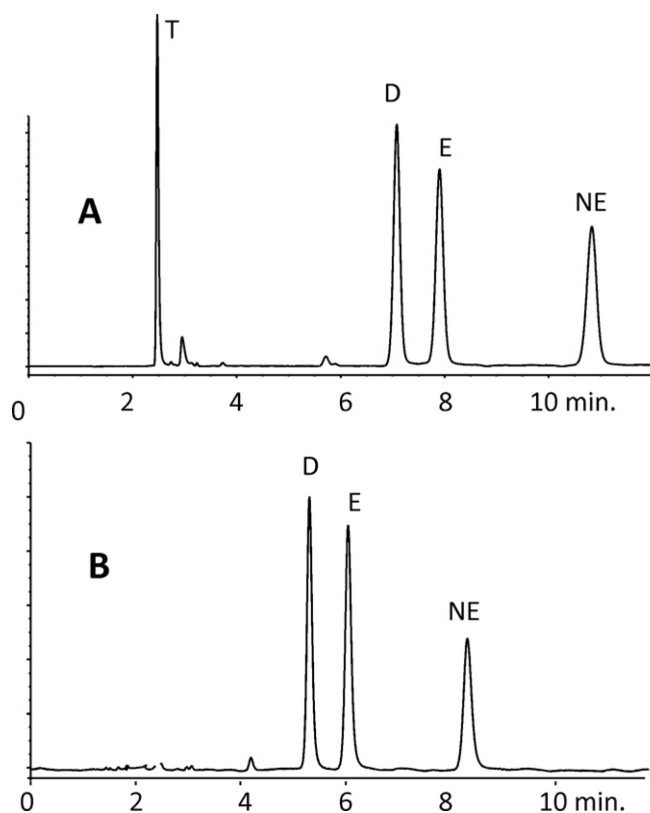

Fig. 4. Analysis of catecholamines and toluene (*T*, void volume marker) using (A) 5 μm ZIC–HILIC column 250 mm × 4.6 mm. Mobile phase 6.25 mM ammonium formate pH 3.0 in 75% ACN. (B) 3.5 μm XBridge Amide 150 mm × 4.6 mm. Mobile phase as (A) but with 85% ACN.

were significantly different at ${}^{s}_{W}$pH 4.6 and ${}^{s}_{W}$pH 6.3 respectively. Formic acid (used to adjust the pH of ammonium formate) is a much weaker acid in 75% ACN than in water. It seems more likely that the increase in retention is due to an increased ionisation of the underlying silanol groups on the phase at higher pH, as it has strongly acidic bonded sulfonic groups that should be fully ionised at either value. There appears to be a negative impact on peak shape of working at the higher pH, with e.g. column efficiency for *NE* decreasing by a factor of about 50% and peak asymmetry increasing from ~1.0 at pH 3.0 to ~2.1 at pH 4.6. The interpretation of these data in terms of ionic interactions is complex however, as the pK_a of silanols is also likely to be affected by the presence of the high concentration of ACN.Secondly, the effect of total ammonium formate concentration in the mobile phase was investigated over the range 3.75–25 mm while maintaining the pH at 3.0 by adjustment with formic acid; Table 2 shows corresponding retention and peak shape data. Peak shapes were good in each mobile phase with optimum efficiencies indicated with 6.25 mM buffer of 14,000 12,600 and 13,800 for *D*, *E* and *NE* respectively. Peak asymmetries for all 3 solutes were close to 1.0. Fig. 5(A) shows a plot of *k* for each solute vs the reciprocal of the buffer cation concentration. For all 3 solutes, retention decreased as the buffer concentration increased, indicative of an ion exchange contribution to retention [14]. For a pure ion-exchange process, strictly linear plots are expected. However, slight curvature can be detected in the plots, which shows that the buffer concentration also has some effect on non-ionic retention processes. For example, it is possible that higher buffer concentration increases the “HILIC retention” (retention due to partition into the aqueous layer and/or adsorption onto polar column sites) which acts in opposition to the decrease in ionic retention it also causes [14,23]. Thus the points were fitted to a second order polynomial

Table 2
Effect of buffer concentration on retention and peak shape of catecholamines using ZIC–HILIC column with mobile phase ammonium formate pH 3.0 in 75% ACN at 1 mL/min, T = 30 °C.

	t_R (min)	N	A_s	USP T_f
3.75 mM				
Dopamine	7.6	14,200	1.0	1.0
Epinephrine	8.5	9500	1.6	1.3
Norepinephrine	11.7	12,800	0.91	1.0
6.25 mM				
Dopamine	7.1	14,000	1.0	1.0
Epinephrine	8	12,600	1.0	1.0
Norepinephrine	10.8	13,800	0.93	1.0
12.5 mM				
Dopamine	6.4	13,400	1.1	1.1
Epinephrine	7.3	12,300	1.1	1.1
Norepinephrine	9.7	17,700	0.93	0.98
25 mM				
Dopamine	6.0	13,600	1.1	1.1
Epinephrine	6.7	12,400	1.1	1.1
Norepinephrine	8.8	6700	0.94	0.90

(coefficient of determination R^2 = 0.9998, 0.9994 and 0.9976 for *D*, *NE* and *E* respectively). Extrapolation of the plots to infinite buffer concentration (1/[NH_4^+] = 0) can be used to deduce the contribution to retention in the absence of ion exchange. However, it should be

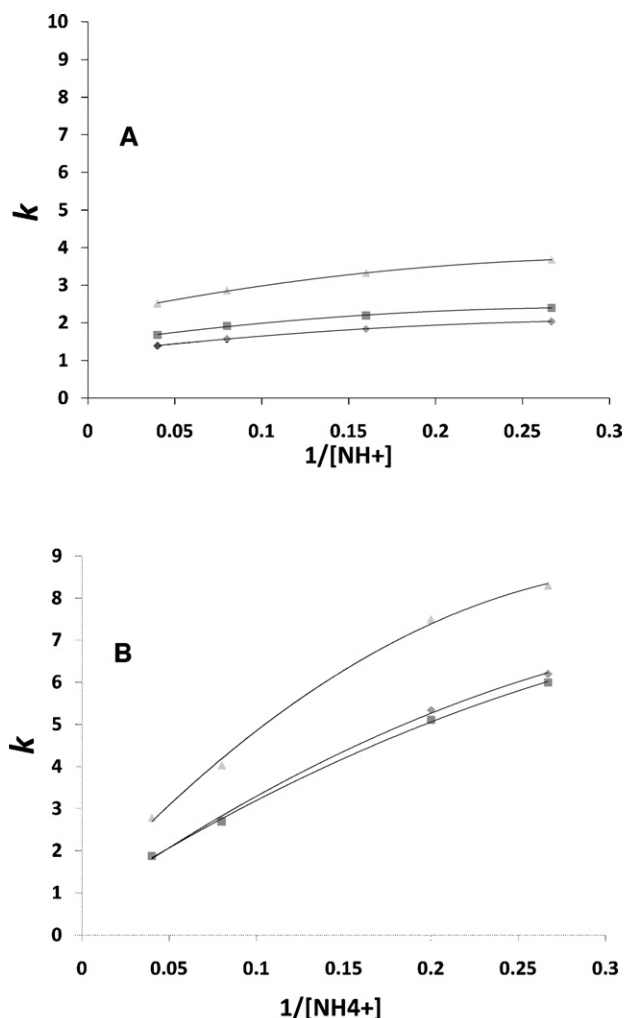

Fig. 5. (A) Plots of *k* vs the inverse of buffer cation concentration using ZIC–HILIC column. Mobile phases ammonium formate pH 3.0 (3.7–25 mM) in 75% ACN. (B) As for (A) but using ZIC–pHILIC column. Peak identities: diamonds = *D*, squares = *E*, triangles = *NE*.

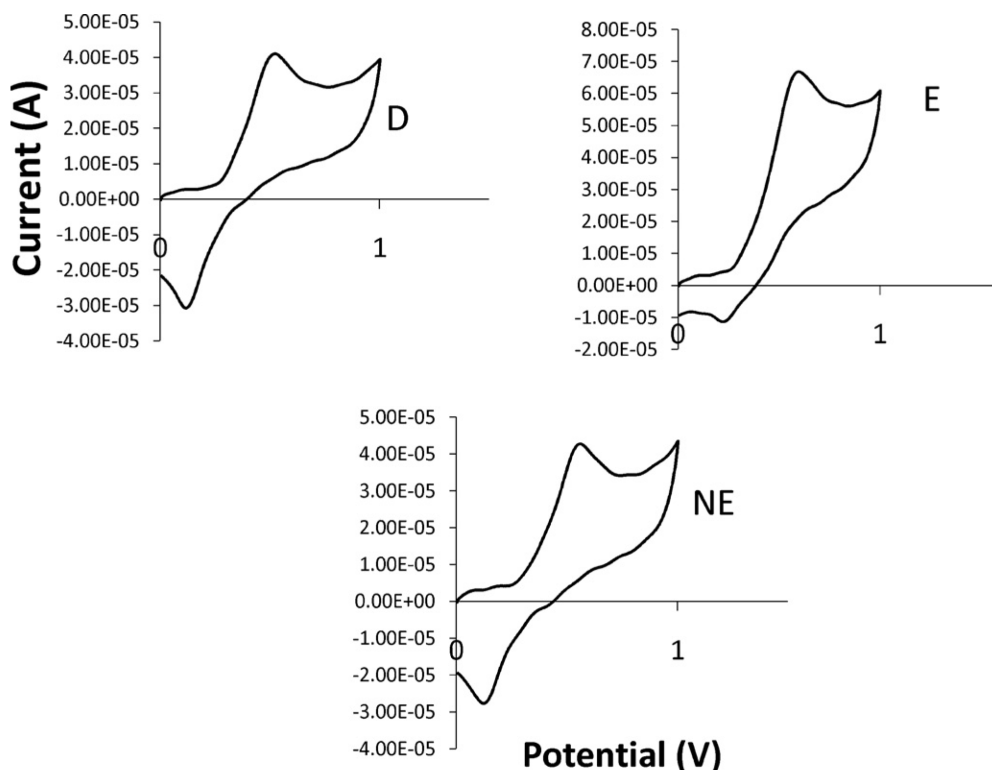

Fig. 6. Cyclic voltammograms of catecholamines (concentration 1 mM). Solution 12.5 mM ammonium formate pH 3.0 in 75% ACN.

emphasised that the complexity of the mechanism and the resulting curvature of the plots compromises the accuracy of calculations of the contribution of the various processes to the overall retention. Nevertheless, for *D*, *E* and *NE*, this HILIC contribution was estimated to be about 58, 60 and 59% respectively at an ammonium formate concentration of 3.75 mM and 86, 86 and 86% respectively at an ammonium formate concentration 25 mM. Clearly, the contribution of ionic processes is less at higher buffer concentration, as the buffer competes with the solute for ionised column groups.

Thirdly, it is interesting to consider the influence of the underlying silica on retention for the zwitterionic ZIC–HILIC column. Some information can be gained by comparing the performance with that of an organic polymer-based column bonded with the same groups (ZIC–pHILIC column) as shown in Fig. 5(B). Curved plots were again obtained by fitting the data to a second order polynomial expression (coefficient of determination R^2 = 0.9984, 0.9978, 0.9991 for *D*, *NE*, and *E* respectively). However the slope of the plots was much steeper than for the silica based column. The HILIC contribution was estimated to be 11, 14 and 8% for *D*, *E* and *NE* respectively at buffer concentration of 3.75 mM and 37, 44 and 25% respectively at buffer concentration 25 mM. Clearly, the HILIC contribution to retention of the polymer based column is much smaller than that of the silica based column, resulting in a much larger proportion of its retention being due to ionic processes. It is possible that the more hydrophobic matrix of the polymer column does not allow the formation of as extensive a water layer as surrounds the ionic groups on the surface of the silica column. Thus the contribution of HILIC retention processes to the overall retention diminishes, leaving the influence of ion exchange as being more significant. However, caution must be exercised in such interpretations, as the exact details of column preparation are proprietary, and the silica and polymer columns may differ in other ways. It is interesting to note that *D* elutes after *E* on the polymer column, but before *E* on the silica column (see Fig. 5). Clearly the balance of the different retention mechanisms on the different columns can affect selectivity. The efficiencies generated by the polymeric column were only about half that of the silica column and the selectivity of the separation was poorer, so no further work was carried out on this column.

Finally, the effect of temperature on the separation of the catecholamines on the silica-based zwitterionic column was briefly examined over the temperature range 25–40 °C. Using a mobile phase composition of 6.25 mM ammonium formate buffer pH 3.0 in 75% ACN at a constant flow rate of 1.0 mL/min, increased temperature was found to have a relatively minor effect on column performance, with efficiencies increasing on average by 10–15% at the highest temperature compared with the lowest temperature. Peak shape data using the same mobile phase at temperatures of 40 °C and 30 °C is shown in Tables 1 and 2, respectively. We preferred 30 °C for routine use in order to prolong column life.

3.3. *Electrochemical detection of catecholamines*

3.3.1. *Cyclic voltammetry of catecholamines*

All previous experiments were performed using UV detection. However, the detection limits with UV are too high for practical use in urine determination, as the concentration of catecholamines is typically in the ultra-trace region. We chose coulometric detection as this technique generally gives better sensitivity than amperometric detection as the whole of the sample passing through the detector is oxidised or reduced. As there are very few reports of the use of such a detector with HILIC-type eluents, we studied in some detail the optimum conditions for detection.

Electrochemical detection can involve a single oxidation or reduction process. However, dual electrode detection is also possible, where the products of the first electrochemical process are then reduced or oxidised. This approach can give greater detection selectivity and sensitivity when the detector cell can be operated at low applied potentials i.e. close to 0 V. Cyclic voltammetry was performed to investigate the electrochemical behaviour of *D*, *E*

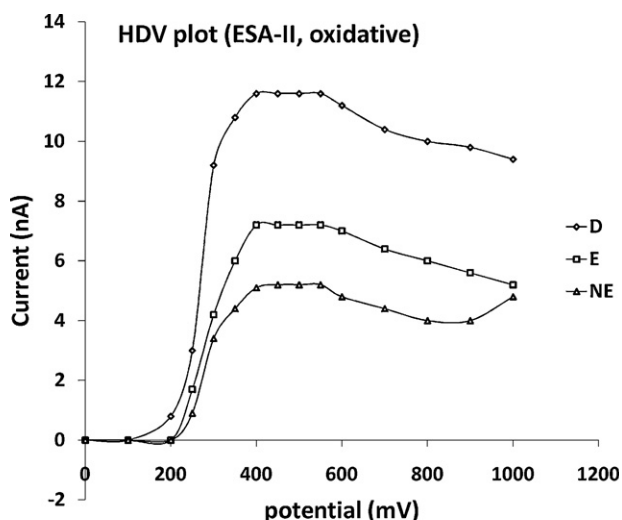

Fig. 7. Hydrodynamic voltammograms for catecholamines obtained with the Coulochem II detector in the oxidative mode. Mobile phase 6.25 mM ammonium formate pH 3.0 in 80.5% ACN. A slightly weaker mobile phase was used compared with the conditions of Fig. 5 in order to improve resolution between *D* and *E* peaks.

and *NE* in a mobile phase consisting of 12.5 mM ammonium formate adjusted to pH 3.0 (by addition of formic acid) in 75% ACN. Each solute gave an anodic peak corresponding to the oxidation of *D*, *E* and *NE* to the related quinone at a potential of +0.504 V, +0.573 V and +0.542 V (vs Ag/AgCl reference electrode) respectively (see Fig. 6). On reversal of the potential, cathodic peaks occurred at around +0.139 V, +0.248 V and +0.139 V (vs Ag/AgCl reference electrode) for *D*, *E* and *NE* respectively. These peaks may correspond to reduction of the quinones back to the parent compounds. The results indicate that the electrode reactions for catecholamines are quasi-reversible under the solution conditions used. Furthermore, the decrease in the peak current ratio (anodic, i_{pa}, peak current to cathodic, i_{pc}, peak current) from 1.8 to 1.2 with increase in the scan rate from 20 mV/s to 200 mV/s respectively (results not shown) indicates that the oxidation reaction appears to be followed by a chemical reaction. This behaviour has also been reported by Hawley et al. [25] and Ciolcowski et al. [26].

3.3.2. Hydrodynamic voltammetry (HDV)

In cyclic voltammetry, the potentials for oxidation and reduction are measured when the solvent (mobile phase) containing catecholamines is stagnant. In hydrodynamic voltammetry (HDV) the measurements are made using the HPLC detector itself, with flowing mobile phase. Also the measurements are made at discrete values of the applied potential, as would be used in the final detection method (rather than continuously varied as in CV). The optimum potentials (at plateau region) were found to be +500 mV in single electrode oxidative mode (see Fig. 7), +500 mV and −200 mV for generator and detector (Fig. 8) cells respectively in the redox (dual electrode) mode.

3.4. Sample cleanup using solid phase extraction (SPE)

Phenylboronic acid (PBA) SPE columns provide a convenient means of cleanup of catecholamine samples. The PBA functionality forms a very strong and reversible (pH dependent) covalent bonding with compounds containing a cis-diol moiety (e.g. catecholamines, nucleic acids and carbohydrates) with release of a water molecule [20]. The column functionality is first converted to an active boronate group at alkaline pH (pH 10). The sample is applied; impurities can be removed by washing with an alkaline solution. Finally the compound of interest is eluted with an

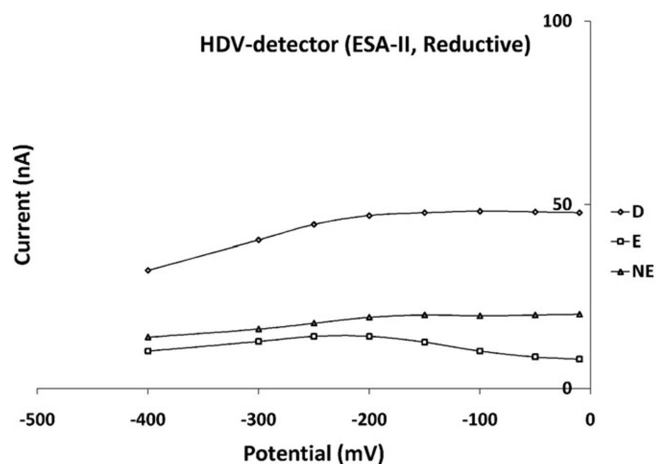

Fig. 8. Hydrodynamic voltammograms for catecholamines obtained with the Coulochem II detector in the redox mode. The oxidation potential was +500 mV. Other conditions as Fig. 7.

acidic solvent (pH below 4) under which conditions, the catechol containing solute is released and the boronate group is converted to a neutral boronic acid group. We studied the conditions of this cleanup in order to maximise recovery and performance for a final analytical method using HILIC and high concentrations of ACN; note that this procedure has previously only been applied prior to RP separations.

Firstly the effect of the elution solvent on the recovery of catecholamines was investigated. Standard solutions with concentration equivalent to a medium level [9] of catecholamines (300 μg/L, 50 μg/L and 150 μg/L for *D*, *E* and *NE* respectively) in urine were used in this study. 3 mL of sample (containing 1 mL of standard soln + 2 mL of 50 mM phosphate buffer, pH 8.5) was applied on column. The wash solution used was 1 mL of 10 mM phosphate buffer (pH 8.5) containing 10% ACN. Elution solvents (1 mL) containing different proportions of ACN (30–80% ACN v/v) containing 1% formic acid were utilised. The results are shown in Table 3. It can be seen that the recoveries of *D*, *E* and *NE* increased from ∼33 to 53% when using 30% ACN to ∼90% when using 80% ACN as the eluting solvent, presumably due to hydrophobic bonding between the solutes and the column, which is disrupted using higher concentrations of ACN. Clearly, increasing the volume of elution solvent would improve the recoveries, especially when using low concentrations of ACN, but at the expense of dilution of the final injected solution. High concentrations of ACN in the injection solvent are ideal for HILIC as ACN is a weak solvent in this technique. However, a possible downside of the use of eluents which are strong in RP chromatography is that a greater concentration of matrix compounds could be eluted from the SPE cartridge, leading to peaks that might interfere with the final HPLC procedure.

Secondly, the effect of the "wash solution" (at higher pH) used to remove matrix compounds from the cartridge prior to elution of the catecholamines was investigated. (Note that the designation

Table 3
Effect of final elution solvent on recovery of catecholamines from PBA cartridge.

	% recovery		
	30% ACN in 1% FA	50% ACN in 1% FA	80% ACN in 1% FA
Dopamine	33	59	88
Epinephrine	51	61	90
Norepinephrine	53	68	87

Recovery experiments were performed using a standard solution containing a medium concentration of catecholamines (300, 50, 150 μg/L for *D*, *E*, *NE* respectively [9]).

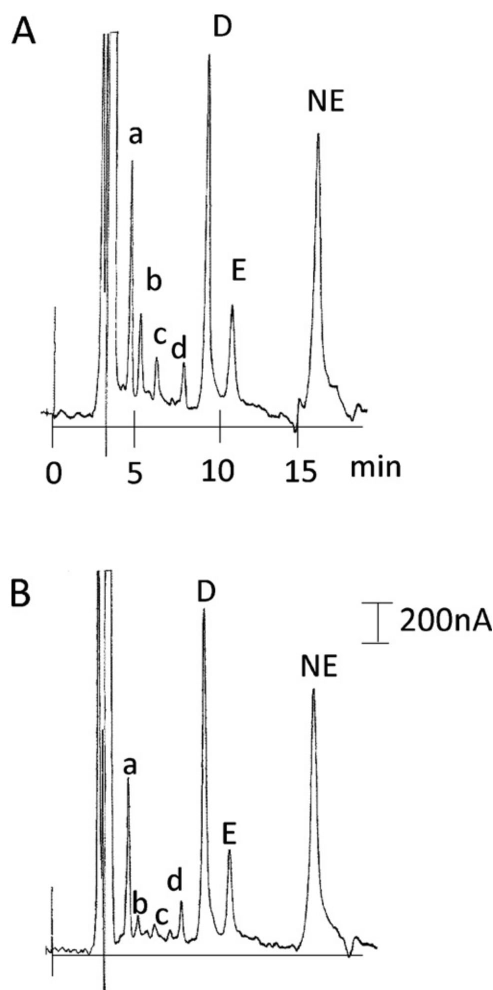

Fig. 9. Effect of wash solution on analysis of urine spiked with *E* and *NE* (concentrations *E* = 44 μg/L, *NE* = 148 μg/L, *D* = 110 μg/L). Column ZIC–HILIC, mobile phase 6.25 mM ammonium formate pH 3.0 in 80.5% ACN. (A) cartridge wash solution 1.0 mL 10% ACN in phosphate buffer pH 8.5. (B) Wash solution 1.0 mL 50% ACN in phosphate buffer pH 8.5. Detection electrochemical, single electrode oxidative mode at +350 mV. 20 μL injection.

"wash solution" is used to refer to a solution used in Step 3 in the protocol to remove matrix compounds from the cartridge, while not removing the analytes. It is distinct from the "elution solvent" used to remove the analytes from the column in Step 4 of the protocol). Other basic amino compounds (that do not contain the catechol group) can also be retained by the PBA cartridge by either weak ionic interactions between the amino group and boronate groups or by hydrophobic interaction [20]. These retained compounds might subsequently be eluted with the analytes, possibly causing interference. However, the catecholamines are retained by strong covalent bonds, which have high energy compared with these ionic or hydrophobic interactions. Hence, it should be possible to wash the column with alkaline solutions containing a significant proportion of ACN to remove the impurities but leave the catecholamines on the cartridge. Nevertheless, the use of higher concentrations of ACN in the wash solution risks loss of the analytes. Fig. 9 compares the analysis of a spiked urine extract where the wash solution was A) 1 mL of 10% ACN in 10 mM phosphate buffer pH 8.5 and B) 1 mL 50% ACN in 10 mM phosphate pH 8.5. Peaks a–d are unidentified matrix constituents. It is apparent that use of 50% ACN minimises the size of these peaks without affecting the size of the peaks of *D*, *E* and *NE*. While these matrix peaks are well resolved from those of the analytes, it is conceivable that they could interfere in meth-

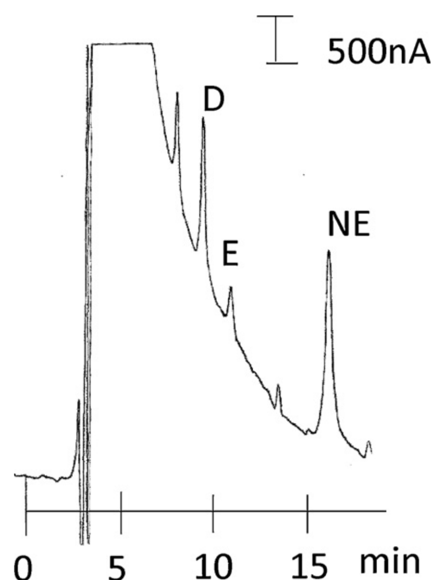

Fig. 10. Analysis of the same spiked urine sample treated as for Fig. 9 (B) but electrochemical detection in the dual electrode mode at potentials of +500 mV and −200 mV.

ods which use a different final HPLC procedure, so there seems no reason not to advocate use of this stronger wash solution. The final optimised scheme for extraction of the catecholamines using SPE is outlined in Section 2.

3.5. Analysis of spiked and unspiked urine in oxidative and redox mode

Good sensitivity was obtained for standard solutions of the catecholamines in either single electrode oxidative (+500 mV) or dual electrode redox mode at the optimum potentials (+500 mV oxidation step; −200 mV reduction step). In the dual electrode mode, the compounds are oxidised at the first electrode, and the products subsequently reduced at the second electrode, producing the analytical response. Similar detection limits were obtained using either system (~15, 20 and 25 pg on column, S/N = 3:1) for *D*, *E* and *NE* respectively. A 20 μL injection was used for this assessment. Urine samples (spiked and unspiked) were analysed in both detection modes. The identities of the peaks attributed to the 3 catecholamines in the unspiked urine were confirmed to be *D*, *E* and *NE* by measuring the peak current ratio at 300, 500 and 900 mV and comparing the ratios obtained from an injection of the pure standards. In order to minimise interference from matrix compounds in the single electrode oxidative mode, the potential was set at +350 mV instead of +500 mV. At this lower potential the sensitivities for *D*, *E* and *NE* were minimally affected but the response to matrix compounds was reduced. Fig. 10 shows the analysis of a spiked urine sample (medium concentration levels-see above) using the dual electrode detection mode. The very large peak in the redox mode for both standards and samples was thought to be due to the presence of oxygen. Indeed this seemed a likely explanation, as degassing the sample extract by bubbling with nitrogen for 20 min reduced the size of the large matrix peak which elutes in the early part of the chromatogram (results not shown); thus oxygen is being reduced at a potential of −200 mV in the detector cell. A further consideration is that in the dual electrode mode, there is increased instrument bandspreading as the second electrode contributes detrimentally to instrument dead volume. Thus it was concluded that the single electrode oxidative mode was the preferred method, and this was used for all subsequent work.

Table 4
Reproducibility of 6 analyses of urine from a healthy male subject, and recoveries of the same urine spiked with the target analytes.

	Concentration (μg/L) in unspiked urine			Concentration (μg/L) added			Concentration (μg/L) found			% recovery		
	D	*E*	*NE*	*D*	*E*	*NE*	*D*	*E*	*NE*	*D*	*E*	*NE*
MEAN	110	10.4	36.0	300	40.0	160	384	48.0	185	91	94	93
s.d.	12.3	1.2	1.5				23.7	1.6	7.2	4.3	3.1	3.7
Rsd (%)	11.3	11.9	4.2				6.2	3.5	3.8	4.7	3.3	4.0

Neubecker and co-workers obtained a detection limit of 50 pg for *NE* using RPLC with MS–MS detection using selected reaction monitoring, using a 5 μL injection, although no other catecholamines were studied [27]. More recently, De Jong and co-workers [9] reported limits of *quantification* (S/N = 10:1) of 222, 27, and 267 pg for *D*, *E* and *NE* respectively using RPLC with tandem mass spectrometry and multiple reaction monitoring, using 100 μL injections. In both cases, these values indicate that the limits of detection obtained with RPLC with tandem mass spectrometry are of the same order as those with electrochemical detection reported above. However, it is possible that improvements in MS detection limits might be obtained if used in conjunction with a HILIC procedure such as the one reported in the present study.

3.6. Precision and recovery of the procedure

Table 4 shows results for a normal urine from a healthy male subject, analysed 6 times by the optimised procedure and found to contain mean concentrations of *D*, *E*, and *NE* of 110, 10.4 and 36.0 μg/L respectively. The same urine was spiked to increase the concentrations by 300, 40 and 160 μg/L for *D*, *E* and *NE* which should result in a medium concentration of the catecholamines in the final sample. The average recovery of *D*, *E* and *NE* was 91, 94 and 93% respectively and the relative standard deviation (Rsd) of the 6 analyses was 4.7, 3.3 and 4.0% respectively. The precision of the results for the unspiked urine at low levels was inferior, but this finding is typical, and the precision of the method at low levels is similar to that found in a previous study [9]. However, highly accurate and reproducible analysis of these very low levels in normal samples is not usually required, as much higher levels are characteristic of diseased states.

4. Conclusions

Bare silica columns gave inadequate separations of the catecholamines either in the conventional HILIC mode or in per-aqueous liquid chromatography, giving poor peak shapes and/or insufficient selectivity. This result was obtained for catecholamines ionised in the mobile phase, although excellent results have been obtained for strongly basic drugs which are also ionised under similar conditions [12]. However, good results were obtained on zwitterionic or amide bonded-phase HILIC columns. Clearly, there is much that is still not understood about the mechanism of HILIC separations, and the complex interplay of the various contributory processes to retention and peak shape. HILIC has advantages for this separation over conventional RP analyses. Ion-pair reagents are unnecessary; these can complicate procedures which use MS as a detection method and furthermore, the high organic content of typical HILIC mobile phases is more compatible with spraying and desolvation in the ES–MS source. In the present work, HILIC was shown to be fully compatible with electrochemical detection, either using a single electrode (oxidative) or dual electrode (redox) mode. However, the single electrode mode gave better results, partially due to interference from dissolved oxygen in the sample, which can interfere with the reduction step. The HILIC separation is also compatible with solid phase extraction based on phenylboronate complexation, and recoveries from these cartridges were improved when using higher concentrations of ACN in the eluting solvent which are fully compatible with HILIC. Furthermore, lipophilic compounds removed from the cartridge using higher levels of ACN are eluted near to the void volume of the column in HILIC, so little interference results. The method was found to be sufficiently sensitive for the analysis of the very low levels of catecholamines found in urine samples from normal subjects, and very suitable for the determination of elevated levels of these compounds at the levels found in diseased states.

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